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Glucosinolates

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Glucosinolates

With 83 Figures and 23 Tables

Springer

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ISBN 978-3-319-25461-6 ISBN 978-3-319-25462-3 (eBook)
ISBN 978-3-319-25749-5 (print and electronic bundle)
DOI 10.1007/978-3-319-25462-3

Library of Congress Control Number: 2017933570

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Glucosinolates, natural S-glycosides, have attained importance in recent years as new class of secondary metabolites of profound physiological properties. Glucosinolates are present in the 16 families of order Brassicales including Brassicaceae which contains several of daily vegetables (cabbage, radish, mustard, cauliflower, broccoli, horseradish, turnip, oilseed rape, etc.). Glucosinolates are accumulated in all plant parts such as root, shoot, stem, and seed and also contain an enzyme called myrosinase (b-thioglucosidase). Glucosinolates have become important parameter to breed and develop new crop varieties for human welfare. They possess wide ranging properties like bacteriocide, antioxidant, bioherbicide and fungicide, and anticarcinogenic; therefore, this book is a timely compilation of state of information about this rapidly developing field.

The book aims to present comprehensive and up-to-date information on this new and developing field. The book comprises of 15 chapters and is divided into three sections, viz.: Part I – Biology, Phytochemistry, Genetics, and Defense; Part II – Biological Activity; and Part III – Analytical and Processing Methods. This comprehensive reference book presents the sources of glucosinolates, genetics and breeding of *Brassica* crops, glucosinolates in food, glucosinolates in plant defense, antimicrobial activity, neuroprotective effects, glucosinolates in atherosclerosis, anticancerous effect and as modulator of drugs, methods of glucosinolates extraction, preparation, processing, and identification by mass spectroscopy. The book will be a valuable source on glucosinolates.

The book is intended to serve the needs of graduate students, scholars, and researchers in the field of botany, agriculture, pharmacy, biotechnology, and phytochemistry; industrial scientists; and those involved in processing and marketing of vegetable products.

This work could not be completed without active support of Springer team who took pains in streamlining the production process. We are particularly indebted to Drs. Lydia Mueller, Sylvia Blago, and Sylvia Jakuscheit for their continuous professional support throughout the project.

January 2017

J.-M. Mérillon
K.G. Ramawat
Editors

Contents

About the Editors

Prof. Dr. Jean-Michel Mérillon received his Pharm.D. (1979), Ph.D. (1984), and HDR (1992) from the University of Tours in France. He joined this same university as assistant professor in 1981 and became associate professor in 1987. In 1993, he moved to the faculty of Pharmacy, University of Bordeaux, France, accepting a position as full professor. He is currently leading the "study group on biologically active plant substances" at the Institute of Vine and Wine Sciences, which comprises 25 scientists and research students. The group has been working on phenolic compounds from vine and wine for many years, mainly complex stilbenes and their involvement in health. Prof. Mérillon has supervised the doctoral theses of 20 students. He is involved in developing teaching on plantbiology, natural bioactive compounds, and biotechnology.

Prof. Mérillon has published more than 150 research papers in internationally recognized journals, resulting in an H index of 38 (documents published between 1996 and 2016). He has coedited books and reference works on secondary metabolites and biotechnology.

Throughout his career, Prof. Mérillon has traveled widely as a senior professor. Scientists from several countries have been and are working in his laboratory, and his research is supported by funding from the Aquitaine Regional Government, the Ministry of Higher Education and Research, and various private companies. In 2004, he founded the technology transfer unit "Polyphenols Biotech," providing support for R&D programs for SMEs and major groups from the cosmetic, pharmaceutical, agricultural, and health-nutrition sectors. Faculty of Pharmacy, Institut des Sciences de la Vigne et du Vin – CS 50008, University of Bordeaux, Villenave d'Ornon, France.

Prof. Dr. Kishan Gopal Ramawat is former professor and head of the Botany Department, M.L. Sukhadia University, Udaipur, India, and can look back on longstanding research experience. He received his Ph.D. in Plant Biotechnology in 1978 from the University of Jodhpur, India, and afterwards joined the university as a faculty member. In 1991, he moved to the M.L. Sukhadia University in Udaipur as associate professor and became professor in 2001. He served as the head of the Department of Botany (2001–2004, 2010–2012); was in charge of the Department of Biotechnology (2003–2004); was a member of the task force on medicinal and aromatic plants of the Department of Biotechnology, Government of India, New Delhi (2002–2005); and coordinated UGC-DRS and DST-FIST programs (2002–2012).

Prof. Ramawat had done his postdoctoral studies at the University of Tours, France, from 1983 to 1985, and later returned to Tours as visiting professor (1991). He also visited the University of Bordeaux 2, France, several times as visiting professor (1995, 1999, 2003, 2006, 2010) and in 2005 Poland in an academic exchange program (2005). Through these visits in France, Prof. Ramawat and Prof. Mérillon established a strong connection, which has resulted in productive collaborations and several book and reference work publications.

Prof. Ramawat has published more than 170 well-cited peer-reviewed papers and articles and edited several books and reference works on topics such as the biotechnology of medicinal plants, secondary metabolites, bioactive molecules, herbal drugs, and many other topics. His research was funded by several funding agencies.

In his research group, Prof. Ramawat has supervised doctoral theses of 25 students. He is an active member of several academic bodies, associations, and editorial boards of journals. Botany Department, M.L.Sukhadia University, Udaipur, India.

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Part I

Biology, Phytochemistry, Genetics, and Defense

Glucosinolates: Novel Sources and Biological Potential

1

Ivica Blažević, Sabine Montaut, Franko Burčul, and Patrick Rollin

Abstract

In this chapter, some of the most recent information on glucosinolate-containing plant families is presented. Glucosinolates (GLs) are structurally homogenous secondary metabolites present in the Brassicaceae, Capparidaceae, Moringaceae, and Resedaceae families, as well as in other less-studied families of the order Brassicales. Based on the GL contents, new subdivisions of GL-containing plants are suggested. It was shown that only a limited number of the reported *ca* 130 GLs are available in fair quantities, acceptable for further investigation of the biological potential. In recent years, degradation products of a limited number of GLs (e.g., gluconasturtiin, glucoraphanin, glucomoringin), mostly isothiocyanates, have been found to possess real pharmacological activity. Some of the biological aspects of GLs and isothiocyanates which have been in recent focus are presented.

Keywords

Glucosinolates • Isothiocyanates • Order Brassicales • Biological activity

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_1

Abbreviations	
AD	Alzheimer's disease
Ala	Alanine
APG	Angiosperm phylogeny group classification
ARE	Antioxidant response element
BCAA	Branched-chain amino acids
DS-GL	Desulfo-glucosinolate
ESI FTICR MS	Electrospray ionization and Fourier transform ion cyclotron resonance mass spectrometry
GC-MS	Gas chromatography–mass spectrometry
GL	Glucosinolate
GSH	Glutathione
HPLC	High-performance liquid chromatography
HPLC-ESI-MS	High-performance liquid chromatography–electrospray mass spectrometry
Ile	Isoleucine
ITC	Isothiocyanate
Leu	Leucine
Met	Methionine
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
Phe	Phenylalanine
Rha	Rhamnose
SeCys	Selenocysteine
SeMet	Selenomethionine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Contents

1 Introduction

Glucosinolates (GLs) represent molecular tags of plants from the order Brassicales. The distribution, biogenesis, and biological activity of GLs, and their most known degradation products isothiocyanates (ITCs), have been reviewed over the last decade by Fahey et al. [1], Bones and Rossiter [2], Clarke et al. [3], and Agerbirk and Olsen [4]. In this chapter, some of the latest developments in the group of natural

products collectively known as the GLs are presented. Emphasis is placed on their natural distribution, abundance level, and biological potential. The lack of commercially available authentic standards for most GLs has been pointed out repeatedly and represents an obvious challenge [3, 5]. In other respects, well-characterized plants (mostly seeds) are also reliable reference materials [4]. Plant species that contain high concentrations of a single or a limited number of GLs represent one of the most suitable sources for the extraction and purification of fair amounts of these compounds. Tables 1 and 2 list the different types of GLs and their sources, which are discussed in the text. Dedicated extractive methods allow one to isolate a number of GLs from adequate plant material, but in many cases, organic synthesis is a necessary alternative to obtain required quantities of GLs. Rollin and Tatibouët's recent review summarizes the known synthetic approaches developed since the early 1960s [6].

In recent years, a number of GL degradation products, mostly ITCs, have been found to possess real pharmacological activity, which will be discussed in the following sections. Although GLs offer a structural variety in the aglycone chain R, which may contain alkenyl, indolyl, hydroxyl, carbonyl, or diverse thiofunctions, only few of them have been studied under the diverse angles including their source as well as their biological potential. Some of the GL-containing plants are recognized for their biological properties, whereas most of them are still not studied. This report includes some of those plants.

2 Chemical Structure and Beyond

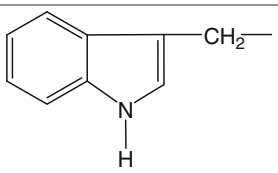
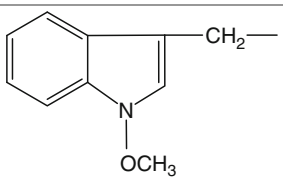
Glucosinolates represent thioglucosidic secondary metabolites occurring in the angiosperm plant families, mainly in the order Brassicales. To date, more than *ca* 130 structurally different GLs have been reported [3, 4]. GLs are water-soluble organic anions that share a common basic structural features (Fig. 1) and bear a side chain which constitution, depending on plant species, is the sole structural variant:

- *β*-D-Glucopyrano unit
- A *O*-sulfated anomeric (*Z*)-thiohydroximate function
- A variable aglycone side chain

The aglycone can originate from one of eight natural amino acids according to which GLs can be classified into: aliphatic (derived from Ala, Leu, Ile, Val, and Met), arylaliphatic (derived from Phe or Tyr), and indolyl GLs (derived from Trp). Many GLs are biosynthesized via extensive changes in the aglycone side chains which is due to a wide variety of chemical modifications such as elongation, hydroxylation, *O*-methylation, desaturation, further glycosylation, oxidation, and acylation [1, 7, 8].

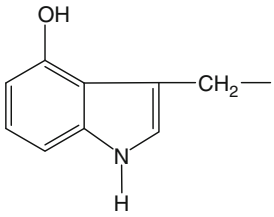
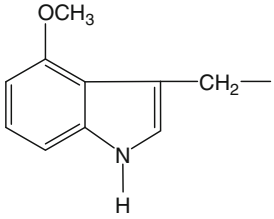
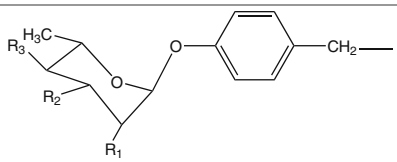
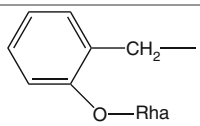
Many GLs are derived from chain-elongated derivatives of Met and Phe. The structures of the several known aliphatic GLs are derived from the elongated and modified side chains of Met homologues (Fig. 2).

Table 1 Structures of the GLs and plant families which can biosynthesize them in large amount

No.	Glucosinolate	Aglycone structure (R-)	Plant family
Aliphatic			
1	Glucocapparin	CH_3-	b, d
2	Glucoputranjivin	CH_3-(CH_3)CH-	a
3	Glucocochlearin	CH_3-CH_2-(CH_3)CH-	a
4	Glucocleomin	CH_3-C(OH)(CH_3)-CH_2-CH_2-	d
5	3-(Hydroxymethyl)pentyl GL	CH_3-CH_2-CH(CH_2OH)-$(CH_2)_2$-	a
6	Sinigrin	CH_2 = CH-CH_2-	a, b
7	Gluconapin	CH_2 = CH-$(CH_2)_2$-	a, e
8	Glucobrassicanapin	CH_2 = CH-$(CH_2)_3$-	a
9	Progoitrin	(*R*)-CH_2 = CH-CH(OH)-CH_2-	a
10	*Epi*progoitrin	(*S*)-CH_2 = CH-CH(OH)-CH_2-	a
11	Glucoibervirin	CH_3S-$(CH_2)_3$-	a
12	Glucoerucin	CH_3S-$(CH_2)_4$-	a
13	Glucoberteroin	CH_3S-$(CH_2)_5$-	a
14	Glucoiberin	CH_3SO-$(CH_2)_3$-	a
15	Glucoraphanin	CH_3SO-$(CH_2)_4$-	a
16	Glucoalyssin	CH_3SO-$(CH_2)_5$-	a
17	Glucohirsutin	CH_3SO-$(CH_2)_8$-	a
18	Glucoarabin	CH_3SO-$(CH_2)_9$-	a
19	Glucocamelinin	CH_3SO-$(CH_2)_{10}$-	a
Arylaliphatic			
20	Glucotropaeolin	C_6H_5-CH_2-	a, c, g, l
21	Gluconasturtiin	C_6H_5-CH_2-CH_2-	a,
22	Glucosinalbin	*p*OH-C_6H_4-CH_2-	a, l
23	Glucolepigramin	*m*OH-C_6H_4-CH_2-	f
24	Glucoaubrietin	*p*CH_3O-C_6H_4-CH_2-	a, h, l
25	Glucolimnanthin	*m*CH_3O-C_6H_4-CH_2-	a, f, l
26	Glucobarbarin	*S*-C_6H_5-CH(OH)-CH_2-	a
27	*Epi*glucobarbarin	*R*-C_6H_5-CH(OH)-CH_2-	a, i
Indolyl			
28	Glucobrassicin		a, i
29	Neoglucobrassicin		a, j, k

(*continued*)

Table 1 (continued)

No.	Glucosinolate	Aglycone structure (R-)	Plant family
30	4-Hydroxyglucobrassicin		a
31	4-Methoxyglucobrassicin		a
***O*-Glycosylated**			
32	Glucomoringin ($R_1 = R_2 = R_3 = OH$)		a, g
33	Glucomoringin monoacetyl-isomer I, II, or III (R_1, R_2, or $R_3 = OAc$)		g
34	2-(α-L-Rhamnopyranosyloxy) benzyl		i

a Brassicaceae, *b* Capparidaceae, *c* Caricaceae, *d* Cleomaceae, *e* Gyrostemonaceae, *f* Limnanthaceae, *g* Moringaceae, *h* Pentadiplandraceae, *i* Resedaceae, *j* Salvadoraceae, *k* Tovariaceae, *l* Tropaeolaceae

Arylaliphatic and indolyl GLs have also been identified together with the *O*-Glycosylated GLs, e.g., containing L-rhamnose as additional sugar moiety linked to the aromatic ring.

GC-MS of GL breakdown products (mostly ITCs) and HPLC analysis of the desulfo-GLs, described in the ISO 9167–1 official method, are extensively used for their identification and quantification [9]. However, some GL breakdown products and desulfo-GLs are unstable in the applied conditions (temperature, pH value, time, and sulfatase enzyme). Some GLs, such as long-chain unsaturated GLs (C8-C10) (Fig. 2), were identified solely by GC-MS of their ITCs [10]. 2-(Methylsulfanyl) ethyl GL was reported without documentation, although it might be expected as a biosynthetic intermediate in plants accumulating 2-(methylsulfinyl)ethyl GL [4]. Even in recent literature, there are occasional reports of GL identification solely based on *m/z* values from HPLC-MS without the use of authentic references.

Table 2 Database of GLs quantity present in plant species of different families

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
Akaniaceae					
Bretschneidera sinensis	**Total**	**0.4–9.2**	**μmol g^{-1} DW**		[27]
Aliphatic					
2-Hydroxy-2-methylpropyl	Glucoconringiin	0.1-0.9	μmol g^{-1} DW	ba, br, fr, l	[27]
Arylaliphatic					
Benzyl	Glucotropaeolin	0.2–7.1	μmol g^{-1} DW	ba, br, fr, l	[27]
4-Methoxybenzyl	Glucoaubrietin	0.6	μmol g^{-1} DW	fr	[27]
4-Hydroxy-3-methoxybenzyl	Glucobretschneiderin	0.6	μmol g^{-1} DW	fr	[27]
4-Hydroxybenzyl	Glucosinalbin	≤0.1	μmol g^{-1} DW	ba, br, l	[27]
Brassicaceae					
Armoracia rusticana	**Total**	**1.6–117.5**	**μmol g^{-1} DW**		[135, 136]
Aliphatic					
Prop-2-enyl	Sinigrin	0.2–111.9	μmol g^{-1} DW	i, l, sp, r	[135–137]
But-3-enyl	Gluconapin	<0.1–73.5	μmol g^{-1} DW	i, l, sp, r	[135, 137]
Pent-4-enyl	Glucobrassicanapin	<0.1–0.8	μmol g^{-1} DW	i, l, sp, r	[135, 137]
3-(Methylsulfinyl)propyl	Glucoiberin	0.2–0.6	μmol g^{-1} DW	i, l, sp, r	[135, 137]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	0.1–2.6	μmol g^{-1} DW	l, sp, r	[135–137]
(*R*)-2-Hydroxy-2-phenylethyl	Glucobarbarin	<0.1	μmol g^{-1} DW	r	[137]
Indolyl					
Indol-3-ylmethyl	Glucobrassicin	0.1–2.1	μmol g^{-1} DW	i, l, sp, r	[135–137]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1–1.1	μmol g^{-1} DW	i, l, sp, r	[135–137]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.2–0.6	μmol g^{-1} DW	pl	[136]

Aurinia leucadea	**Total**	**26.0–90.1**	**μmol g^{-1} DW**		[35, 37]
Aliphatic					
But-3-enyl	Gluconapin	26.3–48.3	μmol g^{-1} DW	fl, l, s, st	[35, 37]
5-(Methylsulfinyl)pentyl	Glucoalyssin	4.8–38.2	μmol g^{-1} DW	fl, l, r, s, st	[35, 37]
5-(Methylsulfanyl)pentyl	Glucoberteroin	0.5–18.8	μmol g^{-1} DW	fl, l, r, s, st	[35, 37]
Pent-4-enyl	Glucobrassicanapin	3.6–14.7	μmol g^{-1} DW	fl, l, s, st	[35, 37]
4-(Methylsulfinyl)butyl	Glucoraphanin	0.4–3.7	μmol g^{-1} DW	fl, l, r, s, st	[35, 37]
4-(Methylsulfanyl)butyl	Glucoerucin	0.4–2.2	μmol g^{-1} DW	l, r, st	[35, 37]
1-Methylpropyl	Glucococchlearin	0.9–1.6	μmol g^{-1} DW	fl, l, s, st	[35, 37]
Arylaliphatic					
Benzyl	Glucotropaeolin	0.3	μmol g^{-1} DW	l	[35, 37]
Aurinia sinuata	**Total**	**21.7–86.4**	**μmol g^{-1} DW**		[35]
Aliphatic					
5-(Methylsulfinyl)pentyl	Glucoalyssin	6.5–62.3	μmol g^{-1} DW	r, s	[35]
5-(Methylsulfanyl)pentyl	Glucoberteroin	17.2	μmol g^{-1} DW	s	[35]
Pent-4-enyl	Glucobrassicanapin	6.9–15.2	μmol g^{-1} DW	r, s	[35]
Camelina alyssum	**Total**	**28.5**	**μmol g^{-1} DW**		[138]
Aliphatic					
10-(Methylsulfinyl)decyl	Glucocamelinin	18.0	μmol g^{-1} DW	s	[138]
9-(Methylsulfinyl)nonyl	Glucoarabin	8.1	μmol g^{-1} DW	s	[138]
11-(Methylsulfinyl)undecyl		2.4	μmol g^{-1} DW	s	[138]
Camelina microcarpa	**Total**	**19.8**	**μmol g^{-1} DW**		[138]
Aliphatic					
10-(Methylsulfinyl)decyl	Glucocamelinin	14.2	μmol g^{-1} DW	s	[138]
11-(Methylsulfinyl)undecyl		4.3	μmol g^{-1} DW	s	[138]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
9-(Methylsulfinyl)nonyl	Glucoarabin	1.3	μmol g^{-1} DW	s	[138]
Camelina pilosa	**Total**	**32.2**	**μmol g^{-1} DW**		[138]
Aliphatic					
10-(Methylsulfinyl)decyl	Glucocamelinin	20.0	μmol g^{-1} DW	s	[138]
11-(Methylsulfinyl)undecyl		11.1	μmol g^{-1} DW	s	[138]
9-(Methylsulfinyl)nonyl	Glucoarabin	1.1	μmol g^{-1} DW	s	[138]
Cardamine pratensis	**Total**	**7.3–124.4**	**μmol g^{-1} DW**		[44]
Aliphatic					
3-(Hydroxymethyl)pentyl		17.1–114.6	μmol g^{-1} DW	l, r, s	[44]
1-Methylpropyl	Glucocochlearin	0.1–10.7	μmol g^{-1} DW	l, r	[44]
1-(Hydroxymethyl)ethyl		0.1–6.3	μmol g^{-1} DW	l, r	[44]
1-(Hydroxymethyl)propyl		0.1–5.5	μmol g^{-1} DW	l, r	[44]
3-Methylpentyl		<0.1–4.7	μmol g^{-1} DW	l, r, s	[44]
1-Methylethyl	Glucoputranjivin	0.1–0.4	μmol g^{-1} DW	l, r	[44]
Arylaliphatic					
4-Hydroxybenzyl	Glucosinalbin	0.5–38.1	μmol g^{-1} DW	l, r	[44]
Benzyl	Glucotropaeolin	<0.1–35.3	μmol g^{-1} DW	l, r	[44]
4-Methoxybenzyl	Glucoaubrietin	0.1–8.6	μmol g^{-1} DW	l, r	[44]
Indolyl					
Indol-3-ylmethyl	Glucobrassicin	0.2–7.0	μmol g^{-1} DW	l, r, s	[44]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	<0.1–2.3	μmol g^{-1} DW	l, r, s	[44]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1–0.2	μmol g^{-1} DW	l, r	[44]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1–2.2	μmol g^{-1} DW	l, r	[44]
1,4-Dimethoxyindol-3-ylmethyl	1,4-Dimethoxyglucobrassicin	0.1	μmol g^{-1} DW	r	[44]
Coincya longirostra	**Total**	**35.3–69.8**	**μmol g^{-1} DW**		[33]

Aliphatic					
But-3-enyl	Gluconapin	2.0–34.0	μmol g^{-1} DW	l, r	[33]
(*R*)-2-Hydroxybut-3-enyl	Progoitrin	0.1–10.0	μmol g^{-1} DW	l, r	[33]
4-(Methylsulfanyl)butyl	Glucoerucin	8.0	μmol g^{-1} DW	r	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	0.1–1.6	μmol g^{-1} DW	l, r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	l, r	[33]
Prop-2-enyl	Sinigrin	<0.1	μmol g^{-1} DW	l	[33]
Pent-4-enyl	Glucobrassicanapin	<0.1	μmol g^{-1} DW	l	[33]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	0.1–28.0	μmol g^{-1} DW	l, r	[33]
4-Hydroxybenzyl	Glucosinalbin	0.2	μmol g^{-1} DW	l, r	[33]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.2–10.0	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	0.2–10.0	μmol g^{-1} DW	l, r	[33]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.2	μmol g^{-1} DW	l	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.2	μmol g^{-1} DW	l	[33]
Coincya monensis	**Total**	**22.2–41.9**	**μmol g^{-1} DW**		[33]
Aliphatic					
But-3-enyl	Gluconapin	0.3–3.0	μmol g^{-1} DW	l, r	[33]
(*R*)-2-Hydroxybut-3-enyl	Progoitrin	1.5	μmol g^{-1} DW	r	[33]
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	1.4	μmol g^{-1} DW	l	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	0.9	μmol g^{-1} DW	r	[33]
4-(Methylsulfanyl)butyl	Glucoerucin	0.2	μmol g^{-1} DW	r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	r	[33]
Prop-2-enyl	Sinigrin	<0.1	μmol g^{-1} DW	l	[33]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	19.0	μmol g^{-1} DW	r	[33]
4-Hydroxybenzyl	Glucosinalbin	10.0–17.0	μmol g^{-1} DW	l, r	[33]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
Indolyl					
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.2–5.0	μmol g^{-1} DW	l, r	[33]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.2–5.0	μmol g^{-1} DW	l, r	[33]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.2	μmol g^{-1} DW	l	[33]
Indol-3-ylmethyl	Glucobrassicin	0.2	μmol g^{-1} DW	l	[33]
Coincya rupestris	**Total**	**41.6–114.0**	**μmol g^{-1} DW**		[33]
Aliphatic					
But-3-enyl	Gluconapin	5.0–38.0	μmol g^{-1} DW	l, r	[33]
(*R*)-2-Hydroxybut-3-enyl	Progoitrin	0.5–26.0	μmol g^{-1} DW	l, r	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	0.3–1.6	μmol g^{-1} DW	l, r	[33]
4-(Methylsulfanyl)butyl	Glucoerucin	2.0	μmol g^{-1} DW	r	[33]
1-Methylpropyl	Glucococchlearin	0.4–1.6	μmol g^{-1} DW	l, r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	l, r	[33]
3-(Methylsulfinyl)propyl	Glucoiberin	<0.1	μmol g^{-1} DW	l	[33]
Prop-2-enyl	Sinigrin	<0.1	μmol g^{-1} DW	l, r	[33]
Pent-4-enyl	Glucobrassicanapin	<0.1	μmol g^{-1} DW	l	[33]
Arylaliphatic					
4-Hydroxybenzyl	Glucosinalbin	<0.1	μmol g^{-1} DW	l, r	[33]
2-Phenylethyl	Gluconasturtiin	<0.1–67.0	μmol g^{-1} DW	l, r	[33]
Benzyl	Glucotropaeolin	<0.1	μmol g^{-1} DW	l, r	[33]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.3–6.0	μmol g^{-1} DW	l, r	[33]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.3–6.0	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	0.3	μmol g^{-1} DW	l, r	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.3	μmol g^{-1} DW	l, r	[33]

Degenia velebitica	**Total**	**9.9–99.1**	**µmol g^{-1} DW**		[36]
Aliphatic					
5-(Methylsulfanyl)pentyl	Glucoberteroin	1.3–88.0	µmol g^{-1} DW	fl + l, r, s, st	[36]
5-(Methylsulfinyl)pentyl	Glucoalyssin	6.4–8.4	µmol g^{-1} DW	fl + l, s, st	[36]
4-(Methylsulfanyl)butyl	Glucoerucin	2.7	µmol g^{-1} DW	s	[36]
Pent-4-enyl	Glucobrassicanapin	2.2	µmol g^{-1} DW	fl + l	[36]
Arylaliphatic					
4-Methoxybenzyl	Glucoaubrietin	0.4	µmol g^{-1} DW	st	[36]
Dithyrea wislizenii	**Total**	**1.8**	**µmol g^{-1} DW**		[139]
Aliphatic					
6-(Methylsulfanyl)hexyl	Glucolesquerellin	1.5	µmol g^{-1} DW	fr	[139]
6-(Methylsulfinyl)hexyl	Glucohesperin	0.2	µmol g^{-1} DW	fr	[139]
7-(Methylsulfanyl)heptyl		<0.1	µmol g^{-1} DW	fr	[139]
5-(Methylsulfanyl)pentyl		<0.1	µmol g^{-1} DW	fr	[139]
Erucastrum canariense	**Total**	**18.7–43.3**	**µmol g^{-1} DW**		[33]
Aliphatic					
Prop-2-enyl	Sinigrin	14.0–34.0	µmol g^{-1} DW	l, r	[33]
3-Methylpenthyl		2.1–3.0	µmol g^{-1} DW	l, r	[33]
But-3-enyl	Gluconapin	0.2–0.3	µmol g^{-1} DW	l, r	[33]
4-(Methylsulfanyl)butyl	Glucoerucin	<0.1	µmol g^{-1} DW	l	[33]
3-(Methylsulfinyl)propyl	Glucoiberin	<0.1	µmol g^{-1} DW	l, r	[33]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	6.9	µmol g^{-1} DW	r	[33]
Indol-3-ylmethyl	Glucobrassicin	1.5	µmol g^{-1} DW	l	[33]
Fibigia triquetra	**Total**	**62.5–135.4**	**µmol g^{-1} DW**		[38]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
Aliphatic					
4-(Methylsulfanyl)butyl	Glucoerucin	3.5–76.7	μmol g^{-1} DW	fl + l, s, st	[38]
But-3-enyl	Gluconapin	32.6–66.7	μmol g^{-1} DW	fl + l, s, st	[38]
4-(Methylsulfinyl)butyl	Glucoraphanin	8.0–23.4	μmol g^{-1} DW	fl + l, s, st	[38]
1-Methylpropyl	Glucocochlearin	1.3–4.3	μmol g^{-1} DW	fl + l, s, st	[38]
1-Methylethyl	Glucoputranjivin	0.7–2.5	μmol g^{-1} DW	fl + l, s, st	[38]
Pent-4-enyl	Glucobrassicanapin	0.7	μmol g^{-1} DW	s	[38]
Guiraoa arvensis	**Total**	**61.1–160.1**	**μmol g^{-1} DW**		[33]
Aliphatic					
Prop-2-enyl	Sinigrin	61.0–150.0	μmol g^{-1} DW	l	[33]
But-3-enyl	Gluconapin	0.1	μmol g^{-1} DW	l, r	[33]
3-(Methylsulfinyl)propyl	Glucoiberin	<0.1	μmol g^{-1} DW	l, r	[33]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	<0.1–5.0	μmol g^{-1} DW	l, r	[33]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1–5.0	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	<0.1	μmol g^{-1} DW	l	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1	μmol g^{-1} DW	l	[33]
Hemicrambe fruticulosa	**Total**	**46.4–64.9**	**μmol g^{-1} DW**		[33]
Aliphatic					
But-3-enyl	Gluconapin	7.0–59.0	μmol g^{-1} DW	l, r	[33]

(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	0.9–8.1	μmol g^{-1} DW	l, r	[33]
1-Methylpropyl	Glucococchlearin	0.7–1.3	μmol g^{-1} DW	l, r	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	≤0.1	μmol g^{-1} DW	l, r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	l, r	[33]
Prop-2-enyl	Sinigrin	<0.1	μmol g^{-1} DW	l, r	[33]
3-(Methylsulfinyl)propyl	Glucoiberin	<0.1	μmol g^{-1} DW	l	[33]
Pent-4-enyl	Glucobrassicanapin	<0.1	μmol g^{-1} DW	l, r	[33]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	2.0–28.0	μmol g^{-1} DW	l, r	[33]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.4–1.3	μmol g^{-1} DW	l, r	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.4–1.3	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	0.4	μmol g^{-1} DW	l	[33]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.4	μmol g^{-1} DW	l	[33]
Hirschfeldia incana	**Total**	**2.8–30.3**	**μmol g^{-1} DW**		[33]
Aliphatic					
(*R*)-2-Hydroxybut-3-enyl	Progoitrin	0.2–8.3	μmol g^{-1} DW	l, r	[33]
But-3-enyl	Gluconapin	2.0–3.0	μmol g^{-1} DW	l, r	[33]
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	0.2	μmol g^{-1} DW	l	[33]
Pent-4-enyl	Glucobrassicanapin	<0.1	μmol g^{-1} DW	r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	r	[33]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	0.6–15.0	μmol g^{-1} DW	l, r	[33]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	2.0	μmol g^{-1} DW	r	[33]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	2.0	μmol g^{-1} DW	r	[33]
Isatis canescens	**Total**	**79.5–103.3**	**μmol g^{-1} DW**		[53]
Aliphatic					

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
But-3-enyl	Gluconapin	33.5–65.1	μmol g^{-1} DW	fl	[53]
Indolyl					
Indol-3-ylmethyl	Glucobrassicin	33.1–68.8	μmol g^{-1} DW	fl	[53]
Isatis indigotica	**Total**	**16.0–45.0** **123.2–152.0**	**μmol g^{-1} DW** **μmol g^{-1} FW**		[140] [141]
Aliphatic					
(*R*)-2-Hydroxybut-3-enyl	Progoitrin	2.5–15.0 27.7–64.2	μmol g^{-1} DW μmol g^{-1} FW	l, s s	[55, 57, 140] [141]
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	0.1–1.3 18.6–115.4	μmol g^{-1} DW μmol g^{-1} FW	l, s s	[55, 57, 140] [141]
But-3-enyl	Gluconapin	<0.1–0.3 0.8–32.9	μmol g^{-1} DW μmol g^{-1} FW	l s	[57, 140] [141]
	(*R*)-glucoisatisin/ (*S*)-*epi*glucoisatisin	2.1 0.4–1.0	μmol g^{-1} DW μmol g^{-1} FW	s s	[57] [141]
Indolyl					
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.4–40.0 2.4–3.4	μmol g^{-1} DW μmol g^{-1} FW	l, s s	[55, 57, 140] [141]
Indol-3-ylmethyl	Glucobrassicin	<0.1–4.4	μmol g^{-1} FW	s	[141]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.1–0.3	μmol g^{-1} FW	s	[57, 141]
Kremeriella cordylocarpus	**Total**	**13.8–77.6**	**μmol g^{-1} DW**		[33]
Aliphatic					
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	<0.1–1.0	μmol g^{-1} DW	l, s	[33]
But-3-enyl	Gluconapin	<0.1–0.6	μmol g^{-1} DW	l, r	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	<0.1	μmol g^{-1} DW	l, r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	l, r	[33]
Arylaliphatic					

4-Hydroxybenzyl	Glucosinalbin	11.0–76.0	μmol g^{-1} DW	l, r	[33]
2-Phenylethyl	Gluconasturtiin	0.2	μmol g^{-1} DW	r	[33]
Benzyl	Glucotropaeolin	<0.1–0.1	μmol g^{-1} DW	l	[33]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	<0.1–2.0	μmol g^{-1} DW	l, r	[33]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1–2.0	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	<0.1	μmol g^{-1} DW	l, r	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1	μmol g^{-1} DW	l, r	[33]
Lepidium fendleri	**Total**	**27.5**	**μmol g^{-1} DW**		[142]
Aliphatic					
3-(Methylsulfinyl)propyl	Glucoiberin	27.5	μmol g^{-1} DW	s	[142]
Lepidium meyenii	**Total**	**3.8–69.5**	**μmol g^{-1} DW**		[143, 144]
Aliphatic					
5-(Methylsulfinyl)pentyl	Glucoalyssin	0.1–11.2	μmol g^{-1} DW	h, l, s, sp	[143, 144]
Arylaliphatic					
Benzyl	Glucotropaeolin	0.2–40.9	μmol g^{-1} DW	h, l, s, sp	[143–145]
3-Methoxybenzyl	Glucolimnanthin	0.2–19.3	μmol g^{-1} DW	h, l, s, sp	[143, 144]
4-Hydroxybenzyl	Glucosinalbin	0.2–12.6	μmol g^{-1} DW	h, l, s, sp	[143, 144]
4-Methoxybenzyl	Glucoaubrietin	0.7–6.4	μmol g^{-1} DW	h, l, sp	[144]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1–7.6	μmol g^{-1} DW	h, l, s	[143, 144]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1	μmol g^{-1} DW	h, l	[143, 144]
Indol-3-ylmethyl	Glucobrassicin	<0.1	μmol g^{-1} DW	h, l	[144]
Noccaea caerulescens	**Total**	**0.8–186.2**	**μmol g^{-1} DW**		[146]
Aliphatic					
Prop-2-enyl	Sinigrin	<0.1–68.4	μmol g^{-1} DW	l, s	[46]
Arylaliphatic					
4-Hydroxybenzyl	Glucosinalbin	<0.1–83.0	μmol g^{-1} DW	l, s	[46]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
***O*-Glycosylated**					
4-α − L − Rhamnopyranosyloxybenzyl*	Glucomoringin*	<0.1–111.0	μmol g^{-1} DW	l, s	[46]
Cheesemanii / Pachycladon	**Total**	**90.1**	**μmol g^{-1} DW**		[147]
Aliphatic					
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	47.9	μmol g^{-1} DW	l	[147]
Prop-2-enyl	Sinigrin	37.1	μmol g^{-1} DW	l	[147]
6-(Methylsulfinyl)hexyl	Glucohesperalin	2.0	μmol g^{-1} DW	l	[147]
7-(Methylsulfinyl)heptyl	Glucoibarin	1.3	μmol g^{-1} DW	l	[147]
4-(Methylsulfanyl)butyl	Glucoerucin	0.5	μmol g^{-1} DW	l	[147]
3-(Methylsulfanyl)propyl	Glucoibervirin	0.4	μmol g^{-1} DW	l	[147]
3-(Methylsulfinyl)propyl	Glucoiberin	0.3	μmol g^{-1} DW	l	[147]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.6	μmol g^{-1} DW	l	[147]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.3	μmol g^{-1} DW	l	[147]
Pachycladon exile	**Total**	**59.4**	**μmol g^{-1} DW**		[147]
Aliphatic					
3-(Methylsulfinyl)propyl	Glucoiberin	46.1	μmol g^{-1} DW	l	[147]
3-(Methylsulfanyl)propyl	Glucoibervirin	9.6	μmol g^{-1} DW	l	[147]
7-(Methylsulfinyl)heptyl	Glucoibarin	1.6	μmol g^{-1} DW	l	[147]
6-(Methylsulfinyl)hexyl	Glucohesperalin	0.9	μmol g^{-1} DW	l	[147]
4-(Methylsulfinyl)butyl	Glucoraphanin	0.8	μmol g^{-1} DW	l	[147]
4-(Methylsulfanyl)butyl	Glucoerucin	0.2	μmol g^{-1} DW	l	[147]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.2	μmol g^{-1} DW	l	[147]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1	μmol g^{-1} DW	l	[147]

Pachycladon novae-zelandiae	**Total**	**102.0**	**µmol g^{-1} DW**		[147]
Aliphatic					
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	42.1	µmol g^{-1} DW	l	[147]
Prop-2-enyl	Sinigrin	31.8	µmol g^{-1} DW	l	[147]
4-(Methylsulfinyl)butyl	Glucoraphanin	10.2	µmol g^{-1} DW	l	[147]
3-(Methylsulfinyl)propyl	Glucoiberin	7.2	µmol g^{-1} DW	l	[147]
But-3-enyl	Gluconapin	3.9	µmol g^{-1} DW	l	[147]
4-(Methylsulfanyl)butyl	Glucoerucin	3.3	µmol g^{-1} DW	l	[147]
8-(Methylsulfinyl)octyl	Glucohirsutin	2.5	µmol g^{-1} DW	l	[147]
3-(Methylsulfanyl)propyl	Glucoibervirin	1.8	µmol g^{-1} DW	l	[147]
7-(Methylsulfinyl)heptyl	Glucoibarin	1.1	µmol g^{-1} DW	l	[147]
7-(Methylsulfanyl)heptyl		<0.1	µmol g^{-1} DW	l	[147]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.9	µmol g^{-1} DW	l	[147]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.3	µmol g^{-1} DW	l	[147]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.1	µmol g^{-1} DW	l	[147]
Pringlea antiscorbutica	**Total**	**52.9–148.7**	**µmol g^{-1} DW**		[34]
Aliphatic					
But-3-enyl	Gluconapin	26.1–66.2	µmol g^{-1} DW	l, s	[34]
n-Butyl		5.6–11.8	µmol g^{-1} DW	l, s	[34]
4-(Methylsulfanyl)butyl	Glucoerucin	10.1	µmol g^{-1} DW	s	[34]
Prop-2-enyl	Sinigrin	2.9–6.4	µmol g^{-1} DW	l, s	[34]
4-(Methylsulfinyl)butyl	Glucoraphanin	3.9–5.4	µmol g^{-1} DW	l, s	[34]
Arylaliphatic					
Benzyl	Glucotropaeolin	12.9–50.3	µmol g^{-1} DW	l, s	[34]
Sinapis boivinii	**Total**	**39.3–43.0**	**µmol g^{-1} DW**		[33]
Aliphatic					
But-3-enyl	Gluconapin	27.0–35.0	µmol g^{-1} DW	l, r	[33]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
Prop-2-enyl	Sinigrin	4.0–8.0	μmol g^{-1} DW	l, r	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	≤0.1	μmol g^{-1} DW	l, r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	l	[33]
3-(Methylsulfinyl)propyl	Glucoiberin	<0.1	μmol g^{-1} DW	l	[33]
Pent-4-enyl	Glucobrassicanapin	<0.1	μmol g^{-1} DW	l	[33]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	6.0	μmol g^{-1} DW	r	[33]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1–1.1	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	<0.1–1.1	μmol g^{-1} DW	l, r	[33]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	<0.1	μmol g^{-1} DW	l	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1	μmol g^{-1} DW	l	[33]
1,4-Dimethoxyindol-3-ylmethyl	1,4-Dimethoxyglucobrassicin	<0.1	μmol g^{-1} DW	r	[33]
Sinapis pubescens ssp. indurata and pubescens	**Total**	**34.8–85.4**	**μmol g^{-1} DW**		[33]
Aliphatic					
But-3-enyl	Gluconapin	27.0–41.0	μmol g^{-1} DW	l, r	[33]
Prop-2-enyl	Sinigrin	<0.1	μmol g^{-1} DW	l	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	≤0.1	μmol g^{-1} DW	l, r	[33]
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	≤0.1	μmol g^{-1} DW	l, r	[33]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1	μmol g^{-1} DW	l, r	[33]
Pent-4-enyl	Glucobrassicanapin	<0.1	μmol g^{-1} DW	l, r	[33]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	20.0–58.0	μmol g^{-1} DW	r	[33]
Indolyl					

4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.2–0.8	μmol g^{-1} DW	l, r	[33]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.2	μmol g^{-1} DW	l	[33]
Indol-3-ylmethyl	Glucobrassicin	0.2	μmol g^{-1} DW	l	[33]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.2	μmol g^{-1} DW	l	[33]
Sinapis flexuosa	**Total**	**12.6–39.0**	**μmol g^{-1} DW**		[33]
Arylaliphatic					
4-Hydroxybenzyl	Glucosinalbin	12.0-39.0	μmol g^{-1} DW	l, r	[33]
Indolyl					
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1-0.6	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	<0.1	μmol g^{-1} DW	l	[33]
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	<0.1	μmol g^{-1} DW	l	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1	μmol g^{-1} DW	l	[33]
Trachystoma labasii	**Total**	**65.1–70.5**	**μmol g^{-1} DW**		[33]
Aliphatic					
Prop-2-enyl	Sinigrin	54.0–70.0	μmol g^{-1} DW	l, r	[33]
But-3-enyl	Gluconapin	0.1	μmol g^{-1} DW	l, r	[33]
4-(Methylsulfinyl)butyl	Glucoraphanin	<0.1	μmol g^{-1} DW	l, r	[33]
3-(Methylsulfinyl)propyl	Glucoiberin	<0.1	μmol g^{-1} DW	l, r	[33]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.1–8.0	μmol g^{-1} DW	l, r	[33]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	0.1	μmol g^{-1} DW	l, r	[33]
Indol-3-ylmethyl	Glucobrassicin	0.1	μmol g^{-1} DW	l, r	[33]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	0.1	μmol g^{-1} DW	l, r	[33]
Capparidaceae					
Boscia senegalensis	**Total**	**2.0–440.0**	**μmol g^{-1} DW**		[68, 148]
Aliphatic					
Methyl	Glucocapparin	2.0–440.0	μmol g^{-1} DW	fr, l, s	[68, 148]
Capparis ovata	**Total**	**11.4–84.6**	**μmol g^{-1} DW**		[65, 67, 149]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
Aliphatic					
Methyl	Glucocapparin	1.3–80.4	μmol g^{-1} DW	bu, fl, l, s, ys	[65, 67, 149]
Prop-2-enyl	Sinigrin	<0.1–29.8	μmol g^{-1} DW	bu, fl, l, ys	[67, 149]
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	0.1–8.2	μmol g^{-1} DW	bu, fl, l, ys	[67, 149]
But-3-enyl	Gluconapin	0.1–2.1	μmol g^{-1} DW	bu, fl, l, ys	[67, 149]
3-(Methylsulfinyl)propyl	Glucoiberin	0.1–2.1	μmol g^{-1} DW	bu, fl, l, ys	[67, 149]
5-(Methylsulfinyl)pentyl	Glucoalyssin	<0.1–1.1	μmol g^{-1} DW	bu, ys	[149]
(*R*)-2-Hydroxybut-3-enyl	Progoitrin	0.1–1.1	μmol g^{-1} DW	bu, fl, l, ys	[67, 149]
(*R*)-2-Hydroxypent-4-enyl	Gluconapoleiferin	0.1–0.8	μmol g^{-1} DW	bu, ys	[149]
Pent-4-enyl	Glucobrassicanapin	0.1–0.2	μmol g^{-1} DW	bu, ys	[149]
Arylaliphatic					
4-Hydroxybenzyl	Glucosinalbin	0.3–0.9	μmol g^{-1} DW	bu, fl, l, ys	[67]
2-Phenylethyl	Gluconasturtiin	0.1–0.4	μmol g^{-1} DW	bu, ys	[149]
Indolyl					
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1	μmol g^{-1} DW	bu, ys	[149]
Indol-3-ylmethyl	Glucobrassicin	<0.1–8.2	μmol g^{-1} DW	bu, fl, l, ys	[67, 149]
Capparis spinosa subsp. rupestris	**Total**	**6.8–127.1**	**μmol g^{-1} DW**		[5, 13, 65, 66, 149]

Aliphatic					
Methyl	Glucocapparin	5.4–127.1	μmol g^{-1} DW	bu, s, ys	[5, 13, 65, 66, 149]
5-(Methylsulfinyl)pentyl	Glucoalyssin	0.1–0.5	μmol g^{-1} DW	bu, ys	[149]
(*R*)-2-Hydroxypent-4-enyl	Gluconapoleiferin	0.1–0.5	μmol g^{-1} DW	bu, ys	[149]
Pent-4-enyl	Glucobrassicanapin	0.1–0.4	μmol g^{-1} DW	bu, ys	[149]
But-3-enyl	Gluconapin	0.1–0.3	μmol g^{-1} DW	bu, ys	[149]
(*S*)-2-Hydroxybut-3-enyl	*Epi*progoitrin	0.1–0.3	μmol g^{-1} DW	bu, ys	[149]
Prop-2-enyl	Sinigrin	0.1–0.3	μmol g^{-1} DW	bu, ys	[149]
3-(Methylsulfinyl)propyl	Glucoiberin	0.1–0.2	μmol g^{-1} DW	bu, ys	[149]
(*R*)-2-Hydroxybut-3-enyl	Progoitrin	0.1–0.2	μmol g^{-1} DW	bu, ys	[149]
Glycinylmethyl	Glycinyl-glucocapparin	<0.1	μmol g^{-1} DW	bu	[13]
Isopropyl/*n*-propyl		<0.1	μmol g^{-1} DW	bu	[13]
Mercaptomethyl	Mercapto-glucocapparin	<0.1	μmol g^{-1} DW	bu	[13]
Disulfanylmethyl	Disulfanyl-glucocapparin	<0.1	μmol g^{-1} DW	bu	[13]
Trisulfanylmethyl	Trisulfanyl-glucocapparin	<0.1	μmol g^{-1} DW	bu	[13]
Arylaliphatic					
2-Phenylethyl	Gluconasturtiin	0.1–0.2	μmol g^{-1} DW	bu, ys	[149]
Indolyl					
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1–2.0	μmol g^{-1} DW	bu, ys	[13, 149]
Indol-3-ylmethyl	Glucobrassicin	<0.1–0.5	μmol g^{-1} DW	bu, ys	[13, 149]
Crataeva religiosa	**Total**	**37.3**	**μmol g^{-1} DW**		[150]
Aliphatic					
Methyl	Glucocapparin	37.3	μmol g^{-1} DW	l	[150]
Caricaceae					
Carica papaya	**Total**	**75.0–259.2**	**μmol g^{-1} DW**		[5, 151]
Arylaliphatic					
Benzyl	Glucotropaeolin	75.0–259.2	μmol g^{-1} DW	lx, p, s	[5, 151]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
		0. 3–20.0	μmol g^{-1} FW	p, s	[152–154]
Carica pentagona	**Total**	**0.1–10.0**	**μmol g^{-1} DW**		[5]
Arylaliphatic					
Benzyl	Glucotropaeolin	0.1–10.0	μmol g^{-1} DW	s	[5]
Cleomaceae					
Cleome hassleriana	**Total**	**15.3–24.1**	**μmol g^{-1} DW**		[80]
Aliphatic					
Methyl	Glucocapparin	9.9–15.7	μmol g^{-1} DW	s	[80]
2-Hydroxy-2-methylbutyl	Glucocleomin	5.4–8.4	μmol g^{-1} DW	s	[80]
Peritoma arborea	**Total**	**13.8–42.9**	**μmol g^{-1} FW**		[79]
Aliphatic					
Methyl	Glucocapparin	13.8–42.9	μmol g^{-1} FW	bu, l, s	[79]
Gyrostemonaceae					
Gyrostemon ramulosus					
Aliphatic					
But-3-enyl	Gluconapin	25.0–50.0	μmol g^{-1} DW	s	[5]
4-(Methylsulfinyl)butyl	Glucoraphanin	0.1–10.0	μmol g^{-1} DW	s	[5]
Limnanthaceae					
Limnanthes alba subsp. alba and versicolor	**Total**	**29.5–204.2**	**μmol g^{-1} DW**		[81, 82]
Arylaliphatic					
3-Methoxybenzyl	Glucolimnanthin	29.5–204.2	μmol g^{-1} DW	s	[81, 82]
Limnanthes douglasii		**75.0–100.0**	**μmol g^{-1} DW**		
Arylaliphatic					
3-Methoxybenzyl	Glucolimnanthin	75.0–100.0	μmol g^{-1} DW	s	[5]

Limnanthes floccosa subsp. bellingeriana, pumila, grandifolia, and californica	**Total**	**86.8–168.0**	**μmol g^{-1} DW**		[81]
Arylaliphatic					
3-Hydroxybenzyl	Glucolepigramin	52.0–134.4	μmol g^{-1} DW	s	[81]
3-Methoxybenzyl	Glucolimnanthin	17.4–67.2	μmol g^{-1} DW	s	
Limnanthes gracilis subsp. gracilis and parishii	**Total**	**75.2–186.0**	**μmol g^{-1} DW**		[81]
Arylaliphatic					
3-Methoxybenzyl	Glucolimnanthin	75.2–186.0	μmol g^{-1} DW	s	[81]
Limnanthes montana	**Total**	**74.3–175.6**	**μmol g^{-1} DW**		[81]
Arylaliphatic					
3-Methoxybenzyl	Glucolimnanthin	74.3–175.6	μmol g^{-1} DW	s	[81]
Moringaceae					
Moringa oleifera		**up to 600.0**	**μmol g^{-1} DW**		[155]
Aliphatic					
4-(Methylsulfinyl)butyl	Glucoraphanin	<0.1	μmol g^{-1} DW	l, p, r	[84]
Arylaliphatic					
Benzyl	Glucotropaeolin	1.3–50.7	μmol g^{-1} DW	l, p, r	[83, 84, 156]
4-Hydroxybenzyl	Glucosinalbin	<0.1	μmol g^{-1} DW	l, p, r, s	[5, 84, 156]
***O*-Glycosylated**					
4-(α-L-Rhamnopyranosyloxy)benzyl	Glucomoringin	28.5–462.3	μmol g^{-1} DW	ba, l, r, s, st	[5, 83, 84, 156]
4-(α-L-Rhamnopyranosyloxy)benzyl glucosinolate monoacetyl-*isomer III*		2.0–81.9	μmol g^{-1} DW	fl, l, st	[83, 84, 156]
4-(α-L-Rhamnopyranosyloxy)benzyl glucosinolate monoacetyl-*isomer I*		1.3–8.8	μmol g^{-1} DW	fl, l, st	[83, 84, 156]
4-(α-L-Rhamnopyranosyloxy)benzyl glucosinolate monoacetyl-*isomer II*		1.5–4.6	μmol g^{-1} DW	fl, l	[83, 84, 156]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
3-Hydroxy-4-(α-L-rhamnopyranosyloxy)benzyl		<0.1–1.1	μmol g^{-1} DW	l, p, r	[84]
Moringa stenopetala	**Total**	**19.4–448.3**	**μmol g^{-1} DW**		[83]
Arylaliphatic					
Benzyl	Glucotropaeolin	68.1–68.8	μmol g^{-1} DW	r, st	[83]
***O*-Glycosylated**					
4-(α-L-Rhamnopyranosyloxy)benzyl	Glucomoringin	9.3–448.3	μmol g^{-1} DW	ba, l, s, st	[83]
4-(α-L-Rhamnopyranosyloxy)benzyl glucosinolate monoacetyl-*isomer III*		8.3–19.2	μmol g^{-1} DW	l, st	[83]
4-(α-L-Rhamnopyranosyloxy)benzyl glucosinolate monoacetyl-*isomer I*		1.0–1.5	μmol g^{-1} DW	l	[83]
4-(α-L-Rhamnopyranosyloxy)benzyl glucosinolate monoacetyl-*isomer II*		0.8	μmol g^{-1} DW	l	[83]
Pentadiplandraceae					
Pentadiplandra brazzeana	**Total**	**2.2–109.1**	**μmol g^{-1} DW**		[87]
Arylaliphatic					
4-Methoxybenzyl	Glucoaubrietin	38.1–109.1	μmol g^{-1} DW	r, s	[87]
Benzyl	Glucotropaeolin	8.1	μmol g^{-1} DW	r	[87]
3,4-Dimethoxybenzyl		2.1	μmol g^{-1} DW	l	[87]
3-Methoxybenzyl	Glucolimnanthin	1.6	μmol g^{-1} DW	r	[87]
Indolyl					
Indol-3-ylmethyl	Glucobrassicin	0.1	μmol g^{-1} DW	l	[87]
Resedaceae					
Caylusea abyssinica					

Arylaliphatic					
(*R*)-2-Hydroxy-2-phenylethyl	*Epi*glucobarbarin	10.0–25.0	μmol g^{-1} DW	s	[5]
2-Phenylethyl	Gluconasturtiin	0.1–10.0	μmol g^{-1} DW	s	[5]
(*S*)-2-Hydroxy-2-phenylethyl	Glucobarbarin	<0.1	μmol g^{-1} DW	s	[5]
Reseda lutea	**Total**	**25.0–50.0**	**μmol g^{-1} DW**		[5]
***O*-Glycosylated**					
2-(α-L-Rhamnopyranosyloxy)benzyl		25.0–50.0	μmol g^{-1} DW	s	[5]
Reseda luteola					
Arylaliphatic					
(*R*)-2-Hydroxy-2-phenylethyl	*Epi*glucobarbarin	25.0–50.0	μmol g^{-1} DW	s	[5]
(*S*)-2-Hydroxy-2-phenylethyl	Glucobarbarin	0.1–10.0	μmol g^{-1} DW	s	[5]
Indolyl					
Indol-3-ylmethyl	Glucobrassicin	0.1–10.0	μmol g^{-1} DW	s	[5]
Reseda odorata	**Total**	**10.0–25.0**	**μmol g^{-1} DW**		[5]
Indolyl					
Indol-3-ylmethyl	Glucobrassicin	10.0–25.0	μmol g^{-1} DW	s	[5]
Salvadoraceae					
Azima tetracantha	**Total**	**0.1–55.5**	**μmol g^{-1} DW**		[5, 25]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	4.6–50.6	μmol g^{-1} DW	l, r, s, st + t	[5, 25]
Indol-3-ylmethyl	Glucobrassicin	0.1–10.0	μmol g^{-1} DW	l, r, s, st + t	[5, 25]
N-Hydroxyindol-3-ylmethyl	*N*-Hydroxyglucobrassicin	1.0	μmol g^{-1} DW	r	[25]
Tovariaceae					
Tovaria pendula	**Total**	**4.1–13.1**	**μmol g^{-1} FW**		[89]
Indolyl					
N-Methoxyindol-3-ylmethyl	Neoglucobrassicin	0.1–11.2	μmol g^{-1} FW	l	[89]

(*continued*)

Table 2 (continued)

Family/Species/GL group	GL trivial name	Range	Unit	Plant organ	Ref.
N-Acetylindol-3-ylmethyl	*N*-Acetylglucobrassicin	0.2–3.8	μmol g^{-1} FW	l	[89]
Indol-3-ylmethyl	Glucobrassicin	0.2–1.3	μmol g^{-1} FW	l	[89]
4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	<0.1–0.2	μmol g^{-1} FW	l	[89]
4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	<0.1–0.2	μmol g^{-1} FW	l	[89]
Tropaeolaceae					
Tropaeolum majus	**Total**	**5.9–130.0**	**μmol g^{-1} DW**		[157, 158]
Arylaliphatic					
Benzyl	Glucotropaeolin	48.0–100.0	μmol g^{-1} DW	l, r, s, tu	[5, 157]
3-Methoxybenzyl	Glucolimnanthin	0.1–50.0	μmol g^{-1} DW	tu	[158]
4-Hydroxybenzyl	Glucosinalbin	<0.1–5.3	μmol g^{-1} DW	tu	[158]
Indolyl					
Indol-3-ylmethyl	Glucobrassicin	0.1–10.0	μmol g^{-1} DW	s	[5]
Tropaeolum peregrinum					
Aliphatic					
1-Methylethyl	Glucoputranjivin	<0.1–2.0	μmol g^{-1} DW	l, r	[33]
1-Methylpropyl	Glucocochlearin	0.6	μmol g^{-1} DW	l	[33]
Arylaliphatic					
4-Hydroxybenzyl	Glucosinalbin	0.5–125.0	μmol g^{-1} DW	l, r, s	[5, 33]
4-Methoxybenzyl	Glucoaubrietin	<0.1–50.0	μmol g^{-1} DW	l, r, s	[5, 33]
Benzyl	Glucotropaeolin	0.1–10.0	μmol g^{-1} DW	r, s	[5, 33]
Tropaeolum tuberosum	**Total**	**36,5–90,0**	**μmol g^{-1} DW**		[159]
Arylaliphatic					
4-Methoxybenzyl	Glucoaubrietin	36.5–90.0	μmol g^{-1} DW	tu	y

ba bark, *br* branch, *bu* buds, *fl* flower, *fr* fruit, *h* hypocotyl, *i* inflorescence, *l* leaf, *lx* latex, *p* pulp, *pl* plantlet, *r* root, *s* seed, *sp* sprout, *st* stem, *t* thorn, *tu* tuber, *ys* young shoot, *μmol g^{-1} DW* μmol g^{-1} dried weight, μmol g^{-1} *FW* μmol g^{-1} fresh weight, * not unequivocally defined structure

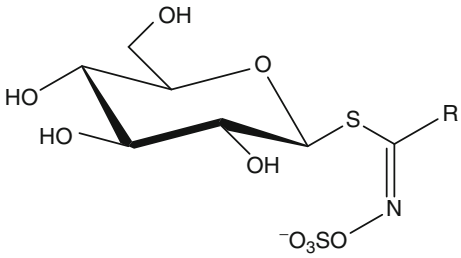


Fig. 1 General structure of glucosinolates

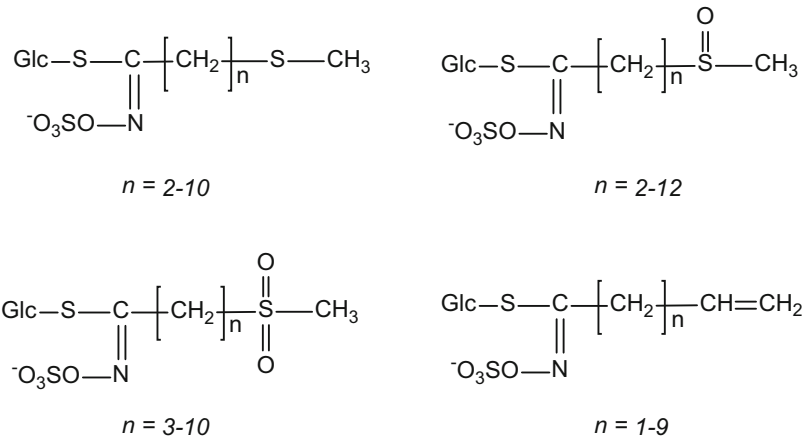


Fig. 2 General structure of chain-elongated aliphatic GLs [3]

However, the use of NMR is necessary for the distinction of isomers and it is indispensable for the structure elucidation of previously not isolated and uncharacterized intact GLs [5, 11].

Acylated GLs (either on the side chain or on the glucopyrano unit) by benzoic, cinnamic, *p*-coumaric, isoferulic, or sinapic acid, as well as some very uncommon and unique structures are also identified [3, 4], but only in very low quantity, so specific techniques were suggested for their detection [12, 13].

Table 1 includes GLs which structures are undoubtedly known and are found in fair amounts in families which include plant species that can be considered as their natural sources.

The metabolism of Brassicaceae plants can accept selenium instead of sulfur into S assimilation pathway. Se assimilation results in the production of the selenoamino acids: selenomethionine (SeMet) and selenocysteine (SeCys). As a result, the so-called seleno-GLs, defined as GL-like metabolites, are biosynthesized. They were reported from a selenium-tolerant crucifer such as broccoli (*Brassica oleracea* var. *italica*) but only when exposed to unusually high levels (100 μmol/L) of sodium

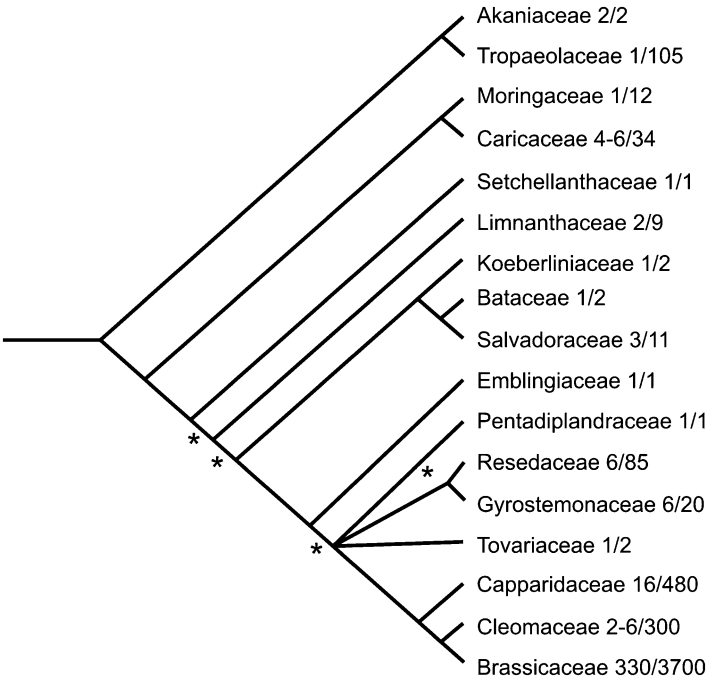


Fig. 3 Slightly modified phylogenetic tree of the order Brassicales according to the APG III system (by permission from Stevens, Peter F.) [22], with numbers representing the approximate numbers of genus and species in each family, * – denotes branches with 50–80% support; all other branches have >80% support

selenite and sodium selenate added to nutrient solutions [14, 15]. Recently, several Se-containing GLs were reported in Se-treated different *Brassica* spp., i.e., glucoselenoiberverin, glucoselenoerucin, glucoselenoberteroin, glucoselenoraphanin, and glucoselenonasturtiin [15–18]. However, the detection of these GLs, or their aglycones, converted into isoselenocyanates was done by using LC-MS, and GC-MS, respectively, and thus not unambiguously identified [16].

3 Occurrence and Novel Sources

Nearly all the GL-producing angiosperm plant families are found in the order Brassicales (Fig. 3). The Putranjivaceae (Malpighiales) is an enigmatic family, notable for being the only lineage outside of the Brassicales in which plants are supposed to possess the GL biochemical pathway [19]. Some other families outside of the order Brassicales, like Phytolaccaceae (Caryophyllales), Pittosporaceae (Apiales), and Rubiaceae (Gentianales) which were reported to contain GLs, and not characterized by proper methodology, should be reexamined [1, 20, 21].

3.1 Glucosinolate Content in Plants

Fahey et al. [1] summarized GL data for different species, but without indication of quantities. *Brassica* vegetables such as cabbage, broccoli, Brussels sprouts, cauliflower, and various root vegetables (e.g., radish, turnip) are economically important members of the Brassicaceae family and thus the most investigated GL-containing plants. McNaughton and Marks [23] developed a food database of total GLs in fresh, frozen, boiled, and cooked cruciferous vegetables. Verkerk et al. reviewed cruciferous vegetables for the major GLs together with the GL content and the influence on human health [24]. A comprehensive analysis of GLs was reported by Bennett et al. [25] which included quantities in seeds. This sound seed-screening has brought to light clear subdivisions based on the GL content:

(i) Only short- to medium-chain-length aliphatic GLs (C-3, or C-3 and C-4 with traces of C-5)
(ii) Only long-chain aliphatic GLs
(iii) Only simple arylaliphatic GLs [such as benzyl (**20**), 4-hydroxybenzyl (**22**), 2-phenylethyl (**21**) GLs]
(iv) Highly substituted arylaliphatic GLs (such as 3,4-dihydroxybenzyl, 3,4-dimethoxybenzyl, and 3,4,5-trimethoxybenzyl GLs)

The GL content in plants varies between cultivars, plant individuals, and part of the plants, due to factors such as genetics, environment, and plant nutrients. GLs can be found in the root, seed, leaf, and stem of the plant, while youngest tissues contain the highest amount. Characterization and quantification of the phytochemical composition in plant organs, such as leaf and seed, provide a good insight into the possible functional uses of these materials in agriculture as potential pesticides and in animal and human diets as functional nutraceuticals [26]. Dedicated extractive methods allow one to isolate a number of GLs from adequate plant material, but in many cases, organic synthesis is a necessary alternative to obtain fair quantities of GLs [6].

Although novel sources are revealed continuously by investigating families other than Brassicaceae, there exists no database reviewing those studies. Table 2 summarizes the GL quantities analyzed in all families of the order Brassicales, except for the Brassicaceae plants previously covered by Bennett et al. [25] and Verkerk et al. [24].

3.1.1 Families in the Order Brassicales

Akaniaceae

The Akaniaceae family comprises two monotypic genera of trees, *Bretschneidera* native to southwest of China, Vietnam, and Taiwan and *Akania* native to eastern Australia (Fig. 4) [22].

Montaut et al. recently quantified the GLs in different tissues of *Bretschneidera sinensis* [27]. This plant is rare and threatened in mainland China. Arylaliphatic GLs, derived from Phe, were mostly found (Table 2), with glucotropaeolin (**20**) as the

Fig. 4 Akaniaceae world distribution map

major one. The total quantity was very low (highest in the fruit), suggesting that this species is not a good GL source. Spectroscopic data are reported for 3-hydroxy-4-methoxybenzyl GL (glucobretschneiderin), a new GL isolated from the fruit in minor amount [27]. In addition, the aliphatic GL, 2-hydroxy-2-methylpropyl GL, was also quantified in *B. sinensis*.

Akania bidwillii, endemic to eastern Australia, was analyzed by Mithen et al., but no quantification was reported [28]. Arylaliphatic GLs were also found, with methoxy-hydroxy-benzyl isomers (major), hydroxybenzyl isomers, methoxybenzyl, and benzyl GLs. However, no aliphatic GLs were detected.

Brassicaceae

This dominant family encompasses over 330 genera and 3,700 species distributed primarily in the temperate and alpine areas of all continents except Antarctica (Fig. 5) [29].

The largest genera are *Draba* (365 species), *Cardamine* (200 species), *Erysimum* (225 species), *Lepidium* (230 species), followed by *Alyssum* (195 species), *Arabis* (120), *Boechera* (110), *Physaria* (105: inc. *Lesquerella*), *Rorippa* (85), *Heliophila* (80), *Isatis* (80), *Noccaea* (80), *Thlaspi* (55), *Biscutella* (55), *Matthiola* (50), *Descurainia* (50), *Hesperis* (45), *Sisymbrium* (45: only Old World), and *Brassica* (38), etc. The family contains well-known species such as *Brassica oleracea* (broccoli, cabbage, cauliflower, kale, etc.), *Brassica rapa* (turnip, Chinese cabbage, etc.), *Brassica napus* (rapeseed, etc.), *Raphanus sativus* (common radish), *Armoracia rusticana* (horseradish), *Arabidopsis thaliana* (thale cress, a model organism), and many others.

Aliphatic GLs

GLs found in these species are derived mostly from the chain-elongated forms of Met, combined with post-synthesis extensive modifications of the side chain [28]. According to Bennett et al.'s report [5], as well as Verkerk et al.'s review of GL

Fig. 5 Brassicaceae world distribution map

concentration ranges [24], C3-C5 aliphatic GLs were a common characteristic of most *Brassica* species. The highest quantities were found in seeds, referring mostly to sinigrin (**6**) (>200 μmol g^{-1} DW), glucoiberin (**14**) (up to 200 μmol g^{-1} DW), gluconapin (**7**) (up to 125 μmol g^{-1} DW), glucoraphanin (**15**) (up to 100 μmol g^{-1} DW), glucoerucin (**12**) and progoitrin (**9**) (up to 50 μmol g^{-1} DW), and Glucoibervirin (**11**) and glucobrassicanapin (**8**) (up to 25 μmol g^{-1} DW). Broccoli seeds are one of the most used sources of **15**, a precursor of the biologically relevant *R*-sulforaphane. In a recent report, De Nicola et al. exploited Tuscan black kale seeds, rich in **15** (5.1% w/w), as a suitable source for gram-scale production of this GL. In a typical experiment, 3.10 g of pure **15**, i.e., 1.09 g of enantiopure *R*-sulforaphane, was obtained from 150 g of defatted Tuscan black kale seed meal [30]. As previously mentioned, selenized *Brassica* produce seleno-GLs. It is proposed that biofortification of *Brassica* crops with Se can further increase their chemopreventive activities [31]. Matich et al. investigated the accumulation of seleno-GLs in different tissues of cauliflower, broccoli, and rape and reported the forage rape roots and the broccoli florets to be the best sources [16]. A high-GL broccoli floret, containing 29.4 μg g^{-1} FW of total methylsulfanylalkyl GLs (MeS-GLs), produced after Se-intake produced 32.4 μg g^{-1} FW of total MeSe-GLs mostly seleno-glucoerucin (30 μg g^{-1} FW) [16].

Several recent reports (Table 2) on the sources of aliphatic GLs include *Armoracia rusticana* sprouts [32] and *Guiraoa arvensis* root [33] which are recognized as good sources of **6** (>100 μmol g^{-1} DW) (Table 2). These plants can be considered as the first (i) Bennett et al. subdivision [5], supporting this categorization.

Some of the recent reports also include GLs in endemic species of the Brassicaceae family. Kerguelen cabbage (*Pringlea antiscorbutica*), a subantarctic endemic perennial plant, from the Kerguelen phytogeographical area in the southern Indian Ocean, is an edible Brassicaceae plant. Having amounts of glucotropaeolin (**20**) (up to 50.3 μmol g^{-1} DW) unusually high for Brassicaceae plant and **7** (up to

66.2 μmol g^{-1} DW) as the major GLs is considered a good dietary source of GLs (Table 2) [34]. This is an example that combines the first and third categories (i + iii), given by Bennett et al. [5], representing a new subdivision.

The endemic plants included in the *Alysseae* tribe of the Croatian native flora (*Aurinia* species, *Degenia velebitica*, and *Fibigia triquetra*) are found to have a GL content ranging from 9.9 to 135.4 μmol g^{-1} DW in different plant parts. Having especially high content in the seeds (over 4.0% w/w with the highest, 6.1% w/w in *F. triquetra*), these *Alysseae* are found to represent a good novel GL source [35–38]. *Aurinia sinuata* and *A. leucadia* can be found in mountainous areas of Central and Southern Europe, Russia, and Turkey. The major GLs found in the seeds of the investigated plants were glucoalyssin (**16**) (38.2 μmol g^{-1} DW) and **7** (39.1 μmol g^{-1} DW) in *A. leucadea*, while **16** (62.3 μmol g^{-1} DW) and **8** (17.2 μmol g^{-1} DW) in *A. sinuata. Degenia velebitica* and *Fibigia triquetra* represent rare Croatian paleostenoendemic species (Fig. 6). The natural habitat of *D. velebitica*, the only species of the genus *Degenia*, is restricted to only several localities on the South and Mid Velebit massif. *D. velebitica* seed, having 88.0 μmol g^{-1} DW of glucoberteroin (**13**) (*ca.* 90% of total GLs), represents a good source of this compound [36]. *F. triquetra*, sometimes confused with *D. velebitica*, is found wild-growing only on the rocky grounds of Dalmatia. The major GLs found in *F. triquetra* are **12** (76.7 μmol g^{-1} DW), **7** (66.7 μmol g^{-1} DW), and **15** (23.4 μmol g^{-1} DW) [38]. To a certain extent, these informations bring a different light to the first Bennett et al.'s subdivision, differentiating in C3-C5 GLs possible combination.

Within the Brassicaceae, long-chain aliphatic thiofunctionalized GLs (C7-C10) are generally restricted to a few species such as *Arabis*, *Biscutella*, *Camelina*, *Capsella*, *Nasturtium*, as well as certain wild *Lepidium* and *Sinapis* species [1, 5]. The leaves of three *Pachycladon* species, endemic to the South Island of New Zealand and Tasmania, are found to biosynthesize C7 and/or C8 GLs (Table 2). *Biscutella laevigata* seed was shown to be a very good source of 8-(methylsulfinyl) octyl GL (**17**) (75.0–100.0 μmol g^{-1} DW) [5]. Different *Camelina* spp. seeds are reported as a source of 10-(methylsulfinyl)decyl GL (up to 25.0 μmol g^{-1} DW)

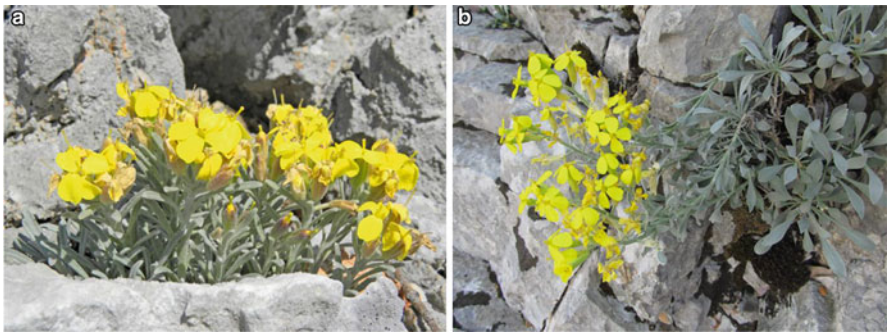


Fig. 6 Paleostenoendemic plants growing only in Croatia (Europe): (**a**) *Degenia velebitica* (Degen) Hayek (Photo by permission of Berislav Horvatić [39]) and (**b**) *Fibigia triquetra* (DC.) Boiss. ex Prantl (Photo by Ivica Blažević)

[5, 33]. In addition, the seeds of *Camelina pilosa* contain the highest quantity of 11-(methylsulfinyl)undecyl GL (11.1 μmol g^{-1} DW) (Table 2). These reports are in agreement with the third (iii) Bennett et al.'s subdivision. On the contrary, Agerbirk et al. investigated *Nasturtium officinale* (watercress) of different origin and it was concluded to be polymorphic for GL profile and chromosome numbers [40]. Long-chain methylsulfanylalkyl (C5-C7) and methylsulfinylalkyl (C8-C10) GLs were found (6–28 μmol g^{-1} DW) the highest being 9-(methylsulfinyl)nonyl GL (**18**) (21.1 μmol g^{-1} DW) but in combination with high levels of arylaliphatic GLs – dominantly gluconasturtiin (**21**) (7.8 – 52.8 μmol g^{-1} DW) [40].

Some GLs are reported only through their breakdown products, such as the presence of long-chain C7-C10 unsaturated ITCs in autolyzates of *Nasturtium montanum* [10]. Bennett et al. reported the content of C7-C10 thiofunctionalized GLs in the seed of several *Arabis* spp. [5]. Blažević et al.'s investigation of *Arabis turrita* of volatile hydrolysis products isolated from different plant parts (seed, root, leaf) revealed mainly long-chain olefinic (C6-C11) and thiofunctionalized (C8-C10) GLs. The suggested occurrence of alkenyl GLs in a plant, like in the case of *N. montanum* also accumulating similar Met-derived GLs, seemed likely. However, HPLC-ESI-MS of intact GLs confirmed only thiofunctionalized C8-C10 GLs in *A. turrita*, and it was suggested that thiofunctionalized ITCs can be converted into olefinic ITCs under high temperature during GC-MS analyses [41]. Thus, the presumption of native unsaturated C8-C10 GLs in the case of *N. montanum* or *A. turrita* is questionable.

Alkyl GLs are also detected in a few species belonging to the Brassicaceae family [5, 34]. It is suggested that methyl GL (glucocapparin, **1**), the simplest GL identified in plants, does not occur in this family [28]. However, Griffiths et al. reported, next to the major glucobrassicin (**28**) (98%), a small amount of **1** (2%) on the leaf surface of *Isatis tinctoria* (containing 0.12 μmol of total GLs per g of leaf material) [42]. In addition, by using GC-MS analysis of its degradation product (methyl ITC), **1** was reported in the seed of *Wasabi japonica* and *Armoracia lapathifolia* Gilib [43].

GLs derived from Val, Leu, Ile, and dihomo-Ile are also found. *Lunaria* spp., *Sisymbrium officinale*, and *Dentaria pinnata* are found to be a source of glucoputranjivin (**2**) (up to 50 μmol g^{-1} DW). A novel GL, 3-(Hydroxymethyl) pentyl GL, was present in high levels in *Cardamine pratensis* from eastern North America and in commercially obtained seeds (114.6 μmol g^{-1} DW), but was not found in *C. pratensis* plants from southern Scandinavia [44].

Arylaliphatic GLs

The arylaliphatic GLs are derived from the aromatic parental amino acids Phe and Tyr and are commonly found in plants belonging to the Brassicaceae family. One of the highest reported concentrations of glucosinalbin (**22**) (250 μmol g^{-1} DW) was from *Sinapis alba* seeds [45]. It was previously mentioned that, besides the high content of C-4 to C-9 aliphatic GLs, *P. antiscorbutica* and *N. officinale* also represent a good source of **20** and **21**, respectively, (up to 50 μmol g^{-1} DW) [34]. Some of the novel good sources of arylaliphatic GLs, given in Table 2, are for **22** *Noccaea caerulescens* seed and *Kremeriella cordylocarpus* leaves (83.0 and 76.0 μmol g^{-1}

DW, respectively) [33, 46] and for **21** *Coincya rupestris* root (67.0 μmol g^{-1} DW) [33]. *Barbarea* spp. seem to be a good source of **21** (up to 100 μmol g^{-1} DW) [5, 47, 48], glucobarbarin (**26**) (up to 90 μmol g^{-1} DW), and *epi*glucobarbarin (**27**) (up to 55 μmol g^{-1} DW) [5, 47]. Among these, only *K. cordylocarpus* and *Barbarea* spp. are in agreement with the third (iii) Bennett et al.'s subdivision. Other plants, as in the case of *P. antiscorbutica*, combine the first and third subdivisions of GL content (i + iii), supporting the new category, combining a short aliphatic and simple arylaliphatic GLs.

Some highly substituted arylaliphatic GLs, such as 3,4-dimethoxybenzyl GL and 3,4,5-trimethoxybenzyl GL, are restricted to a few genera, mostly belonging to the Brassicaceae family, such as *Coronopus*, *Lepidium*, and *Matthiola* [1, 49, 50]. 3,4,5-Trimethoxybenzyl GL, identified after its degradation volatile, has been previously reported as the sole GL in the seeds of few investigated *Lepidium* spp. [50–52]. *Coronopus squamatus* seeds of Spanish origin were found to be a good source of 3,4,5-trimethoxybenzyl GL (50–75 μmol g^{-1} DW) [5]. Due to the presence of this GL, a very close relationship between the *Coronopus* and *Lepidium* genera was suggested [49].

Indolyl GLs

Indolyl GLs found in cruciferous plants are derived from Trp and possess a diversely substituted heterocyclic moiety. Four main indolyl GLs have been identified in most cultivated *Brassica* species: glucobrassicin (**28**), neoglucobrassicin (**29**), 4-hydroxyglucobrassicin (**30**), and 4-methoxyglucobrassicin (**31**). *Isatis canescens* flower buds displayed a high **28** content (up to 60 μmol g^{-1} DW) and represent a good source of this GL. The purification method consisted of two chromatographic steps, made possible to obtain **28** (purity of 92–95%), with a yield of 21 g kg^{-1} [53]. It seems that *Isatis* spp. (Table 2) display a high content of short aliphatic and indolyl GLs. Some species can produce fair amounts of some additional arylaliphatic or indolyl GLs, next to the short and medium aliphatic GLs, as in the case of *Cardamine pratensis*, *Coincya* spp., *Hemicrambe fruticulosa*, *Lepidium meyenii*, *Isatis* spp., *Noccaea caerulescens*, *Pringlea antiscorbutica*, and *Sinapis pubescens* (Table 2). Thus, an additional subdivision can be suggested next to the ones of Bennett et al., combining aliphatic with arylaliphatic or indolyl GLs.

1,4-Dimethoxyglucobrassicin was firstly isolated and confirmed structurally from the roots of *Barbarea vulgaris* R. Br. ssp. *arcuata* which contained 0.53.1 μmol g^{-1} DW of this GL [54]. This rare side chain was also recently detected in the root of *Cardamine pratensis* (0.1 μmol g^{-1} DW) [44].

Extracts of *Isatis tinctoria* were found to contain *N*-sulfoglucobrassicin (11.3 – 13.5 μmol g^{-1} DW) [55]. In addition, *Isatis* spp. are found to contain the unusual combination of an aliphatic and an indolyl moiety, known as glucoisatisin/*epi*glucoisatisin (*ca.* 1.5 μmol g^{-1} DW) [56, 57].

O-Glycosylated GLs

A *O*-glycosylated GL, containing L-rhamnose, as additional sugar moiety linked to the aromatic ring, i.e., 4-α-rhamnosyloxybenzyl GL (glucomoringin; **32**, Table 1),

was identified in *Noccaea caerulescens*. This was the first report of **32** in this Brassicaceae species where it was the main GL in 10 out of 13 populations collected from different locations in Europe, with the highest concentration in the seeds up to 111.0 μmol g^{-1} DW (Table 2) [46].

O-Acylated GLs

Uncommon GLs displaying *O*-acylated sites on the D-glucopyrano unit and/or on the hydroxyl groups present in the side chain are also detected among Brassicaceae plants. Those carboxylic esters usually result from acylation by benzoic, cinnamic, *p*-coumaric, isoferulic, or sinapic acid, mostly conjugated at the primary position of the D-gluco unit. However, these GLs, mostly found in the seeds, are detected in very low quantities or their quantity was not reported [3, 4, 12, 40, 47, 57–60].

Recently, Survay et al. reported the 6-*p*-coumaroyl derivative of **15** isolated from broccoli florets (0.04 μmol g^{-1} FW) [59]. However the above structure might be regarded as questionable after retraction of a following paper by the same authors, due to "structure elucidation issues." In *Arabidopsis* seeds, esterification on the side chain of hydroxylated GLs leads to the accumulation of benzoylated and sinapoylated GLs and some of them are identified: 3- and 4-benzoyloxypropyl GLs, 3- and 4-sinapoyloxypropyl GLs, 6'-benzoyl-4-methylsulfanylbutyl GL, 6′-benzoyl-4-methylsulfinylbutyl GL, and 6′-benzoyl-4-benzoyloxybutyl GL (<6.0 ng mg^{-1} DW) [61, 62]. From the seeds of *Barbarea* spp., five 6′-isoferuloyl esters of **28**, **21**, (*S*)-2-hydroxy-2-phenylethyl GL (**26**), (*R*)-2-hydroxy-2-phenylethyl GL (**27**), and (*R*)-2-hydroxy-2-(4-hydroxyphenyl)-ethyl GL were isolated, and their structures were elucidated [47]. The acyl group was in all cases found to be a *trans* isoferuloyl group at the primary position of the D-glucopyrano unit. The highest level of 6′-isoferuloylglucobarbarin was found in *B. vulgaris* mature seeds (3 μmol g^{-1} DW) [47]. GLs with diverse acylation patterns are much less abundant and cannot be identified solely by applying low resolution MS [47]. Thus, *Barbarea vulgaris* seed extracts were analyzed by reversed-phase liquid chromatography coupled with electrospray ionization and Fourier transform ion cyclotron resonance mass spectrometry (RPLC-ESI FTICR MS), which represents a new procedure for detecting and identifying intact acylated GLs found in trace amounts in natural plant samples [12].

Capparidaceae

The Capparidaceae family consists of 16 genera and 480 species, which are mostly distributed in warm regions all around the world. The largest genera include *Capparis* (250), *Maerua* (100), *Boscia* (37), and *Cadaba* (30) (Fig. 7) [22].

Methyl GL (glucocapparin, **1**) is the principal GL accumulating in many species of the Capparidaceae and Cleomaceae. However, its biosynthesis has not been explored. It may derive from Ala, as a novel GL precursor possibly through the action of CYP79, or it may arise as a result of the single or multiple cleavage of side chains derived from Met or branched-chain amino acids (BCAA: Leu, Ile, Val) or elongated Phe [28].

The genus *Capparis* is native to the Mediterranean basin, but is now widely distributed from Atlantic coasts (Morocco) to Black Sea lands and Caspian Sea.

Fig. 7 Capparidaceae world distribution map

Cultivation of some species of this genus, especially *C. spinosa* and *C. ovata*, has been reported from the plain areas and deserts of Afghanistan, India, Indonesia, Nepal, Pakistan, North Africa, Southwest Asia, Australia, and Southern Europe up to an elevation of 1100 m [63]. Generic limits found in Capparidaceae are discussed by Hall et al., suggesting that the New World *Capparis* will need a new name [64]. The sound qualitative GL profiling in Old World and New World Capparidaceae was reported by Mithen et al. [28]. Old World taxa of the Capparidaceae were reported to have high levels of **1**, with three exceptions, namely, *Boscia longifolia* and *Capparis tomentosa* which showed high levels of hydroxymethylbutyl and hydroxyethyl GL, respectively, and *Maerua triphylla*, where **28** was the most abundant [28].

In most reports on *Capparis spinosa*, the highest **1** content was found in the seeds (up to 127 μmol g^{-1} DW) [5, 65, 66]. ESI FTICR MS was applied for LC profiling of intact GLs (specially for the ones with low abundance) in flower buds of *C. spinosa* [13]. In addition to the GLs already found (**1**, isopropyl/*n*-propyl-GLs, mercaptoglucocapparin, **28**, and **30**) [3, 13], the uncommon glycinyl-glucocapparin and two sulfur-rich GLs (disulfanyl-glucocapparin and trisulfanyl-glucocapparin) were also reported (Table 2) [13].

Next to **1**, *C. ovata* can also contain a relatively high amount of **6** (up to 29.8 μmol g^{-1} DW in the seeds, Table 2) [67]. The GL content in *B. senegalensis*, monitored throughout the whole year in leaves and fruits collected in 4 localities in Senegal, ranged from 2 to 440 μmol g^{-1}. The highest amount was recorded in January (dry season) and the lowest between August and November (rainy period) [68].

Whereas all Old World Capparidaceae contain **1**, Mithen et al. reported that New World Capparidaceae, including New World *Capparis*, contain either **1** or several distinctive and perhaps unique GLs of complex and unresolved structures, indicating continued innovation in GL biosynthesis [28]. This research has brought to light

clear differences between New World and Old World *Capparis* and can be related to the subdivisions based on GL content given by Bennett et al. [5]. On the basis of GL qualitative profiles, New World specimens of the Capparidaceae were divided into three groups:

(a) With high levels of **1** – which can be included as a new subdivision given by Bennett et al.
(b) Lack of **1** but presence of GLs with molecular weights consistent with chain-elongated Met or BCAA-derived GLs, including certain GLs, such as oxoheptyl and oxooctyl GLs found in *C. scabrida* and C10-C11 isomers of methylsulfonyldecyl and methylsulfonylundecyl GLs found in *Steriphoma peruvianum*. These GLs do not occur in other families, but can be related to Bennett et al.'s subdivision (ii), i.e., plants that produce long-chain aliphatic GLs.
(c) Several taxa, such as *Morisonia* and *Atamisquea*, have potentially novel GLs that do not correspond to any known structures, though the structural identity must remain provisional until further studies [28].

Caricaceae

The Caricaceae are a family of dicotyledonous angiosperms found in mostly tropical (Andean) America (three genera in Mexico) and Africa (*Cylicomorpha* only). It is made up of several genera (4–6) with 34 species or more: *Vasconcellea* (22), *Jacaratia* (7), *Carica* (5), *Jarilla* (3), and *Cylicomorpha* (2) (Fig. 8) [22].

Analysis of the rbcL gene sequences by Rodman et al. demonstrated that the family Caricaceae belongs to the main GL-containing order Brassicales and that Caricaceae are apparently a sister family of the Moringaceae [69–72]. DNA sequencing of the nuclear 18S ribosomal RNA gene has yielded the same result [73]. Benzyl GL (**20**) is the major GL found in the family Caricaceae [1]. The modification in the biosynthetic route to GLs includes (i) chain elongation of

Fig. 8 Caricaceae world distribution map

aliphatic and aromatic amino acids by inserting methylene groups into their side chains and (ii) metabolic modification of the amino acids (or chain-extended derivatives of amino acids) that takes place via an aldoxime intermediate, the same as for cyanogenic glycosides. However, the co-occurrence of GLs and cyanogenic glycosides in the same plant is very scarce [74, 75]. *Carica papaya* L., the most known plant of this family, includes, next to **20**, the cyanogenic glucosides, prunasin, sambunigrin, and tetraphyllin B [76]. Aside from *C. papaya*, *Alliaria petiolata* (M. Bieb.) Cavara and Grande (Brassicaceae) is the only other known exception of a plant containing both GLs and cyanogenic glucosides which, next to **6** also contains large quantities of hydroxynitrile glucoside alliarinoside [75]. In immature papaya fruit pulp, the amount of **20** ranged from 163.1 to 259.2 μmol g^{-1} DW of latex fluid. No myrosinase was found in papaya latex. To explain the variability of benzyl ITC formation in the macerated immature fruit pulp, the author speculated that papain (a proteolytic enzyme also found in the papaya latex) prevents uncontrolled enzymatic production of benzyl ITC by inactivating small quantities of myrosinase in the latex through proteolysis [77].

Cleomaceae

The Cleomaceae is a small pantropical family. Although historically treated as a subfamily of Capparidaceae sensu lato, Cleomaceae are easily distinguished from closely related Capparidaceae sensu stricto [78]. This family comprises about 300 species including mostly *Cleome* (275, including *Podandrogyne*) and *Cleomella* (25) genera [22]. These genera were previously included in the family Capparidaceae, but were raised to a distinct family when DNA evidence suggested that the genera included in it are more closely related to the Brassicaceae than they are to the Capparidaceae. They are found in tropical and warm temperate areas, especially in America (Fig. 9).

Fig. 9 Cleomaceae world distribution map

The seeds of *Peritoma arborea* (formerly *Isomeris arborea*, syn. *Cleome isomeris*) have been previously analyzed for their GL content and shown to contain only **1** (up to 43 μmol g^{-1} DW) [79]. Mithen et al. analyzed GLs of *Cleome spinosa* and *Cleome gynandra* seeds, and only **1** was detected [28], concluding the previous reports on *Cleome* genus [1]. *Cleome hassleriana* seed contained **1** (up to 15 μmol g^{-1} DW) and 2-hydroxy-2-methylbutyl GL (up to 8 μmol g^{-1} DW) known as glucocleomin (**4**) [80].

Gyrostemonaceae

The Gyrostemonaceae comprises 6 genera: *Gyrostemon* (12), *Borthwickia* (1), *Codonocarpus* (3), *Cypselocarpus* (1), *Tersonia* (2), and *Walteranthus* (1). All are endemic, to temperate parts of Australia (except in the north, Fig. 10) [22].

The information of GL quantity in this family is included only in Bennett et al.'s report. *Gyrostemon ramulosus* seeds contained a high amount of **7** (25.0–50.0 μmol g^{-1} DW) and **15** (0.1–10.0 μmol g^{-1} DW) [5]. In contrast to Bennett et al.'s report, after qualitative analysis of GLs in extensive number of herbarium tissues and some from seeds, Mithen et al. reported the likely occurrence of the following chain-elongated and branched-chain GLs in *Gyrostemon* spp., (with chromatography data presented only for *G. sheathii*): methylethyl, methylpropyl, pentyl (likely to be methylbutyl), and hexyl (likely to be methylpentyl) [28]. Bennett et al.'s report also mentioned quantities of C5 hydroxyalkyl GL in *Tersonia cyathifolia* and *Codonocarpus* species (10.0–50.0 μmol g^{-1} DW), which, on the basis of literature data, could be either 1-methyl-3-hydroxybutyl, 2-hydroxy-2-methylbutyl, or 2-hydroxypentyl GL [5]. Combining LC with NMR would allow these GL isomers to be identified. Indolyl GLs were also detected, while no Phe-derived GLs were present, which is consistent with previous reports [1, 28].

Fig. 10 Gyrostemonaceae world distribution map

Limnanthaceae

The Limnanthaceae family includes two genera, *Limnanthes* (8) and *Floerkea* (1) growing in temperate North America (Fig. 11) [22].

The GLs identified in the seeds are likely derived from Phe. 3-Methoxybenzyl GL (**25**) also known as glucolimnanthin was found in high amount in all seeds as common GL (30–204 μmol g^{-1} DW). These species are a good source of this GL [5, 81, 82]. The GL profile in *L. floccosa* seeds revealed high content of 3-hydroxybenzyl GL (**23**) (52.0–134.4 μmol g^{-1} DW), even more abundant than **25** [81]. Mithen et al. stated that *Limnanthes* seeds also contain **20**, and dimethoxybenzyl GL, while one report mentioned 2-hydroxy-3-methylpropyl GL [1, 28]. The whole family nicely fits in Bennett et al.'s only simple arylaliphatic subdivision [5].

Moringaceae

The Moringaceae family includes only the genus *Moringa* (12) growing in India Africa, and Madagascar. *Moringa oleifera* is quite widely cultivated (Fig. 12) [22].

All species of *Moringa* contain very high amounts of 4-(α-L-rhamnopyranosyloxy)benzyl GL (**32**) in all plant parts. The highest amount was reported in *M. oleifera* and *M. stenopetala* seeds, *ca.* 450 μmol g^{-1} DW, representing over 25% of the dried plant material. This GL was also found as dominant in the leaf (9.3-122.9 μmol g^{-1} DW), root (3.5–71.6 μmol g^{-1} DW), stem (23.8–28.5 μmol g^{-1} DW), and bark (17.9–38.7 μmol g^{-1} DW) [83]. In addition, the leaf of *Moringa* spp. were also found to contain lower levels of three isomeric *O*-acetylated 4-(α-rhamnopyranosyloxy)benzyl GLs (**33**) [83]. The roots of *M. oleifera* and *M. stenopetala* were found to contain high concentrations of **20** (up to 75.0 μmol g^{-1} DW). A recent study of *M. oleifera* showed the presence of very minor not previously reported aliphatic GLs, such as **14** and **15** [84].

Fig. 11 Limnanthaceae world distribution map

Pentadiplandraceae

The Pentadiplandraceae family possesses only one genus, *Pentadiplandra*, which contains a single species, *Pentadiplandra brazzeana* Baillon. *P. brazzeana* is a plant found in Western and Central Africa (Fig. 13) [22].

Although some botanical studies of *Pentadiplandra* genus indicated a strong phylogenetic similarity with the GL-containing *Tovaria pendula* Ruiz and Pavón (Tovariaceae) [85], the analyses using rbcL, ndhF, and matK sequences showed a weak resolution of the relationship of Pentadiplandraceae and Tovariaceae [86]. De Nicola et al.'s analysis of GLs in different plant parts revealed mostly arylaliphatic GLs having the highest content of glucoaubrietin (**24**) in the seeds, i.e., 109.1 μmol g^{-1} DW, representing it as a good source of this GL [87]. The total

Fig. 12 Moringaceae world distribution map

Fig. 13 Pentadiplandraceae world distribution map

GLs in the roots were 48.0 μmol g^{-1} DW which also contained the highest quantity of the same GLs, together with, **20** and **25** (Table 2) [87]. The leaf, with low GL content of 2.2 μmol g^{-1} DW, contained 3,4-dimethoxybenzyl GL, **20**, and **28**. The same qualitative profile of GLs was reported by Mithen et al. [28]. According to the GL content, this family nicely fits in Bennett et al.'s only simple arylaliphatic subdivision [5].

Resedaceae

The Resedaceae family is represented by six genera (about 85 species), distributed worldwide [22]. The genus *Reseda* (68 species) is the largest genera in the Resedaceae. Natural areas of occurrence are warm temperate and dry subtropical parts in the world, especially Mediterranean, Middle East, North African regions, and also Southern Africa, S.W. North America, and S.W. China [22]. With the exception of 4 *Reseda* species (*R. alba*, *R. lutea*, *R. luteola*, and *R. phyteuma*), which are worldwide weeds, almost all of the *Reseda* species are narrowly distributed in the Mediterranean basin (Fig. 14) [88].

Bennett et al. investigated the GLs in the seeds of *Reseda* species and reported arylaliphatic and indolyl type GLs in *R. luteola* and *R. odorata*, while a *O*-glycosylated GL was only found in *R. lutea* [5].

Two arylaliphatic GLs, glucobarbarin (**26**) and *epi*glucobarbarin (**27**), were identified in *R. luteola* ranging 0.1–10.0 and 25.0–50.0 μmol g^{-1} DW, respectively [5]. The same report included another member of the Resedaceae family, *Caylusea abyssinica*, which also contained **21**, next to the same arylaliphatic GLs and had the highest reported **27** content (10.0–25.0 μmol g^{-1} DW) [5]. Mithen et al. investigated the GL profile in fresh leaves and seeds of *Reseda* species and reported hydroxyphenylethyl GL (*R*-/*S*-isomer not given) and **21** being present in significant amounts such as in *R. luteola* [28]. Glucobarbarin (**26**) was identified as the major GL of the total leaf surface of *R. luteola* and it accounted for over 90% of the total

Fig. 14 Resedaceae world distribution map

leaf surface GLs (0.5 μmol g^{-1} FW) [42]. Mithen et al. also reported that *R. suffruticosa* tissues predominantly contained **21** and simple BCAA-derived GLs [28]. Among indolyl type, only **28** was detected in *R. odorata* seed, ranging 10.0–25.0 μmol g^{-1} DW [5].

Bennett et al. reported that, in contrast to other *Reseda* spp., only *R. lutea* occasionally contains *O*-glycosylated 2-(α-L-Rhamnopyranosyloxy)benzyl GL (**34**) with the concentration ranging 25.0–50.0 μmol g^{-1} DW [5]. Mithen et al. investigated the GL profile in fresh leaves and seeds of *Reseda* spp. and found **34** as predominant in *R. lutea*, *R. odorata*, *R. phyteuma*, and *R. stricta* [28].

Salvadoraceae

The Salvadoraceae family comprises 3 genera (*Azima*, *Salvadora*, and *Dobera*) and 11 species. Plants of this family occur in Africa (including Madagascar), Southeast Asia, and West Malaysia. They are often found in hot and dry areas (Fig. 15) [22].

Azima tetracantha roots and seeds were found to be sources of indolyl GLs, particularly rich with neoglucobrassicin (**29**), i.e., 46.5 and 50.6 μmol g^{-1} FW, respectively [25]. Indolyl GLs, although not quantified, were found as major components in *Salvadora* and *Dobera*, along with **20** and hydroxybenzyl GL in *Salvadora*. [28]

Tovariaceae

The Tovariaceae family contains one genus, *Tovaria*, and two species of annual herbs that grow in the tropical America area (Fig. 16) [22].

The GL profile of *Tovaria pendula* leaves is mostly constituted of indolyl GLs [89]. Salvadoraceae, as well as Tovariaceae, have only quantified indolyl GLs which justifies the additional subdivision to Bennett et al.'s system – i.e., only indolyl GLs.

Later, Mithen et al. reported a qualitative GL profile in which 28, methoxyindolylmethyl and N-acetyl-3-indolylmethylGLs had predominated, along with 20, and

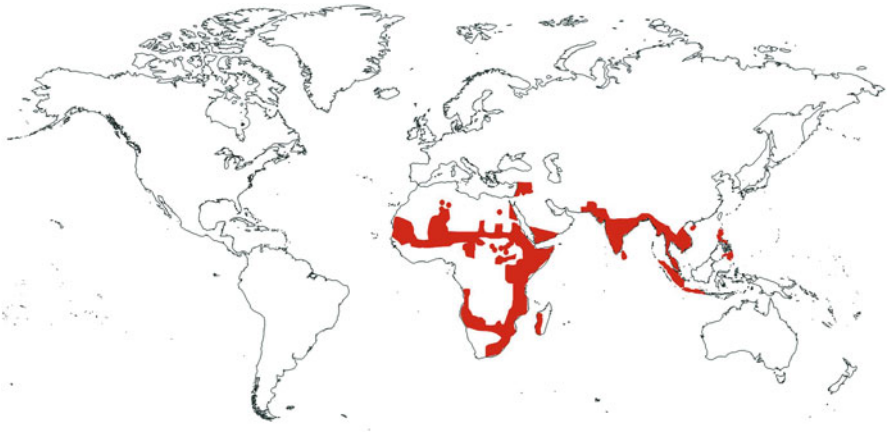


Fig. 15 Salvadoraceae world distribution map

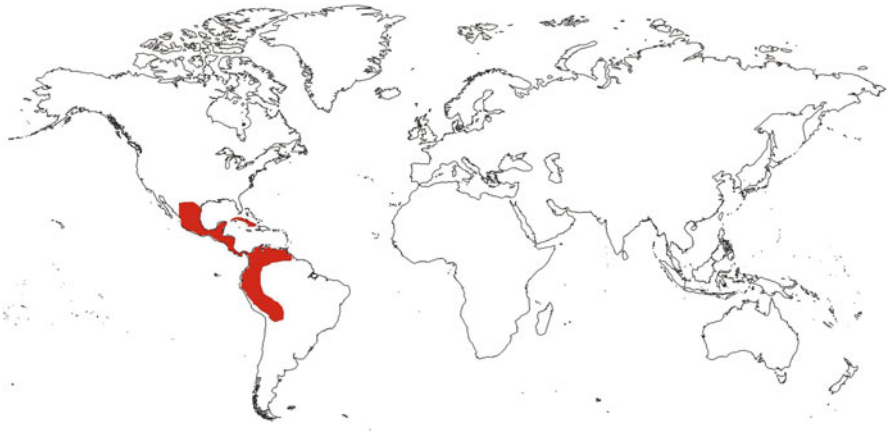


Fig. 16 Tovariaceae world distribution map

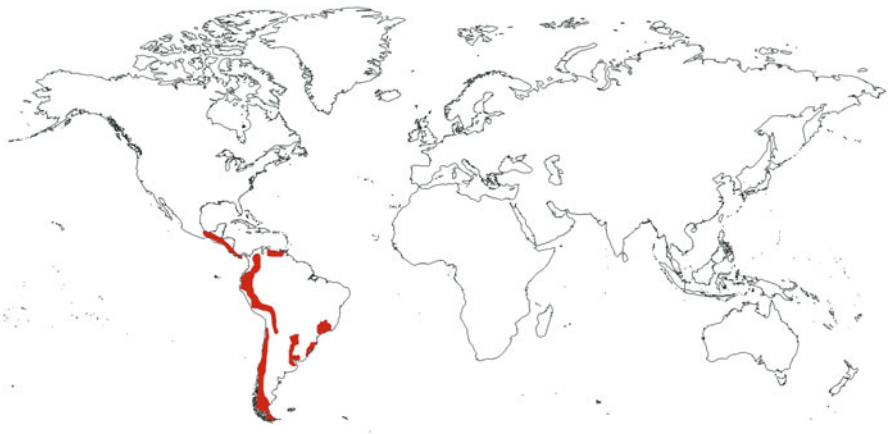


Fig. 17 Tropaeolaceae world distribution map

methylethylGLs [28]. Low levels of pentyl (or methylbutyl) GL, probably derived from chain-elongated BCAA, were also detected [28].

Tropaeolaceae

The Tropaeolaceae family contains one genus, *Tropaeolum* (105 species), that grows in the New World, especially in Andean parts (Fig. 17) [22].

The GL profile of *T. majus*, *T. peregrinum*, and *T. tuberosum* included arylaliphatic GLs. Glucotropaeolin (**20**) and **24** were found as dominant GLs in *T. majus* seeds, i.e., 100.0, and 50.0 μmol g^{-1} DW, respectively. On the other hand, *T. peregrinum* seeds contained high content of **22** and **24**, with 125.0 and 50.0 μmol g^{-1} DW, respectively. Glucoaubrietin (**24**) was the only arylaliphatic GL found in the

seeds of *T. tuberosum*, with content up to 90.0 μmol g^{-1} DW. This family fits into an only simple arylaliphatic subdivision.

3.1.2 Other Families in the Order Brassicales

Reports of other families included in the order Brassicales (Bataceae, Emblingiaceae, Koeberliniaceae, and Setchellanthaceae) are very scarce.

Bataceae

The Bataceae family comprises two species, *Batis argillicola* and *Batis maritima*. These plants are distributed in N. Australia and S. New Guinea, tropical America, and the Galapagos Islands [22]. No GL quantification was reported for these plants to date, as far as the authors know. The most recent report on the analysis of *B. maritima* revealed small amounts of **28** and **29** [28].

Emblingiaceae

The Emblingiaceae family includes only *Emblingia calceoliflora* growing in W. Australia [22]. The only record of GL qualitative profile was given by Mithen et al., who reported hydroxy and hydroxymethoxybenzyl GLs in the leaf tissue [28]. This finding supports the assignment of this taxon to the Brassicales.

Koeberliniaceae

The Koeberliniaceae family includes one genus with two species growing in Central and S.W. North America and Bolivia [22]. Mithen et al.'s study of *Koeberlinia spinosa* Zucc. (originated from Bolivia and USA) revealed that no GL was present [28], which was consistent with a previous report [73]. This is one family in the Brassicales that lacks GLs, though other evidences firmly place it in that order.

Setchellanthaceae

The Setchellanthaceae family contains only one species, *Setchellanthus caeruleus*, a shrub endemic to Mexico, restricted to two small, arid or subarid, and disjunct areas fully 1,000 km apart. The only report by Mithen et al. included qualitative analyses of GLs with the major **2** and hydroxybenzyl GLs and minor hydroxymethylpropyl and methylpropyl GL isomers [28].

3.1.3 Families Outside the Order Brassicales

The nonexistence of GLs in some species of Capparidaceae and species of the Koeberliniaceae family is not the only surprise. The well-known report by Daxenbichler et al. included information of GL-bearing plants outside the order Brassicales [50]. However, these early reports of GLs included the identification of unstable *p*-hydroxybenzyl ITC and ITCs containing an indolyl moiety under thiocyanate ion release. Such was the case of *Bursaria spinosa* var. *incana* (order Apiales, family Pittosporaceae) and *Phytolacca americana* (order Caryophyllales, family Phytolaccaceae). *Phytolacca dioica* was reported also to contain **20** from the corresponding benzyl ITC [50]. Hu et al. reported GLs in the roots of *Phytolacca acinosa* [20]. Some reports were discounted after subsequent investigations, such as

in the case of edible mushrooms (*Agaricus bisporus*) [90], *Plantago* species (order Lamiales, family Plantaginaceae) [91], and *Theobroma cacao* (order Malvales, family Malvaceae) [92].

On the other hand, the genus *Drypetes* (order Malpighiales, family Putranjivaceae) is the only one accepted to possess the GL biochemical pathway outside the order Brassicales. The latest reported study of the essential oil from the bark of *Drypetes gossweileri* (Putranjivaceae) from Gabon found a high concentration of benzyl ITC and benzyl cyanide that can result from **20** degradation [21]. The same report included an investigation of the essential oil from the root and bark of *Rinorea subintegrifolia* (order Malpighiales, family Violaceae), which suggested that the plants of the Violaceae family also have the ability to biosynthesize GLs. The most recent report from Hu et al. revealed that *Croton tiglium*, *Phyllanthus emblica*, *Euphorbia humifusa*, *E. lathyris*, and *E. kansui* from the Euphorbiaceae family and *Knoxia valerianoides* and *Gardenia jasminoides* from the Rubiaceae family contain GLs [20]. However, the existence of GLs should be reexamined, with more accurate approaches using standards and more modern techniques (HPLC-MS, NMR) which are indispensable for structure elucidation with regard to GLs outside the order Brassicales.

4 Biological Potential

This database which includes all the families in the order Brassicales along with Bennett et al.'s. and Verkerk et al.'s works represents the insight into the GL quantity profile in GL-containing species investigated to date. GLs degrade into various compounds, i.e., ITCs and nitriles, but also in other depending on various conditions. This can be induced by the enzyme myrosinase (EC 3.2.1.147), but also thermally and chemically, which is discussed in the review papers by Bones and Rossiter [2], and Hanschen, et al. [93].

In addition to plant myrosinases, GLs may undergo hydrolysis induced by myrosinase found in specialist aphids [94, 95], by myrosinase-like enzymes in the bacteria found in the intestinal tract as well as others [96–100]. Although it is found that the microbiome can hydrolyze GLs, it seems to be far less efficient than plant myrosinase and it is possibly limited to few commensal bacteria. Several GLs have been found to degrade by using different bacterial strains such as *Bacteroides* [101], *Bacillus* [102], *Bifidobacterium* [103], *Clostridium* [97], *Enterobacter* [104, 105], *Enterococcus* [106], *Escherichia* [106–108], *Lactobacillus* [99, 106–108], *Lactococcus* [108], *Staphylococcus* [102], and *Streptomyces* [102]. Those investigations included **6** [99, 101–103, 107, 109], **20** [103, 109], and recently **12**, **14**, and **15** [106, 108] which were degraded by different bacterial strains yielding different amounts of ITCs, nitriles, and/or other metabolites. In some cases interconversion into another GL occurred [106, 108]. Currently, it is unknown whether the bacteria have more than one enzyme capable of hydrolyzing GLs [110].

ITC-containing volatile extracts obtained from GL-containing plants [37, 111–114] as well as pure ITCs showed wide broad spectra of antimicrobial activity [115]. Sulforaphane and several other ITCs were found to be very active against a large number of clinical isolates of *Helicobacter pylori* strains, many of which were resistant to conventional antibiotics [116–118]. However, the antimicrobial mechanisms of ITCs are not well understood. It is known that ITCs are highly reactive electrophiles which bind reversibly to thiols (e.g., glutathione or bacillithiol found in bacteria), but when free thiols are not available, they accumulate and cause damage [119, 120]. Questions arise whether some of those bacteria found in human gut get killed by the ITCs that they produce, as well as if the ones that have no protective system against ITCs are also killed [119].

Earlier studies dealt with the reduction of the GL content from plants due to their bitterness and some antinutritional properties such as goiter, growth retardation, poor egg production, and liver damage in animals [7]. However, studies showed that, due to the structural differences of GLs and corresponding ITCs, they can be used in pest control and that they possess a wide array of bioactivities which are the result of host defense system triggered by ITCs. Allyl ITC, benzyl ITC, phenylethyl ITC, sulforaphane, and moringin are the most widely studied ITCs. Aside previously mentioned antimicrobial activity [115], they show anticarcinogenic [121, 122], antioxidant and prooxidant activity [123], neuroprotective [124], and other activities.

The information on the GL quantitative profile which also includes plants outside of the Brassicaceae family enables the creation of a database for estimation of the dietary intake, which in turn would improve epidemiological work on association of GLs via their breakdown products, mostly ITCs, with various biological activities [125]. Passive diffusion is the most prominent mechanism of ITCs cellular uptake which is a major limiting factor for their therapeutic efficacy [122]. At low levels, ITCs trigger a health defense system in mammals through their capacity to modulate the activities of phase I (e.g., cytochrome P450s) and phase II (e.g., GSH-*S*-transferase, UDP-glucuronyl transferase) biotransformation enzymes [7, 122, 124]. The key trigger is considered to be the activation of Keap1-Nrf2-ARE pathway and Nrf2 accumulation and transport to the nucleus. This transcription factor upregulates a broad range of xenobiotic-metabolizing enzymes, antioxidant enzymes, and others, including GSH-*S*-transferases.

Above all, ITCs are known for their anticancer activity. Showing their strong potential against various cancers (lung, breast, colon, prostate, ovary), few ITCs have advanced to the clinical trials as potential drug candidates [122]. Studies in vitro, as well as preclinical studies, on animal models suggest critical features of cancer cells such as suppression of cellular proliferation, angiogenesis, metastasis, etc. [122], as approaches in fighting against cancer. By depletion of GSH, ITCs act as prooxidants, i.e., they can indirectly generate reactive oxygen species (ROS), which, as a consequence, induce apoptosis of cancer cells [122, 123].

ITCs are able to pass blood–brain barrier and thus exert neuroprotective effects. They showed activity in both in vitro and in vivo models of neurodegeneration [126–129], but mechanisms involved in the pathogenesis of neurodegenerative

diseases remain puzzling [130]. A wide variety of neurodegenerative diseases, including Alzheimer's (AD), Parkinson's, and Huntington's diseases as well as multiple sclerosis, amyotrophic lateral sclerosis, etc., share common characteristics such as oxidative stress, misfolded proteins, excitotoxicity, inflammation, and neuronal loss [124, 130]. Among all neurodegenerative disorders, AD accounts for 60% to 70% of cases of dementia [131, 132]. Cholinesterase inhibitors serve as a strategy for the treatment of AD (as well as some other diseases) and are main pharmacotherapeutics, while very little information on ITCs as cholinesterase inhibitors is available [133, 134] and further studies should be conducted. Strong evidences suggest that their beneficial effects could be mainly ascribed to the ability to activate Nrf2-ARE pathway. ITCs can also modulate other pathways, such as inflammation and apoptosis, which could be involved in neurodegenerative disease development.

5 Conclusions

After the insight of GL content reports of all GL-producing plants in the order Brassicales, the Bennett et al.'s subdivisions based on GL content seem to be mostly supported. However, this can be revised a bit and extended solely by the highest GL content into:

(i) Only methyl GL
(ii) Only short- to medium-chain-length aliphatic (C3-C5 GLs)
(iii) Only long-chain aliphatic GLs
(iv) Only simple arylaliphatic (such as benzyl (**20**), 4-hydroxybenzyl (**22**), 2-phenylethyl GL (**20**))
(v) Highly substituted arylaliphatic GLs such as 3,4-dihydroxybenzyl, 3,4-dimethoxybenzyl, and 3,4,5-trimethoxybenzyl GLs
(vi) Only indolyl GLs
(vii) Only short- to medium-chain-length aliphatic (C3-C5 GLs) or only long-chain aliphatic (C8-C10) along with simple arylaliphatic or indolyl GLs

Other GLs present in plants could be considered as specific, or as chemotaxonomic markers, as they do not occur as dominant compounds. On the other hand, some plants in the order Brassicales seem to lack the ability to produce GLs (Koeberliniaceae and some Capparidaceae plants). In addition, outside the order Brassicales, some plants are suggested to produce GLs, but due to the methodology used, this should still be considered cautiously.

Further elucidation of the protective mechanisms of food and the identification of active constituents in GL-containing plants is needed. Additional studies are also needed to determine the amount of ITCs or their metabolites that reach target tissues, and the concentrations needed to exert biological effects.

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Genetics and Breeding of *Brassica* Crops

2

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Abstract

Brassica genus includes very common crops including oilseeds (oilseed rape, mustard) and vegetables (broccoli, cauliflower, Brussels sprouts, cabbage, turnip, Chinese cabbage, pak choi, etc.). Glucosinolates are the major class of secondary metabolites in the family Brassicaceae, and their hydrolytic products have beneficial effects in plant protection and human health. Knowledge on the genetics and inheritance of these compounds may be used to modify the content and the profile of glucosinolates. In this review, we summarize the identification of the main genes related to glucosinolate synthesis in crops of the *Brassica* genus using different tools, such as syntenic information with the model plant *Arabidopsis*, whole-genome sequence information, or identification of quantitative trait loci. Breeding programs to decrease total glucosinolate content of seed (oilseeds, mustards) or to increase the content of a specific glucosinolate (glucoraphanin in broccoli) through conventional breeding or genetic engineering are also reviewed. Besides, recent studies on genetics of glucosinolates in other crops that do not belong to *Brassica* (*Raphanus*, *Sinapis*, etc.) are also presented.

Keywords

Brassica genus • Brassicaceae • Genes • Regulation • Breeding • QTLs

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_2

Contents

1 Introduction

Brassicaceae family represents a monophyletic group including approximately 350 genera and 3,700 species, with many economically important plants used as vegetable source (edible and condiment), oilseeds, forage, or ornamental. This family includes very common known species such as *Brassica oleracea* (broccoli, cauliflower, Brussels sprouts, cabbages, etc.), *Brassica rapa* (turnip, Chinese cabbage, pak choi, etc.), *Brassica napus* (rapeseed, leaf rape), *Sinapis alba* (white mustard), and *Raphanus sativus* (radishes). *Brassica* crops were probably domesticated in the Neolithic period thousands of years ago. Ancient Greeks, Romans, Indians, and Chinese valued and used them extensively as a source of food and medicinal compounds. The evolution of *Brassica* crops was deeply marked by the particular regional preferences over the centuries. Nowadays, these crops are cultivated across the world with a production of vegetable and oilseed forms in 2012 estimated in 90 and 65 million tons, respectively, in more than 150 countries [1].

Glucosinolates (GSLs) are amino acid-derived natural plant products found exclusively throughout the Capparales order and the major class of secondary metabolites found in the family Brassicaceae. The hydrolytic breakdown products of GSLs, especially isothiocyanates (ITCs), have beneficial effects, such as preventing cancer in humans, reducing risk of degenerative diseases [2], or enhancing plant protection to abiotic and biotic stresses [3]. GSLs also exhibit certain adverse effects, for example, goiter on animal health [2], which caused the deliberate reduction of GSLs levels in *B. napus* seeds in the past. However, there is no evidence for any goitrogenic effect on humans from *Brassica* consumption [4].

Approximately, 15 GSLs are common in the genus *Brassica*, while other 30 GSLs could be present in the different species of the genus [2] (Table 1). Besides, other Brassicaceae show significant levels of GSLs. More than 150 out of the

Table 1 Principal glucosinolates identified in *Brassica* vegetable crops

	Aliphatic GSL										Indolic GSL				Aromatic GSL
Crop	GIB	PRO	SIN	GAL	GRA	GNA	GBN	GIV	GER	GNL	GBS	NGBS	4HGBS	4MGBS	GST
Brassica oleracea															
White cabbage	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Savoy cabbage	+	+	+	–	+	+	+	+	–	–	+	+	–	+	+
Red cabbage	+	+	+	–	+	+	–	+	–	–	+	+	–	–	–
Kale	+	+	+	–	+	+	–	+	–	–	+	+	+	+	+
Collard	+	+	+	–	–	–	–	+	+	–	+	–	–	–	–
Tronchuda cabbage	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+
Broccoli	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
Brussels sprouts	+	+	+	–	+	+	–	+	–	–	+	+	–	–	–
Cauliflower	+	+	+	–	+	–	–	+	–	–	+	+	–	–	–
Kohlrabi	+	+	+	+	+	+	–	+	–	–	+	+	+	+	–
Brassica rapa															
Turnip	+	+	–	–	–	+	+	–	+	+	+	+	+	+	+
Turnip greens	+	+	–	+	+	+	+	+	–	+	+	+	+	–	+
Turnip tops	+	+	–	–	–	+	+	+	–	–	+	+	+	–	+
Chinese cabbage	+	+	–	–	–	+	+	–	–	+	+	+	–	+	+

(*continued*)

Table 1 (continued)

	Aliphatic GSL										Indolic GSL				Aromatic GSL
Brassica napus															
Swede	–	+	–	–	+	–	+	–	–	+	+	+	+	+	+
Leaf rape	–	+	–	+	–	+	+	+	–	+	+	+	–	+	+

GIB glucoiberin (3-methylsulfinylpropyl), *PRO* progoitrin (2-hydroxy-3-butenyl), *SIN* sinigrin (2-propenyl), *GAL* glucoalyssin (5-methylsulfinylpentyl), *GRA* glucoraphanin (4-methylsulfinylbutyl), *GNA* gluconapin (3-butenyl), *GBN* glucobrassicanapin (4-pentenyl), *GIV* glucoiberverin (3-methylthiopropyl), *GER* glucoerucin (4-methylthiobutyl), *GNL* gluconapoleiferin (2-hydroxy-4-pentenyl), *GBS* glucobrassicin (3-indolylmethyl), *NGBS* neoglucobrassicin (1-methoxy-3-indolylmethyl), *4HGBS* 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl), *4MGBS* 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl), *GST* gluconasturtiin (2-phenylethyl)

200 GSLs [5] have been identified nowadays in species from Brassicaceae family that do not belong to the *Brassica* genus, indicating that different mechanisms of genetics regulation and/or biosynthesis must be present in other species and genus. In the last decades the main pathway of GSLs biosynthesis has been well understood in the model species *Arabidopsis thaliana* (*Arabidopsis*), which also belongs to Brassicaceae, and several works investigated this pathway in economic important crops belonging to *Brassica* genus. These studies are mainly based on the discovery of homologous genes of those of *Arabidopsis*, since the pathway seems to be rather well conserved in the Brassicaceae family [6].

2 Glucosinolate Synthesis

All GSLs share a chemical structure consisting of a β-D-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester, plus a variable R group derived from one of eight amino acids [7]. GSLs can be classified by their precursor amino acids and the types of modification to the R group. Compounds derived from Ala, Leu, Ile, Met, or Val are called aliphatic GSLs, those derived from Phe or Tyr are called aromatic GSLs, and those derived from Trp are called indole GSLs. The R groups of most GSLs are modified from these precursor amino acids, with methionine undergoing an especially wide range of transformations [3]. Most of the R groups are elongated by one or more methylene moieties. Both elongated and non-elongated R groups are subject to a wide variety of transformations, including hydroxylation, *O*-methylation, desaturation, glycosylation, and acylation [7].

The sequence of the biosynthesis of GSLs has been well studied and is divided in three steps: amino acid chain elongation, core structure, and secondary transformations.

2.1 Amino Acid Chain Elongation

Initially, the parent amino acid is deaminated to form the corresponding 2-oxo acid. Next is a three-step cycle in which the 2-oxo acid condenses with acetyl-CoA to form a substituted 2-malate derivative, which then isomerizes via a 1,2-hydroxyl shift to a 3-malate derivative that undergoes oxidation-decarboxylation to yield a 2-oxo acid with one more methylene group than the starting compound. After each turn of the cycle, the extended 2-oxo acid can be transaminated to form the corresponding amino acid and enter the second phase of GSL formation. Or it can undergo additional cycles of acetyl-CoA condensation, isomerization, and oxidation-carboxylation, resulting in further elongation. Up to nine cycles are known to occur in plants. Methylthioalkylmalate synthase (MAM), bile acid: sodium symporter family protein 5 (BASS5), and branched-chain aminotransferase (BCAT) are involved in this step [7, 8].

2.2 Core Structure

The biosynthesis of the core GSL structure involves intermediates common to all GSLs. The intermediates in the pathway from the amino acid to the core structure include N-hydroxy amino acids, aldoximes, *aci*-nitro or nitrile oxide compounds, S-alkyl thiohydroximates, thiohydroximic acids, and desulfoGSLs. The genes responsible for all these steps, except the S-alkylation, have been identified since 2000. Core structure formation of GSLs is accomplished in five steps via oxidation by cytochromes P450 of CYP79 and CYP83, followed by C-S-lyase, S-glucosyltransferase, and sulfotransferase [7, 8].

2.3 Secondary Transformations

The initially formed parent GSL is subject to a wide range of further modifications of the R group. The R group of GSLs derived from methionine and its chain-elongated homologues is especially subject to further modifications, such as the stepwise oxidation of the sulfur atom in the methylthioalkyl side chain leading successively to methylsulfinylalkyl and methylsulfonylalkyl moieties. Methylsulfinylalkyl side chains can be further modified by oxidative cleavage to afford alkenyl or hydroxyalkyl chains. The indolic GSL secondary modifications involve a series of hydroxylations and methoxylations catalyzed by several CYP family enzymes [9, 10]. Aliphatic and indolic are the major GSLs in the *Brassica* genus, and studies have been mainly focused on the elucidation of the genetic regulation of these compounds. So far little information is available about the genetic regulation of aromatic GSL. For this reason, we will focus on the genetics of aliphatic and indolic GSLs.

3 Genetic Regulation of Aliphatic Glucosinolates

Models of the major loci controlling the synthesis of aliphatic GSLs were initially postulated in *Brassica* in the 1990s by four articles labeled genetics of aliphatic GSLs (I, II, III, and IV) [11–14]. In the first one [11], authors evaluated the segregation of glucosinolate phenotypes using recombinant populations of *B. napus*. The results were consistent with a model in which alleles at a single locus (GSL-PRO) regulate the presence or absence of propyl GSLs, and those at two other loci (GSL-ELONG-C and GSL-ELONG-A) regulate side-chain elongation of the amino acid derivative which results in the production of butyl and pentyl GSLs. Using the same *B. napus* population, Parkin et al. [14] investigated the hydroxylation of alkenyl GSLs showing that there are two loci responsible of this step in leaves and seeds. GSL-OH-*C* at linkage group LG 13 has a major effect, while the homoeologous locus at LG 3 (GSL-OH-A*)* has a minor effect. In the third study, Mithen et al. [13] showed in *A. thaliana* two side-chain modifications produced by alleles in the same locus or in two close loci in the chromosome 4: the conversion of

methylsulfinylalkyl to alkenyl GSLs (GSL-ALK) and the conversion of methylsulfinylpropyl GSLs to hydroxypropyl GSL (GSL-OHP). Cloning and functionality studies of the GSL-ALK in *B. oleracea* were carried out by Li and Quirós [15] in two recombinant inbred line populations. In the last paper of this series, Giamoustaris and Mithen [12] used a backcross population from a cross between two *B. oleracea* relatives to find the model in which 3-methylthiopropyl GSL is sequentially converted to 3-methylsulfinylpropyl, and then to 2-propenyl GSL, by the action of dominant alleles at two loci (GSL-OXID and GSL-ALK). By RFLP mapping, these two loci were positioned on the same LG homologous to the *B. napus* LG 19, loci further confirmed by Hall et al. [16], in *Arabidopsis* and wild *Brassica* species.

Based on these studies and others in *Arabidopsis*, a model where there are four major biosynthetic loci was suggested: GSL-AOP, −ELONG, −OH, and –OX. Contrarily to *Arabidopsis*, where glucosinolates up to eight carbons occur, in *Brassica* there are glucosinolates with three, four, and five carbons. For this species, Li et al. [17] suggested a model for GSLs with three and four carbons. In this model, the presence of the dominant allele of the GSL-ELONG gene will result in four-carbon (4C) GSLs, while the presence of the dominant allele for GSL-PRO will result in three-carbon (3C) GSLs. In 2002, a homologous of the GSL-ELONG mapped in *Arabidopsis* was found and sequenced in *B. oleracea*, determining the presence of 4C GSL [18]. Major GSL genes were definitively found and sequenced in *B. oleracea* by comparison between a bacterial artificial chromosome (BAC) from *B. oleracea* with its homologous region in *Arabidopsis* [19]. The region in *Arabidopsis* contains a gene family involved in the synthesis of GSL corresponding to three 2-oxoglutarate-dependent dioxygenase (AOP) genes: AOP3 (GS-OHP), AOP (GS-ALK), and AOP1 (null allele). In *B. oleracea*, two of the genes are duplicated; AOP1.1, AOP1.2, AOP2.1 (BoGSL-ALKa), AOP2.2 (BoGSL-ALKb), and AOP3 are missing.

The GSL-OX locus is responsible for the oxygenation of a methylthioalkyl GSL to its methylsulfinylalkyl structure [13]. In some *Arabidopsis* populations, there are multiple QTLs controlling GSL-OX, and a forward genetics screen of *Arabidopsis* mutants also identified multiple modifier loci complicating genetic approaches to identify the causal basis of this polymorphism [20]. Hansen et al. [21] performed a fine-scale mapping of natural variation identifying a subset of candidate genes which co-expressed with the known aliphatic GSL biosynthetic genes. They identified a crucifer specific family of five flavin monooxygenases that control the conversion of methylthioalkyl to methylsulfinylalkyl GSLs. The sequence of two of these genes was further identified in *B. rapa* by Wang et al. [8].

In 2009, Zang et al. [22] performed a genome-wide identification of GSL synthesis and regulation based on sequencing of expressed sequence tags (ESTs) and bacterial artificial chromosomes (BACs) of Chinese cabbage, a *B. rapa* cultivar, in conjunction with the *Arabidopsis* sequence. They found 44 genes that contain all the homologues of *Arabidopsis*, except for CYP79F2, $FMO_{GS\text{-}OX2\_4}$, and AOP3. These genes are present in various copy numbers. Thus, they showed that a high collinearity in the GSL biosynthesis pathway exists between *Arabidopsis* and *B. rapa* despite the difference in gene copy numbers [22]. A more comprehensive

study was later performed using the assembled genome sequence of *B. rapa* [8]. Authors found 102 genes related to GSL synthesis in *B. rapa*, and all genes but one were mapped to the ten chromosomes. Most of the *Brassica* GSL genes exist in more than one copy, and 93% of GSL genes exhibit synteny between *B. rapa* and *Arabidopsis*. In addition, copy number variation of these genes correlates with a triplication event in *B. rapa*, and the GSL gene content can explain the GSL profiles and accumulation in *B. rapa* [8].

The sequences of most of the GSL genes were found in *Brassica* by comparative genomics with the *Arabidopsis* genome sequence. In the last years, several studies were made to prove the function of the main GSL genes in *Brassica* crops. In this sense, the genes CYP83A1 and CYP83B1, which catalyze the oximes to give aliphatic, aromatic, or indolic GSLs, were cloned from leaves of pak choi (*B. rapa*), and their expression was studied in different tissues. The expression levels of these genes were consistent with the GSL accumulation in several cultivars and tissues of this species [23]. In other study, aliphatic GSLs were genetically manipulated through homoeologous recombination in backcross lines [24]. A resynthesized *B. napus* line, from a cross between *B. rapa* and *B. oleracea*, was backcrossed with a Chinese cabbage-doubled haploid line, followed by a marker-assisted selection for nonfunctional gene in each backcross generations (BC_3F_2). Reduction in 5C aliphatic GSLs (gluconapoleiferin, glucoalyssin, and glucobrassicanapin) was observed in progenies of the recurrent parent that carried the GSL-ELONG$^-$ gene. The results suggest that the functional allele had been replaced by the nonfunctional GSL-ELONG$^-$ allele from *B. oleracea*. In *B. juncea* (AABB genome), Augustine et al. [25] identified four MYB28 homologues, two from *B. rapa* (AA) and two from *B. nigra* (BB) genomes. Four genes encode functional MYB28 proteins and resulted in similar aliphatic GSL composition and content. Later, they identified four CYP83A1 homologues and, by expression analysis, confirmed that the four retained ubiquitous, overlapping but distinct expression profiles in different tissue and cell types of *B. juncea* [26]. Another study was focused on the expression of the multiple paralogs of aliphatic GSL regulators, such as BrMYB28 and BrMYB29 genes in *B. rapa* ssp. *pekinensis* by quantitative real-time PCR (qRT-PCR) analysis in different tissues and at various developmental stages. An overlapping gene expression pattern between the BrMYBs as well as their downstream genes (DSGs) was found at different developmental stages. Among the BrMYB28 and BrMYB29 paralogous genes, the BrMYB28.3 and BrMYB29.1 genes were dominantly expressed in most of the developmental stages, compared to the other paralogs of the BrMYB genes [27].

The study of the function of GSL genes in *B. oleracea* has received less attention until recently. The study of two databases, Bolbase and Ensembl Plants, showed 84 genes in *B. oleracea* orthologous to those identified in *B. rapa*, related to GSL biosynthesis, transcriptional regulation, and breakdown. Yi et al. [28] evaluated the expression of these genes in 12 different genotypes of four different groups of *B. oleracea*. All of these genes were expressed in different tissues. Most of them were expressed in leaves of cabbage or kale and florets of cauliflower and only eight expressed in stems of kohlrabi.

4 Genetic Regulation of Indolic Glucosinolates

Indolic GSLs constitute an important group of tryptophan-derived secondary metabolites in the Brassicaceae family where they function as defense compounds. During the last years, substantial progress in the biosynthesis, transportation, and functional properties of indolic GSLs has been achieved using the model plant *Arabidopsis* [29–34]. To date, genes from the biosynthetic pathway in *Brassica* have been identified using syntenic information with the model plant *Arabidopsis* based on sequencing of expressed sequence tags (ESTs), bacterial artificial chromosome (BAC) libraries, whole-genome sequence information, as well as metabolic engineering of *B. napus* [35, 36], *B. rapa* [8, 10, 22, 23, 37, 38], and *B. oleracea* [39].

The conversion of amino acids to aldoximes is catalyzed by the cytochrome P450 monooxygenase CYP79B2 or CYP79B3 gene products, converting the precursor amino acid tryptophan to indole-3-acetaldoxime [40]. Next, aldoximes are oxidized to activated compounds (either nitrile oxides or *acid* nitro compounds) by CYP83A1 and CYP83B1 [32]. The function of this CYP family of genes involved on the first steps of the pathway was addressed in *B. rapa* performing metabolic engineering. Zang et al. [38] introduced *Arabidopsis* cDNA coding CYP79B2/CYP79B3 and CYP83B1 in Chinese cabbage plants. They analyzed the GSL content of independent lines transformed with each double and triple construct, and they found that overexpression of CYP79B3 or CYP83B1 did not affect indole GSL accumulation levels. However, when CYP83B1 was overexpressed together with CYP79B2 and/or CYP79B3, the transformed plants accumulated higher levels of glucobrassicin, hydroxyglucobrassicin, and methoxyglucobrassicin. Thus, coordinate expression of the two consecutive enzymes is needed to divert the flux into indole GSL biosynthesis in *B. rapa*. Moreover, overexpression of CYP79B2 or CYP79B3 causes indole-3-acetic acid (IAA) overproduction suggesting a principal role of these genes on both auxin and indole GSL biosynthesis. Further studies isolated and characterized BrCYP83B1 from leaves of pak choi (*B. rapa*), and the expression pattern in different organs and cultivars was studied [23]. Recently, Gao et al. [39] described, by transcriptome analysis, this CYP family of genes in *B. oleracea* (BoCYP79B2/BoCYP79B3 and BoCYP83B1). Based on amino acid sequence analysis and sequence alignment, these authors also showed that these key GSL synthetic genes within Brassicaceae family are highly conserved.

The step between thiohydroximic acids to desulfoGSLs is regulated by glucosyltransferases of the UGT74 family, UGT74B1, and UGT74C1 [30]. The enzyme UGT74B1 transforms the indolylmethyl-thiohydroximate to the indolylmethyl-desulfoGSL in the indolic GSL pathway. Overexpressing BnUGT74B1 in *B. napus* lines increased both the aliphatic and indolic GSL levels and showed less severe disease symptoms and tissue damage after inoculation with *Sclerotinia sclerotiorum* and *Botrytis cinerea* [36]. Homologues of this gene were identified from cDNA and BAC libraries in *B. rapa* [22] although they were missing in *B. oleracea* [39].

The sulfotransferases (SOTs) play a crucial role in the GSL biosynthesis, by catalyzing the final step of the core GSL formation. Three SOTs were characterized

(SOT16, SOT17, and SOT18) in *Arabidopsis*. All three enzymes use desulfoGSLs as substrates, but have different affinities [31]. In this model plant, SOT16 prefers indolic desulfoGSLs as substrate [31, 41]. The recent publication of the complete *B. napus* genome [42] enabled a detailed investigation of its complete SOT protein family. Based on sequence analysis, Hirschmann and Papenbrock [35] identified four homologues of the *Arabidopsis* SOT16 in *B. napus* genome. In vitro, substrate specificity assays of BnSOT16 revealed similar substrate preferences as their *Arabidopsis* orthologs.

Side-chain modification in indole GSLs occurs through hydroxylations and methoxylations catalyzed by several enzymes. Four main indolic GSLs have been identified in most cultivated *Brassica* species: the unmodified 3-indolylmethyl GSL (glucobrassicin) and its downstream relatives 4-hydroxy 3-indolylmethyl GSL (hydroxyglucobrassicin), 4-methoxy-3-indolylmethyl GSL (metoxyglucobrassicin), and N-methoxy-3-indolylmethyl GSL (neoglucobrassicin) [3]. The members of the CYP81F family (CYP81F1, CYP81F2, CYP81F3, and CYP81F4) are involved in the 4-hydroxylation of indolic GSLs. CYP81F2 catalyzes the conversion of glucobrassicin to hydroxyglucobrassicin, which in turn is converted to metoxyglucobrassicin by one or several as yet unknown methyltransferase(s) [9]. Recently, functional characterization of the two identified isoforms coding for CYP81F4 in the *B. rapa* genome was performed using expression analysis and heterologous complementation of the respective *Arabidopsis* mutant [10]. Moreover, transcriptome analysis has led to the identification of BoCYP81F4, BoCYP81F1, and BoCYP81F3 in *B. oleracea* [39].

In *Arabidopsis*, indolic GSL biosynthesis is regulated by a complex network of transcription factors (TFs) of MYB family: MYB34, MYB51, and MYB 122 [34]. Although all of them upregulate transcription of the core GSL biosynthetic genes, MYB34 and MYB122 also function as stimulators of auxin biosynthesis [43], whereas MYB51 specifically regulates indolic GSL biosynthesis and plays a role in biotic stress responses [44]. Wang et al. [8] identified homologous *Arabidopsis* MYBs related with indolic GSLs using BLASTN and BLASTP, on the basis of the draft of *B. rapa* genome and annotated genes. They reported that each TF of *B. rapa* showed more than 70% sequence identity when compared with corresponding transcription factors in *Arabidopsis*. Similar studies based on EST data set and RNA-seq analysis identified BoMYB34 in kale [45] and BoMYB51 in broccoli seeds and sprouts [39].

In addition, TFs could respond to specific environmental stimulus altering plant metabolic activities. Elicitation studies with signaling molecules and mechanical damage have shown an enhancement of GSL levels in several *Brassica* species. Studies in *Arabidopsis* as well as in *B. oleracea* (broccoli), *B. napus* (rutabaga, cabbage, oilseed rape), *B. rapa* (turnip), and *Raphanus sativus* (China rose radish and red radish) have suggested that methyl jasmonate and wounding could have a distinct impact on indolic GSL levels [46–50]. In concordance with previous *Arabidopsis* studies [29], it has been reported that also in *B. rapa* MYBs encoding indolic GSLs, TFs are upregulated upon hormone treatment [51] in *B. rapa*. Chinese cabbage plants treated with jasmonate, abscisic acid, salicylic acid, and ethanol

increase the expressions of MYB34 and MYB122, subsequently altering the expression levels of CYP79B2/CYP79B3 and the SOT16 finally resulting in enhanced accumulation of indolic GSLs [51]. Thus, indolic GSL types and contents in different organs of the plant are strongly affected by environmental conditions.

5 Modifying GSL Content in *Brassica* Crops: Conventional Breeding and Transgenic Approaches

In the last decades, there was a strong interest in developing methods and procedures to alter levels of specific GSLs in *Brassica* plants as certain GSLs have desirable properties in flavor, insect protection, biofumigation, and cancer prevention, whereas others have undesirable properties such as bitterness and goiter disease in animals [52]. Therefore, the increase of beneficial GSLs and the reduction of detrimental GSLs are a target in *Brassica* improvement either to obtain crops with high value and improved food quality [3, 53, 54] or to obtain useful material to study the biological effects of these compounds. In addition to their structural diversity, there is a high-GSL diversity between families, genera, species, subspecies, and different accessions of subspecies [55–57]. This diversity provides the potential to produce new varieties with optimal GSL composition and content. Different approaches, through conventional breeding or genetic engineering, have been used to modify the level of GSLs in cruciferous vegetables.

5.1 Conventional Breeding

5.1.1 Oilseed Crops

Oilseed rape (*Brassica napus* L.) is one of the most important crops in the world. It is grown primarily for its oil which is used both for nutritional and industrial purposes. After oil extraction, the residual seed meal is limited in its usefulness as a protein source because of their GSLs and erucic acid content [58]. The presence of GSLs in rapeseed had hindered the use of rapeseed meal in livestock industries due to anti-nutritional effects of its hydrolysis products in animals. GSLs affected the thyroid gland of animals fed on rapeseed meal and reduced its palatability. Therefore, breeding programs for *Brassica* oil crops have often been related to reduce the seed GSL content. Early forms of domesticated rapeseed contained a high concentration of GSL (100 to 180 μmol/g) in their oil-free seed meal. As a result, in the 1970s, plant breeders searched germplasm collections for low GSL contents. The first modification of GSL content in the seeds took place by conventional plant breeding. Low erucic acid and low GSL content varieties of *B. napus* were obtained by introgression from other *B. napus* cultivars [59]. The initial advance came with the discovery that in the Polish spring rape (*B. napus*) cultivar "Bronowski," a genetic block was operated to prevent the GSL accumulation in the seeds. This variety was discovered by the Agriculture Canada Research Station in Saskatoon. This sole genetic source of the low GSL trait has been used to develop all the low

GSL cultivars in *B. napus* and *B. rapa* worldwide by introgression. The world's first double low (low erucic acid and low GSL content) *B. napus* and *B. rapa* cultivars, Tower and Candle, respectively, were developed by pedigree selection in 1970s which led to a new era for *Brassica* crop production and its consumption. These new varieties were designated "canola" ("zero erucic acid, zero GSL"). The canola name is derived from Canadian oil low acid (Canola Council of Canada, 2010a, http://www.canola-council.org/canola_the_official_definition.aspx). The official definition of canola refers to any rapeseed with less than 2% of erucic acid in the oil and less than 30 μmol/g of air-dried oil-free meal. This definition was changed in 1995 so the limit was reduced 15 μmol/g of GSL in oil-free seed meal. Consequently, plant breeders have nearly eliminated erucic acid from the seed oil and have dramatically reduced the level of seed GSLs via conventional breeding, allowing the nutritious seed meal to be used as an animal feed supplement. The development of low erucic acid and low GSL cultivars has also been undertaken for other oilseed *Brassica* species (e.g., *B. juncea*) and in other parts of the world for the quality improvement of their oils and seed meals.

Cultivars or lines with low-seed GSLs were selected successfully without pernicious effects on the GSL content of other tissues (Mithen [4]). The genotypes with low-seed GSLs do not necessarily have low GSL content in vegetative tissues. Lines with high-GSL content may have low/high leaf GSL content. There was no correlation between the GSL content of leaves, stems, and seeds. Thus, GSL synthesis and accumulation seem to be under tissue-specific control, and the effect of mutation which blocks accumulation of GSLs in seeds is tissue restricted [60]. Depending on the final use of the crop, this fact gives the possibility to improve cultivars with a double use: reduced GSL content in seeds for animal feed or oil production and enhanced GSL content in vegetative organs for human consumption.

5.1.2 Vegetable Crops

Brassica breeders have been concerned to increase the nutritional profile of the vegetables by increasing GSL content, particularly glucoraphanin, but also other GSLs related to health properties such as glucoiberin, glucoerucin, and the indolic glucobrassicin. Conventional breeding in vegetable *Brassica* crops is exemplified by the production of broccoli with enhanced glucoraphanin content due to the substantial body of epidemiological evidence that relate health benefits of broccoli and biological activity of sulforaphane, the ITC derived from glucoraphanin [61]. Sulforaphane has been shown in several in vitro and animal studies to have potentially health-promoting activities associated with an anticarcinogenic activity [62, 63]. In the 1990s, UK groups held a screening of diverse wild *B. oleracea* species ($n = 9$) and found that *Brassica villosa* species contained a high concentration of glucoraphanin. Faulkner et al. [64] showed that hybrids formed by crossing broccoli inbreds and this wild relative species showed enhanced levels of glucoraphanin and expressed a higher induction potential of phase II enzymes than the broccoli inbred lines themselves. Later, Mithen et al. [65] reported an enhanced ITC production in broccoli after the introgression of two genomic segments from *B. villosa* L., through several breeding cycles. It was shown that a genome segment of *B. villosa* located on

linkage group 5 enhanced 3C GSLs, whereas segments on linkage groups 2 and 9 enhanced all methionine-derived GSLS. In addition to enhanced levels of GSLs, these genotypes have enhanced conversion of GSLs to ITCs through a reduction in nitrile production.

The high-glucoraphanin hybrids possessed a high expression of the MYB28 allele introgressed from *B. villosa* segment. These hybrids contained 2.5–3 times as much glucoraphanin as standard hybrids. The US BroccoSprouts patent (broccoli sprouts obtained from GSL-rich genotypes) is an early application of these studies [66]. The high-glucoraphanin hybrids from the USA and the Netherlands program are commercialized as Beneforté® broccoli (a registered trademark of Seminis Vegetable Seeds, Inc.). An experimental high-glucoraphanin F1 hybrid from the UK program, identified as HG1, has been used in human intervention studies [67].

Other breeding and selection procedures have been successfully performed for GSL content in different *Brassica* species. For example, marrow stem kale was successfully improved by low contents of indole GSLs using a full-sib family selection program [57]. On the other hand, divergent mass selection has been used as a useful tool in plant breeding to generate groups of individuals that share the same genetic background but with extreme values for a particular GSL. Stowe et al. (2011) used this type of selection in a rapid cycling variety of *B. rapa*, and they found that it is possible to modify the total GSL content of leaves. The research group at Misión Biológica de Galicia (MBG-CSIC) carried out several divergent selection programs in a local population of kale (*B. oleracea* var. *acephala*) selecting in parallel for the three major GSLs present in this population, sinigrin, glucoiberin, and glucobrassicin in leaves; in turnip greens (*B. rapa*) selecting for high and low gluconapin content; and in leaf rape (*B. napus*) selecting for high and low GSL content with the aim to obtain base germplasm to study the biological effect of these GSLs. In the *B. oleracea* selection, authors found a side effect of divergent selection performed in leaves in the GSL content of other plant organs as flower buds and seeds and indirect effects of divergent selection performed for the two aliphatic GSLs in the content of other GSLs (unpublished data).

5.2 Transgenic Approaches

The biosynthetic pathway of GSLs has been elucidated in detail, and many of the corresponding genes have been cloned and characterized in *Arabidopsis* and *Brassica*. Based on this knowledge, genetic engineering to modify the content of GSLs is currently carried on in cruciferous crops acting on different steps of the synthesis and degradation of GSLs. Transgenic technologies to overexpress or suppress single or multiple genes allow for rapid and directed engineering of plant metabolism to achieve specific plant traits. Genetic modification by transgene introduction provides an additional route to altering both the levels and types of GSLs.

In this sense, Liu et al. [54] obtained *B. napus* seeds enriched in glucoraphanin through RNAi silencing of the GSL-ALK gene. Introgression of the *Arabidopsis* genes related to the core biosynthesis of aliphatic and indolic GSLs into Chinese

cabbage lines was successfully performed [68]. The overexpression of these genes resulted in the accumulation of some aliphatic (gluconapin and glucobrassicanapin) and some indolyl (glucobrassicin and 4-methoxyglucobrassicin) GSLs in the transgenic lines.

Metabolic engineering of GSLs in plants can be achieved through various approaches targeting either the biosynthetic or the regulatory genes of GSL biosynthetic pathway. An attractive approach is the modulation of transcription factors, which appears to be more effective for the control of metabolic pathways than that of genes encoding single enzyme in plants. More than 20 genes with potential regulatory function in GSL metabolism have been identified in the model plant *Arabidopsis*, in the past few years. Further efforts toward this direction will certainly provide the required insights to facilitate the modification of the complex GSL biosynthesis of plants in the near future. Besides, production of GSLs in non-cruciferous plants has been achieved in *Nicotiana benthamiana* by genetic engineering [69] which opens the field of GSL-enriched foods in different vegetable products, focused, for example, in cancer prevention.

The success of plant breeders in reducing levels of GSLs by conventional breeding in *B. napus* and the unpopularity of genetic-modified (GM) crops in some oilseed rape-growing countries has reduced the incentive among the commercial sector to target GSL reduction by genetic modification. Likewise, obtaining high-GSL broccoli by genetic engineering may not be acceptable to its potential consumers. However, conventional breeding is time-consuming, less straightforward, and less predictable than modern and directed genetic engineering. The development of molecular markers using sequenced genome information of *Brassica* crops and *Arabidopsis* will speed marker-assisted selection of target GSLs to increase beneficial GSLs in *Brassica* vegetables.

6 Identification of QTLs Related to GSL Biosynthesis

As previously explained, the route of glucosinolate synthesis is well known in *Arabidopsis* and *Brassica*, especially due to the works made in the last decade. Nevertheless, the importance of glucosinolates in the animal and human consumption has been pointed out since the 1970s. Early attempts to localize genomic region implied in GSL synthesis were done in the 1990s, and they were partially focused on the search for QTLs. During these decades, with the development of molecular markers, several works have attempted to localize QTLs related to GSL inheritance. The genotype at marker loci linked to QTLs can be used to predict the parental alleles at the QTLs themselves and thus enable breeders to select for specific desirable genotypes [70]. Besides, detecting QTLs is a first step in searching and cloning the genes underlying the traits of interest. All the knowledge about genes regulating biosynthesis of GSLs in *Arabidopsis*, the existing homology among *Brassica* species, and the availability of the genome sequences of the main *Brassica* crops are tools which have helped on the search for candidate genes for GSL biosynthesis in *Brassica* crops. QTL analysis has been applied extensively in

breeding for GSL content in *Brassica* crops. In this section an overview of this type of analysis is shown, dissected by species.

6.1 Oilseed Crops

First attempts to elucidate the genetic control of total seed GSL contents in *B. napus* were done in the 1990s, in germplasm related to Bronowski. Several authors [58, 71] found between two and four major QTLs controlling the total level of GSLs in seeds. Later, Howell et al. [70] detected four QTLs, GLN1–GLN4, that together explain at least 76% of the phenotypic variation in the accumulation of GSLs in seeds. The major QTLs GSL-1 and GSL-2 and the minor QTL GSL-4 found by Toroser et al. [58] correspond to GLN1, GLN2, and GLN4, respectively. The QTLs gsl-1, gsl-2, and gsl-3 of Uzunova et al. [71] correspond to GLN1, GLN3, and GLN2, respectively. Low GSL spring and winter cultivars related to Bronowski had a specific RFLP fragment identified by probe wg3f7 which is linked to the GSL-1 defined by Toroser et al. [58]. High-GSL cultivars possessed a specific RFLP fragment identified by probe wg7a8, which is linked to the QTL GSL-2 [72]. Summarizing, four major QTLs on chromosomes A09, C02, C07, and C09, which were detected independently by several authors [58, 70, 71, 73], positioned at four common regions at 3.2, 50.0, 39.9, and 2.8 Mb of A09, C02, C07, and C09, form the basis of the major reduction in seed GSL content which has been achieved in worldwide canola breeding during the past three decades [74]. More recently, 105 mQTLs for 16 traits which included total and individual GSLs in leaves and seeds were detected by Feng et al. [75]. Nine mQTLs for total GSLs in seeds correspond to QTL identified by several authors [58, 70, 71, 76].

Further reductions in seed GSLs require combination of these main effect loci with additional QTLs that have less prominent effects. Fu et al. [74] identified 43 minor QTLs across environments for total seed GSL content in two mapping populations involving low GSL parents. Inheritance of QTLs of seed GSL content is also related to maternal effects. Nine QTLs found by Xu et al. [77] had significant embryo additive main effects, embryo dominant main effects, and/or maternal additive main effects and could explain 83.8% and 89.7% of their phenotypic variation, respectively.

Breeding for reducing seed GSL content could have a negative impact in seed yield and resistance to pest and diseases. Six QTLs for seed GSL content were detected by Quijada et al. [76] in mapping populations derived from crosses between spring and winter oilseed rape. The allele coming from the winter oilseed rape in one of the QTLs that increased seed yield was linked in coupling to a QTL allele for high-GSL content, suggesting that the transition of rapeseed into canola could have resulted in the loss of favorable seed yield alleles. Zhao and Meng [73] studied the relationship of GSL content in seed and *Sclerotinia* resistance in leaves of a mapping population including Bronowski germplasm. Three QTLs were identified for seed total GSL content. Fifteen loci were found to be responsible for different types of GSLs. No significant correlation was detected between the total GSL content in the

seeds and the disease resistance in leaves. However, tight linkage exists between a QTL for indolic GSL content and QTLs for the level of disease resistance. However, it did not indicate pleiotropy because the distance of the peaks between the two types of QTLs was too wide. This linkage might be broken if a recombination happened at a place between the two loci.

Association mapping is a valuable tool for the dissection of QTLs controlling complex traits in crop plants. Two different association mapping approaches, GWAS and candidate QTL (cQTL), were used to identify SNP markers associated with seed GSL content in a *B. napus* winter oilseed rape association panel (89 inbred lines) [78]. In the GWAS approach, 17 SNP markers were identified to be significantly associated with seed GSL content. As a result of the cQTL analysis, four SNP markers were significantly associated with total GSL content. Two of them were mapped to a previously identified QTL on chromosome A9 [70, 73]. Wurschum et al. [79] performed a multiple-line cross QTL mapping and joint linkage association mapping in a set of 391 doubled haploid (DH) rapeseed progenies, which were derived from nine crosses among ten parental lines, fingerprinted with 253 SNPs. Both approaches detected several additive QTLs for GSL content.

Improvement of quality of the meal through the development of low GSL lines has been a major objective in the breeding of oilseed rape and mustard (*B. juncea*). Most of the breeding efforts in this crop have been limited to the exploitation of variability available in the adapted pool of elite germplasm. Seeds of rapeseed and mustard are a source of edible oil and have a protein-rich seed meal. High GSLs in the seed meal when fed to poultry and livestock pose health risks [80]. Hybridizations within a narrow germplasm base have resulted in slackening response to selection and consequently yield stagnation.

Mahmood et al. [81] studied QTLs controlling seed GSL content and found five QTLs explaining 29.5–45.1% of the total phenotypic variance. Four QTLs associated with individual GSL content (GSL-A2a, GSL-A2b, GSL-F, GSL-B3) could be successfully employed in a MAS breeding program to alter the GSL profile of *B. juncea*. Gupta et al. [82] analyzed QTL for total and individual GSLs in leaves and seeds of *B. juncea*. Three QTLs for total GSLs were found in leaves explaining 15–21% phenotypic variance on linkage groups J6 and J14. One QTL in J18 explained phenotypic variance of 17% for GSLs in seeds, which is coincident with a QTL reported by Ramchiary et al. [83]. In seeds, the most significant QTL for individual GSLs was identified in J9 for glucoiberin. In leaves, major QTL explained 42% of phenotypic variance for gluconapin.

Future increase in productivity requires introduction of novel alleles from varied ecological niches of Eastern Europe and China [82]. Natural variability of *B. juncea* could be broadly classified into two diverse gene pools—the Indian and the east European types [80]. Ramchiary et al. [83] introgressed low GSL alleles from an east European gene pool *B. juncea* line, Heera, into an Indian gene pool variety, Varuna, and performed a QTL analysis for seed GSLs in early and advanced generation populations. Later, Bisht et al. [84] based on the abovementioned work reported fine mapping of loci involved with the low GSL trait in *B. juncea*. Three QTLs, *J2Gsl1*, *J3Gsl2*, and *J9Gsl3*, located in the linkage groups A2, A3, and A9 of *B. juncea*,

respectively, are the most important loci for breeding low GSL, and all probability could be the "Bronowski gene(s)" which have been introgressed from *B. napus* into *B. juncea*. Candidate genes BjuA.GSL-ELONG.a, BjuA.GSL-ELONG.c, BjuA.GSL-ELONG.d, and BjuA.MYB28.a mapped in the confidence interval of several QTLs. QTLs for total and individual GSLs were found by Rout et al. [80] in two mapping populations. The first one was derived from a cross between high and low European GSL lines. The second mapping population was derived from a cross between Indian lines with high and low GSL content. QTLs for individual and total GSLs in seed were mapped in both populations, and a meta-analysis was applied to merge QTLs from both and to obtain consensus QTLs.

Brassica rapa is another species that can be used as an oilseed crop (oilseed rape). Rahman et al. [85] dissected the genetic control of seed GSL content in oilseed rape, finding three QTLs on linkage groups A2, A7, and A9. The QTL located on A9 is coincident with other QTLs found in the linkage group 9 of the A genome of *B. juncea* [83, 84].

6.2 Vegetable Crops

QTLs for vegetable forms of *B. rapa* (turnip, turnip tops, cima di rapa, turnip, pak choi, Chinese cabbage) have been extensively studied. Lou et al. [86] found QTLs for GSL composition and accumulation in *B. rapa* leaves in two mapping populations involving yellow sarson varieties, pak choi, and Kairiyou Hakata, a Japanese turnip. Sixteen QTLs controlled aliphatic GSLs, three controlled indolic GSL concentration, and three regulated aromatic GSLs.

The integration of metabolomics with transcriptomic and genomic platforms has frequently been used as a strategy to identify candidate genes. Del Carpio et al. [87] studied the profile of GSLs and transcript abundance of genes related to GSL synthesis in the leaves of plants from a mapping population derived from a cross between a yellow sarson and a pak choi plant. Significant mQTLs were detected for 13 GSLs, showing co-localization mostly in genomic regions on linkage groups A03 and A09. Forty-two from 94 probes, representing 25 candidate genes, showed at least one eQTL. These genes had both cis- and trans-eQTL in most hotspots in the genomic regions at A03 and A09, with most of the genes showing trans-eQTL effects at the position of MYB29 in A03 and both UGT74B1 and MYB28 in A09. Bagheri et al. [88] employed nuclear magnetic resonance (NMR) to analyze the genetic variation for a range of secondary metabolites in *B. rapa*. In total, six QTLs for GSLs were mapped to A3, A5, A9, and A10, with two in A3 and two in A5 possibly co-locating. The progoitrin QTL presented on A3 at 95 cM co-located with the map positions of MYB28/MYB29.

Attention has been paid in recent times in breeding for high-glucoraphanin content in *B. oleracea* crops. Fourteen QTLs for total and individual GSLs were found by Brown et al. [89] in a mapping population of broccoli. GSL-ALK was suggested as the gene underlying variation for GSL12 in C09, while GSL-PRO was associated to a locus in C05 and GSL-ELONG to GSL03 in C02. Eighteen

meta-QTLs for individual and total GSL content in leaves, flower buds, and seeds were detected by Sotelo et al. [90] in a mapping population derived from a cross between a broccoli and Chinese kale inbred lines. GSL-PRO, GSL-ALK, and GSL-OH were proposed as the genes underlying variation for QTL-5.1, QTL-9.2, and QTL-3, respectively. Genes from the indolic GSL pathway (CYP79B2, CYP79B3, ATR1, and CYP81F2) were proposed as candidate genes for QTLs: QTL-1.2, QTL-7.4, QTL-8.1, and QTL-2.1, respectively.

7 Genetic Regulation in Other Genus of the Brassicaceae Family

Radish (*Raphanus sativus* L.) is probably the Brassicaceae species most studied besides the *Brassica* genus. This vegetable is a common crop in Asia where it is cultivated as a root vegetable. Genomic studies have revealed that the genus *Raphanus* is genetically related to the *B. oleracea/rapa* linkage; however, syntenies between these species are complicated, suggesting extensive genome rearrangements during speciation [91]. Wang et al. [6] performed a de novo transcriptome sequencing experiment in *Raphanus* using a next-generation sequencing (NGS)-based Illumina paired-end Solexa platform and identified 94 unigene orthologs to previously identified genes involved in the GSL biosynthetic pathway and 257 unigenes predicted to encode MYB transcription factors that potentially could regulate that pathway. The major biochemical characteristic of this species is the high content on 4-methylthio-3-butenyl GSL (4MTB-GSL), which accounts for more than 90% of the total GSL content [92, 93]. 4MTB-GSL, with common names glucoraphasatin, dehydroerucin, or glucodehydroerucin, is a four-carbon aliphatic GSL predominantly contained in roots [94]. Zou et al. [95] used a NGS-based strategy to develop a highly saturated genetic map to identify candidate genes involved in the biosynthesis of glucoraphasatin. Three QTLs were consistently identified in this experiment. Two of these QTLs were associated to candidate genes (RsMAM3 and RsBCAT4) involved in the amino acid chain elongation, the earliest step of the GSL biosynthetic pathway (Fig. 1). Interestingly, no candidate gene was found for the QTL that accounted for the largest contribution of the phenotypic variance. Recently, Ishida et al. [96] identified a spontaneous mutant with low content of glucoraphasatin from a landrace of white radish that was used to develop a new cultivar (DPL5) with no detectable glucoraphasatin content. Total GSL content was remarkably reduced in this mutant, and instead of glucoraphasatin, glucoerucin accounted for more than 90% of the GSL content. Genetic studies reveal that this trait is regulated by a single recessive gene. Authors postulate that this gene, located at the end of LG 1, encodes for a dehydrogenase enzyme that produces glucoraphasatin from glucoerucin by a dehydrogenation reaction between C3 and C4.

White or yellow mustard (*Sinapis alba* L.) is a close relative of *Brassica nigra* and is largely grown as a condiment crop due to the specific seed's GSL profile [97, 98]. Similarly to biosynthesis in oilseed rape, the silique cell wall is the major

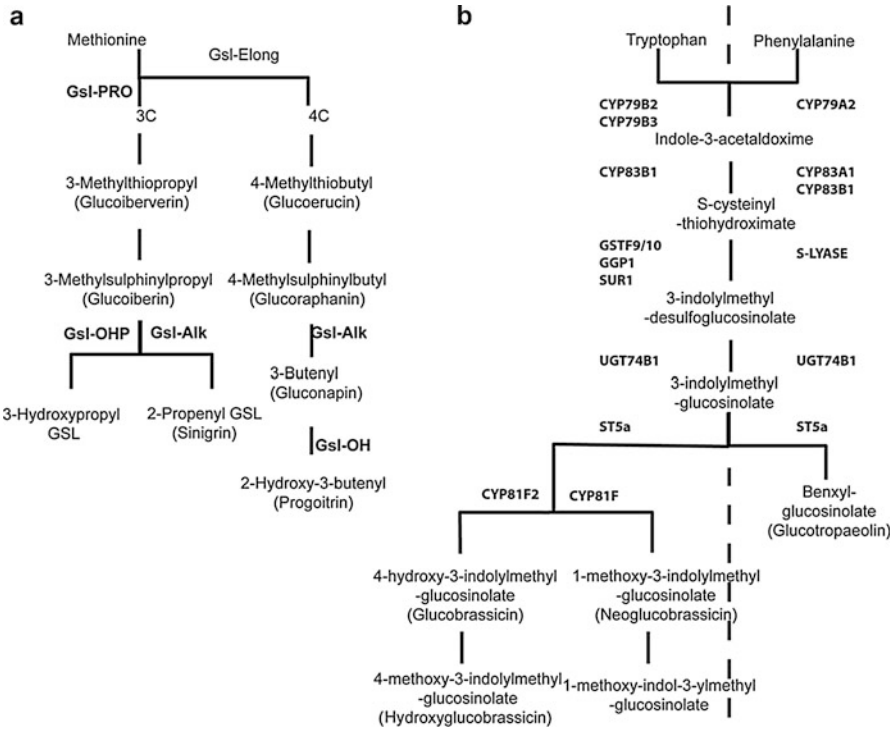

Fig. 1 A biochemical genetic model of the biosynthesis of aliphatic glucosinolates (**a**) and indolic glucosinolates (**b**) in Brassicaceae including the major genes controlling this process

site for GSL synthesis in *S. alba*, and these GSLs are subsequently translocated to the seeds [99]. The dominant individual GSL present in *S. alba* is sinalbin, an aromatic GSL, that accounted for more than 80% of the total GSL content [98]. Genetic studies show that a single dominant gene model is suitable to explain GSL content variation in yellow mustard [100]. However, a more exhaustive study shows that variations of sinalbin and glucobrassicin fit with this model, whereas variations of hydroxybrassicin and progoitrin exhibit a continuous distribution suggesting polygenic regulation [101]. The LG 2 of *Sinapis* seems to be a hotspot in the regulation of GSL biosynthesis. Four QTLs were identified in an overlapped section of the extreme of this linkage group regulating the biosynthesis of sinalbin, brassicin, hydroxibrassicin, and progoitrin. Whether these four QTLs are controlled by the same gene or by the linkage of different genes remains to be clarified [101]. In agreement with the former genetic models, the QTLs identified regulating the content of sinalbin and glucobrassicin explain a large proportion of the phenotypic variance, the 93.1% and 68.8%, respectively, whereas the QTLs regulating the variation of hydroxybrassicin and progoitrin explain only the 35.1% and 20.4% of the phenotypic variance. An additional QTL regulating the biosynthesis of progoitrin was identified in the LG 11, which explains a 19.2% of the total variation.

Also belonging to the mustard family, winter cress (*Barbarea vulgaris* R. Br.) shows a characteristic GSL profile. Three different chemotypes in wild populations have been described based on the GSL content [102, 103]. These chemotypes contained the same set of GSLs but show differences in the quantitative profile. Van Leur et al. [103] describe two types of wild populations; the so-called BAR type contains glucobarbarin as the predominant GSL, whereas in the NAS type, gluconasturtiin is the prominent GSL. Genetic analysis indicates that differences between these two chemotypes fit with a simple Mendelian inheritance model of the GSL type, coded by a single gene, with the BAR type dominant over the NAS type. Authors hypothesize that, in the NAS-type plants, an enzyme converting gluconasturtiin into glucobarbarin is damaged, has a lower expression level, or is missing. In wild populations of eastern Denmark, Agerbirk et al. [102] describe a third chemotype of *B. vulgaris*, in which glucosibarin is the predominant GSL. Glucosibarin and glucobarbarin are two isomeric forms of 2-hydroxy-2-phenylethyl GSL. These authors distinguish two types of wild populations; the G type contains predominantly the S-isomer (glucobarbarin) and therefore could be equivalent to the BAR type previously described, just considering the GSL profile, whereas the P type contains predominantly the R-isomer (glucosibarin). Kuzina et al. [104] crossed two populations of each chemotype and constructed a genetic map based on 100 AFLP and 31 microsatellite marker. A QTL associated to glucosibarin level was located on linkage group 4 which co-localize with a QTL associated to flea beetle resistance and a QTL associated to the high levels of glucobarbarin in the linkage group 5 with a significant dominant effect.

Several GSLs sparsely present in the *Brassica* genus have been reported in other genus of the Brassicaceae family, for instance, glucohesperin, glucoputranjivin, or glucococochlearin, and have been reported in *Boechera stricta* [105] or 4-mercaptobutyl and glucoerucin in the *Eruca* genus [106, 107]; however, few genetic studies have been developed in these genera.

8 Conclusions

In the last decades, knowledge about the importance of glucosinolates in the human and animal consumption, as well as their role in the plant defense, has been increased. This fact led to a high number of works to understand the genetic bases of glucosinolate synthesis. Major genes are well known in *Brassica* crops, but there are other genetic factors, showed by QTL analysis, that will need more research. Other aspects, such as the interaction of glucosinolate genes and pathways with environmental signals, are a field with an increasing importance nowadays.

Other species from the Brassicaceae family, apart from the genus *Brassica*, are recognized as a good source of healthy compounds and may be incorporated into the diet. These species have different glucosinolate patterns. The study of genetics underlying these traits is a recent and open field which will render interesting results in a close future, for breeding in other brassicaceae. Finally, the extensive knowledge about genetics and mode of inheritance of glucosinolates is being incorporated in

breeding programs to modify the content of these healthy metabolites. The sequencing of the main *Brassica* crops and their existing homologies with *Arabidopsis* will speed this process.

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Glucosinolates in Food

3

Marco Possenti, Simona Baima, Antonio Raffo, Alessandra Durazzo, Anna Maria Giusti, and Fausta Natella

Abstract

Glucosinolates are secondary plant metabolites that have attracted researcher's attention due to their potential chemopreventive activity. More than 120 different glucosinolates have been identified in plants, and several of these compounds have been studied for the potential anti-cancerogenic effect of their metabolic breakdown products (mainly ITCs).

Glucosinolates are peculiar of vegetables belonging to Brassicaceae family but are present also in few other species (capers, papaya, and moringa) used for human consumption. The type and concentration of glucosinolates in food are highly variable depending on several factors, such as genetics, cultivation site, cultivar, growth conditions, developmental stage, plant tissue, post-harvest handling, and food preparation methods. As types and concentration are also the main determinant of their biological activities, estimates of their content in food are essential tool to understand if a certain diet is adequate to deliver qualitatively and quantitatively appropriate glucosinolates and ITCs.

The aim of this chapter is to describe qualitative and quantitative glucosinolate distribution among commonly eaten food, as well as the effect of the post-harvest handling on the glucosinolate food content.

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_4

Keywords
Glucosinolates • Isothiocyanates • Brassicaceae • Food content • Processing

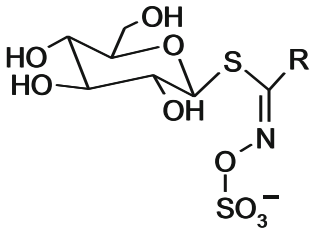
Abbreviations

4-GDB	4-[β-D-Glucopyranosyldisulfanyl] butyl glucosinolate
DMB	Dimeric 4-mercaptobutyl-glucosinolate
DW	Dry weight
FW	Fresh weight
GLS	Glucosinolate
ITC	Isothiocyanate

Contents

1 Introduction

Glucosinolates (GLSs) are secondary metabolites of vegetables that have attracted a great interest, due to their possible effects on human health. They are β-thioglucoside *N*-hydroxysulfates with a side chain (R) and a sulfur-linked β-D-glucopyranose moiety. GLSs have a similar basic structure consisting of a D-thioglucose group linked to a sulfonated aldoxime group and a variable side chain derived from amino acids.



GLSs have been divided in three classes based on the structure of different amino acids precursors:

- Aliphatic GLSs, derived from methionine, isoleucine, leucine, or valine
- Aromatic GLSs, derived from phenylalanine or tyrosine
- Indole GLSs, derived from tryptophan

The GLSs synthesis is regulated according to the different developmental stages of the organs and tissues and by the environment. Although more than 120 different GLSs have been identified, only some of these are present in plants in high quantity. GLS profile varies between species and ecotypes as well as between and within individual plants due to the developmental stage, tissue, photoperiod, and environmental conditions.

The pungent flavor of the raw edible plants containing GLSs is due to the products of GLS enzymatic degradation, which are formed through the activity of an endogenous enzyme, myrosinase (thioglucohydrolase). Myrosinase is localized in the cytoplasm instead GLSs are stored in the vacuole. After cell rupture, induced by chewing during human consumption or by herbivores, insects, or fungal wounding/penetration, GLSs and myrosinase come in contact and hydrolysis occurs. A similar interaction between GLS and myrosinase is obtained by tissue damage during freezing, thawing, and chopping of edible plants.

The glucosinolate-myrosinase system generates thioglucose, sulfate, and an unstable intermediate which rearranges spontaneously into several degradation products. The final composition of the product mix depends on pH, availability of

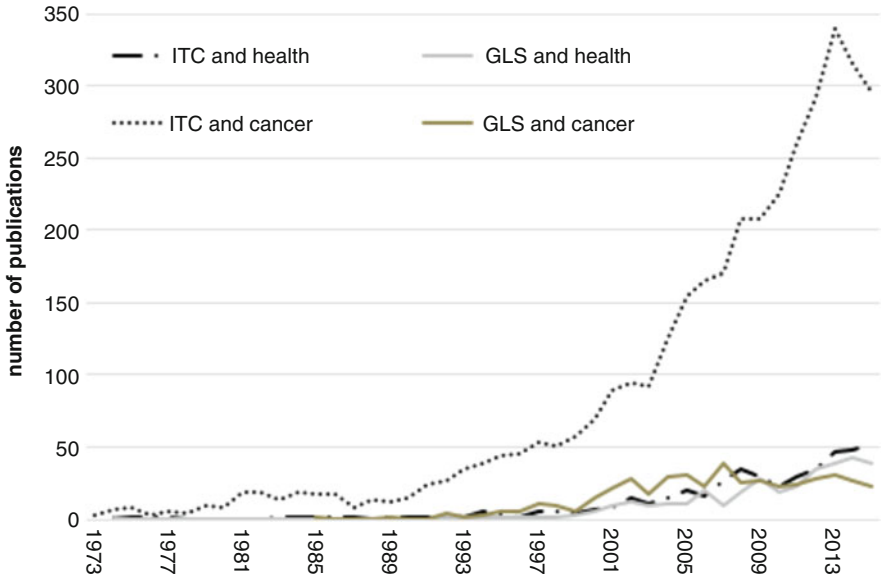


Fig. 1 Chronology of the number of publications appearing in Scopus between 1970 and 2015 with the keywords "glucosinolate," "isothiocyanate," "health," and "cancer"

ferrous ions, and the presence of myrosinase-interacting proteins. In the wide range of biological active breakdown products, there are nitriles, isothiocyanates (ITCs), thiocyanates, epithionitriles, and vinyl oxazolidinethiones.

The GLS breakdown products play an important role in the interactions between plants and insects/herbivores; in fact, they can act both as poisons and deterrents to generalist insects/herbivores or, on the contrary, as signaling molecules to attract specialist insects/herbivores laying their eggs or feeding on that specific plant [1, 2]. Several studies have shown that GLSs determine a growth inhibition or represent a feeding deterrent to a wide range of herbivorous animals such as birds, slugs, and generalist insects [3, 4]. Moreover, it has been observed that volatile hydrolysis products of GLSs can attract natural enemies of herbivores such as parasitoids and therefore determine an indirect protection of the plant [5, 6].

In the last decades, it was found that the GLS breakdown products might have beneficial effects on human health, and this is, probably, the reason that has determined a steadily increase in the number of studies on GLS and ITC. Figure 1 shows the number of publication per year obtained through a search in Scopus with the keywords "glucosinolate" or "isothiocyanate" and "health" or "cancer." In particular, the keyword combination "isothiocyanate and cancer" yields 3691 scientific articles evidencing the great scientific interest on the anti-cancerogenic activity of these compounds (Scopus, January 2016).

2 Biological Activities of GLS Breakdown Products

In the past years, it has become clear that the consumption of food containing GLSs and/or their degradation products could affect human health. Initially, GLSs have been studied for their toxic and goitrogenic activity, and, now, it has become clear that the metabolic product of progoitrin can inhibit the accumulation of iodide in the thyroid gland predisposing to goiter. A similar goitrogenic activity has been reported for thiocyanate ions that are GLS breakdown products common to several GLSs [7]; thiocyanate ions are iodine competitors and can inhibit iodine uptake reducing thyroid hormone synthesis [8]. Then a high progoitrin and thiocyanate diet could be potentially hazardous for hypothyroid patients, especially in those parts of the world characterized by a low iodine supply. Due to the toxic and goitrogenic activity of these compounds (especially when used for animal feed), in the 1990s, the plant breeders started to select plant with low GLS content [9]. However, more recently, this trend has been completely reverted because of the demonstration that several of these compounds (especially ITCs) possess interesting health protective capacities, particularly anti-cancerogenic activity (see Table 1). In fact, in vitro and in vivo studies have reported that GLS breakdown products can affect several stages of cancer development, including the induction of detoxification enzymes (Phase II) and the inhibition of activation enzymes (Phase I) [10]. These enzymes play central roles in the metabolism, elimination, and detoxification of carcinogen and pro-carcinogen molecules (xenobiotics). In order to minimize the toxic effect of xenobiotics, Phase I enzymes introduce polar groups into the toxic molecules (sometimes activating them to more hazardous compounds), while Phase II enzymes inactivate these molecules through conjugation reactions that produce more polar and less toxic molecules. Then, the modulation of Phase I and Phase II enzymes operated by GLS metabolic products can prevent the formation of endogenous and exogenous carcinogens, in turn avoiding carcinogenesis initiation. In addition to modulation of Phase I and Phase II enzymes, GLS breakdown products can affect cancer initiation, progression, and development through many different mechanisms:

1. Blocking the cell cycle [11]
2. Inducing apoptosis [12, 13]
3. Inhibiting angiogenesis [14]
4. Inhibiting metastasis and migration of cancer cells [15]
5. Decreasing the sensitivity to estrogen (through the downregulation of estrogen receptor) and then affecting estrogen-dependent cancer (such as breast cancer) [16]
6. Inhibiting the NF-kB pathway and then acting as anti-inflammatory agents [17]

Besides anti-cancerogenic activity, the GLS breakdown products have shown protective effects against cardiovascular disease, neurodegeneration, diabetes, and several inflammatory disorders [18]. However, due to their high reactivity, several ITCs can be toxic and act as promoter of carcinogenesis [18, 19], even if the

Table 1 Biological activity of the main GLS hydrolytic products

Glucosinolates	Main breakdown products	Biological effects	Reference
Aliphatic			
Glucobrassicanapin	4-Pentenyl-ITC	In vitro antimicrobial and antibacterial activity	[20, 21]
Glucocapparin	Methyl-ITC	Antiproliferative in cancer cell	[22]
Progoitrin/epiprogoitrin	5-Vinyl-2-oxazol-dinethione → oxazolidine-2-thione	Goitrogenic activity in animal model (inhibitor of thyroxine synthesis)	[23]
Sinigrin	AllylITC (AITC)	Antiproliferative in cancer cell Induction of cell cycle arrest and apoptosis Antimetastatic activity in animal model Anti-angiogenic activity in animal model Anti-cancerogenic activity in animal model	[24] [25] [26] [27] [26]
Glucoalyssin	Alyssin (5-(methylsulfinyl)-pentyl ITC)	Induction of apoptosis in cancer cell	[28]
Glucoerucin	Erucin (**4**-(methylthio)-3-butyl ITC) (MTBITC)	Induction of Phase II enzyme Anti-genotoxic activity in cancer cell Antiproliferative in cancer cell Anti-inflammatory in vitro and in animal model	[29] [30] [31] [32]
Dehydroerucin (glucoraphasatin)	4-Methylsulfanyl-3-butenyl ITC (MIBITC)	Induction of Phase II enzyme Antiproliferative in cancer cell Induction of apoptosis in cancer cell	[33] [34] [35] [36]
Glucoerysolin	Erysolin	Induction of Phase II enzyme Anti-genotoxic activity in cancer cell Induction of apoptosis in cancer cell	[29] [30] [28]

Glucoiberin	Iberin	Inducer of Phase II enzyme	[37]
Glucoiberverin	Iberverin 4-Methylthio-3-butenyl-ITC	Anti-mutagen activity Antiproliferative in cancer cell Anti-cancerogenic activity in animal model	[38] [39] [35]
Glucoraphanin	Sulforaphane	Induction of Phase II enzyme Induction of cell cycle arrest and apoptosis Inhibition of histone deacetylase Anti-cancerogenic activity in animal model Antimetastatic activity in animal model Anti-inflammatory effects in vitro and in vivo Inhibition of angiogenesis Hypocholesterolemic effect in animal model Neuroprotective effect in vitro and in vivo Antibacterial activity against *Helicobacter pylori*	[29] [11] [40] [41] [42] [43] [44] [45] [46] [47] [10]
Indolic			
Glucobrassicin	Indole-3-carbinol	Induction of Phase II enzyme Anti-proliferative in cancer cell Induction of apoptosis in cancer cell Modulation of estradiol metabolism Anti-cancerogenic activity in animal model	[48] [49] [50] [51] [52]
	Indole-3-acetonitrile	Mutagen precursor	[53]
	Thiocyanate ions (SCN^-)	Goitrogenic activity (iodine competitors)	[23]

(*continued*)

Table 1 (continued)

Glucosinolates	Main breakdown products	Biological effects	Reference
Aromatic			
Glucomoringin	Moringin	Anti-cancerogenic activity in animal model Anti-inflammatory activity in vitro and in vivo Neuroprotective effect in vitro and in vivo	[54] [55] [56]
Gluconasturtiin	Phenethyl-ITC (PEITC)	Inhibition of Phase I enzymes Induction of Phase II enzyme Anti-proliferative in cancer cell Induction of apoptosis in cancer cell Anti-cancerogenic activity in animal model Anti-metastatic activity in animal model Inhibition of angiogenesis in vitro and ex vivo Down-regulation of estrogen receptor in vitro Anti-inflammatory effects in vitro	[57] [58] [59] [60] [61] [62] [63] [15] [16] [64, 65]
Glucosinalbin	4-Hydroxybenzyl-ITC →4-hydroxybenzyl alcohol + thiocyanate ions (SCN^-)	Goitrogenic activity (iodine competitors)	[23]
Glucotropaeolin	Benzyl-ITC (BITC)	Induction of Phase II enzyme Anti-proliferative in cancer cell Cancer cell apoptosis inducer Anti-cancerogenic activity in animal model Inhibition of angiogenesis in animal model Anthelmintic activity in in vitro model	[66] [67] [68] [69] [14] [70]

concentration needed to induce genotoxic and mutagenic activity is generally higher than those reached in tissue after usual human consumption.

Because the biological activities of GLS breakdown products depend on type (see Table 1) and concentration, the health effect of the GLS-containing food/diet consumption is strongly dependent on the types and amount of GLS present in that particular food/diet. In this respect, the knowledge of GLS dietary intake becomes crucial to understand if diet is adequate to deliver qualitatively and quantitatively appropriate GLS breakdown products.

An essential tool to achieve this objective is the knowledge of GLS composition of food and the post-harvest modification of this composition.

3 Glucosinolates in Food

GLSs occur only in dicotyledonous angiosperms and are common in the order of Capparales, which includes the Brassicaceae family, where they are highly prevalent. Several members of the Brassicaceae family are marketed for animal and human consumption, and they are usually eaten by almost all the populations around the world.

Although GLSs are typical of *Brassica* vegetables, they are not confined to this family, and at least 500 species of non-cruciferous dicotyledonous angiosperms have been reported to contain one or more GLSs, but just few of these species are edible and used for human consumption (capers, papaya, and moringa). These species generally possess a distinctive GLS in very high amount (e.g., glucocapparin in cappers or glucomoringin in moringa).

The GLS-containing foods greatly differ for the GLS distribution, and generally each food contains just few GLS in significant amount; this means that the GLS profile of broccoli is highly different from those of kale or cauliflower. Furthermore, the GLS content can differ quite significantly even between plants belonging to the same species, as great qualitative and quantitative difference can be determined by the cultivar. GLSs are also tissue specific, and great difference in their level can be observed among different parts of the same plant (generally the highest concentration is found in seeds). Within the same vegetable, different GLS profile and amount can be determined by the growth stage; in fact, sprouts are richer than adult plants. Finally, within any particular type of vegetable, the GLS content is affected greatly by growth conditions (from soil to climate), post-harvest treatment, and food processing. While the post-harvest handling may affect indiscriminately all GLSs, environmental clues act prevalently on indolic GLSs, that being induced by stressful growth conditions are also the most variable.

All these factors contribute to the great variability of data found in the several surveys carried out for the determination of GLS content in food [71, 72].

The aim of this chapter is to describe this variability and provide information on qualitative and quantitative distribution of GLS among foods, considering both Brassicaceae and other vegetables and using the most recent literature data. A paragraph describing the effect of post-harvest treatments will help to understand

the possible changes in GLS composition between the raw vegetable and the food as it is actually consumed.

4 Vegetables

4.1 Broccoli (*Brassica oleracea* convar. *botrytis* var. *cymosa*) (commonly *Brassica oleracea* ssp. *italica*)

Broccoli evolved in Europe from a wild cabbage plant. Reports of broccoli growing wild on the shores of the Mediterranean Sea are found in Latin texts since the time of the Roman Empire. For this reason, this plant variety was named *italica*.

The common names broccoli, sprouting broccoli, and calabrese (whose name comes after the region of Calabria in Italy) are frequently considered equivalent, but actually the terms are not synonymous and are inappropriately used in the markets as they refer to different types of plants. Calabrese represents only a small part of the *italica* group, but it has been the most intensively developed and currently it is represented by many cultivars. It produces large (10–20 cm) green heads with thick stalks and is a cool season annual crop harvested from midsummer to mid-autumn. Otherwise, sprouting broccoli has a larger number of heads with many thin stalks and is grown over the winter season and harvested until early spring. Besides morphology and seasonality, there is a considerable diversity within this group also in the color of florets that can be green, purple, or white.

Broccoli is consumed worldwide by people from both Western and Eastern cultures [71] and is one of the most important fresh and processed horticultural crops worldwide. A great number of cultivars, adapted to grow at a wide range of latitudes and seasons (FAOSTAT, http://faostat.fao.org/), are grown in China (the major producing and exporting country), in USA, and Europe.

The GLS content of broccoli ranges from 47 to 806 mg/100 g FW (Table 1), with an average value of 296 mg/100 g FW. The GLS profile of broccoli is distinct from that of other *B. oleracea* vegetables in that glucoraphanin is the predominant GLS observed, followed by progoitrin and glucoiberin among aliphatic GLS and glucobrassicin among indolic GLS. The content of indolic GLS is variable among different studies, probably due to the influence of environmental conditions, but it contributes to almost half of the total GLS on average (Fig. 2). Remarkably, higher levels of progoitrin have been reported in the primary commercial Asian cultivars with respect to the broccoli cultivars widespread in Europe and America [73].

A great variation of glucoraphanin content has been observed among different broccoli accessions both within a single study (usually less than 10-fold) and between different studies (up to 40-fold).

In an attempt to increase its intake with the diet, great efforts have been made to find the higher glucoraphanin producer among the best performing cultivars in different horticultural conditions or to stimulate its production within a specific cultivar through the application of elicitors [73–75]. A high glucoraphanin

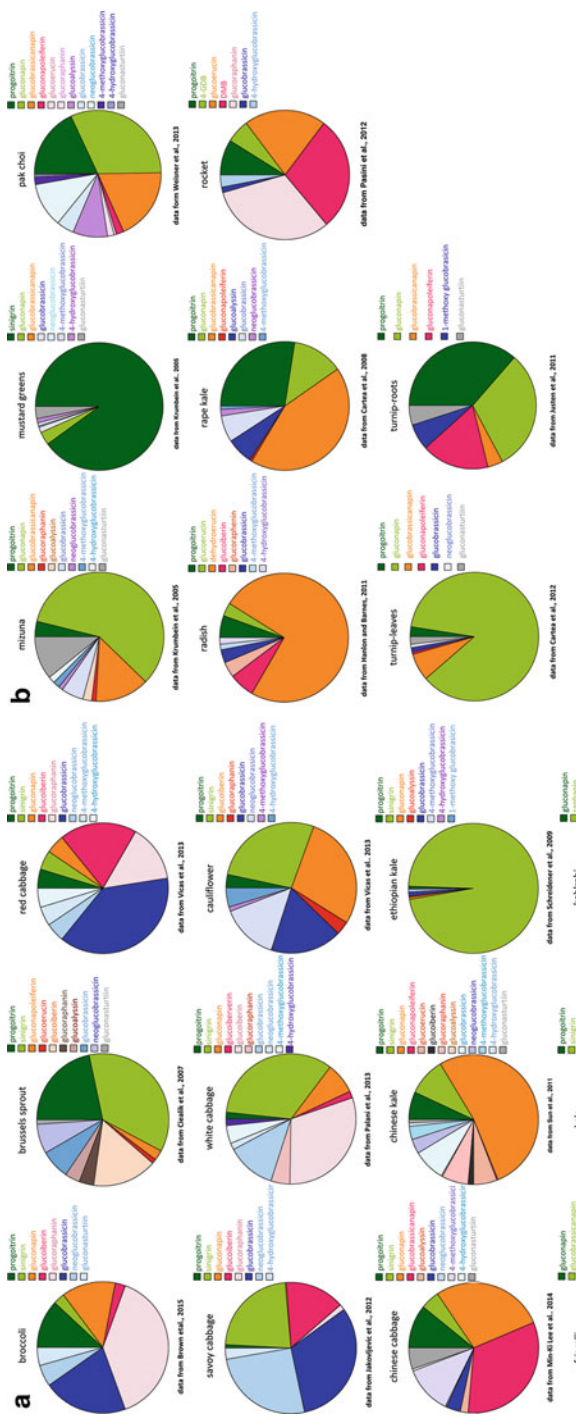


Fig. 2 Glucosinolates' profile of *Brassica* vegetables

proprietary broccoli, marketed under the brand name Beneforte®, has been developed also through the introgression of chromosomal regions from *B. villosa* [76].

4.2 Brussels Sprouts (*Brassica oleracea* var. *gemmifera*)

The term Brussels sprouts is generally used for the edible, leafy, green buds of the plant belonging to the *gemmifera* group of *Brassica oleracea*, which requires a mild cool climate and is widely grown in Europe and North America. Brussels sprouts are usually eaten cooked, mainly boiled, but also steaming, stir-frying, grilling, and roasting are common methods for the domestic consumption.

As recent data on GLS content of Brussels sprouts are rather fragmented (generally just one cultivar per paper was analyzed), non-homogenous, and generally lower than those measured in earlier studies [71], further studies are needed to better establish their mean GLS content.

Based on data collected from recent studies, the mean GLS content of Brussels sprouts ranges from 18 to 390 mg/100 g FW (Table 2), and progoitrin, sinigrin, and glucoiberin represent its main GLSs (Fig. 2), even though different proportions of these GLSs have been reported [77].

4.3 Cabbage (*Brassica oleracea* convar. *capitata*)

Cabbage is an herbaceous biennial plant with leaves forming a compact head; for this reason, it is also known as head cabbage or heading cabbage. Cabbage belongs to the *Brassica oleracea* var. *capitata* group, in which various types of cabbages are comprised, such as white head cabbage (*Brassica oleracea* convar. *capitata* var. *alba*), red cabbage (*Brassica oleracea* convar. *capitata* var. *rubra*), and savoy cabbage (*Brassica oleracea* convar. *capitata* var. *sabauda*). They differ in size, shape, and color and possess a typical flavor.

Cabbages are cultivated worldwide, being economically and commercially important for their cheapness, availability in local markets, and consumer preference. Indeed, they are widely used in human diet, eaten as fresh vegetables in salads or after boiling and stir-frying. It is also consumed as fermented product, for the preparation of various recipes such as sauerkraut and kimchi.

The GLS profile of cabbages differs depending on type. The most abundant GLSs found in red cabbage are sinigrin, glucoiberin, and glucobrassicin; however, the GLS profile is highly different among the different varieties (Fig. 2).

Among cabbages, the white cabbage appears to contain the highest level of GLSs, with a mean total value of 148 mg/100 g FW. This value is almost double those observed in red cabbage and savoy cabbage (81 mg/100 g FW and 88 mg/100 g FW, respectively) (Table 2).

Table 2 Estimated total glucosinolate content of the most widely consumed foods (mg/100 g FW)

Common name	Botanical classification	Organ	No. of accessions	Weighted mean[a]	GLS			References
					Mean	Min	Max	
***Brassica* vegetables**								
Broccoli	*B. oleracea* convar. *botrytis* var. *cimosa*	Inflorescences	**220**	**328**				
	(commonly var. *italica*)		1		105			[96]
			3		198	142	245	[78]
			12		181	57	452	[108]
			2		234	205	256	[109]
			2		774			[110]
			1		184			[111]
			23		237			[74]
			5		806	286	858	[112]
			3		592			[76]
			5		386	212	495	[73]
			143		326	47	931	[73]
			1		702	473	1000	[113]
			6		566	204	1266	[81]
			7		281	88	551	[114]
			1		47			[115]
			2		488			[116]
			3		97	30	58	[117]
Brussels sprouts	*B. oleracea* var. *gemmifera*	Leaf buds	**5**	**142**				
			1		19			[77]
			1		392			[118]

(*continued*)

Table 2 (continued)

Common name	Botanical classification	Organ	No. of accessions	Weighted mean[a]	GLS			References
					Mean	Min	Max	
			1		128			[119]
			1		27			[120]
			1		144			[121]
Cabbage, white	*B. oleracea* convar. *capitata* var. *alba*	Leaves	**87**	**148**				
			1		44			[122]
			1		42	34	49	[123]
			2		14	10	17	[124]
			70		172	126	250	[125]
			1		7	3	10	[80]
			6		76	50	92	[81]
			1		64			[126]
			5		36	23	63	[127]
Cabbage, red	*B. oleracea* convar. *capitata* var. *rubra*	Leaves	**7**	**81**				
			1		17			[128]
			5		107	56	152	[81]
			1		19	12	26	[80]
Cabbage, Savoy	*B. oleracea* convar. *capitata* var. *sabauda*	Leaves	**5**	**88**				
			2		121	40	196	[81]
			1		75	52	98	[129]
			2		62	55	70	[130]
Cauliflower	*B. oleracea* convar. *botrytis* var. *botrytis*	Inflorescences	**21**	**71**				
	White		**12**	**71**				

			2		106	42	171	[78]
			3		103	83	119	[82, 131]
			1		109			[79]
			1		91			[120]
			1		151			[80]
			4		16	13	19	[81]
	Green		**3**	**65**				
			2		85	23	148	[82, 131]
			1		23			[81]
	Romanesco		**4**		**48**			
			1		137			[82, 131]
			3		19			[81]
	Purple		**2**	**154**				
			1		200			[82, 131]
			1		108			[81]
Chinese cabbage	*B. rapa* ssp. p*ekinensis*	Leaves	**88**	**193**				
	also known as		23		176			[132]
	B. campestris ssp. *pekinensis*		1		246			[84]
			1		59			[133]
			62		203			[85]
			1		49			[96]
Chinese kale	*B. oleracea* var. *alboglabra*	Florets/stems	**28**	**38**				
		Leaves	27		26	13	50	[87]
		Stems	27		34	11	87	[87]
			1		150			[86]
Ethiopian kale	*B. carinata*	Leaves	**4**	**43**				
			1		37			[134]
			1		39			[135]

(*continued*)

Table 2 (continued)

Common name	Botanical classification	Organ	No. of accessions	Weighted mean[a]	GLS			References
					Mean	Min	Max	
			2		48			[88]
Friarelli or	*B. rapa* ssp. *campestris*	Florets/shoots	**8**	**96**				
Cime di rapa or	also known as		2		93			[136]
turnip rape	*B. rapa* ssp. *sylvestris L.*		5		97			[89]
	Janch. var. *esculenta* Hort.		1		100			[137]
Kale	*B. oleracea* convar. *acephala*	Leaves	**222**	**143**				
			153		170	72	345	[138]
			40		39			[139]
			1		177			[140]
			24		155	17	313	[90]
			1		84			[96]
			1		53			[141]
			1 green		165			[142]
			1 red		79			[142]
Kohlrabi	*B. oleracea* var. *gongylodes*	Stem tuber	**8**	**296**				
			1		74			[143]
			2		234			[91]
			2		102			[81]
			1		50			[80]
			2		784			[144]
Mustard greens or	*B. juncea*	Leaves	**8**	**121**				
Indian mustard			2		53	26	113	[94]
			1		112	69	155	[95]

			2		201	49	354	[145]
			1		62			[146]
			1		209			[96]
			1		73	62	80	[98]
Mizuna or	*B. rapa* ssp. *nipposinica* var.	Leaves	**3**	**20**				
Japanese mustard	*chinoleifera*		2		18	5	32	[94]
			1		25	19	32	[98]
Pak choi or	*B. rapa* ssp. *chinensis*	Leaves	**31**	**101**				
Bok choy	also known as		13		207	107	318	[99]
	B. campestris ssp. *chinensis*		3		8	6	13	[94]
			2		15	12	18	[147]
			1		57			[148]
			3		74	36	112	[149]
			7		9	5	16	[100]
			2		18	15	21	[150]
Radish	*Raphanus sativus*	Roots	**31**	**505**				
			1		309			[143]
			8		411			[151]
			17		627			[152]
			3		441			[153]
			1		22			[154]
			1		49			[96]
Rape kale	*B. napus* ssp. *napus* var. *pabularia*	Leaves	**88**	**102**				
			36		116	6	299	[155]
			36		65	7	258	[156]
			16		151			[156]
Rocket	*Diplotaxis tenuifolia*	Leaves	**30**	**31**				

(*continued*)

Table 2 (continued)

Common name	Botanical classification	Organ	No. of accessions	Weighted mean[a]	GLS			References
					Mean	Min	Max	
	also known as		16		17	2	39	[103]
	"wild" rocket		7		64	37	86	[102]
			5		11	10	25	[107]
			1		70	62	79	[105]
			1		76			[104]
	Eruca vesicaria ssp. *sativa*	Leaves	**93**	**40**				
	also known as		16		10	3	22	[103]
	"cultivated" rocket		25		56	26	96	[102]
			1		93	72	106	[157]
			32		14	6	20	[107]
			4		78	48	99	[158]
			1		65	59	71	[105]
			13		95	48	137	[104]
			1		34			[159]
Turnip	*B. rapa* var. *rapa*	Leaves	**59**	**737**				
			10		639			[160]
			45		796			[161]
			4		313			[162]
		Roots	**4**	**265**				
			4		265			[162]

Other foods								
Caper	*Capparis*	Flower buds	**4**	**359**				
	C. ovata		1		894			[163]
			1		123			[164]
	C. spinosa		1		404			[163]
			1		13			[165]
Moringa or	*Moringa oleifera*	Leaves	**2**	**325**				
drumstick tree			1		96			[166]
			1		555			[167]
		Seed pulp	**2**	**4724**				
			1		8626			[166]
			1		822			[167]
Papaya	*Carica papaya*	Fruit	**3**	**37**				
			1		≈10			[168]
			1		≈100			[169]
			1		0			[170]

Data of the original study were converted to mg/100 g FW
[a]Mean weighted by the number of accessions of each study

4.4 Cauliflower (*Brassica oleracea* convar. *botrytis* var. *botrytis*)

Cauliflower is a traditional European crop that is largely cultivated also in North America and Asia. The crop is characterized by a hypertrophy of flower branches, that probably has given rise to the name cauliflower (that consists of two Latin words, namely, "caulis" which means stem and "floris" which means flower). It is available year-round but is especially abundant in the spring and fall. Cauliflower cultivars show great variability: the varieties most known and widespread are white, green, Romanesco, and purple, each characterized by a peculiar leaf shape and color. Most of the cauliflower cultivars are white and possess a big head, which represents the edible portion (also known as curd) and is made up of abortive flowers, the stems of which are short, pulpy, and crowded. Less than 10 years ago though, a green cauliflower was developed by crossing cauliflower with broccoli; in this new cultivar, the green color is determined by the presence of chlorophyll (like in broccoli). Green cauliflower flavor is sweet, mild, and nutty and free of the bitterness sometimes found in white cauliflower.

The Italian variety referred to as "broccoli Romanesco" or "Romanesco cabbage" belongs to the group of green cauliflower and is characterized by a unique and unusual growth patterns with multiple compact heads forming a pyramid of spiraling and pointed cones.

The purple cultivar characterized by the presence of the anthocyanin pigments (that confer the characteristic color) is available year-round with a peak season during the winter months. Similar to the green variety, the purple cauliflower is characterized by a mild and sweet flavor.

Generally, cauliflower is used as a cooked vegetable, but it can be eaten also in its raw form.

The predominant GLSs found in cauliflower are sinigrin, glucoiberin, and glucobrassicin (Fig. 2). In white cauliflower, the total GLSs content varied from 91 mg/100 g FW to 109 mg/100 g FW (mean value of 78 mg/100 g FW) [78–82] (Table 2). Glucoiberin is the predominant GLS ranging from 3 to 43 mg/100 g FW (mean value of 23 mg/100 g FW), followed by sinigrin (mean value of 22 mg/100 g FW) and glucobrassicin (mean value of 14 mg/100 g FW) [80, 81] (Fig. 2).

The mean GLS content in green cauliflower is 65 mg/100 g FW (Table 2), and glucoiberin is the most abundant GLS (mean value of 14.4 mg/100 g FW) [81–83]. Romanesco shows a mean GLS content of 48 mg/100 g FW (Table 2), and glucobrassicin is the predominant GLS (mean value of 14.3 mg/100 g FW) [81, 82]. In purple cauliflower, the mean GLS content is 154 mg/100 g FW (Table 2), and glucobrassicin is the most abundant compound (the mean value of 124 mg/100 g FW) [78, 82].

4.5 Chinese Cabbage (*Brassica rapa* ssp. *pekinensis*, Also Known as *Brassica campestris* ssp. *pekinensis*)

The *pekinensis* group is the more common of the two different groups of *Brassica rapa* used as leafy vegetables in Asia (*pekinensis* and *chinensis*), and, within these

two groups, there is a wide range of varieties. The binomial name *Brassica campestris* is also used to refer to these cultivars.

Chinese cabbage is one of the most important and popular vegetables in Asia, especially in China, Japan, and Korea. It is the main ingredient of "kimchi," which is the traditional fermented food of the Republic of Korea with a consumption *pro capite* of about 120–150 kg of kimchi per year. Analysis performed on fresh leaves of many Korean varieties identified glucobrassicanapin, gluconapin, and 4-methoxyglucobrassicin as the main GLS compounds [84, 85] (Fig. 2). The total GLS content varies largely among varieties of Chinese cabbage, from 49 to 246 mg/100 g FW, with a mean value of about 190 mg/100 g FW (Table 2).

4.6 Chinese Kale (*Brassica oleracea* var. *alboglabra*)

Chinese kale, also known as Chinese broccoli or Kai-lan, is a popular Chinese green vegetable, widely distributed in Southern China and Southeast Asia, even if it is also present in Japan, Europe, and America. It is grown for its bolting thick stems, characterized by a tender and crisp texture and an appreciated flavor, while the consumption of other edible parts of the plant, such as leaves and sprouts, is becoming increasingly popular. It is generally eaten after stir-frying, steaming, or boiling.

The mean value of the total GLSs is 38 mg/100 g FW [86, 87] (Table 2), while the most abundant GLS is gluconapin, followed by sinigrin, glucobrassicin, progoitrin, glucoraphanin, and glucoerucin (Fig. 2).

4.7 Ethiopian Kale (*Brassica carinata*)

Ethiopian kale, also known as Ethiopian mustard, is one of the major traditional leafy vegetables in East Africa (especially in Ethiopia), and its leaves are widely consumed in various parts of Africa. The plant is traditionally grown in kitchen gardens, but it is also grown in field and used as an oilseed or fodder crop. Leaves and tender stems are eaten fresh in salad, boiled in soup, or marinated, while seeds are used as spice or crushed to produce vegetable oil for cooking or for industry.

The mean total GLS content in Ethiopian kale is 43 mg/100 g FW (Table 2) and sinigrin is its predominant and almost sole GLS (Fig. 2); in fact, it accounts for more than 95% of the total level, while glucobrassicin, gluconapin, glucoalyssin, progoitrin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, and 1-methoxyglucobrassicin are found in very small amount [88].

4.8 Friarielli or Cime di rapa or Turnip Rape; (*B. rapa* ssp. *campestris* Also Known as *B. rapa* ssp. *sylvestris L. Janch*. var. *esculenta Hort.*)

The group *Brassica rapa* includes also a plant cultivar that is commonly cultivated in Southern Italy, where it is traditionally known as *friarielli* or *cime di rapa* depending

from the region of origin (Campania and Puglia, respectively). Outside of Italy, it is also known with the names of *broccoli rabe*, *raab*, *Italian turnip*, or *turnip rape*.

The edible parts of the plant, represented by its turnip tops or shoots (sprouts plus inflorescences), are generally consumed after cooking: boiled, steamed, braised, or served with pasta or sausages.

The few data reporting the GLS composition of this local *Brassica* variety indicate a mean total GLS content of about 95 mg/100 g FW (Table 2). The most abundant GLSs are the aliphatic gluconapin, glucobrassicanapin, and the indolic glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin (Fig. 2). Progoitrin has been found only in one ecotype, while glucoraphanin is completely absent [89].

4.9 Kale (*Brassica oleracea* var. *acephala*) and Rape Kale (*Brassica napus*)

Many different crops are described worldwide as "kale," some of them being used for human consumption while other being grown for fodder. Generally, kale is defined as a vegetable of the plant species *Brassica oleracea* var. *acephala* characterized by having leaves (colors ranging from light green to violet brown) along the stem not forming a head. However, also some cultivars of *Brassica napus* are generally known as kale, the Hanover salad, the red Russian, Siberian, and rape kale being good examples. These kinds of kale are generally more tender and have a milder flavor than the *Brassica oleracea* kales and are well-known varieties in the USA and in Europe (particularly appreciated in northwestern Spain and in northern Portugal where they are known as "nabicol" and "couve nabica").

Kale is grown along a wide range of latitudes, as it is robust and can tolerate a broad range of agricultural and climatic conditions (such as cold temperature). It is frequently consumed after boiling or as an ingredient for stew and soup; however, it may be eaten also raw for salad use.

Based on recent data, the GLS content of kale (*B. oleracea* var. *acephala*) ranges from 17 to 345 mg/100 g FW, with an average value of 143 mg/100 g FW (Table 2). Aliphatic GLSs represent the main contributor to the whole GLS profile, while glucoiberin, sinigrin, and glucobrassicin represent the most abundant GLS (Fig. 2), but different proportions of them have been observed in different studies. Generally, glucoraphanin is present in low amount; however, some kale varieties, such as black kale, can contain high glucoraphanin level [90].

Rape kale (*B. napus*) contains similar total GLS level (range, 6–298 mg/100 g FW; average value, 102 mg/100 g FW) (Table 2), but the GLS pattern is quite different; in fact, the principal GLSs are glucobrassicanapin, progoitrin, and gluconapin, while indolic GLSs are present in lower amount (Fig. 2).

4.10 Kohlrabi (*Brassica oleracea* var. *gongylodes*)

Kohlrabi is a biennial, herbaceous plant grown as an annual crop that produces an edible turnip-like swollen stem at the base of the plant body. It is closest in form to wild cabbage (*Brassica oleracea* ssp. *oleracea*), which is the progenitor of all the *B. oleracea* varieties. Kohlrabi was cultivated since the sixteenth century in the northwestern coast of Europe. Today it is produced in Europe, North America, many parts of Asia (India, China, Korea, and Vietnam), and North Africa. Depending on the cultivar, kohlrabi may be purple, red, or white-green in the skin, but the flesh is always white. GLS content varies among the different parts of the kohlrabi (skin and flesh), and generally the red varieties have a higher total content of GLSs than the green ones [81, 91, 92]. Beside these differences among varieties (ranging from 50 to 784 mg/100 gFW), GLS content data demonstrate that kohlrabi possesses a very high total GLS content, with a mean value of 295.5 mg/100 g FW (Table 2). The principal GLSs are raphanin and gluconapin (Fig. 2).

4.11 Mustard Greens (*Brassica juncea*)

Brassica juncea is an important crop in many parts of the world, being extensively cultivated in Asia, especially in China and India, primarily for oilseed production but also for the production of the leafy vegetable known as mustard greens. Young leaves of mustard greens are consumed raw in salads or mixed with other salad greens, while older leaves and stems may be used fresh or processed for potherbs or cooked in a variety of recipes. A remarkable genetic variability has been reported among strains of *Brassica juncea* accessions grown in different areas of the world, reflecting significant differences in the chemical composition of their seeds [93].

The total level of GLSs found in mustard green leaves ranges from 26 to 354 mg/100 g FW, with an average level of 121 mg/100 g FW (Table 2). From the few quantitative data reported in the literature, it seems that the European-type Canadian cultivars of *Brassica juncea* are characterized by relatively low levels of GLSs (26 to 155 mg/100 g FW) [94, 95] when compared to cultivars grown in Asia (208 to 354 mg/100 g FW) [96, 97], paralleling well-established differences in the composition of the seeds [93].

The GLS profile is mainly characterized by aliphatic GLSs, such as sinigrin (predominant in European cultivars), gluconapin (predominant in Indian cultivars), and glucobrassicanapin, with minor concentration of indole GLS (glucobrassicin, 4-hydroxyglucobrassicin, and 4-methoxyglucobrassicin) and aromatic GLS (gluconasturtiin) (Fig. 2).

4.12 Mizuna (*Brassica rapa* subsp. *nipposinica* var. *chinoleifera* Also Known as *Brassica juncea* var. *japonica*)

Mizuna also known as Japanese mustard is traditionally consumed in Japan. The plant produces many stalks bearing dark green leaves, characterized by a pungent, mild peppery, and spicy flavor. It is consumed raw in salads or cooked in soups, stir-fries, and hot pots and pickled.

Few data reported in the literature show a total GLS content ranging from 5 to 32 mg/100 g FW, with an average level of about 20 mg/100 g FW (Table 2). The GLS profile is dominated by the aliphatic fraction (gluconapin and glucobrassicanapin), but also indole (glucobrassicin and its derivatives) and aromatic (gluconasturtiin) GLSs have been reported [94] (Fig. 2). Only low levels (less than 2 mg/100 g FW) of glucoraphanin have been observed [94, 98].

4.13 Pak Choi (*Brassica rapa* ssp. *chinensis* Also Known as *Brassica campestris L.* ssp. *Chinensis* var. *communis*)

Pak choi is an Asian leafy vegetable, also known as Chinese celery cabbage, bok choy, and horse's ear, which has traditionally been highly used in China, but whose consumption is also increasing in Europe in salads or Asian-style cooking [99]. Sometimes the species *Brassica rapa* ssp. *chinensis* is reported as *Brassica campestris L.* ssp. *chinensis* var. *communis*, but the two names refer to the same species. The fully developed vegetable looks like a short and thick celery, with glossy and crisp green leaves, whose flavor is somewhere between mild cabbage and spinach, and white or pale green chunky stalks. Leaves, preferably if young, can be eaten raw in salads or cooked by brief steaming or stir-frying.

While an average total GLS content of 100 mg/100 g FW results from recent data reported in the literature (Table 1), a remarkable variability can be observed in the total GLS content found in the different studies, ranging from 8 [94, 100] to 207 mg/100 g FW [99] (Table 2). This marked variation may be due not only to different genetic backgrounds but also to differences in the developmental stages considered in these studies: prolonging plant growing time, in environmentally controlled conditions, from 10 to 25 days, has been shown to markedly increase the total GLS content of the leaves at harvest [100].

The aliphatic GLSs are the predominant group in pak choi leaves, with gluconapin, glucobrassicanapin, progoitrin, and glucoalyssin giving the main contribution, whereas indolic GLSs (neoglucobrassicin and glucobrassicin) and aromatic GLSs (gluconasturtiin) are also present at a lower level [99] (Fig. 2).

4.14 Radish (*Raphanus sativus*)

Radish is a crop of the Brassicaceae family, cultivated in China, Korea, Europe, and America since the fifth century BC. The different varieties of radish diverge in size,

shape, and color. The round radish varieties are more popular in Western countries, and they have many different colors. Instead, the "daikon," a radish with a long white taproot, is more popular in Asia. In the past centuries, radish has been used as medicinal foods for a lot of diseases, but, nowadays, it is commonly used for dietary consumption. The major deterring factor of its use is the bitter and pungent flavor caused by GLSs and particularly by the high content of dehydroerucin [101]. The total GLS content varies extensively among varieties of radish ranging from 22 to 627 mg/100 g FW (mean value of 505 mg/100 g FW) (Table 2). Besides dehydroerucin, other GLSs present in radish are glucoiberin, progoitrin, glucoraphanin, glucobrassicin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin (Fig. 2).

4.15 Rocket (*Eruca vesicaria* ssp. *sativa* and *Diplotaxis tenuifolia*)

Rocket (or arugula, rucola, and roquette) is the collective name for a group of leafy vegetable crops, all belonging to the Brassicaceae family and native to the Mediterranean geographic area. While traditional consumption of rocket in this area dates back to ancient times, in the past few decades, it has gained increasing popularity in the salad vegetable market in many countries all over the world [102, 103]. Two main species are cultivated as salad crops: *Eruca vesicaria* (more precisely, the subspecies *sativa*), belonging to the genus *Eruca*, and generally referred to as "salad" or "cultivated" rocket, and *Diplotaxis tenuifolia*, belonging to the genus *Diplotaxis*, often referred to as "wild" rocket. The two species have similar chemical composition and appearance, and one can be easily mistaken for the other by a nonexpert eye and before a certain level of maturity has been achieved. Both species share the distinctive pungent flavor of the leaves that makes them an appreciated ingredient of vegetable salads (simple or in mixtures). Even though rocket is most commonly consumed as raw leaves, food use of cooked leaves and also of flowers and sprouted seedlings has also been reported [104, 105].

The total GLS content of rocket leaves ranges from 2 to about 140 mg/100 g of fresh weight (Table 2), with an average value of 40 mg/100 g FW and 31 mg/100 g FW for *Eruca vesicaria* subsp. *sativa* and *Diplotaxis tenuifolia*, respectively. The average values reported in Table 2, obtained on a global number of more than 120 varieties, are noticeably lower than the range (72–106 mg/100 g FW) previously reported by Verkerk and colleagues [72], which was obtained on leaves from a single cultivar. As for other vegetables from the Brassicaceae family, the total GLS content is markedly affected by many environmental conditions and physiological factors [102]. In particular, GLS content seems to markedly increase throughout the multiple harvests that are allowed by the ability of this crop to regrow the leaves repeatedly after cutting [106].

As regards GLS profile, it is generally dominated by the three aliphatic-derived compounds glucosativin, in the monomeric and the two dimeric forms (4-[β-D-glucopyranosyldisulfanyl]-butyl-GLS and dimeric 4-mercaptobutyl-GLS (DMB)), glucoerucin, and glucoraphanin, even though very different proportions of these three have been reported [102, 107]. In addition, other aliphatic GLSs (glucoalyssin,

progoitrin/epiprogoitrin, glucoiberverin), aromatic (glucosinalbin), and indole-derived GLS (4-OH-glucobrassicin, glucobrassicin) give a minor contribution to the global profile (Fig. 2).

4.16 Turnip (*Brassica rapa* var. *rapa*)

The turnip is a root vegetable grown in temperate climates worldwide for human consumption or to feed livestock. In some countries the turnip leaves are eaten as "turnip greens" (the leaves harvested in vegetative period) or "turnip tops" (the fructiferous stems with the flower buds and the surrounding leaves); they are a common side dish in the USA (especially in the southeastern of the country), Italy, Spain, and Portugal, but turnip is consumed in Asia too. The edible parts are consumed boiled and generally served with meat.

The total GLS content varies among the plant organs, being higher in leaves (737 mg/100 g FW on average) than in roots (265 mg/100 g FW on average) (Table 2). Gluconapin is the most abundant GLS compound in all the edible parts of the plant body, but roots contain also progoitrin and gluconapoleiferin in relevant amount (Fig. 2).

4.17 Sprouts (Various Brassicaceae spp.)

Edible sprouts are tasty easy-to-eat food traditionally consumed in Asian countries. In the past years, the consumption of edible sprouts in salads or in Asian-style cooking has been growing enormously also in Western countries.

Among the large variety of seeds and ready-to-eat sprouted seeds offered by the market, those of the Brassicaceae family, mainly broccoli (*Brassica oleracea* ssp. *italica*) and radish (*Raphanus sativus*), are among the most popular for the presence of GLSs. With respect to their conventional mature vegetable counterpart, sprouts present several advantages. First, sprouts can be grown, simply and inexpensively, all year-round, thus encouraging vegetable consumption. Second, due to their physiological state, sprouts are an enriched source of phytochemicals; in fact, GLSs have been found to be higher in Brassicaceae sprouts than in the corresponding mature vegetables [96, 171]. Third, they are consumed raw, thus avoiding the loss of GLSs usually associated to food processing and cooking. On the other hand, great care must be taken to avoid mold and bacterial contaminations that can occur during sprouts' growth and storage as a great number of serious food-borne illness outbreaks associated to the consumption of raw sprouted seeds have been reported in many different countries [172, 173]. During germination, the combined effect of reactivation of metabolic pathways and dilution due to tissue expansion dramatically affects the content of bioactive molecules in sprouts [174]. This means that GLSs' content is higher in seeds and decreases over the germination period [175–177]. For this reason, sprouts between 5 and 8 days old are considered optimum for consumption, balancing biomass yield and size with GLS

content. As for adult plants, the GLS content of sprouts is influenced by both genetic and environmental factors, including production and storage conditions [175]. In an attempt to identify the best performers in terms of GLS content, an increasing number of studies have been undertaken aiming at comparing different genotypes under standardized growth conditions [75, 175, 178, 179]. Significant differences, both qualitative and quantitative, have been reported in the GLS content of sprouts of different cultivars and species of Brassicaceae. Overall, the GLS pattern of sprouts reflects that of the mature edible part, but almost all the crops exhibited higher concentrations of GLS in sprouts than in adult parts (the mean total GLS level ranging from 297 to 1297 mg/100 g FW in sprouts and from 193 to 736 mg/100 g FW in adult plants, respectively) (Tables 2 and 3). The total aliphatic GLS concentration also was found generally higher in sprouts than in the mature vegetable. In fact, aliphatic GLSs represented more than 90% of the total GLSs in sprouts of most crops, whereas they range from 57% to 98% of the total GLSs in adult parts. The relatively higher concentrations of indolic GLS in mature edible parts are probably due to the induction of indolic GLS synthesis in the presence of stressful growth conditions.

5 Processed Foods

5.1 Colza and Canola oil

Colza (or rapeseed) oil is a vegetable oil obtained from the seeds of oilseed rape (*Brassica napus*, *Brassica rapa*, *Brassica juncea*). Already in the thirteenth century, the oil was used for street lighting in the Northern Europe countries, while its food use was developed in the mid-nineteenth century, but it had not a lot of success because of its effects on health. In fact, from 1960 to 1970 years, several studies on animals have shown potential health risks (lipidosis, infarction, and cardiac lesions) associated with the use of colza oil [182–187]. The potentially toxic element was found to be the erucic acid present in concentrations ranging between 30% and 60% depending on the cultivar, the harvesting period, and other factors. The GLSs were responsible for the bitter taste of the oil and for the goitrogenic effect of the meal remaining after oil extraction; due to this effect, the use of meal was drastically reduced in animal feed use. In order to use colza oil in food industry, researchers identified and/or developed different mutants of the original plant. The first and most important varieties of *Brassica* able to produce an oil with a low content of erucic acid (called "double zero") have been developed in Canada (University of Manitoba) in 1974. By the use of this new variety, the standard Canola oil has been developed, the name evoking Canadian oil, which is an oil with less than 2% erucic acid and less than 30 μmol of GLSs per gram of defatted and dried meal (the seed must contain <30 μmol/g of any mixture of 3-butenyl GLS, 4-pentenyl GLS, 2-hydroxy-3-butenyl GLS, and 2-hydroxy-4-pentenyl GLS per gram DW [188]).

Table 3 Estimated total glucosinolate content of sprouts (7–12 days old, light-grown; mg/100 g FW)

Common name	Botanical classification	No of accessions	GSL			References
			Weighted mean[a]	Min	Max	
Broccoli	*B. oleracea* convar. *botrytis* var. *cimosa* (commonly var. *italica*)	9	**1297**	137	2757	[75, 96, 174, 175, 177–181]
Brussels sprouts	*B. oleracea* var. *gemmifera*	1	**1013**			[178]
Cabbage, white	*B. oleracea* convar. *capitata* var. *alba*	2	**1069**	753	1385	[178, 181]
Cabbage, red	*B. oleracea* convar. *capitata* var. *rubra*	3	**833**	516	1102	[174, 175, 178]
Cabbage, savoy	*B. oleracea* convar. *capitata* var. *sabauda*	1	**1299**			[178]
Cauliflower	*B. oleracea* convar. *botrytis* var. *botrytis*	2	**1178**	886	1470	[96, 178]
Chinese cabbage	*B. rapa* ssp. p*ekinensis*	1	**297**			[96]
Garden cress	*Lepidium sativum*	2	**695**	174	1216	[175, 178]
Kale	*B. oleracea* convar. *acephala*	4	**1206**			[96, 174, 178, 179]
Kohlrabi	*B. oleracea* var. *gongylodes*	2	**829**	668	990	[175, 178]
Mustard greens	*B. juncea*	1	**759**			[96]
Mustard	*Sinapis alba*	3	**834**	548	1138	[175, 178, 181]
Pak choi	*B. rapa* ssp. *chinensis*	14	**642**	353	1156	[96, 99]
Portuguese tronchuda cabbage	*B. oleracea* var. *costata*	1	**598**			[174]
Radish	*Raphanus sativus*	16	**676**	38	2036	[96, 151, 175, 176, 178, 179]
Rocket	*Eruca vesicaria* ssp. *sativa*	1	**742**			[178]
Rutabaga	*B. napus* var. *napobrassica*	3	**458**	144	842	[75, 175, 178]
Turnip	*B. rapa* var. *rapa*	4	**698**	164	1544	[75, 175, 178]

Data of the original study were converted to mg/100 g FW
[a]Mean weighted by the number of accessions of each study

Canola oil is generally consumed in its liquid form as salad oil, salad dressing, and cooking oil (pure or in blends with other oils), while a minor amount of this oil is used for margarine or shortening production.

5.2 Mustard

Mustard is a popular and widely consumed condiment generally used for sausages and meats and also as ingredient for sandwiches and salad preparation. It is prepared from a mixture of some *Brassica* seeds that are usually grinded with wine, vinegar, water, salt, and spices. The seeds of *Brassica nigra*, *Brassica juncea*, and *Sinapis alba* are the most frequently used, but also those of *Brassica carinata*, *Brassica carista*, and *Brassica eruca* can be used in some mustard preparation.

During the preparation process, the GLSs contained in the seeds are degraded by myrosinase to produce ITCs (responsible for the pungent flavor), so that no GLSs can be found in the end product [189, 190]. The type and level of GLS degradation products in mustard and, as consequence, the intensity of the associated pungent flavor greatly depend on the type of GLSs present in the seeds used for its production [189].

5.3 Sauerkraut

Sauerkraut is a very popular fermented product obtained from fermentation of shredded and salted white cabbage. It is the most well-known German food, but it is largely consumed in all Europe (especially Eastern Europe) and in many parts of the USA and Canada. It can be eaten raw or after cooking.

During the fermentation process, cabbage GLSs are degraded, with formation of ITCs and other degradation products (such as indole-3-acetonitrile as well as indole-3-carbinol that reacts with ascorbic acid forming ascorbigen) [123, 191], so that no detectable amounts of GLSs are present in sauerkraut at the end of the fermentation process [126]. The type and the level of GLS degradation products are strongly dependent on the content of native GLSs of the raw materials, as well as on the fermentation conditions.

6 Other Food

6.1 Caper (*Capparis spinosa* and *Capparis ovata*)

Capparis is a plant genus of the Capparaceae family that includes several species, among which the most common and studied are *Capparis spinosa* and *Capparis ovata*. These aromatic plants are largely distributed in the Mediterranean regions but are also present in Asia and Australia.

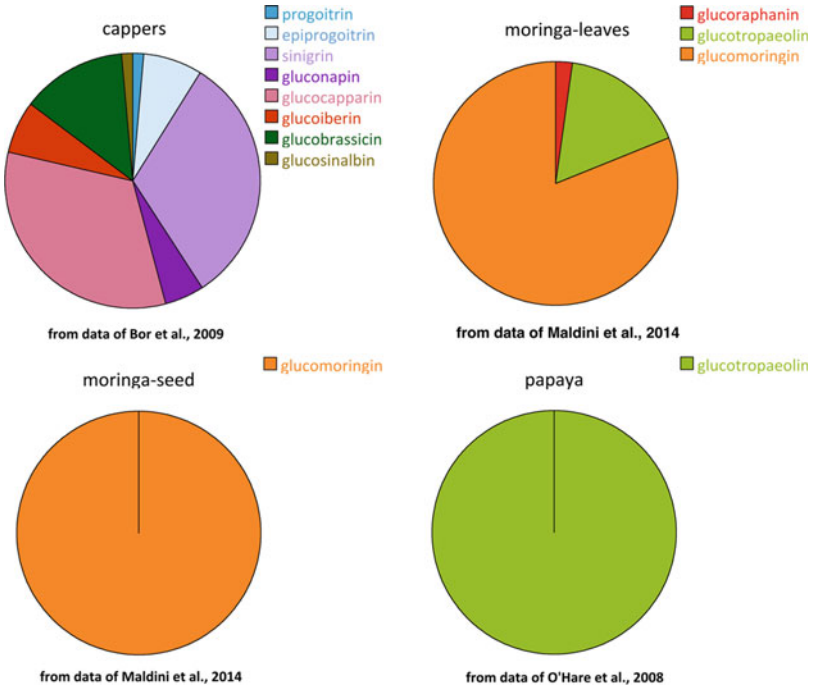


Fig. 3 Glucosinolates' profile of other food

Flower buds and fruits of *Capparis*, known as capers, are used as food ingredient, condiment, or seasoning and are highly appreciated for their particular aroma.

The few available data indicate that capers contain quite high level of GLSs (the mean total amount is about 360 mg/100 g FW that is higher than those found in many *Brassica* species); however, the intake by weight of this ingredient is so small that they do not seem to represent a good dietary source of these compounds. Glucocapparin is the distinctive GLS of this plant; in fact, it is almost exclusively present in *Capparis* genus. It is the major GLS in capers accounting for 40–80% of the total GLS [163, 164]; the aliphatic GLSs are particularly abundant, while indolic GLSs occurred only in small amount (Fig. 3). Finally, the few available data seem to indicate that there are great quantitative differences in GLS content between the two most studied species, *C. ovata* being richer than *C. spinose* [163] (Table 2).

6.2 Moringa or Drumstick Tree (*Moringa oleifera*)

Moringa oleifera is a fast-growing, medium-sized and drought-resistant tree native to many tropical and subtropical countries. Today it is widely grown in India, Africa, Central and South America, Hawaii, and throughout Asia.

Seeds, leaves, oil, sap, bark, roots, and flowers are widely used in traditional Indian medicine since a long time. In addition, many parts of the plant are commonly used for human consumption (principally fruit and leaves and also flowers, roots, and seed oil) in India, Pakistan, the Philippines, Hawaii, and many parts of Africa.

Very young pods are consumed raw, while mature pods are used in preparation of soups and stews. The younger leafy tips and tender leaves, as the young flowers and the flower buds, are consumed as side dish and in salad. Moringa young leaves are a very rich source of GLSs, with a mean content of 325 mg/100 g FW. Glucomoringin is the main GLS found in Moringa, it is found almost exclusively in this species, and it accounts for about 78% and 99% of the total GLS in leaves and seed pulp, respectively [166, 192] (Fig. 3).

6.3 Papaya (*Carica papaya*)

Papaya (*Carica papaya*) belongs to the family of Caricaceae and is one of the most cultivated plants in tropical and subtropical regions. It is grown in nearly all countries of the tropical Americas (Central and South America and the state of Hawaii) and in India, Sri Lanka, various Asian countries, as well as the Antilles and tropical Africa. Papaya is available year-round, and the ripe fruit is largely consumed as such or to make fruit salad, drinks, jam, jelly, candies, etc.

While *Capparis* and *Moringa* are close relatives of the Brassicaceae family (they all belong to the order of Capparales), *C. papaya* is one of the few edible species not related to Brassicaceae containing GLSs. Glucotropaeolin seems, at the moment, the unique GLS present in this plant (Fig. 3).

The content of glucotropaeolin is quite different in the papaya tissues; it is very high in seed and leaf, while it is lower in the edible flesh [168, 169]. The glucotropaeolin content in the pulp gradually decreases during fruit maturation [168], so that its content in fruit is greatly dependent on the maturation stage. This can explain the great data variability observed among studies: from 0 [170] to about 100 mg/100 g FW [169]. Because glucotropaeolin imparts an unpalatable flavor to the fruit (“nasturtium” flavor), the fruits high in glucotropaeolin tend to be disliked by consumers and then less consumed.

6.4 Honey

Honey represents the unique animal-derived food present in this list of GLS-containing food. In fact, while GLS degradation products have been reported outside the plant kingdom, for example, in cow milk [193], honey is the only animal product containing intact GLSs. These GLSs come from the pollen of plants belonging to the Brassicaceae family (e.g., *Brassica napus* or *Diplotaxis tenuifolia*) that are used for honey production [194]. It has been demonstrated that pollens of these plants contain low and variable amount of GLSs (from 0 to about 200 μg/100 g FW) [195], and very low amount of GLSs has been found in some single flower

honey [196]. However, the GLS amount is so low that honey cannot represent a dietary source of GLSs.

6.5 Impact of Post-Harvest Factors on GLS Content

In the above sections, GLS content has been mainly discussed as determined in the freshly harvested vegetables, but it is important to keep in mind that this original content can be, even substantially, affected by the several steps of the food supply chain occurring before actual consumption: transport, storage, industrial processing, and home preparation [72, 197–199]. It is generally agreed that the impact of post-harvest handling and storage is in most cases less pronounced than the effects of processing and preparation, even though the extent of these changes critically depends on the type of vegetables, the processing method, and the specific processing conditions applied [199]. Transport and storage are key steps in post-harvest life of vegetables before consumer purchase and environmental conditions in these steps are generally optimized with reference to visual quality. Vegetables of *Brassica* species exhibit a broad variety of plant structures, from roots to stems, sprouts, leaves, and inflorescences, with an associated variety in physiology and biochemistry and, hence, shelf life. In this respect, broccolis are very perishable vegetables, whereas cabbage and Brussels sprouts are less susceptible to senescence and decay and can sustain a longer shelf life [72]. Time and temperature are the most important factors affecting GLS content during transport and storage: broccoli inflorescences stored for 5 days at 4 °C show a relatively limited decrease in the total GLS content (from 4 to 16%), whereas a much marked degradation occurs at 20 °C (from 64% to 79%) [200]. A drastic reduction (55%) of glucoraphanin content may even occur after 3 days of storage at ambient temperature, while the beneficial effect of cold storage takes place in broccoli stored both in open boxes and packaged in plastic bags [201]. With regard to broccoli, it has been found that the degree of total GLS losses generally corresponds to the degree of visual quality degradation [201]. Interestingly, different classes of GLS are affected in a different way by the same storage conditions: while glucoraphanin losses are higher (up to 82% after 5 days at 20 °C) than average GLS losses, indole GLS tends to be less strongly affected by storage [200], and in some studies their content increases during storage at 10 °C [202] or ambient temperature, while all other GLSs decrease [203].

The impact of freezing without previous inactivation of myrosinase may be remarkable: the global freezing-thawing cycle has been reported to reduce by more than 30% the total GLS content in various *Brassica* vegetables [204] and to completely deplete it in sprouts of sea kale [205]. GLS content of blanched vegetables is not affected by storage in a freezer [197], even though the preliminary blanching process may have a more or less strong impact (from 2% to 74% losses) depending on blanching time and conditions [72].

In many cases, *Brassica* vegetables are subjected to industrial or domestic processing before consumption. There is a great variety of preparation methods worldwide, ranging from boiling to steaming, microwave cooking, stir-frying, and

fermentation, the combination of procedures and ingredients changing across regions and cultures [199]. In addition, variability in home preparation is further widened by individual preferences about sensory properties of cooked vegetables, which dictate time-temperature settings of the applied domestic cooking processes. Among cooking methods, boiling is probably the most common used for *Brassica* vegetables, at least in Western countries, and in some instances it may produce a substantial depletion of the GLS content present in the raw vegetable. Losses of GLS due to boiling are strongly influenced by the time of cooking, ratio of vegetable to water, and type of vegetable and mainly occur through leaching into cooking water [199]. Regarding the effect of the type of vegetable, it has been observed a progressive increase of GLS losses from 58% to 65%, 75%, and 77%, moving from Brussels sprouts to green cabbages, cauliflowers, and broccoli, respectively, after 30 min of boiling [204]. Much lower, or no, losses have been observed when shorter time is applied to boiling of broccoli florets (3 to 8 min) and Brussels sprouts (10 min), whereas significant losses (38%) have still been found after a relatively short boiling time (10 min) on cauliflowers [120, 206]. Steaming, by preventing direct contact to boiling water, results as a much more effective method in preserving the original GLS content of the freshly harvested vegetable: tests carried out on cabbages, broccoli, cauliflowers, and Brussels sprouts, by varying cooking time up to 20 min, have shown either slight decreases or increases in total GLS content [199]. The increase of GLS content may be ascribed to an increased extractability of GLS from the food matrix in the analytical process due to the effect of the heat treatment. Microwave cooking has, in general, an intermediate impact when compared to boiling and steaming, but, similar to the other methods, the degree of GLS losses depends on the specific conditions of cooking applied, in terms of time, power output, and addition of water prior to microwave processing. The level of microwave power is linked to the extent of losses of the total GLS in broccoli florets, whereas slight decreases of total GLS content have been observed on Brussels sprouts, cabbages, and cauliflowers when applying varying power levels [199]. In some case the application of microwave processing may result in a marked increase (up to 180%) of total GLS content, as observed on red cabbage, probably due to an increased extractability from the plant tissue after heating [207]. Addition of water to the vegetable promotes leaching of GLS into the cooking water [208]. Stir-frying is one of the most popular processing methods in several Asian countries; however, relatively few data are available in the literature about the effects of this method of cooking. Somewhat inconsistent results have been reported, ranging from slight losses to substantial reduction of total GLS content in broccoli florets after varying times and conditions of stir-frying [199].

Fermentation, as mentioned above, is a quite popular processing method not only in some European countries, for the preparation of sauerkraut, but also in Asian countries, for the preparation of a variety of fermented products [199]. In the preparation of sauerkraut, fermentation results in complete degradation of GLS just after 7 days from the start of processing and in the formation of GLS bioactive breakdown products, such as sulforaphane, ascorbigen, indole-3-carbinol, and many others, whose levels undergo further changes along the following storage time

[126]. The content of the GLS breakdown products in the food at the time of consumption depends on the level of native GLS in the raw material and also on the conditions of fermentation (application of starter bacteria cultures, the type of bacteria strains applied) [72].

7 Conclusions

Among all known phytochemicals, GLSs are of special interest both for their health-related effects and for their relatively high food concentration (ranging from 20 to about 5000 mg per 100 g FW). However, specific recommendations for increasing GLS-containing food consumption have not been established yet [209]. Among the reasons leading to this lack of specific dietary guidelines, there is the persistence of a number of uncertainties in the causal relationship between GLS-rich food consumption and the health output. First, GLS/ITCs associated to both beneficial and detrimental health effects coexist in the same food. Second, there are insufficient data about GLS-containing food consumption. Third, the use of cruciferous consumption as a proxy for GLS intake represents an epidemiological study confounder [210]. In fact, cruciferous vegetables contain other bioactive molecules in addition to GLS. Moreover, estimation of GLS daily dietary intake is especially difficult due to the lack of a comprehensive GLS food composition database, taking into consideration also the food processing-induced changes. All these factors contribute to the insufficient strength of epidemiological evidence (see as examples the relation between esophageal and endometrium cancer and cruciferous vegetable consumption [211]).

Therefore, a clear qualitative and quantitative description of the presence of GLSs in food represents a prerequisite in order to define GLS dietary guidelines.

Acknowledgments This work was supported by Italian Ministry of Agriculture, Food & Forestry (MiPAAF) grants “NUTRIGEA” (DM 30281 23/12/2009).

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Accumulation of Glucosinolates in Broccoli

4

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Abstract

Broccoli is a rich source of health-promoting antioxidants and anticarcinogenic glucosinolates, which has long been recognized for their outstanding benefits to human nutrition and plant defense. The composition and content of glucosinolate are closely associated with the flavor and anticancer activity of broccoli. Up to now, broccoli is among a few *Brassica* vegetables, in which the biosynthetic pathway of glucosinolate has been widely studied and has attracted extensive attention. Recent studies in glucosinolate research have also identified the genetic variations, as well as the functions of individual glucosinolate profiles and their degradation products in broccoli, which provide the basic aims and powerful strategies for breeding of broccoli varieties with optimal glucosinolate composition and content. To fully exploit the potentially beneficial effects of broccoli, it is important to investigate the glucosinolate variation and metabolism across the whole food chain, from preharvest production to post-harvest storage, processing, and cooking. This chapter provides a general overview of glucosinolate biosynthetic pathway, as well as the genetic variation and function of individual glucosinolate profiles in broccoli, highlights the recent advances in glucosinolate accumulation of broccoli upon different preharvest and post-harvest handlings, and discusses their potential application in broccoli breeding, production, storage, processing, and consumption.

Keywords

Glucosinolate • Broccoli • Light • Chemical regulation • Post-harvest handling • Processing • Cooking

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_16

Abbreviations

6-BA	6-Benzylaminopurine
CA	Controlled atmosphere
EBR	Epibrassinolide
ESP	Epithiospecifier protein
GS	Glucosinolate
I3C	Indole-3-carbinol
ITC	Isothiocyanate
JA	Jasmonic acid
LDPE	Low-density polyethylene
M_0	No holes
M_1	Two microholes
M_2	Four macroholes
MAP	Modified atmosphere packaging
MDA	Malondialdehyde
MeJA	Methyl jasmonate
NSP	Nitrile-specifier protein
PEF	Pulsed electric field
RH	Relative humidity

Contents

1 Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is an economically important vegetable crop in many countries and has been highly valued by consumers due to its flavor as well as its nutritional components like minerals, vitamins, polyphenols, and other beneficial phytochemicals, particularly potent anticarcinogenic glucosinolates (GSs) [1–4]. Epidemiological studies have suggested that the consumption of broccoli could lower the risk for the development of certain forms of cancers, which have been attributed to glucosinolates and their degradation products [5, 6].

Glucosinolates, a group of sulfur- and nitrogen-containing secondary metabolites, mainly belong to the Brassicaceae family. The general structure of glucosinolate consists of a β-D-thioglucose group, a sulfonated oxime group, and a side chain derived from amino acids [7]. More than 200 glucosinolates have been identified so far [8] and can be divided into three classes on the basis of their derived amino acid precursors: aliphatic glucosinolates, indole glucosinolates, and aromatic glucosinolates. Usually, glucosinolates with different structures can be degraded into various biologically active breakdown products in broken tissues and living cells of plant, as well as gastrointestinal tract of mammalian [9–14], which contribute to the flavor, anticarcinogenic activity, and resistance of broccoli [15]. As maintaining and enhancing good health through dietary habits have been proposed to deal with increased lifestyle diseases in current society, optimal glucosinolate profile and content in broccoli are expected. The purpose of this chapter is to review the research findings related to glucosinolate biosynthetic pathway and variation among different broccoli genotypes, as well as the regulation of glucosinolate accumulation along the whole food chain for better retention of glucosinolates in broccoli.

2 Biosynthetic Pathway of Glucosinolates in Broccoli and *Brassica* Plants

So far, the biosynthetic pathway of glucosinolates has been successfully elucidated in *Arabidopsis* and summarized in several review papers [7, 16]. Generally, aliphatic glucosinolate biosynthesis consists of three separate steps: (i) chain elongation of precursor amino acid, (ii) development of the core glucosinolate structure, and (iii) secondary modifications of the amino acid side chains [7, 16, 17]. There are five reactions required for precursor amino acid elongation: an initial and final transamination, acetyl-CoA condensation, isomerization, and oxidative decarboxylation [18, 19]. In this process, branched-chain amino acid aminotransferase (BCAT), bile acid transporter 5 (BAT5), methylthioalkylmalate synthase (MAM), isopropylmalate isomerase (IPMI), and isopropylmalate dehydrogenase (IPMDH) are involved [20–26]. The core glucosinolate structure formation begins with oxidation of precursor amino acids by cytochrome P450 monooxygenases (cytochrome P450s) of the CYP79 family and then CYP83 family, followed by further metabolic process catalyzed by *C-S* lyase, glucosyltransferase, and sulfotransferase [27–35]. The secondary modifications are attributed to several loci, including GS-OX which catalyzes methylthioalkyl to methylsulfinylalkyl glucosinolates, GS-ALK/AOP2 which controls the conversion to alkenyl glucosinolates, GS-OHP/AOP3 which is responsible for the production of 3-hydroxypropyl glucosinolates, and GS-OH which participates in the production of 2-hydroxy-3-butenyl glucosinolate in aliphatic glucosinolate biosynthesis [36–38], as well as cytochrome P450 CYP81Fs and indole glucosinolate methyltransferase (IGMT) in indole glucosinolate biosynthesis [39–42]. Furthermore, several regulators of glucosinolate biosynthesis have also

been identified, such as nuclear-localized calmodulin-binding protein IQD1, DOF transcription factor AtDof1.1, sulfur limitation1 (SLIM1), TU8, and six R2R3-MYB transcription factors as well as three basic helix-loop-helix (bHLH) transcription factors [43–54].

As the genome sequence of *Brassica* crops including *B. oleracea* has been reported [55], genes related to glucosinolate biosynthesis in *Brassica* crops were identified gradually with the assistance of bioinformatics. The aliphatic glucosinolate biosynthesis is described in Fig. 1 based on previous studies and genome sequence. *BoGSL-ELONG*, *BoGSL-ELONGL*, *BoGSL-PRO*, and *BoGSL-PROL*, which control chain elongation of precursor amino acid in *B. oleracea*, have been cloned or inferred [56–59]. Li and Quiros [56] have conducted the genetic

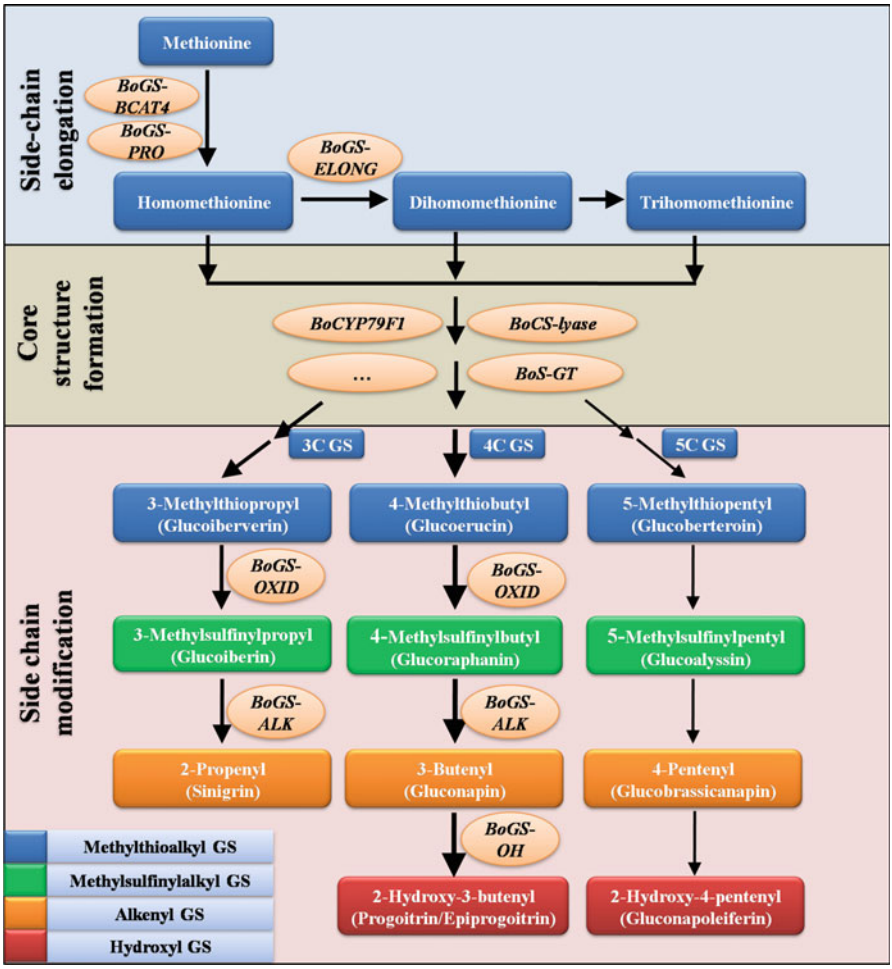

Fig. 1 Aliphatic glucosinolate biosynthesis in *Brassica oleracea*

analysis, expression, and molecular characterization of *BoGS-ELONG*, which was responsible for the synthesis of four-carbon glucosinolates in *B. oleracea*. However, *BoGS-ELONGL*, which corresponds to *MAM-L* in *Arabidopsis,* was probably nonfunctional, at least in broccoli variety 'Early Big' [58]. In addition, genetic analysis suggested that *BoGS-PRO* was associated with the biosynthesis of three-carbon side-chain glucosinolates [57], while the function of *BoGS-PROL* has not been assessed yet [59]. *BoGS-ALK* was also cloned and demonstrated to be responsible for alkenylation in side-chain modification in glucosinolate pathway [57]. Furthermore, *BoCYP79F1*, *BoCS-lyase*, *BoS-GT* (S-glucosyltransferase), *BoGS-OH*, and the candidate gene for *BoGS-OXID* have also been inferred [60, 61].

Although *Brassica* crops share the similar pathway of glucosinolate biosynthesis with *Arabidopsis*, the genes involved are different in sequence identities with their counterparts in *Arabidopsis* as well as with other *Brassica* crops. For instance, *MAM* family contains high copies in *B. rapa* and *B. oleracea*, however, with different expression patterns, resulting in the fact that the major aliphatic glucosinolates are 3C and 4C glucosinolates in *B. oleracea* while 4C and 5C glucosinolates in *B. rapa* [55].

3 Genetic Variation and Function of Glucosinolate Profiles in Broccoli

3.1 Genetic Variation of Glucosinolate Composition and Content

According to previous reports, there are up to 17 different kinds of glucosinolates identified in broccoli (Table 1) with a genetic variation in their composition and content [62–65]. Nevertheless, glucoraphanin is the predominant methionine-derived aliphatic glucosinolate in most varieties. In an early report, Carlson et al. [62] determined the variation of nine glucosinolate profiles in six commercial cultivars and found that glucoraphanin contents were from 29.2 to 88.3 μmol 100 g^{-1} fresh weight (FW). In 1999, Kushad et al. [64] identified 14 glucosinolates in 50 broccoli accessions, and glucoraphanin levels were in a scope from 0.8 to 21.7 μmol g^{-1} dry weight (DW). Similarly, Farnham et al. [66] demonstrated that the amount of glucoraphanin varied from 0.04 to 2.94 μmol g^{-1} FW in 71 pure lines and 5 hybrid checks. The results from Chinese broccoli germplasms indicated that the glucoraphanin concentrations ranged from 0.06 to 24.17 μmol g^{-1} DW in 143 pure lines and from 1.57 to 5.95 μmol g^{-1} DW in 5 commercial varieties [65], while progoitrin levels in 5 commercial cultivars differed from 1.77 to 6.07 μmol g^{-1} with a mean of 3.20 μmol g^{-1} DW. However, reports about high levels of progoitrin or epiprogoitrin were few in primary broccoli cultivars from America [62, 64, 67], England [68], Germany [69], Portugal [70], and Spain [71]. Likewise, relatively higher concentration and variation in indole glucosinolate were reported by most surveys [65, 68–71] except for Kushad et al. [64]. Generally, the content of aromatic glucosinolates, mainly gluconasturtiin, is much lower than aliphatic or indole group. Moreover, some specific lines with altered glucosinolate profiles were observed in broccoli germplasms [65]. Former studies have demonstrated that the accumulation

Table 1 Trivial and chemical name of glucosinolates identified in broccoli

Trivial name	Chemical name (side chain)	**Corresponding** isothiocyanate **(ITC)/indole**	References
Aliphatic glucosinolate			
3-Carbon chain length			
Glucoiberverin	3-Methylthiopropyl	Iberverin	[72]
Glucoiberin	3-Methylsulfinylpropyl	Iberin	[73]
Sinigrin	2-Propenyl	Allyl isothiocyanate	[74]
4-Carbon chain length			
Glucoerucin	4-Methylthiobutyl	Erucin	[75]
Glucoraphanin	4-Methylsulfinylbutyl	Sulforaphane	[76]
Gluconapin	3-Butenyl	3-Butenyl isothiocyanate	[77]
Progoitrin	(2R)-2-hydroxy-3-butenyl	Oxazolidine-2-thione	[78]
Epiprogoitrin	(2S)-2-hydroxy-3-butenyl	Oxazolidine-2-thione	[79]
5-Carbon chain length			
Glucoalyssin	5-Methylsulfinylpentyl	–	–
Glucobrassicanapin	4-Pentenyl	4-Pentenyl isothiocyanate	[80]
Gluconapoleiferin	2-Hydroxy-4-pentenyl	–	–
Aromatic glucosinolate			
Gluconasturtiin	2-Phenylethyl	Phenethyl isothiocyanate	[81]
Indole glucosinolate			
Glucobrassicin	3-Indolylmethyl	Indole-3-carbinol	[82]
Neoglucobrassicin	*N*-methoxy-3-indolylmethyl	*N*-methoxyindole-3-carbinol	[83]
4-Hydroxy glucobrassicin	4-Hydroxy-3-indolylmethyl	–	–
4-Methoxy glucobrassicin	4-Methoxy-3-indolylmethyl	4-Methoxyindole-3-carbinol	[84]

of aliphatic glucosinolates in broccoli head is mainly regulated by genetic factors, while the content of indole glucosinolates is greatly affected by environment and environment × genotype interaction [63, 85, 86]. Recently, the identification of two glucosinolate transporters, GTR1 and GTR2, which are response for the transportation of glucosinolates in *Arabidopsis*, provided us a useful means to control the allocation of glucosinolates in different tissues [87].

The breakdown pathway as well as products of glucosinolates is a leading area in glucosinolate research in recent years. The classical breakdown pathway in plant depends on classic myrosinase (β-thioglucoside glucohydrolase, TGG) [17], which is localized in separate plant cells or in separate intracellular compartments with glucosinolates in intact plant tissue while tissue damage brings them together and initiates TGG-catalyzed glucosinolate hydrolysis [88, 89]. In addition, an atypical myrosinase, PENETRATION2 (PEN2), is thought to be involved in a new indole glucosinolate catabolic pathway in living plant cell [39, 40, 90]. Furthermore, intact glucosinolates could also been degraded by the resident microflora of the gastrointestinal tract to form ITCs, amines, or nitriles depending on the type of bacterial myrosinase-like activity involved [10–14].

In *Arabidopsis*, there are six myrosinase genes with different gene sequences and expression patterns [9]. Besides, some specific proteins, such as epithiospecifier protein (ESP) and nitrile-specifier protein (NSP), are also involved in the hydrolysis of glucosinolates, affecting the final breakdown products. Mithen et al. [91] compared the ability to induce phase II detoxification enzymes in two broccoli lines at similar glucosinolate level with 'Marathon', a standard commercial broccoli cultivar. They found one line showed 80 times the ability to induce quinone reductase compared to 'Marathon', while the other showed little induction. The detection of breakdown products showed that plenty of ITCs (95%) were degraded from glucoiberin and glucoraphanin, the predominant glucosinolates of broccoli, in the former line, while high levels of nitriles, and only 1% ITCs, were formed from glucoiberin and glucoraphanin in the later line. The results of sequences for *B. oleracea* sp. *capitata* indicated that the *B. oleracea* species also had a complex glucosinolate-myrosinase system [55]. In vitro, a recombinant broccoli ESP from cv. Packman directed myrosinase-dependent degradation of epiprogoitrin toward the formation of epithionitrile and glucoraphanin to sulforaphane nitrile instead of sulforaphane [92]. In addition, the variety of breakdown products from aliphatic glucosinolates, mainly glucoraphanin, in floret and sprout among different broccoli varieties was observed in some studies [91–93]. These researches indicated that the typical glucosinolate-myrosinase was much complex upon tissue damage, and genetic effect played an important role in hydrolysis of glucosinolates, especially aliphatic glucosinolates.

3.2 Biological Functions of Individual Glucosinolate Profiles

Glucosinolates and their hydrolysis products in broccoli have been considered to be bioactive, while some of them may have adverse effects for human and animal. There are in vitro and in vivo evidences that ITCs play multiple roles in cancer prevention. For example, some ITCs from methionine- and aromatic-derived glucosinolates such as sulforaphane, allyl isothiocyanate, and phenethyl isothiocyanate can strongly inhibit phase I enzymes and induce phase II enzymes, as well as promote cell cycle arrest and apoptosis in various cancer cell lines [94–100]. Indole-3-carbinol (I3C), a breakdown product from indole glucosinolate, has been found to exert chemoprotective activity through altering estrogen metabolism [101, 102]. Moreover,

ITCs, notably sulforaphane, make contributions to cancer protection via inhibition of tumor invasion and angiogenesis [103], anti-inflammatory [104], as well as immunomodulatory activities [98]. In addition to these cancer chemopreventive properties, ITCs also play important roles in protection of the central nervous system [105], preventing against cardiovascular disease [106, 107] and bacterial infection [76].

As we have mentioned above, glucosinolates could be degraded to not only ITCs but also nitriles due to different hydrolytic environments. Previous research verified that quinone reductase and glutathione S-transferase activities in hepatic, colonic mucosal, and pancreatic were induced by high doses of sulforaphane but not by sulforaphane nitrile. Quinone reductase activity of sulforaphane and sulforaphane nitrile treated Hepa 1c1c7 cells also suggested that production of sulforaphane rather than sulforaphane nitrile could increase the potential chemoprotective effects of broccoli [108].

Glucosinolates and their breakdown products also show some adverse effects besides their healthy benefit functions. Indole derivatives (e.g., I3C) have been classified as bifunctional inducers [109, 110]. It has been reported that I3C inhibited the development of cancer in animals when given before or with a carcinogen, whereas enhanced development of cancer was observed when administered after a carcinogen in some cases [5]. Certain glucosinolates, such as sinigrin, progoitrin, and gluconapoleiferin, are responsible for bitter taste [111], which may decrease consumer acceptance. Furthermore, progoitrin has been considered as natural toxicants for its derivative has goitrogenic effects on mammals [112]. However, the amount of progoitrin in broccoli is quite low, and the occurrence of these substances is dependent on many cofactors. In addition, this malnutritional effect can also be avoided by normal iodine intake [113].

Glucosinolates and their hydrolytic products have been demonstrated to be crucial in plant defense response. They can stimulate feeding or oviposition by crucifer-specialist herbivores [16, 114–116]. Aliphatic glucosinolates were reported to be important for resistance of plants to pests [117], and sulforaphane functioned in nonhost resistance in *Arabidopsis-Pseudomonas* pathosystem [118]. Moreover, PEN2-dependent potential hydrolysis products of 4-methoxy glucobrassicin were found to activate innate immunity, leading to protection against fungal penetration [39, 40], while TGG-catalyzed degradation of indole glucosinolates attenuated mycotoxin fumonisin B1-induced programmed cell death in *Arabidopsis* [119].

4 Breeding of Broccoli Varieties with Optimal Glucosinolate Composition and Content

A high-glucoraphanin commercial hybrid, Beneforté®, was developed by Mithen's group through genome introgression from the wild species *Brassica villosa* [120]. In former surveys of this group, a major quantitative trait locus (QTL) on linkage group 2 that determined the concentrations of methionine-derived glucosinolates in the high-glucoraphanin hybrids was found, and a microsatellite marker OI12-F02 originated from a wild species *Brassica villosa* was developed [68, 91]. Recently,

researchers of this group cloned the major dominant QTL and identified an *MYB28* allele resulted in higher expression of *MYB28* in the leaves of high-glucoraphanin hybrids compared to the standard broccoli cultivars. There are three SNPs identified in the *MYB28* allele: two located in the intronic region upstream of exon 3 and one at the 5'-end of exon 3 [120]. Sotelo et al. [121] used a DH mapping population of F_1 from a Chinese kale and a DH broccoli line to detect the significant QTLs in leaves, flower buds, and seeds. They identified three loci controlling the content of aliphatic glucosinolates and four loci manipulating the accumulation of indole glucosinolates. Therefore, besides *MYB28* allele from *Brassica villosa*, there might be other gene/locus variation determining the variety of aliphatic glucosinolate level in broccoli. In addition, significant general combining ability was observed between glucoraphanin concentration and total head content in 2-year assessment of 36 combinations by crossing nine pure parents' lines [122]. Based on putative high-glucoraphanin materials, Gu et al. [86] also obtained sixteen high-glucoraphanin hybrids with acceptable agronomic traits by direct crossbreeding and screened two high-glucoraphanin parental lines.

The basic aims of breeding broccoli varieties with optimal glucosinolate composition and content include the following: (1) to reduce the content of anti-nutritional component, notably 2-hydroxy-3-butenyl glucosinolate; (2) to improve the content of beneficial compositions (glucoraphanin, glucoiberin, and glucoerucin) for better nutritional qualtiy, and to regulate the biosynthetic and hydrolytic pathways of glucosinolate profiles involved in interaction with pathegens and pests for enhanced resistance; (3) to promote the formation of ITCs rather than nitriles; (4) to preserve acceptable agronomic traits for head and considerable seed yield for sprout; and (5) to preserve other beneficial compounds, such as vitamins, minerals, and flavonoids. The related principles/strategies to reach above aims are as follows:

1. To select the parents without alkenyl glucosinolates through direct detection or markers (e.g., *BoGS-ALK* and *BoGS-OH*) selection.
2. To detect glucosinolates directly or analyze the critical genes or markers, which could regulate the aliphatic glucosinolate accumulation in edible parts. For example, the microsatellite marker OI12-F02 or other primers designed for the *BoMYB28* allele, which are located in chromosome 2.
3. Due to no available markers for breakdown products variation, direct detection of breakdown products is required and enough for determination of the ability to deliver aliphatic glucosinolates to correspondent ITCs.
4. Other nutrients are not affected in the final hybrid. Furthermore, glucosinolate metabolism is widely involved in various defense reactions, especially against insects and pathogens. It is needed to consider the adaptability and resistance of glucosinolate hybrid in complex environments.

In addition to genotype, glucosinolate contents in broccoli are also influenced by many factors along the whole food chain from farm to table. Preharvest factors are pivotal to enhancement of glucosinolate accumulation, and suitable post-harvest handlings are essential for maintaining the glucosinolate content of broccoli products

during the storage after harvest, while processing and cooking are other effective ways for glucosinolate retention before consumption.

5 Factors Influencing Glucosinolate Accumulation in Broccoli

5.1 Preharvest Factors

Plenty of investigations have been conducted to search useful tools for modulation of glucosinolate accumulation in broccoli before harvest. Regulation of glucosinolate by light as an environmental factor, as well as by phytohormones, sugars, salinity, and fertilization as chemical regulation, has been widely studied, and many valuable results were obtained.

5.1.1 Light

Light is a crucial environmental factor in plant life, which regulates seed germination, phototropism, and flowering [123, 124]. Recent studies have revealed that light influenced the accumulation of phytochemicals such as vitamin C, phenolic compounds and carotenoids, as well as glucosinolates [125–128]. It has been reported that UV-B radiation caused an induction of glucosinolate accumulation, especially of glucoraphanin and 4-methoxy glucobrassicin in broccoli sprouts and florets [129, 130]. In contrast, preharvest supplemental far-red light resulted in reduction of glucosinolate content, whereas supplemental red and blue light did not show a significant effect in broccoli florets [131].

5.1.2 Phytohormones

According to previous studies, glucosinolate accumulation in broccoli can be manipulated through treatment with several kinds of plant hormones, such as jasmonates, auxin, and brassinosteroids (BRs).

It has been shown that jasmonic acid (JA) and methyl jasmonate (MeJA) enhanced glucosinolate accumulation in broccoli. Liuann et al. [132] reported that significantly increased levels of indole glucosinolates including glucobrassicin, neoglucobrassicin, and gluconasturtiin were observed in 250 μM MeJA-treated broccoli (*B. oleracea* var. Green Magic), while the contents of aliphatic glucosinolates remained unchanged. Similar results were also observed in another individual survey, which demonstrated that the content of indole glucosinolates was increased by 3- to 20-fold upon JA treatment when compared to the control [133].

Since the indole acetaldoxime serves as a common precursor for indole glucosinolate and indole-3-acid (IAA) biosynthesis, close attention has been paid to the cross talk between these two kinds of indole compounds [134]. In broccoli, exogenous auxin treatment generally resulted in substantially higher levels of glucosinolates, especially indole glucosinolate. However, the effects of auxin varied with the types and concentrations used. IAA application at low concentration

(0.1 mg L^{-1}) led to the highest content of glucosinolates, followed by 0.1 mg L^{-1} indole-3-butyric acid (IBA), while 1 mg L^{-1} naphthalene acetic acid (NAA) treatment resulted in the lowest accumulation of glucosinolates [135].

Our previous survey has found that BR treatment downregulates glucosinolate accumulation in *Arabidopsis* [136], but a dose effect of BR existed in broccoli. Our other survey showed that the content of total glucosinolates and glucoraphanin in broccoli sprouts treated with 2 nM epibrassinolide (EBR) plus 40 mmol/L NaCl was increased by 86% and 85%, respectively. However, the reduction in glucosinolate content was observed due to the enhanced activity of myrosinase upon 20 nM EBR treatment alone [137].

5.1.3 Sugars

Sugar is not only an important source of carbon and energy but also an effective signal molecule modulating many developmental and metabolic processes in all phases of plant life cycle [138–143]. Our former studies have shown that glucosinolate accumulation in broccoli sprouts was enhanced by various kinds of sugars including sucrose, glucose, fructose, and mannitol [144, 145], with sucrose being the most effective one. Though mannitol treatment also led to an increase in glucosinolate content, the effect was not as strong as that resulted from sucrose treatment, which suggested that sucrose might function as a signal instead of osmotic stress in inducing glucosinolate accumulation [145]. Moreover, different concentrations of sugars have been demonstrated to have distinct influences on glucosinolate accumulation in broccoli sprouts. A relatively higher concentration of 176 mM sucrose and mannitol treatments dramatically increased glucosinolate content, whereas no significant difference was found after 88 mM sucrose or mannitol treatments [145]. As the glucosinolate level in broccoli is a reflection of two opposing physiological processes, glucosinolate biosynthesis and hydrolysis by myrosinase [146], two reasons may account for increased glucosinolates by sugar treatment. For one thing, the biosynthesis of glucosinolate might be induced by sugar treatment. It has been reported that *Bo-ELONG*, an important biosynthetic gene of aliphatic glucosinolate biosynthesis, was upregulated in broccoli sprouts by sucrose application [144]. For another, the activity of myrosinase was not changed after sucrose treatment, indicating that the hydrolysis of glucosinolate might not be affected by sugar treatment [144].

5.1.4 Salinity

Plants exhibit morphological or physiological alterations when subjected to salt stress. Besides, several reports indicated that salt stress was an important abiotic factor regulating glucosinolate accumulation in broccoli. The research by Carmen et al. [147] demonstrated that the total glucosinolate content in broccoli leaves was significantly increased after salt treatment and application of 80 mM NaCl displayed a more obvious effect when compared with 40 mM NaCl. This result was consistent with the data obtained from another research, which indicated a tendency of increase for the total glucosinolates in broccoli leaves upon NaCl treatment at concentrations of 60 mM and 90 mM [148]. However, studies in broccoli sprouts presented a

contradictory result. We found that application of 40 mM and 80 mM NaCl did not notably enhance the accumulation of glucoraphanin in 4-day-old broccoli sprouts [149]. Similarly, our another study based on 7-day-old broccoli sprouts showed that total glucosinolate level was markedly decreased after treatment with NaCl at concentrations of 20, 40, and 60 mM [150]. These different responses might be due to rapid growth of sprouts in which Na^+ and $C1^-$ ions become utilizable or different broccoli organs used for analysis [150].

5.1.5 Fertilization

The content of glucosinolate, a category of nitrogen- and sulfur- containing secondary metabolites, could be remarkably affected by nitrogen (N) and sulfur (S) fertilization. It has been demonstrated that levels of glucosinolates in broccoli were decreased by N fertilization but increased by N deficiency [151–153]. Schonhof et al. [152] reported that the elevation of glucosinolate content by N deficiency was mainly attributed to the presence of the alkyl glucosinolates, glucoraphanin and glucoiberin. However, in other *Brassica* crops, such as oilseed rape and Indian mustard, the levels of glucosinolates increased upon N application [154, 155]. This distinct response might be due to the difference of species used. In contrast to N fertilization, S fertilization usually led to an increase of glucosinolate content in broccoli in many cases [156]. However, conflicting result has also been demonstrated by another report which showed that broccoli sprouts did not benefit from S fertilization [157].

In addition to N and S fertilization, other fertilizer applications have also been indicated to influence glucosinolate accumulation. It has been reported that total glucosinolate contents in two broccoli cultivars (‘Calabrese’ and ‘Southern star’) were notably increased by organic and bioorganic fertilizers supply, respectively [158]. This is probably because organic and bioorganic manure can serve as alternative mineral fertilizers to improve soil structure [159] and microbial biomass [160].

Selenium (Se), an essential trace element for humans and mammals, often substitutes for S in physiological and metabolic processes in plants due to their chemical and physical resemblance [161]. Selenite and selenate salts, which are taken from the soil by plants through the sulfate absorption pathway, have been shown to improve the antioxidant status of plants. In addition, selenoglucosinolates displayed a higher anticarcinogenic activity than thioglucosinolates [162]. Se fertilization has been reported to exert no significant influence on glucosinolate levels in both broccoli sprouts [163–165] and florets [166]. Hence, Se fertilization is a good approach to accumulating selenium while maintaining glucosinolate content.

5.2 Post-Harvest Handlings

Broccoli is a highly perishable product, whose shelf life and visual quality strongly rely on storage conditions, such as temperature, atmosphere composition, and relative humidity (RH) [146, 167, 168]. Generally, the declines of their visual quality

are accompanied by the loss of nutrients including glucosinolate. Cooling, controlled atmosphere (CA), and modified atmosphere packaging (MAP) are widely used methods to extend the shelf life and reduce the nutrient loss. In addition, inhibiting the action of ethylene by 1-methylcyclopropene (1-MCP) treatment is also an effective way to improve the shelf life and quality of horticultural crops. The effects of these post-harvest handlings on glucosinolates of broccoli are discussed here.

5.2.1 Temperature

Glucosinolate contents generally decrease during post-harvest storage, which is coincided with the loss in visual quality of broccoli, and low temperatures clearly delay the quality decline [167]. Rangkadilok et al. [169] reported a more than 50% decrease in glucoraphanin concentration in 'Marathon' broccoli heads after 7-day storage at 20 °C. In contrast, no considerable decline was found after 7-day storage at 4 °C [169]. These results were consistent with the finding of Rodrigues and Rosa [170] that glucoraphanin levels declined to 82% when principal inflorescence of broccoli was left at 20 °C for 5 days but only 31% at 4 °C. The possible explanation is that plant cells are most likely to rapidly become damaged due to the loss of cellular integrity under high temperature and thus allowed the mixing of myrosinase and glucosinolates, resulting in the degradation and rapid decrease of glucosinolates. However, contradictory results were found in several other surveys. The study of Howard et al. [171] showed that sulforaphane decreased by approximately 50% after 21-day storage at 4 °C, and the severe decrease occurred within the first 7 days after harvest. Interestingly, the content of 4-methoxy glucobrassicin in broccoli florets stored at 10 °C increased from 0.4 μmol · g^{-1} DW to 1.8 μmol · g^{-1} DW after 9-day storage [172]. Likewise, the contents of 4-hydroxy glucobrassicin and 4-methoxy glucobrassicin in broccoli increased notably after chopping and 48 h storage at room temperature [173]. Moreover, the elevated level of 4-methoxy glucobrassicin in broccoli florets during the first day of storage at 20 °C was also observed by Yuan et al. [174]. It seems that the post-harvest enhancement of some indole glucosinolates counteracted the myrosinase-mediated degradation. Verkerk et al. [173] also proposed a stress-induced increase of glucosinolates by yet unknown mechanism, which plays a vital role in maintaining glucosinolate content of broccoli during post-harvest storage.

In addition to refrigeration, freezing is widely used in food industry. Usually, products are blanched before freezing, which can inactivate myrosinase as well as other enzymes causing deterioration. Rodrigues and Rosa [170] regarded freezing as the best method for preserving the glucosinolates in broccoli. However, blanching before freezing led to severe loss of glucosinolates by leaching them into water [175, 176]. Considering this, refrigeration might be the better procedure for storage of broccoli in comparison with freezing.

Although "cool as soon as possible" is the general recommendation for broccoli handling, delays of several hours before cooling may be encountered in post-harvest handling, especially when forced air or hydrocooling is employed instead of immediate liquid ice cooling in the field. Nevertheless, our former study showed that keeping broccoli florets at 20 °C for 6 h before cooling at 5 °C resulted in little

influence on glucoraphanin content as well as shelf life, while keeping them at 20 °C for 24 h led to remarkable decline [168].

5.2.2 Controlled Atmosphere Storage

CA has been widely used to extend the storage period and maintain the quality of horticultural products. Broccoli is one of the commodities that benefit from CA storage [169, 177]. In regard to the changes of glucosinolate contents in broccoli upon CA treatment, discrepant results were obtained in various broccoli cultivars under different storage temperatures combined with diverse O_2 and CO_2 concentrations. Hansen et al. [172] investigated the changes of glucosinolates in 'Marathon' broccoli florets stored under low O_2 and high CO_2. Results showed that the total glucosinolate content increased 42% under air and 21% under 0.5% O_2 + 20% CO_2 during 7-day storage at 10 °C when compared to freshly harvested broccoli. However, the study of Fernández-León et al. [178] demonstrated that CA storage (10% O_2, 5% CO_2) was effective in lowering the decrease of glucosinolates in broccoli heads during cooling as well as room temperature (20 C) storage. Similarly, glucoraphanin concentration in broccoli heads was maintained to a higher level under CA (1.5% O_2 + 6% CO_2) storage at 4 °C than that under air condition [169]. These were consistent with the results of our former survey, which showed that CA conditions with relative higher CO_2 concentrations (10%) and normal O_2 concentrations (21%) facilitated the retention of glucoraphanin content at 5 °C [168]. These results suggested that the elevated CO_2 concentration might favor the induction of glucoraphanin biosynthesis and/or reduction of its degradation by endogenous myrosinases. Dunford and Temelli [179] have proposed that elevated CO_2 concentrations caused myrosinase inactivation, which might explain the decreased glucoraphanin degradation in CA conditions with elevated CO_2 concentrations. Contrarily, marked decline in glucoraphanin content was found under 1% O_2 or 1% O_2 + 10% CO_2 treatments compared to air treatment during 20-day storage at 5 °C [168], and 20% CO_2 treatment led to a 15% decline of total glucosinolate content in 'Marathon' florets during 7-day storage at 10 °C [172]. It is known that CYP79F1, a cytochrome P450 monooxygenase, is responsible for aldoxime production, a key step in biosynthesis of glucoraphanin [16, 30, 180]. Therefore, the oxygen dependence of CYP79F1 action might explain above findings that low concentrations or the absence of O_2 might result in reduced biosynthesis and content of glucoraphanin. Moreover, treatment with 20% CO_2 in the absence of O_2 brought about severe off-odors, injury, and water soaking of the tissue [172]. Thus, normal level of O_2 and enhanced level of CO_2 are better for CA treatment of broccoli.

5.2.3 Modified Atmosphere Packaging

Cooling and CA are effective in storage of broccoli [181]. However, cooling and CA facilities are not always available in developing countries, where high temperatures are often encountered throughout the post-harvest storage, distribution, and marketing phases of broccoli products [167]. In this case, MAP might be a better choice, which is simple, economical, and also effective in delaying the post-harvest deterioration and maintaining the visual quality of broccoli at either low or high

temperature [146, 167, 169, 182]. The investigation of Rangkadilok et al. [169] showed that glucoraphanin content in broccoli heads descended significantly in air packaging at 20 °C with 48% loss at day 3 and 64% loss at day 10, whereas there were no significant changes in glucoraphanin levels in MAP with no holes at 4 °C and two microholes at 20 °C for up to 10 days. In this research, products were wrapped using low-density polyethylene (LDPE) bags (40 μm thick), and the atmospheres of approximately 0% O_2 and 21% CO_2 were reached in the MAP with microholes after 10 days at 20 °C, while the atmospheres inside the MAP (with no holes) were modified to 0.2% O_2 and 15% CO_2 after 10 days at 4 °C [169]. However, the glucoraphanin content dropped 48% in 'Marathon' heads after 7-day storage at 1 °C under MAP using 11 μm LDPE bags, in which the atmospheres reached 17% O_2 and 2% CO_2 [183].

A survey by Schreiner et al. [184] demonstrated that modified atmosphere, 8% O_2 and 14% CO_2, could maintain aliphatic and indole glucosinolates in mini broccoli heads for 7 days after an initial decrease in 4 days in mixed packaging of mini broccoli and mini cauliflower in polypropylene food trays. Similarly, the decreases of aliphatic, indole, and aromatic glucosinolates in 'Parthenon' broccoli florets were more severe when stored in air than that in modified atmospheres under microperforated polypropylene plastic after 12 days at 5 °C [182]. We also investigated the effects of MAP treatments on glucosinolate contents in 'Youxiu' broccoli heads by comparing with non-wrapped florets [146]. The broccoli florets were packaged by using polyethylene bags (40 μm thick) with no holes (M_0), two microholes (M_1), and four macroholes (M_2) and then stored at 4 °C or 20 °C. The results showed that all three MAP treatments slowed the decline of glucosinolates in broccoli florets when compared to those in the control, with M_0 being the most significant, followed by M_1 and M_2 during 23-day storage at 4 °C or 5-day storage at 20 °C.

In conclusion, MAP has potential in maintaining glucosinolates in broccoli at both low and high temperatures, while the differences in types of polyethylene film used, atmosphere reached in MAP, and genotypes of broccoli had distinct effects in preserving glucosinolates. In addition, it was worth noting that not only atmosphere but also RH was modulated by CA and MAP. Glucosinolate retention could benefit from these two factors as they could prevent cell membrane degradation and subsequent mixing of glucosinolates with myrosinase [167], though the precise mechanism involved remains to be further investigated.

5.2.4 1-Methylcyclopropene

1-MCP has been commercialized and widely utilized in post-harvest handling of fruits and vegetables. Ku et al. [185] indicated that application of 1-MCP could increase the storage life of broccoli. 1-MCP has also been found to maintain phytochemicals including glucosinolates in broccoli. We found that 1-MCP treatment at the concentration of 2.5 μl l^{-1} reduced the decreasing rate of total glucosinolates in broccoli florets stored at 20 °C [174]. Similar result was also observed by Fernández-León et al. [178] in broccoli heads under 0.6 μl l^{-1} 1-MCP treatment. Moreover, the content of total glucosinolate was remarkably enhanced by

application of 1-MCP (25 μl l^{-1}) in broccoli florets stored at 15 °C for 5 days [186]. We found that 1-MCP treatment could inhibit the increase of malondialdehyde (MDA) when compared with the control [174]. It is known that MDA is the product of membrane peroxidation, which could damage the structure and integrity of membrane during the senescence of broccoli florets. Thus, lower MDA was beneficial for preventing the mixing of glucosinolates with myrosinase, namely, reducing glucosinolates hydrolysis. Moreover, the biosynthesis of glucosinolate is proved to participate in plant defensive response, and ethylene might play a role in this process [187]. Finally, it is also possible that the blockage of ethylene action by 1-MCP treatment favored glucosinolate biosynthesis or inhibited some ethylene-related degradative pathways. However, more studies are needed to elucidate the regulation mechanism of 1-MCP in glucosinolate metabolism.

In summary, application of 1-MCP is effective in delaying the decrease in glucosinolate contents during storage at low or relatively high temperature. Furthermore, 1-MCP has been proved nontoxic for humans and environment [188], and it is active at very low concentrations with a negligible residue. With no doubt, commercialization of 1-MCP will be a new tool to maintain glucosinolates in broccoli during post-harvest storage.

In addition, light and 6-benzylaminopurine (6-BA) treatments were also used in glucosinolate retention in post-harvest broccoli. Jin et al. [189] reported that the content of total glucosinolates in post-harvest broccoli florets was profoundly boosted by light-emitting diode (LED) green light. The levels of glucosinolate and sulforaphane in broccoli florets were markedly increased after treatment with 6-BA [190]. It is known that LED lighting sources are durable with small size and cool emitting surfaces, which is suitable for plant growth. 6-BA has been considered nontoxic for human and environment by the US Environment Protection Agency (EPA). Therefore, their applications in post-harvest handling of broccoli are potential.

5.3 Processing and Cooking

Broccoli is mostly consumed as a processed food, and various processings before consumption might cause the degradation or transformation of the health-promoting compounds including glucosinolates. Conventional processing and cooking methods generally affect the content of glucosinolate in broccoli by several aspects: (I) glucosinolate leakage into cooking water, (II) enzymatic hydrolysis by myrosinase, and (III) glucosinolate breakdown in thermal conditions [191]. Here, we try to summarize the effects of processing and cooking conditions on glucosinolate and its derivative in broccoli.

5.3.1 Blanch-Freezing

Broccoli is almost exclusively available to the consumer in two forms, fresh broccoli heads and frozen broccoli florets. Freezing is widely used in broccoli processing as it provides cheaper product with longer shelf life. In commercial industrial freezing,

broccoli usually undergoes blanching prior to freezing, a processing method utilizing hot water or steam to inactivate enzymes that may cause degradative changes and thus limit shelf life severely [192]. Unfortunately, blanching also destroyed myrosinase, resulting in the disability to form sulforaphane in pre- and post-cooking in frozen broccoli, and substantially reduced sulforaphane bioavailability [193]. Because of the disnatured myrosinase, blanch-frozen broccoli can retain the same levels of glucosinolates after 90 days of frozen (−20 °C) storage [194]. Furthermore, Alanís-Garza et al. [195] reported that glucosinolate content increased in three tested broccoli cultivars while remained constant in only one cultivar after blanching and freezing. In order to maximize the production of cancer preventative sulforaphane in broccoli florets, blanching step is optimized to maintain myrosinase activity while destroying ESP activity, for a high percentage of glucoraphanin can be converted to a nitrile with ESP as mentioned above. Perez et al. [196] proposed that the optimal blanching conditions to maximize sulforaphane content in broccoli florets were immersion in water at 57 °C for 13 min, coinciding with the minimum glucosinolates and maximum myrosinase activity.

5.3.2 Cooking

Broccoli is always cooked before eating. Domestic cooking methods include boiling, steaming, microwaving, stir-frying, and stir-frying followed by boiling (stir-frying/boiling). All these methods could influence the levels of glucosinolates and their hydrolysis products in broccoli and thus affect its heath protective capacity. Slicing is the common step in food preparation prior to further cooking, which disrupted tissues and facilitated the release of myrosinase, leading to glucosinolate hydrolysis at high degree [146]. General heating methods in cooking such as boiling, microwaving, and steaming would cause decrease of glucosinolate content in broccoli, and the rate of decrease was higher along with increased cooking time [197]. Sones et al. [198] found that the total glucosinolate content of broccoli boiled for 10 min was approximately 40% less than that of fresh broccoli. Moreover, the total glucosinolate content dropped by 62.0% and 67.7% after 5 and 10 min of blanching, respectively, with the greatest decline in glucoraphanin (71.58%) being observed after 1 min of boiling [197]. Boiling with cold start and hot start also shows different glucosinolate retention. A glucosinolate decrease of 50% was observed in boiling-cold start while 41% loss in boiling-hot start, which provide a suggestion for consumers in preferring boiling way [199]. The microwave heating process led to distinct results due to the differences in conditions such as cooking time, power, and the volume of added water [200]. Vallejo et al. [201] reported a reduction of 74% in total glucosinolate concentration after microwaving broccoli at 1000 W for 5 min, which is the result of glucosinolate leaching into the evaporated water. Likewise, a significant loss of glucosinolates (62% reduction in glucoraphanin) was also observed in microwaved broccoli in our former study [202]. As for steaming, our survey showed that it led to a 36.8% loss of total indole glucosinolates and no significant change in the content of total aliphatic glucosinolates [202]. Similarly, steaming caused a decrease of 17% in total glucosinolate content after cooking for 22 min [199]. Interestingly, Gliszczynska-Swiglo et al. [203] found that steaming

elevated glucosinolate content in broccoli when compared with the fresh broccoli. Bongoni et al. [199] also reported that steaming increased total glucosinolates' level by 17% at the end of cooking, which may be attributed to an increase in the extractability of glucosinolates by processing rather than a real increase in their total content. In addition, the contents of phytochemicals including glucosinolate were evaluated when fresh broccoli florets were subjected to stir-frying treatments in various edible oils, and the results indicated that glucosinolate in broccoli stir-fried with extra-virgin olive, soybean, peanut, or sunflower oil was similar to that in the uncooked sample [204]. We compared the effect of all five domestic cooking methods on the retention of glucosinolates in broccoli and found that stir-frying and stir-frying/boiling presented the highest loss of glucosinolates while steaming resulted in the lowest loss [202]. Therefore, steaming method appeared to be the best way to retain glucosinolates in cooking broccoli.

The loss of glucosinolate in broccoli upon cooking is mainly caused by leaching, for numerous glucosinolates were found in cooking media [205, 206]. Furthermore, heating also leads to thermal degradation of glucosinolate. In general, aliphatic glucosinolates are more stable than indole glucosinolates during cooking [197, 201, 202]. The relative thermostability of individual glucosinolates was shown to vary with heating temperature, duo to their respective chemical structures [207, 208]. However, thermal degradation might not be the main cause of glucosinolate loss as the content of glucosinolates dropped considerably within a very short cooking time.

In addition, significant change in sulforaphane content was detected in cooked broccoli. Matusheski et al. [209] showed that sulforaphane production increased when broccoli was cooked at 60 °C for 5 or 10 min, as temperatures higher than 50 °C inactivated ESP. Considering that myrosinase could be inactivated when subjected to 100 °C for 5–15 min, thus cooking at temperature between 50 °C and 90 °C or at 100 °C within 5 min would favor the formation of sulforaphane [209]. This conclusion was also supported by another survey, which found that steaming for 1–3 min produced less nitrile and more sulforaphane yield from a broccoli meal [65]. It has also been reported that microwave heating (900 W) for 0.5 and 0.75 min increased sulforaphane in production while reduced sulforaphane nitrile content. Furthermore, Ghawi et al. [210] found that adding mustard seeds was proved to intensify sulforaphane formation in cooked broccoli, as these seeds contained a more resilient isoform of myrosinase.

5.3.3 Other Processing Methods

An early publication by Rosa et al. [211] reported the effect of dehydration processes on glucosinolates in broccoli. They found that 50–65 °C drying of intact broccoli maintained the original glucosinolate content as well as the myrosinase activity. However, the rehydration process caused the hydrolysis of glucosinolates. Moreover, as ESP is disnatured during drying (50–65 °C), sulforaphane synthesis should be favored in dehydration processes. A recent study suggested that pulsed electric field (PEF) treatment could increase glucosinolate content in both floret and stalk of broccoli, and the optimal condition was 4 kV cm^{-1} for 525 and 1000 μs [212]. Other

processing conditions, such as pH, also had a significant effect on sulforaphane and sulforaphane nitrile production. A neutral or alkaline pH resulted in predominate sulforaphane production, whereas an acidic pH (3.5, typical of salad dressings) led to more sulforaphane nitrile [108].

6 Conclusions

In recent years, a great progress has been achieved in elucidating glucosinolate biosynthetic pathway, as well as its regulation and degradation in broccoli by a combination of molecular, genomic, and bioinformatic approaches, which provides a solid basis for breeding of broccoli for optimal glucosinolate composition and content. However, plant secondary metabolites usually have adverse effects besides beneficial properties, and several glucosinolate breakdown products have anti-nutritional effects. Therefore, more studies are needed to identify the biological activities of the glucosinolate degradation products in greater detail, so that the balance of benefit, risk, and consumer preference can be properly defined.

Currently, metabolic engineering of glucosinolate in *Brassica* crops is underway, providing a feasible method for improving glucosinolate composition and content in these crops [213–217]. A thorough understanding of glucosinolate biosynthetic pathway and its regulatory network is necessary for regulation of glucosinolate in broccoli by molecular breeding and metabolic engineering, which is promising for better nutrition value and resistance.

In addition, a large number of preharvest factors and post-harvest handlings have been clarified to modulate glucosinolate accumulation in broccoli, and attentions are paid to glucosinolate metabolism in broccoli across the whole food chain, from the production to consumption. Suitable environmental conditions such as light, as well as chemical regulations, help to enhance glucosinolate accumulation during production before harvest. Post-harvest handlings including cooling, CA, MAP, and 1-MCP treatments, as well as freezing processing, are effective in attenuating glucosinolate loss during post-harvest package, storage, and processing period, and steaming is the recommended method for best retention of glucosinolate during cooking before consumption in broccoli. However, the underlying mechanism behind regulation of glucosinolate metabolism by different preharvest and post-harvest handlings remains to be further elucidated.

Acknowledgments This work was supported by grants from National Science Foundation of China (NO. 30370974, 31270343, 31470385).

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Regulation of Glucosinolate Metabolism: From Model Plant *Arabidopsis thaliana* to *Brassica* Crops

5

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Abstract

Brassicaceae are blessed with secondary metabolites called glucosinolates which form the defense arsenal of these plants. Glucosinolates and its degradation products are also proved to be beneficial in agriculture and human health even though some are known to be detrimental. The type of glucosinolates and its content displays huge diversity across different species. The glucosinolate diversity is primarily genetically controlled. The profile of glucosinolates also varies depending on the growth stages and external environment of the plant. The environmental factors include type of pest/pathogen attack, nutrient status of the plant, and other abiotic stress factors. The glucosinolate pathway is also linked to other major metabolic and signaling pathways resulting in a complex mechanism of regulation. Even though the regulatory mechanism is not completely understood, the current chapter integrates the knowledge available from the model plant *Arabidopsis* and related *Brassica* crops.

Keywords

Brassica • Glucosinolates • Regulation • Variability • Differential accumulation • Plant defense • Sulfur deficiency • Metabolic cross talk

Abbreviations

ABA	Abscisic acid
BCAT	Branched-chain aminotransferases
CYP	Cytochrome P450
ET	Ethylene
FMO	Flavin monooxygenase

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_3

Glc	Glucose
GSL	Glucosinolate
GUS	β-Glucuronidase
HPLC	High-performance liquid chromatography
IPM	Isopropylmalate
JA	Jasmonic acid
MAM	Methylthioalkyl malate synthase
MeJA	Methyl jasmonic acid
Met	Methionine
QTL	Quantitative trait loci
Trp	Tryptophan

Contents

1 Introduction

Brassicaceae is a large family of plants which consists of >3000 species in 370 genera [1]. Brassicaceae, often referred as Cruciferae, includes agriculturally important mustards, cabbages, broccoli, turnips, cresses, and their many relatives. The genus *Brassica* which comes under this family is remarkable for containing the largest number of agriculturally and horticulturally important crops than any other genus. Crops under the genus *Brassica* are cultivated since ancient times as vegetables (*Brassica oleracea*), oilseeds (*Brassica napus*, *Brassica rapa*, and *Brassica juncea*), and condiments (*Brassica nigra*, *Brassica carinata*, and *B. juncea*). The oldest references for the cultivation of *Brassica* crops come from India, China, and Japan. Six economically important *Brassica* species (*B. rapa*, *B. nigra*, *B. oleracea*, *B. juncea*, *B. napus*, and *B. carinata*) share three major genomes (A, B, and C). *B. napus* ($2n = 38$, AACC), *B. juncea* ($2n = 36$, AABB), and *B. carinata* ($2n = 34$, BBCC) are amphidiploid species resulted from combining genome sets of the low chromosome number species which are *B. rapa* ($2n = 20$, AA), *B. nigra* ($2n = 16$, BB), and *B. oleracea* ($2n = 18$, CC) [2, 3].

Plants are equipped with a diverse array of natural chemicals to cope up with environmental challenges either biotic or abiotic to balance their sessile nature. Among such compounds, glucosinolates (GSL) are one of the widely studied plant secondary metabolites that contribute toward plant fitness. Glucosinolates (mustard oil glucosides) are a diverse group of nitrogen and sulfur-rich anionic secondary metabolites derived from amino acids. Chemical structure of glucosinolates consists of a β-D-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester and a variable R group derived from amino acids (Fig. 1). Glucosinolates in intact form are known to be inactive in nature. Hydrolysis of glucosinolates by β-thioglucoside glucohydrolase (EC 3.2.3.1, myrosinases) yields a variety of products that are responsible for the biological activities of these compounds. In plants, myrosinases are located in specialized cells known as myrosin cells/idioblast cells of the phloem parenchyma [4], whereas the glucosinolates are held separately in the vacuoles or S-cells of most plant tissues. The compartmentalization prevents hydrolysis in natural conditions. This system has been called as "mustard oil bomb" [5] and constitutes a defense system against pests and diseases.

Exist as defense mechanism of plants [6–8], glucosinolates also impart pungency to *Brassica* cultivars and hence find its use in culinary purpose from time immemorial. However, some glucosinolates and their degradation products have antinutritional activities. Hence, development of *Brassica* varieties low in certain glucosinolates is a key thrust in *Brassica* breeding program [9, 10]. A major breakthrough in glucosinolate research was the discovery that degradation product of glucosinolate, glucoraphanin, i.e., sulforaphane, is a nutritional giant with

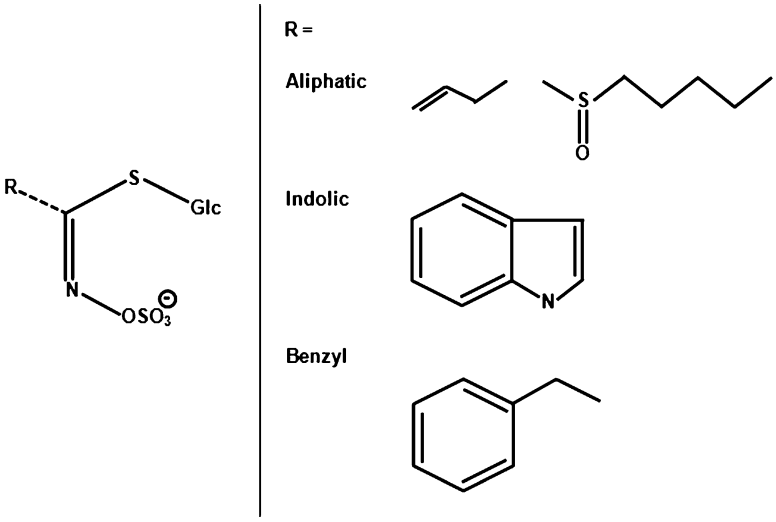


Fig. 1 Basic chemical structure of glucosinolate. *R* variable side chain, *S* sulfur, *N* nitrogen and *Glc* glucose moiety. *Right panel* shows examples of variable side chain (R)

immense healing properties including anticarcinogenic and antimicrobial activities [11, 12]. Because of its multitude of biological activities, glucosinolate biosynthesis and regulation is an active area of research worldwide. Since the model plant *Arabidopsis thaliana* belongs to Brassicaceae, a great extent of information furnished in this chapter is based on *Arabidopsis*.

2 Biosynthesis of Glucosinolates

Glucosinolate biosynthesis is known to be highly complex, and the use of model plant *Arabidopsis* has largely assisted toward our understanding of various steps involved in this biosynthesis [6, 13].

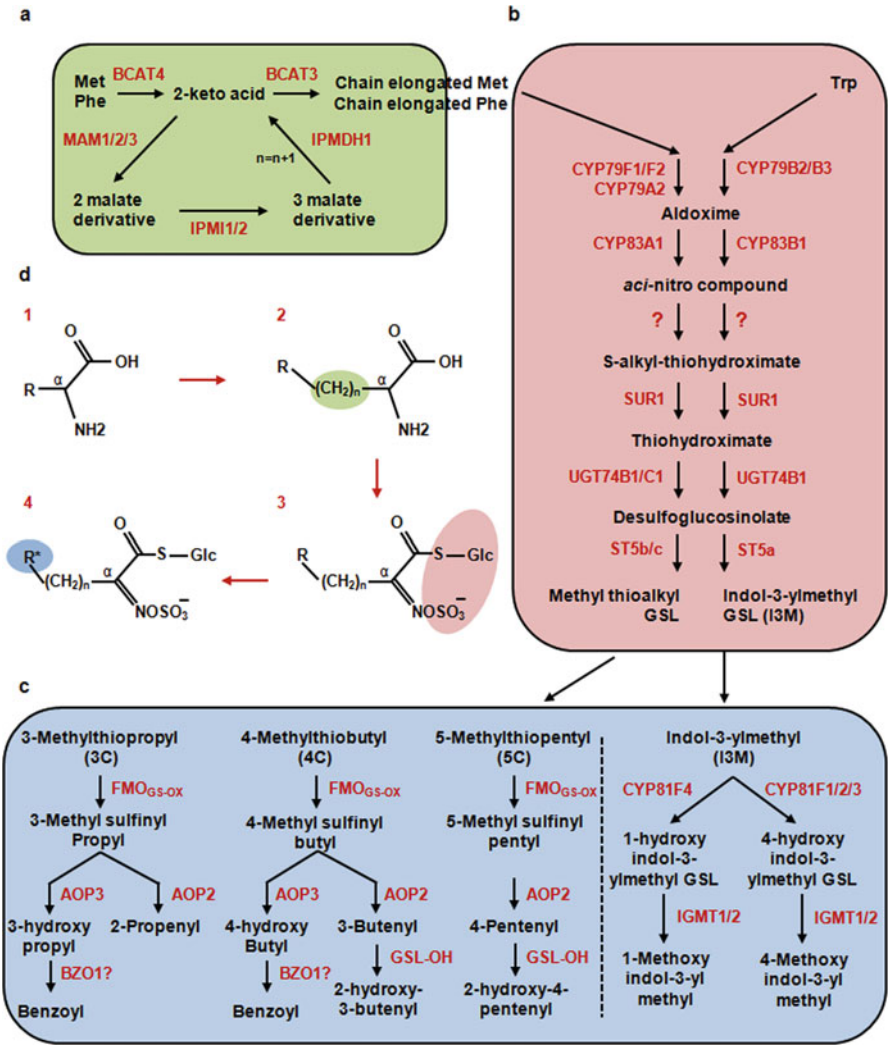
Glucosinolates can be broadly classified based on their biosynthetic precursor amino acid into aliphatic (derived from Ala, Val, Ile, Met, or Val), indolic (derived from Trp), and benzyl (derived from Phe and Tyr) glucosinolates. Till date, more than 130 glucosinolate structures have been identified across various members of Brassicaceae family [14], and *Arabidopsis* itself has about 40 types of glucosinolates, mainly derived from Met and Trp [15]. Table 1 enlists some of the glucosinolates which are commonly present in model plant *Arabidopsis* and cultivated *Brassica* species. In general, the series of reactions involved in glucosinolate biosynthesis can be further divided into three distinct phases, viz., chain elongation of the precursor amino acids, core glucosinolate structure formation, and side-chain modification reactions of the glucosinolate core. However, there is no side-chain elongation that occurs for indolic glucosinolate biosynthesis.

Step I: Side-chain elongation of precursor amino acids: Biosynthesis of glucosinolates initiates in cytosol with the deamination of the specific amino acid to corresponding α-keto acid/2-keto acid/2-oxo acid by branched-chain aminotransferases (BCATs) [6]. For instance, in the case of methionine-derived glucosinolates, the BCAT4 catalyzes the initial step of chain elongation by converting Met to 4-methylthio-2-oxobutanoic acid (MTOB) (Scheme 1a). The initial evidence of BCATs in deamination of Met was suggested by Diebold et al. [16] based on yeast complementation studies. In a more recent study [17], *Arabidopsis bcat4* mutant showed increased accumulation of free Met pool and decreased biosynthesis of aliphatic glucosinolates, suggesting the role of BCAT4 in the first deamination step. After deamination, the resulting α-keto acids are further metabolized in a condensation reaction with acetyl-CoA, catalyzed by methylthioalkyl malate synthase (MAM1, MAM2, and MAM3 localized in *GSL-ELONG* locus) present inside chloroplasts to form substituted 2-malate derivatives [18–20]. MAM1 is involved in the production of glucosinolates derived from 1 to 3 chain elongation cycles (i.e., C3–C5 GSL), whereas MAM2 catalyzes the production of glucosinolates derived from single chain elongation cycle (C3-glucosinolate only). Besides MAM1/2 (At5g23010), the *Arabidopsis GS-ELONG* locus also harbors a tandemly duplicated gene (At5g23020) which encodes another enzyme MAM3 (formerly known as MAM-L), which contributes to the production of all aliphatic glucosinolates, although having higher specificity for longer chain lengths, C5 to C9 [20].

Table 1 Some of the commonly occurring glucosinolates and their abbreviations

Chemical name	Trivial name
Aliphatic GSL	
2-Propenyl	Sinigrin (SIN)
3-Butenyl	Gluconapin (GNA)
4-Pentenyl	Glucobrassicanapin (GBN)
Methyl	Glucocapparin
3-Hydroxypropyl (3OHP)	———
4-Hydroxybutyl	———
2-Hydroxy-3-butenyl	Progoitrin (PRO)
2-Hydroxy-4-pentenyl	Gluconapoleiferin (GNL/NAP)
3-Methylsulfinylpropyl (3MSOP)	Glucoiberin (IBE)
4-Methylsulfinylbutyl (4MSOB)	Glucoraphanin (GRA)
5-Methylsulfinylpentyl (5MSOP)	Glucoalyssin (ALY)
6-Methylsulfinylhexyl (6MSOH)	Glucohesperin
7-Methylsulfinylheptyl (7MSOH)	Glucoibarin
8-Methylsulfinyloctyl (8MSOO)	Glucohirsutin
3-Methylsulfonylpropyl	Glucocheirolin (CHE)
4-Methylsulfinyl-3-butenyl	Glucoraphenin (RAA)
3-Methylthiopropyl (3MTP)	Glucoibervirin (IBV)
4-Methylthiobutyl (4MTB)	Glucoerucin (ERU)
6-Methylthiohexyl (6MTH)	Glucosquerellin
7-Methylthioheptyl (7MTH)	———
8-Methylthiooctyl (8MTO)	———
3-Benzoyloxypropyl (3BzOP)	Glucomalcomiin
4-Benzoyloxybutyl (4BzOB)	———
Benzyl GSL	
Benzyl	Glucotropaeolin (GTL)
2-Phenylethyl (2PE)	Gluconasturtiin (NAS)
p-Hydroxybenzyl	Glucosinalbin/sinalbin
Indolic GSL	
Indol-3-ylmethyl (I3M)	Glucobrassicin (GBC)
4-Hydroxyindol-3-ylmethyl (4-OH)	4-Hydroxyglucobrassicin (4-OH)
1-Methoxyindol-3-ylmethyl (1MOI3M)	Neoglucobrassicin (NEO)
4-Methoxyindol-3-ylmethyl (4MOI3M)	4-Methoxyglucobrassicin (4ME)

The 2-malate derivative is then isomerized into 3-malate derivative which is further converted to homoketo acid by oxidative decarboxylation adding an extra carbon in the side chain than the starting compound (Scheme 1b). The isopropylmalate isomerases (IPMI1 and IPMI2) and isopropylmalate dehydrogenase (IPMDH1) are known to catalyze these reactions [21, 22]. The resulting homoketo acids are now chain elongated by a single methylene group. This molecule can either be transaminated by BCAT to yield homoMet and enter the core glucosinolate pathway or again can pass through additional elongation cycles resulting in



Scheme 1 The glucosinolate biosynthesis pathway. (**a**) Chain elongation of precursor amino acid. (**b**) Formation of glucosinolate core. (**c**) Side-chain modification reactions. The reactions on the left panel show formation of aliphatic glucosinolates and those on right (of **b** and **c**) shows formation of indolic glucosinolates. Enzymes involved in the respective steps are given in red color font. (**d**) General scheme of chemical reactions involved in each step. *1* precursor amino acid, *2* chain-elongated amino acid, *3* glucosinolate core formation, *4* R group undergoing secondary modification reactions

homoketo acids with increased side-chain length up to nine methylene groups [6, 13, 23]. Based on knockdown assay in *Arabidopsis*, BCAT3 was identified as the chloroplast localized enzyme involved in the transamination of 2-oxoacids derived from Met [24]. Thus, BCATs are capable of both deamination and transamination

reactions. These reactions generate a variety of chain-elongated derivatives of methionine.

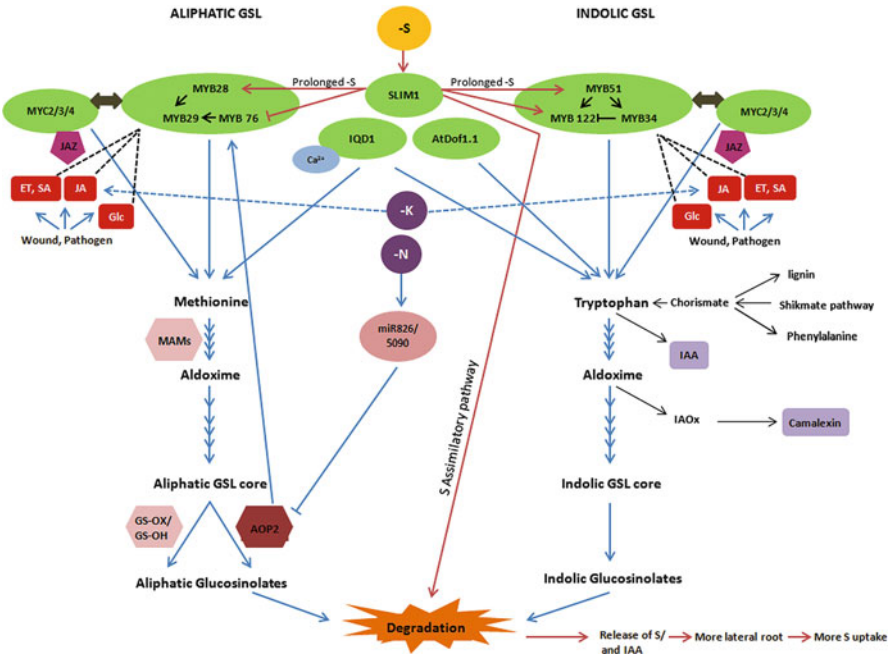
Step II: Formation of glucosinolate core structure: The first chain elongation step of glucosinolate biosynthesis starts in cytosol [17], and later steps occur in chloroplast [6]. However, the core structure formation takes place in the cytosol. This implicates the need for a transporter which can shuttle between the cytoplasm and chloroplast membranes. Recently, Gigolashvili et al. [25] have shown that the bile acid transporter, BAT5, is involved in the transport of MTOB and chain-elongated keto acids across the chloroplast membrane before, during, and after side-chain elongation of keto acids.

Biosynthesis of glucosinolate core structure initiates with the conversion of precursor amino acids (chain elongated in the case of aliphatic) to aldoximes by cytochrome P450 monooxygenases of the CYP79 family (Scheme 1c). The CYP79B2 and CYP79B3 enzymes catalyze the formation of indolic-3-acetaldoxime (IAOx) from Trp [26], whereas CYP79F1 and CYP79F2 are specific to chain-elongated Met substrates [27]. CYP79A2 catalyzes this particular step in the case of benzyl glucosinolates [28]. The aldoximes are subsequently oxidized into *aci*-nitro compounds or nitrile oxides by enzymes of the CYP83 family. In *Arabidopsis*, two nonredundant enzymes, CYP83A1 and CYP83B1, oxidize aliphatic and indolic aldoximes to their corresponding *aci*-nitro compounds [29]. These compounds are strong electrophiles and hence can spontaneously react with thiols to form S-alkylthiohydroximate conjugates [23]. Cysteine was suggested to be the thiol donor for the reaction in vivo; however, it is not clear whether this conjugation is enzyme mediated [30]. More recently, Schlaeppi et al. [31] have shown glutathione as a potential sulfur donor for the reaction. The C-S bond of S-alkylthiohydroximates is then cleaved by a C-S lyase to yield thiohydroximates, pyruvate, and ammonia. Using bioinformatics approach, Mikkelsen et al. [32] have identified SUR1 as the C-S lyase involved in the glucosinolate biosynthesis. From mutant studies, it is also suggested that C-S lyase is a single gene family which lacks side-chain specificity in glucosinolate biosynthesis. Hence, formed thiohydroximates undergo a glucosylation reaction catalyzed by UGT74B1 glucosyltransferase [33] to generate desulfoglucosinolates. Analysis of *ugt74b1* knockout mutant in *Arabidopsis* showed considerably decreased but not completely abolished level of indolic and aliphatic glucosinolates, thus indicating that other UGT activities may also be present in *Arabidopsis* plants. *UGT74C1* has been suggested as a candidate gene having UGT activities involved in glucosinolate biosynthesis and seems to be more specific for Met-derived thiohydroximates [6, 13]. In the last stage of core structure synthesis, desulfoglucosinolates are sulfated by the sulfotransferases (SOT) to form the final glucosinolate core structure [13]. The sulfur donor for the reaction is suggested to be 3′-phosphoadenosine-5′-phosphosulfate (PAPS). Three *Arabidopsis* sulfotransferases, AtST5a, AtST5c, and AtST5b (SOT16, SOT17 and, SOT18, respectively), have been shown to catalyze this reaction. The AtST5a metabolizes tryptophan- and phenylalanine-derived desulfoglucosinolates, whereas AtST5b and AtST5c are specific to aliphatic glucosinolates [34].

Step III: Secondary modifications of glucosinolates: The final stage in glucosinolate biosynthesis is the secondary modifications of the side chain. The side chain undergoes various modification(s) such as acylation, oxidation, alkylation, and/or esterification reactions. Side-chain modifications of the glucosinolates occur through stepwise oxidation of methylthioalkyl moieties to methylsulfinylalkyl and then to alkenyl moieties (Scheme 1c). A subclade of flavin monooxygenase ($FMO_{GS\text{-}OX1–5}$) was characterized which catalyze the conversion of methylthioalkyl glucosinolates into methylsulfinylalkyl glucosinolates [35, 36]. Two α-ketoglutarate-dependent dioxygenases (AOP2 and AOP3) are demonstrated to control the conversion of methylsulfinylalkyl to alkenyl- and hydroxyalkyl glucosinolates, respectively [37, 38]. A 2-oxo acid-dependent dioxygenase, GS-OH, is demonstrated to convert 3-butenyl to 2-hydroxy-but-3-enyl glucosinolate in *Arabidopsis* [39]. The side-chain elongation and side-chain modification reactions contribute to the vast diversity of glucosinolate structures which is described in detail in Sect. 3.2. It has been shown that esterification of Met-derived hydroxyl glucosinolates with benzyl CoA give rise to benzyloxy glucosinolates or sinapoylated to corresponding sinapoyloxypropyl or sinapoyloxybutyl glucosinolates [40]. Using genetic mapping and T-DNA insertion lines in *Arabidopsis*, benzyl oxidase (BZO1) has been identified to possess the benzoyl-CoA ligase activity [41]. Later, it has been proposed that BZO1 is involved in the synthesis of benzoate precursor cinnamoyl-CoA, rather than to generate benzoyl-CoA [42].

3 Regulation of Glucosinolate Biosynthesis

Genome sequencing and various genetic and functional screens in *Arabidopsis* and related *Brassica* crops have led to the identification of almost all the genes involved in every stage of the glucosinolate biosynthesis [6, 13, 23, 43, 44]. The genetic and molecular regulation of glucosinolate biosynthesis is highly complex (or quantitative) and not yet completely understood. In recent years, various functional studies in model plant *Arabidopsis* have led to the identification and functional analysis of key genes toward regulating the glucosinolate content and profile during plant growth and developmental stages and in response to various stress and elicitor treatments [45]. Our understanding of molecular regulation of glucosinolate biosynthesis in polyploid *Brassica* crops is still impeding owing to inherent polyploidy and complex genetic architecture of these crops. Homologs of some of the glucosinolate candidate genes are reported from some of the recently sequenced *Brassica* genomes [43, 44, 46, 47]; however, only a handful of these *Brassica* homologs has been functionally characterized till date [48–56]. Based on the recent information available from *Arabidopsis* and other *Brassica* crops, we have redrawn an updated scheme for the regulation of glucosinolate metabolism in Brassicaceae species, which is described in detail in the coming sections (Scheme 2).



Scheme 2 Regulation of glucosinolate biosynthesis based on current literature. Molecules on green oval ( ) represent transcriptional regulators. Pathway genes having regulatory role are given in hexagons. *Blue lines* indicate direct regulation, whereas *red lines* with *arrows* show glucosinolate regulation under prolonged sulfur deficiency. *Dotted lines* (both *blue* and *black*) indicate interaction either positive or negative but not yet fully understood. Other metabolic pathways having cross talk with indolic glucosinolate pathway are also shown. (⊣) indicate repression. (⇔) represent protein-protein interaction. Regulation among the major MYB transcription factors is also shown

3.1 Direct Transcriptional Regulators of Glucosinolate Biosynthesis

There are several transcription factors identified which regulate the biosynthesis of glucosinolates in model plant *Arabidopsis* and other related *Brassica* species, including ATR1, IQD1, AtDof1.1, and different members of subgroup-12 of R2R3-MYB superfamily.

One of the initial studies using molecular-genetic approach, IQD1, a calmodulin-binding nuclear protein, was shown to regulate aliphatic and indolic glucosinolate biosynthesis in *Arabidopsis* [57]. Analysis of steady-state messenger RNA levels of glucosinolate biosynthesis genes in both gain- and loss-of-function mutants in *Arabidopsis* indicated that IQD1 regulates the expression of multiple genes of both aliphatic and indolic glucosinolate metabolism in a differential manner.

IQD1 was observed to be induced by mechanical stimuli, and hence with the help of previous literature it may be concluded that IQD1 might integrate early wound

and pathogen/elicitor-induced changes in cytoplasmic Ca^{2+} concentrations and thus stimulate a wide array of coordinated defense responses, including the upregulation of glucosinolate biosynthesis [58]. Another central transcriptional regulatory component of glucosinolate biosynthesis proposed is SLIM1 -(**S**ulfur **LIM**itation), which represses the biosynthesis of glucosinolates in response to sulfate deficiency through the activation of the enzymes catabolizing glucosinolates and hence regulating the sulfur (S) assimilatory pathways [59]. It was observed that the *slim1* mutation altered the expression of metabolic and regulatory genes of glucosinolate biosynthetic pathways including the initial step Met chain elongation enzymes, CYP79F1/F2, CYP79B2/B3, and CYP83B1, and the indolic transcription factor MYB34 (ATR1). Recently, SLIM1 was shown to be the upstream regulatory component to all other transcriptional regulators under sulfur deficiency [60, 61]. Skirycz et al. [62] identified transcription factor OBP2, also called as AtDof1.1 (**D**NA-binding-with-**o**ne-**f**inger), as a positive regulator of indolic glucosinolate biosynthesis in *Arabidopsis*. OPB2 was identified initially based on its homology to OPB1 in *Arabidopsis* [63]. It was observed in wild-type *Arabidopsis* plants that expression of OBP2 was stimulated by wounding, methyl jasmonic acid (MeJA) treatment, and in response to insect feeding which are also known to induce expression of glucosinolate biosynthetic genes and a subsequent accumulation of glucosinolates. Also, overexpression of OBP2 in transgenic plants resulted in the upregulation of *CYP79B2/B3* and *CYP83B1* genes involved in the glucosinolate biosynthetic pathway and an increase in indolic glucosinolates. These findings further confirm that OBP2 is involved in the regulatory network controlling indolic glucosinolate biosynthesis in *Arabidopsis* [62].

In recent years, MYB class of transcription factors have been discovered as one of the major regulators of glucosinolate biosynthesis. MYB proteins represent a large family of proteins that include the conserved MYB DNA-binding domain. Out of the three MYB subfamilies, the R2R3-MYB subfamily is largely found in plants which are involved in a variety of plant functions including developmental processes and defense responses [64]. The R2R3-MYB family proteins are further classified into subgroups depending on specific functions they perform. Members of subgroup-12 R2R3-MYBs are transcription factors, which include MYB34, MYB51, MYB122, MYB28, MYB29, and MYB76, and are shown to be important regulators of glucosinolate biosynthesis. MYB34, MYB51, and MYB122 are regulators of indolic glucosinolates, whereas MYB28, MYB29, and MYB76 are involved in the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis* [65]. Recently two other members of R2R3-MYB transcription factors, MYB115 and MYB118 are shown to control benzyloxy glucosinolate pathway in *Arabidopsis* [66].

Bender and Fink [67] initially identified the R2R3-MYB transcription factor, ALTERED TRYPTOPHAN REGULATION 1 (ATR1/MYB34) as a positive regulator of Trp biosynthesis from *Arabidopsis*. In Brassicaceae, indolic glucosinolates are derived from the amino acid Trp which is also the precursor for the plant hormone IAA. Later, Celenza et al. [68] have shown that ATR1 (MYB34) is a key regulator of indolic glucosinolate biosynthesis. Even though overexpression of ATR1 caused increase in IAA levels, more prominent elevation was observed for

indolic glucosinolate levels, along with an upregulation of biosynthetic genes *CYP79B2/B3* which are also involved in Trp metabolism. Furthermore, the *atr1*-defective mutation suppressed Trp gene deregulation in a *cyp83B1* mutant background confirming the role of ATR1 as a key homeostatic regulator of Trp metabolism and hence indolic glucosinolates.

Analysis of transcript levels of genes located near the site of insertion of transposon in the activation-tagged HIG1-1D (an activation-tagged line high in indol-3-ylmethyl glucosinolate; I3M) mutant showed high level of overexpression of At1g18570 compared to the wild type. The product of this gene, a R2R3-MYB transcription factor, was later coined as HIGH INDOLIC GLUCOSINOLATE 1 (HIG1 or MYB51) [69]. To examine the role of HIG1 in glucosinolate biosynthesis, the glucosinolate accumulation pattern of HIG1-1D mutant and HIG1/MYB51 overexpression lines in *Arabidopsis* was analyzed. The HIG1-1D mutant accumulated up to six-fold higher levels of I3M compared to the wild type. Similarly, the overexpression lines also showed an enhancement of I3M levels up to eight-fold compared with the wild type. In addition, there was also an increase in the other indolic glucosinolates like 4MOI3M and 1MOI3M in these lines compared to the wild type. When transcript profiling of the candidate genes of the indolic glucosinolate and Trp biosynthesis was carried out, it was observed that the expression of the genes *DHS1*, *TSB1*, *CYP79B2*, *CYP79B3*, *CYP83B1*, and *AtST5a* was activated by HIG1/MYB51 in trans [69]. The level of major aliphatic glucosinolate 4MSOB was found to be lower in HIG1-1D and in HIG1/MYB51 overexpression lines compared to the wild-type plants which indicate that HIG1/MYB51 could exert opposite effects on the biosynthetic pathways of indolic and aliphatic glucosinolates. Expression of HIG1/MYB51 was shown to respond to mechanical stimuli like touch or wounding, and overexpression of HIG1/MYB51 resulted in increased plant resistance against generalist herbivores. Thus, these findings provide functional evidence that HIG1/MYB51 is a transcriptional regulator of indolic glucosinolate biosynthesis genes in *A. thaliana*.

Another close homolog of MYB51 in *Arabidopsis* which is involved in the regulation of indolic glucosinolates is MYB122. Ectopic overexpression of HIG2/MYB122 in wild-type Col-0 background resulted in high levels of I3M accumulation but not that of other indolic glucosinolates [69]. Attempts to restore the low I3M level of the *hig1-1* knockout mutant by overexpression of HIG2/MYB122 showed no restoration of function. However, the expression of some Trp pathway genes like *ASA1*, *TSB1*, *CYP79B2*, *CYP79B3*, and *CYP83B1* was found to be enhanced along with elevated auxin levels. These results demonstrate that HIG2/MYB122 has the potential to upregulate indolic glucosinolate biosynthesis but only in the presence of a functional HIG1/MYB51 [65].

It was later confirmed that MYB34, MYB51, and MYB122 have distinct but overlapping functions in the regulation of indolic glucosinolate biosynthesis based on the expression analysis of these transcription factors in knockout mutants and overexpression lines. Expression levels of *MYB122* was significantly increased in the *myb34* mutant, and expression of both *MYB34* and *MYB122* was downregulated in *myb51* mutant. However, expression levels of both *MYB34* and *MYB51* were

unaffected in the *myb122* double knockout mutant. In MYB51 overexpression lines, *MYB34* and *MYB122* transcript levels significantly increased, whereas the transcript level of *MYB51* was marginally activated by *MYB34* or *MYB122* overexpression. The results are conclusive of the fact that the role of MYB34 and MYB122 partially overlap but not of MYB34 and MYB51 [70]. It can be hence inferred that MYB51 might be the major regulator of indolic glucosinolate biosynthesis - (Scheme 2).

In *Arabidopsis*, other members of the subgroup 12 of R2R3-MYB transcription factors, MYB28, MYB29, and MYB76, have been identified as positive regulators of aliphatic glucosinolate biosynthesis using an omics-based methodology [71], a trans-activation approach [72, 73], and a quantitative systems biology approach [74]. MYB28 or HIGH ALIPHATIC GLUCOSINOLATE 1 (HAG1) is shown to be the dominant regulator of aliphatic glucosinolate biosynthesis controlling the expression of almost all genes of the pathway [13]. The aliphatic glucosinolate content as well as transcript levels of aliphatic glucosinolate biosynthetic genes were elevated in gain-of-function mutants and decreased in RNAi knockdown mutants of HAG1 in *Arabidopsis* [69, 71, 74]. Strong MYB28/HAG1 promoter activity was observed in reproductive organs and mature leaves of the plants, the main sites of aliphatic glucosinolate accumulation. *Arabidopsis* transgenic lines overexpressing MYB28 showed reduced feeding preference of the generalist lepidopteran pest *Spodoptera exigua*. *MYB28* expression was also found to be induced by glucose both in *Arabidopsis* and *B. juncea* [72, 75], suggesting the existence of transcriptional regulation for integrating carbohydrate availability and defense compound allocation upon biotic stress.

It was also demonstrated that other two members of subgroup 12 of R2R3-MYB transcription factor, namely, HAG2/MYB76 and HAG3/MYB29, were also involved in the biosynthesis of Met-derived glucosinolates [73]. MYB29 plays only a minor role in the regulation of aliphatic glucosinolates. There was no significant change in the expression of glucosinolate biosynthesis genes or glucosinolate content in *myb29* gene knockdown plants suggesting that MYB29 may not be essential for basal level glucosinolate biosynthesis. However, the double knockout mutant *myb28/myb29* abolished the synthesis of aliphatic glucosinolates, indicating the primary importance of both MYB28 and MYB29 in the regulation of aliphatic glucosinolates [13, 74]. In *Arabidopsis*, it was observed that on treatment with MeJA, the expression of *MYB29* was induced but not that of *MYB28*. Expression of the aliphatic glucosinolate biosynthesis genes *CYP79F1*, *CYP79F2*, and *CYP83A1* was also upregulated with the induction of *MYB29*. These results suggest that MYB28 is essential for basal level synthesis of aliphatic glucosinolates and MYB29 probably has a role in the induction of aliphatic glucosinolate biosynthesis genes in response to MeJA signaling [71].

Later, Sonderby et al. [13] proposed a new model for the regulation of aliphatic glucosinolates based on genotypic and systems analysis techniques, which suggests that MYB28 is the major transcriptional regulator involved in the regulation of both short- and long-chain aliphatic glucosinolates, whereas MYB29 and MYB76 mainly act toward short-chain aliphatic glucosinolates. But under certain conditions, MYB29 and MYB76 can also induce long-chain aliphatic glucosinolates in the

absence of MYB28. The model also propose the presence of additional factors which can interact with both MYB29 and MYB76, located probably outside of the tissue, which is needed for the induction of long-chain aliphatic glucosinolates. The findings also show that MYB76 is independent of MYB28 and MYB29 in aliphatic glucosinolate biosynthesis regulation and it plays a role in determining the spatial distribution of aliphatic glucosinolates within the leaf (Scheme 2). Since there is a complex interplay that exists between these three transcription factors in glucosinolate regulation, understanding their individual role is challenging. Therefore, Li et al. [76] generated transgenic lines expressing each aliphatic *MYB* gene driven by its own promoter in the *myb28myb29* mutant background to get a better picture. It was observed that the three MYB genes work differentially in regulating aliphatic glucosinolate profiles in a tissue-specific manner. Orthologs of MYB transcription factors have been identified from other *Brassica* crops, *B. rapa* ssp. *pekinensis* [43, 47, 77], *B. oleracea* var acephala [78], *B. oleracea* var italica [44, 79], and other related *B. oleracea* varieties including kale and kohlrabi [80] and *B. juncea* [81].

Recently, *Arabidopsis* basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 were identified as direct transcriptional regulators of glucosinolate biosynthesis [82]. These transcription factors were well documented previously for their potential to interact with JAZ repressors to control JA mediated responses, including defense against herbivory [83]. A study by Dombrecht et al. [84] using genome-wide transcriptional profiling of wild-type and jasmonate signal transduction mutant *jasmonate-insensitive 1* (*jin1/myc2*) plants followed by functional analysis revealed that MYC2 negatively regulates indolic glucosinolate biosynthesis in response to jasmonic acid (JA) signaling. Later, it was observed that the triple mutant, *myc2 myc3 myc4* (*myc234*), almost completely abolished glucosinolate biosynthesis and expression of glucosinolate biosynthesis genes was significantly reduced. However, the expression of known positive regulators *MYB28* and *MYB29* remained unaltered which shows that this regulation is direct and independent of MYB factors. The pest status of the mutant plants were also altered compared to the wild-type plants. Chromatin immunoprecipitation, yeast two-hybrid, and pulldown experiments showed that MYC2 binds directly to the promoter of several glucosinolate biosynthesis genes and MYC2/MYC3/MYC4 can interact with MYBs involved in both aliphatic and indolic glucosinolate biosynthesis (Scheme 2). The study concludes that MYC2, MYC3, and MYC4 are essential for constitutive and insect-inducible glucosinolate biosynthesis [82].

3.2 Biosynthetic Genes Controlling Glucosinolate Variability

There are over 130 glucosinolate structures documented so far [14], of which *Arabidopsis* posses about 40 types across different accessions [15]. The huge variability in the glucosinolate structures follows a complex regulatory mechanism. The existence of the different glucosinolate structures is also controlled by variability of individual gene functions, especially the genes involved in initial elongation

and side-chain modification reactions (Scheme 2). In addition, the molecular function of genes varies depending on the plant species, accession, allelic condition, and the polymorphic state of the regulatory network controlling it. The huge variation in glucosinolates is believed to be evolved as a counter mechanism against variety of pests and diseases and other life-threatening challenges encountered by the plant.

Based on the available literature, there are four major loci that control variation in aliphatic glucosinolate accumulation namely, *GS-ELONG*, *GS-OX*, *GS-AOP* (contains QTLs *GS-ALK* and *GS-OHP*), and *GS-OH* [37, 38, 85–87]. *GS-ELONG* is responsible for the variation in chain length of glucosinolates and is composed of three genes, *MAM1*, *MAM2*, and *MAM3*. Analysis of glucosinolate profiles and knockout studies showed that MAM1 catalyzes the production of aliphatic glucosinolates with two carbon chains and MAM2 plays an important role in the production of glucosinolates with a single carbon chain. MAM3 catalyzes the formation of all glucosinolate chain lengths especially those derived from one, five, and six elongation cycles, making this enzyme as the major player of glucosinolate chain length diversity across Brassicaceae [18, 20]. Moreover, MAM1 and MAM2 never exist together in the same accession except for Sorbo of *Arabidopsis* [19]. This gene duplication and neo-functionalization in MAM genes hence plays important role toward the diversification of glucosinolate profiles in Brassicaceae.

Through performing analysis on phylogeny and synteny relationships across 13 sequenced Brassicaceae species, a recent study has investigated the evolution of *MAM* genes, which underwent frequent tandem duplications [88]. The syntenic loci of *MAM* genes showed two independent lineage-specific evolution routes and were driven by positive selection after the divergence from *Aethionema arabicum*, an early branching sister group to the core Brassicaceae group. In the lineage I species (*Capsella rubella*, *Camelina sativa*, *A. lyrata*, and *A. thaliana*), the MAM loci have evolved three tandem genes encoding enzymes (MAM1, MAM2, and MAM3) responsible for the biosynthesis of aliphatic glucosinolates with different carbon chain lengths. In contrast, among the species belonging to lineage II (*B. rapa*, *B. oleracea*, *Thellungiella salsuginea*, *T. halophila*, *Schrenkiella parvula*, *Sisymbrium irio*, and *Raphanus sativus*), the MAM loci encode enzymes responsible for the biosynthesis of short-chain aliphatic glucosinolates only (MAM1/MAM2). Further biochemical studies of MAM enzyme variants from Brassicaceae species could be undertaken in the future to understand the biochemical basis of glucosinolate side-chain variability present in this economically important plant lineage.

Another set of loci that determine glucosinolate variability is involved in side-chain modification reactions. Genetic studies in *B. napus* and *Arabidopsis* identified important loci controlling the conversion of methylthioalkyl glucosinolate to methylsulfinylalkyl glucosinolate [87]. Later, this locus was designated as *GS-OX* [38]. Biochemical knowledge combined with the transcriptome co-regulation database analysis in *Arabidopsis* led to the identification of a flavin monooxygenase ($FMO_{GS\text{-}OX1}$), which was mapped to *GS-OX* locus, and catalyzes the conversion of methylthioalkyl glucosinolates to methylsulfinylalkyl glucosinolates [35, 38].

Bacterial overexpression of the recombinant $FMO_{GS\text{-}OX}$ protein and analysis of the glucosinolate content in overexpression and knockout mutants of $FMO_{GS\text{-}OX1}$ in *Arabidopsis* further confirmed $FMO_{GS\text{-}OX1}$ as a major enzyme controlling the conversion of methylthioalkyl glucosinolates in the wild-type plant [35]. Phylogenetic analysis identified a crucifer specific subclade of *FMO* genes, named $FMO_{GS\text{-}OX1\text{–}5}$, which was involved in the conversion of methylthioalkyl to methylsulfinylalkyl glucosinolates [36]. The study also showed that like $FMO_{GS\text{-}OX1}$, $FMO_{GS\text{-}OX2}$ to $FMO_{GS\text{-}OX4}$ are also capable of catalyzing the S-oxygenation independent of chain length and $FMO_{GS\text{-}OX5}$ is specific for 8-methylthiooctyl (8-MTO) glucosinolates [36].

QTL *GS-AOP* is yet another important locus modulating glucosinolate variability and content [37, 86]. *GSL-AOP* is the collective name for two tightly linked loci *GS-ALK* and *GS-OHP*. Fine scale mapping identified three *AOP* genes localized with these loci, namely, *AOP1*, *AOP2*, and *AOP3*. *AOP2* and *AOP3* are believed to be derived from the ancestral gene *AOP1* by gene duplication [37, 89]. Both *AOP2* and *AOP3* encode a 2-oxoglutarate-dependent dioxygenase which is involved in the conversion of methylsulfinylalkyl glucosinolate to methylalkenyl glucosinolate and hydroxylalkyl glucosinolate, respectively. *AOP2* is localized within *GS-ALK* whereas *AOP3* is localized within *GS-OHP* locus [37]. Hence the presence, absence, or the allelic variations of these genes determine the type of glucosinolate accumulated in a particular plant species. For example, sulforaphane, an isothiocyanate derived by the hydrolysis of glucoraphanin, a methylsulfinylalkyl glucosinolate, has immense curing properties in humans including cancer prevention. However, the occurrence of glucoraphanin is limited to few *Brassica* species, owing to the nonfunctional *AOP2* gene. In broccoli, a nonfunctional *AOP2* homolog has been identified which is associated with high glucoraphanin accumulation. In *B. rapa* and *B. juncea*, three and four *AOP2* gene homologs have been identified, respectively, which were found functional and hence explain the reason for the absence of glucoraphanin and presence of high amounts of alkenyl glucosinolates (sinigrin, gluconapin) in these *Brassica* crops [44, 46, 48, 50, 55].

It has been shown that, other than its catalysis function, *AOP2* genes are also involved in the regulation of total aliphatic glucosinolate content as well [38, 90, 91]. Evidence for this feedback regulation mechanism was initially proposed based on the analysis of *Arabidopsis* L*er* X Cvi recombinant inbred lines which showed an enhancement of aliphatic glucosinolates up to three-fold in the *GS-ALK* variant compared to the *GS-OHP* or the *GS-AOP-null* variant which concluded that *GS-AOP* has a significant role in controlling the total aliphatic glucosinolate concentration in the leaf. Similarly, overexpression of *AOP2* gene from *B. oleracea* in *Arabidopsis* ecotype Col-0 which is null for both *AOP2* and *AOP3* resulted in the enhancement of aliphatic glucosinolates by increasing the expression of almost all biosynthetic genes and the major transcriptional regulators such as *MYB28* and *MYB29* [48, 91, 92]. In an effort to enhance glucoraphanin content in oilseed *B. juncea* through the downregulation of *BjuAOP2* gene family, it was observed that total glucosinolate content was also significantly reduced. This was further substantiated by the downregulation of *BjuMYB28* transcription factor gene, the

major positive regulator of aliphatic glucosinolate biosynthesis in *B. juncea* [55]. All these findings corroborate the regulatory role of *AOP2* in aliphatic glucosinolate accumulation. However, the specific role of *AOP3* in controlling aliphatic glucosinolate accumulation is less understood. Recently, using QTL mapping and phenotypic analysis of F2 mapping population expressing different *AOP3* transgenes confirmed the regulatory role of *AOP3* in aliphatic glucosinolate biosynthesis [15]. It was found that *AOP3* interact with *GSL-ELONG* loci to control glucosinolate accumulation.

The *GS-OH* locus is involved in the biosynthesis of hydroxylated alkenyl glucosinolate (2-hydroxybut-3-enyl glucosinolate) [85, 86, 93]. This glucosinolate produced by the oxidation of 3-butenyl glucosinolate show wide variation among different *Brassica* species indicating a variable control mechanism of biosynthesis [39, 94]. Hansen et al. [39] identified a 2-oxoacid-dependent dioxygenase (2-ODD) required for the formation of 2-hydroxybut-3-enyl glucosinolate from the precursor 3-butenyl glucosinolate using the existing natural variation as well as T-DNA mutations available within *Arabidopsis* populations. It was further suggested that 2-ODD could also determine the total content of aliphatic glucosinolates in a particular plant species.

3.3 Tissue-Specific Modulation of Glucosinolate Accumulation

We have seen that huge diversity of glucosinolates exists among Brassicaceae, primarily controlled by the presence, absence, or allelic variations in regulatory and biosynthetic genes. Accumulation of glucosinolates is also regulated both developmentally in various organs and tissues and with the age of the plant [95]. This developmental regulation has important consequences on the plant's defense strategy. In most of the Brassicaceae plants, aliphatic glucosinolates are the predominant glucosinolates found in the aerial tissues, whereas indolic glucosinolates dominates the below ground tissues, i.e., roots. Roots possess higher levels of benzyl glucosinolate 2-phenylethyl (2-PE), compared to shoots possibly for rendering greater effectiveness and toxicity of its hydrolysis products in soil against the microbial invaders. Among the indolic glucosinolates, I3M is predominant in shoots, whereas roots majorly contain methoxy derivatives of indol-3-yl glucosinolate [96].

HPLC analysis of glucosinolate content in various organs of the model plant *Arabidopsis* ecotype Col-0 shows that dormant and germinating seeds contain the highest concentration of glucosinolates, followed by inflorescences, siliques, leaves, and roots. Glucosinolate content in leaf tissues also decline with age of the tissue [97]. The higher accumulation of glucosinolate content in reproductive tissues was later supported by the strong activity of the regulatory gene *MYB28* in the inflorescence as evident by promoter GUS analysis. The GUS activity was found declining with senescence of leaves [72]. Promoter activity of indolic glucosinolate regulators *MYB34* and *MYB51* showed a different expression profile in different tissue types of *Arabidopsis* when analyzed histochemically. In general, *MYB34* showed highest

expression in meristematic tissues and young flowers, which are the main site of indolic glucosinolate biosynthesis and accumulation, whereas *MYB51* promoter activity was highest in the vegetative parts of the plants, especially in mature rosette leaves. *MYB51* expression was also observed in roots, but not in mature flowers or siliques [69].

Tissue-specific expression of transcription factors involved in glucosinolate biosynthesis has been studied in *B. rapa* ssp. *pekinensis* (Chinese cabbage) [77]. Homologs of *Dof1.1* (*Dof1.1-1*), *MYB34* (*MYB34-1, 2, 3*), and *MYB51* (*MYB51-1, 2, 3*) showed highest expression in seeds. *MYB28-1* showed highest expression in flowers whereas *MYB28-2*, *MYB28-3*, and *MYB29* showed peak expression in stem. The other transcriptional regulators *IQD1.1-1* and *MYB122* showed maximum expression in the young leaves. Expression of *IQD1.1-2* and *MYB122-1* was found high in roots. Another study conducted by Baskar and Park [98] in Chinese cabbage showed an overlapping expression pattern between *BrMYB*s and their downstream genes during different developmental stages. *BrMYB28.3* and *BrMYB29.1* gene homologs were found expressed throughout the developmental stages with highest expression in seedlings and roots, respectively. In allotetraploid *B. juncea*, four homologs of *MYB28* have been identified as the major transcriptional regulator of aliphatic glucosinolate which exhibited a differential expression pattern in different tissue types [81]. All four *BjuMYB28* genes showed expression throughout leaf development but had a lower expression during the younger stages. Among the homologs, two *BjuMYB28* genes, namely, *BjuBMYB28-1* and *−2*, had relatively higher transcript accumulation in the primary and young leaves with the onset of the reproductive phase. A significantly higher expression of all four *BjuMYB28* was observed in the mature and inflorescence leaves. The expression of all the four *BjuMYB28* genes was highest in siliques. In addition sub-functionalization of homologs was also observed within leaves. The expression partitioning of regulators can be correlated with differential accumulation of glucosinolates, but not always true.

3.4 Environmental Regulation of Glucosinolate Biosynthesis

As glucosinolates play a vital role in plant fitness, the induction of glucosinolates is certainly regulated by plant-environment interactions. Both biotic and abiotic factors can contribute toward the selective activation of glucosinolates leading to its altered accumulation, in the plant organs, depending on the external stimuli. Plants are often exposed to multiple challenges simultaneously during their lifecycle. The defense mechanism is also regulated differentially when the plant is under a particular stress or a combination of stress factors. These factors result in a complex and interdependent mechanism of regulation of defense chemicals to cope up with such situations (Scheme 2).

It is well established that incidence of pest and diseases affect glucosinolate accumulation [7, 8, 99–103]. Profiles of glucosinolates also vary greatly with the type of pests and pathogens. According to literature, indolic glucosinolates are known to be more important for inducible resistance even though the roles of

aliphatic glucosinolates are also undebatable [100, 103]. Although a large number of studies have been carried out in this direction, only a few which describe the molecular basis of glucosinolates-pests-pathogens interaction is focused in this section. A study correlating the signaling pathways modulating *Arabidopsis* glucosinolate accumulation and plant responses to insect attack using two phloem-feeding aphids *Myzus persicae* (generalist) and *Brevicoryne brassicae* (specialist) and two lepidopteran pests *S. exigua* Hubner (generalist) and *Pieris rapae* (specialist) found that insect feeding causes increase in short-chain aliphatic methylsulfinyl glucosinolates in all the first three cases, whereas *P. rapae* did not alter aliphatic glucosinolate content, but indolic glucosinolates increased slightly. However, gene expression associated with aliphatic glucosinolate biosynthesis increased after feeding by all species, indicating that glucosinolate accumulation is not always regulated at the level of these gene transcripts even though glucosinolate levels were altered during herbivory [104, 105].

It has been demonstrated that infestation of *M. persicae* (green peach aphid) on *Arabidopsis* causes an overall decrease in the glucosinolate content. However, production of indolic glucosinolate like 4-methoxyindol-3-ylmethyl glucosinolate is detected at the site of infection [100]. Kus´nierczyk et al. [106] studied the changes in gene expression levels of three *A. thaliana* ecotypes, Landsberg erecta (Ler), Wassilewskija (Ws), and Cape Verde Islands (Cvi) differing in their glucosinolate profiles, in response to feeding by aphids *M. persicae* and *B. brassicae* using oligonucleotide microarrays and qRT-PCR analysis. In all three ecotypes, indolic glucosinolate genes were upregulated up on both generalist and specialist feeding. The upregulation of the indolic glucosinolate biosynthesis pathway genes was highest in Ler, suggesting a different defense strategy. A concomitant increase in the indolic glucosinolates was also observed. In another case study in *Arabidopsis*, glucosinolate mutants with two important pest of Brassicaceae, the diamond back moth *Plutella xylostella* (specialist utilizing glucosinolate for host recognition) and *Heliothis armigera* (generalist pest), glucosinolate induction was observed. *P. xylostella* feeding induced both aliphatic and indolic glucosinolates, in most of the plant lines whereas feeding *H. armigera* mostly induced indolic glucosinolates [107]. Appel et al. [108] observed that infestation of *S. exigua* caterpillars on *Arabidopsis* elicited expression of genes involved in aliphatic glucosinolate biosynthesis, including *MAMs*, *UGT74B1*, *UGT74C1*, *CYP79F1*, *CYP83A1*, and *AOP2* and indolic glucosinolate genes like *CYP79B1*, *CYP79B2*, and *CYP79B3*. The expression of myrosinase-encoding genes, *thioglucoside glucohydrolase 1* and *2* (*TGG1, TGG2*), was found downregulated. In a recent study, it was found that infestation by cabbage moth *Mamestra brassicae* increased the levels of indol-3-yl-methyl, 1-methoxy-indol-3-yl-methyl, and total glucosinolates in both wild-type *B. napus* and *MINELESS* (lacking myrosin cells) seedlings. The mRNA transcript levels of the *B. napus* homologs of *CYP79B2*, *CYP79B3*, *CYP83B1*, *GSTF9*, and *SUR1*, key genes involved in indolic glucosinolate biosynthesis showed significant upregulation in *MINELESS* seedlings fed with *M. brassicae* [109]. These studies support the existence of differential regulation of glucosinolate biosynthesis and

accumulation in different *Brassica* species which is also influenced by the species of pest.

Glucosinolate profiles are altered during pathogen attack. Schlaeppi et al. [110] have analyzed the involvement of Trp, indolic glucosinolates, and camalexin in the resistance against *Phytophthora brassicae* of *Arabidopsis*. Transcript profiling revealed that genes involved in the biosynthetic pathway of all the three compounds were coordinately induced in by *P. brassicae* infection. In a study conducted by Bednarek et al. [101], it was observed that MYB51 upregulates indolic glucosinolate biosynthesis in response to Flg22, a microbe-associated molecular pattern (MAMP) molecule (these molecules play important role in plant resistance to pathogens), as evident by the significant downregulation of indolic biosynthetic genes like *CYP79B2*, *CYP79B3*, *CYP83B1*, *SUR1*, *SUR2*, *UGT74B1*, and *AtST5a* in myb51 mutant upon Flg22 induction in contrast with *ATR1/MYB34*. Two genes, *PEN2* and *PEN3*, were found necessary for glucosinolate induction. Many other studies also showed the upregulation of indolic glucosinolate biosynthetic genes and transcriptional factors during pathogen attack [111–113]. Reports on induction of aliphatic glucosinolates by pathogens are limiting. One study suggests that in *B. rapa*, infection of *Xanthomonas campestris* pv. campestris (Xcc) induces the production of aliphatic glucosinolate gluconapin, even though its involvement in resistance is not clear [102, 114]. On the other hand, overexpression of *BnMAM1*, *BnCYP83A1*, and *BnUGT74B1* in *B. napus* conferred greater resistance to fungal pathogens *S. sclerotiorum and Botrytis cinerea* [115] which shows the involvement of these genes in pathogen resistance.

The glucosinolate-mediated defense response is coordinated by various signaling molecules like jasmonic acid (JA), salicylic acid (SA), or ethylene (ET). These plant hormones have a well established role in eliciting plant defense response up on pest and pathogen attack. Apart from these hormones, there are other signaling molecules like glucose and mechanical injury/wounding that can also induce glucosinolate biosynthesis. Kiddle et al. [116] was among the pioneer in demonstrating the induction of glucosinolates in response to SA application. Soil application of SA induced glucosinolates, especially the benzyl glucosinolate 2-PE, in the leaves of oilseed rape (*B. napus*). Following this, next report came in the subsequent year, showing that spraying of *B. napus* plants with methyl jasmonate overaccumulated indolic glucosinolates especially 3-indolyl-methyl and 1-methoxy-3-indolyl-methyl-glucosinolates in a dose-dependent manner [117]. Brader et al. [118] demonstrated that jasmonate signaling is required for the induction of indolic glucosinolate biosynthesis in *Arabidopsis* through the activation of biosynthetic pathway genes in response to attack by the bacterial pathogen *Erwinia carotovora*. Pathogenic elicitors increased indolic glucosinolate content, particularly 3-indolyl-methyl-glucosinolate in the infected plants. The response is suggested to be mediated by JA as evident by the lack of induction in the jasmonate-insensitive mutant *coi1-1*. However, in this study, ET and SA could not elicit such a remarkable induction. A classic study by Mikkelsen et al. [119] describes the elicitation of glucosinolates in response to JA and SA. Wild-type *Arabidopsis* plants were treated with MeJA and

2,6-dichloro-isonicotinic acid (an SA analog), and the glucosinolate profiles were analyzed. The results showed that after MeJA treatment, the content of indolic glucosinolates was increased by three- to four-fold; particularly, the *N*-methoxy-indol-3-ylmethyl glucosinolate accumulated up to ten-fold in response to MeJA treatment. Upon SA treatment, 4-methoxy-indol-3-ylmethyl glucosinolate was also found to be overaccumulated. In contrast, among aliphatic glucosinolates, only 8-methylthiooctyl glucosinolate and 8-methylsulfinyloctyl glucosinolate levels were elevated. There was a corresponding transcriptional activation of indolic glucosinolate biosynthetic genes like *CYP79B2* and *CYP79B3* in response to jasmonate and SA. The study draws the conclusion that since different indolic glucosinolate biosynthesis genes are induced by jasmonate and SA, indolic glucosinolates might be responsible for an induced defense response, and the aliphatic glucosinolates might be primarily genetically regulated and not by biotic elicitors.

Cross talk between the signaling molecules and glucosinolate biosynthesis is very complex and can depend on the feeding behavior of the pest. It has been previously reported that JA and SA possess antagonistic roles [119, 120]. Most of the studies describe the induction using exogenously applied signaling molecules. Mewis et al. [104] evaluated the impact of constitutive and insect-induced changes in glucosinolates following attack by two phloem-feeding aphids (*M. persicae* and *B. brassicae*) and a chewing insect *S. exigua* using mutant and transgenic plants having changes in the JA (*coi1*), SA (*npr1*, non-expressor of PR genes 1), and ET (*etr1*, ethylene receptor 1) signaling pathways. Blocking of JA signaling in *coi1* reduced constitutive glucosinolate concentrations. However, blocking of SA signaling at the mediator protein *npr1* mutant increased glucosinolate content while enhanced SA signaling in *hrl1* (hypersensitive response-like) mutants reduced constitutive glucosinolates. There was no significant impact on constitutive glucosinolate contents on blocking ET signaling (ET Resistant, *etr1*) and reducing SA concentrations in nahG transgene. It was hence proposed that increased glucosinolate accumulation in response to insect feeding require functional NPR1 and ETR1 but not COI1 or SA. NPR is suggested to be central regulatory protein in this complex interaction.

Transcriptional regulation of glucosinolates in response to JA, SA, and ACC (the ethylene precursor) is reported from *Arabidopsis*. Promoter GUS lines of the major aliphatic regulator MYB28 was generated, and the seedlings were treated with the abovementioned signaling molecules [72]. However, none of these molecules could induce MYB28 activity. Interestingly, MYB28 was found negatively regulated by SA. In another study, the aliphatic glucosinolate regulator MYB29 was found to be induced by JA signaling [71]. Recent update on cross talk between indolic glucosinolate regulators and the signaling molecules in *Arabidopsis* suggests that MYB51 is the major regulator of indolic glucosinolate biosynthesis upon SA and ET signaling and MYB34 is the key regulator upon ABA and JA signaling. The role of MYB122 is rather minor toward JA/ET-induced glucosinolate biosynthesis. However, all the three MYB factors are necessary for the induction of indolic glucosinolates in association with signaling molecules during plant defense [70].

Response of IQD1 was also studied in conjunction with these signaling molecules JA, SA, and ACC, the results of which showed no significant effect on transcript levels of this regulator [57]. However, AtDof1.1 was induced by JA [62].

Research studies have also suggested that glucose is an important signaling molecule that can induce glucosinolate transcriptional regulatory networks, integrating carbohydrate availability and hormone action during biotic stress [72]. It might be due to the fact that biotic stress, wounding, or infection by pathogens can alter the carbohydrate status of plant tissues. An extracellular invertase is suggested to be involved in carbon partitioning and integrating sugar, stress, and hormone signals [121]. Using promoter GUS lines in *Arabidopsis*, a significant induction of the native *MYB28* promoter activity up on glucose treatment was observed [72]. Microarray data in *Arabidopsis* also shows that glucose induction can upregulate *MYB28* [122]. Miao et al. [123] studied the effect of glucose on aliphatic glucosinolate biosynthesis in *A. thaliana* using mutants related to aliphatic glucosinolate biosynthesis and regulation, as well as glucose signaling. The study showed a significant enhancement in the individual and total glucosinolate contents up on glucose treatment, which was further explained by profound upregulation of *MYB28* and *MYB29*, the two key transcriptional regulators of aliphatic glucosinolate biosynthesis. There was no enhancement observed for the double mutant *myb28myb29*. Moreover, the *glucose-insensitive* mutant (*gin2-1*) and the ABA-insensitive 5 mutant (*abi5-7*) showed substantial decrease in the content of total aliphatic glucosinolates. Concomitantly, the expression of *MYB28* and *MYB29* was also downregulated compared to the wild type in these lines. Another sugar-insensitive RGS1 (regulator of G-protein signaling) mutant (*rgs1-2*) also showed reduced accumulation of total aliphatic glucosinolates compared to the wild type. The results are conclusive of the fact that both HXK1- and RGS1-mediated sugar signaling regulate aliphatic glucosinolate biosynthesis through the involvement of transcription factors, MYB28, MYB29, and ABI5.

Interactions between JA, SA, and glucose in the regulation of glucosinolate biosynthesis genes were also reported. A recent report in *Arabidopsis* demonstrated that exogenous application of JA could enhance the glucose-induced glucosinolate biosynthesis, whereas the synergistic effect of SA and glucose on glucosinolate induction was comparably less [124]. The enhancement in the glucosinolate accumulation was supported by the upregulation of glucosinolate biosynthesis genes and transcription factors. Among the candidate genes, the expression of *CYP79B2*, *CYP79B3*, *CYP79F1*, *CYP79F2*, *CYP83A1*, *CYP83B1*, *UGT74B1*, and *UGT74C1* was upregulated. The expression of transcriptional regulators, *MYB28*, *MYB29*, *MYB34*, and *MYB122*, also showed enhancement. Induction of indolic and aliphatic glucosinolates after treatment with JA and Glu in JA-insensitive mutants (*coi1*, *jar1*, and *jin1*) and glucose-insensitive mutants (*rgs1-2* and *abi5-7*) was reduced. These results suggest that COI1, JAR1, JIN1, RGS1, and ABI5 are key components involved in the regulation of glucosinolate accumulation in response to JA and glucose treatments.

Among other phytohormones, brassinosteroids also affect glucosinolate biosynthesis [125]. 24-Epibrassinolide (EBR) has been shown to decrease glucosinolate

biosynthesis in *Arabidopsis*. EBR significantly decreased the contents of both aliphatic glucosinolates including glucoiberin, glucoraphanin, and glucoerucin, and the indolic glucosinolates glucobrassicin and neoglucobrassicin. Glucosinolate estimation in BR-deficient mutant *cpd* showed significant enhancement in glucosinolate content, whereas the *DWF4-ox* transgenic plants overexpressing the BR biosynthetic gene *DWF4* showed drastic reduction. It has been also shown that two major components in BR signal transduction BZR1 and BES1 are responsible for BR-mediated glucosinolate downregulation possibly by downregulating transcriptional regulators MYB28, MYB34, and MYB122 [125]. Mechanical damage or wounding is another stimulus which can regulate glucosinolate accumulation. Wounding mimic herbivory, even though few studies defend that, in herbivory, subsequent cascade of reactions may vary as insects probably have evolved strategies for counteracting the plants defense mechanism [126]. In *Arabidopsis*, it has been shown that mechanical stimuli such as touch or wounding can also induce the expression of *MYB28* and *MYB51* but not *MYB34* or *MYB122* [69, 72]. In *Arabidopsis*, expression of IQD1 and AtDof1.1 was also found wound inducible [57, 62].

Other than *Arabidopsis*, effect of elicitors has been reported from other *Brassica* species [96], with variable results. It was shown in *B. napus* that wounding can induce indolic glucosinolates [127]. Treatment of pak choi plants (*B.* ssp. *chinensis*) with different signaling molecules such as MeJA, JA, and methyl salicylate showed induction of glucosinolates only under JA [128]. Among the glucosinolates, only indolic glucosinolates were induced, with a dramatic enhancement of 1-methoxy-indol-3-ylmethyl glucosinolate. The molecular basis of the elevated glucosinolates were studied by the expression analysis of *Arabidopsis* orthologs of some of the candidate genes and major transcriptional regulators of indolic glucosinolates such as *CYP79B2/B3*, *ST5a*, *MYB34*, *MYB51*, and *MYB122*. The high glucosinolate phenotype was well correlated with the enhanced expression of these genes [128]. Similarly, JA and sugars have been found to enhance total glucosinolate levels in sprouting *B. oleracea* (broccoli), *B. napus* (rutabaga cabbage), *B. rapa* (turnip), and *Raphanus sativus* (China rose radish) and red radish [129]. In a recent study, effect of biotic elicitors on glucosinolate accumulation was studied in polyploid *B. juncea* [75]. Effects of JA, SA, glucose, and wounding were studied in a time course experiment. Total glucosinolates were enhanced under these stress conditions with an optimum accumulation observed 24 h post treatment. In general, all the aliphatic glucosinolates were enhanced mostly by glucose treatment, whereas indolic glucosinolates were highly induced under all the treatments except for glucose. Among the pool of glucosinolates, it was found that the indolic glucosinolates have more profound effect following the elicitor treatments. Indolic glucosinolates like 1-methoxy-indol-3-ylmethyl and its precursor indol-3-methyl glucosinolates showed strongest induction. Among the signaling molecules, JA was found to be the strongest inducer of indolic glucosinolates. Mechanical injury had the most pronounced effect on total glucosinolates accumulation. Expression profiling of multiple homologs of MYB transcriptional regulators of aliphatic and indolic glucosinolate biosynthesis showed differential expression patterns,

suggesting a complex, coordinated, and overlapping effects toward glucosinolate accumulation in allotetraploid *B. juncea*.

3.5 Minerals and Other Abiotic Determinants of Glucosinolate Metabolism

The availability of macro- and micronutrients is vital for crop production. Nutrient status of the soil not only affects primary metabolism but also the secondary metabolism. Nutritional status also affects the feed value of the plants by invaders. Among the major nutrients, NPK (nitrogen, phosphorus, and potassium), effect of N and P can be both beneficial and detrimental with respect to plant defense [130]. Deficiency of potassium (K) makes the plant more susceptible to pest attack, as K deficiency increases the concentrations of sugars and amino acid which can attract the invaders [131]. The effect of other macro- and micronutrients are also depicted. Among all these, the well-studied nutrients with respect to glucosinolate regulation are described in this section.

Since glucosinolates are derived from amino acids, N status can play a major role in regulation of glucosinolate biosynthesis. MicroRNA profiling under nitrogen starvation showed that miR826 was induced under N deficiency. Interestingly, this microRNA was found to target the *AOP2* gene which has a role in aliphatic glucosinolate regulation [132]. Later, the same group [133] identified another miRNA (miR5090) from the complementary transcript of the *MIR826* gene, evolved from the inverse duplication of their common target gene, *AOP2*. Transcript levels of miR826, miR5090, and *AOP2* transcripts in 10-day-old *Arabidopsis* (accession Cvi having functional AOP2) seedlings grown in N-deficient medium showed enhanced expression of both miR826 and miR5090, whereas the expression of *AOP2* showed a decline, suggesting that *AOP2* transcript level was negatively correlated with miR826 and miR5090 under N starvation. As expected, glucosinolate content was also declined. Most glucosinolate biosynthesis-associated genes were also downregulated under N starvation conditions. This nutrient-mediated regulation of glucosinolate candidate genes might be important for conserving the available N for vital metabolism of plants under stress conditions.

K is an important mineral having effect on glucosinolate accumulation. K deficiency induced JA and both indolic and aliphatic glucosinolates in *Arabidopsis* through COI1-dependent and COI1-independent pathways, respectively [134]. Transcript analysis of K-starved plants showed induction of genes involved in the biosynthesis of glucosinolates and JA signaling. Transcript and glucosinolate profiles of K-deficient plants were similar to herbivore attacked plants. The results suggest that the under K deficiency, JA pathway is induced as a means of fitness strategy of the plant by elevating glucosinolate content, thereby enhancing defense potential of the plant against insect pests.

Glucosinolates are S-rich secondary metabolites. Due to their high sulfur content and role in defense, glucosinolates are hypothesized to accumulate less under S-limiting condition, as plants utilize the available S for protein synthesis and

essential functions instead of synthesizing secondary metabolites [135]. Hence, glucosinolates can also be considered as S reserve of Brassicaceae plants [30]. Induction of glucosinolates under S application is well documented in many cruciferous plants [136, 137]. Zhao et al. [138] showed that S deficiency reduced the accumulation of the alkenyl glucosinolates compared to that of indolic glucosinolates in *B. napus*. The condition was reversed up on application of S, in which alkenyl glucosinolates were largely induced. The authors suggest that higher induction of alkenyl glucosinolates in response to S might be due to the requirement of Met for their biosynthesis, as compared with the indolic glucosinolates which are derived from Trp. Similar study in turnip also showed that aliphatic glucosinolates, particularly 3-butenyl and 5-pentenyl, were strongly regulated on S application [139]. In broccoli (*B. oleracea var. italica*), differential accumulation pattern of glucosinolates under S-rich condition is reported, wherein aliphatic glucosinolates showed higher level of induction compared to indolic glucosinolates [140]. Since it is very clear that S plays an important role in glucosinolate regulation, much research was focused on discovering molecular basis of this interaction. Transcriptional regulation of glucosinolate biosynthesis genes have been reported under S starvation [136, 141].

Combining metabolomics and transcriptomics data of *Arabidopsis* grown under S deficiency, sulfotransferase genes clustering together with known glucosinolate biosynthesis genes were identified as candidate genes involved in glucosinolate biosynthesis [142]. Coexpression analysis under S deficiency also identified MYB28 as the major transcriptional regulator of aliphatic glucosinolate biosynthesis in *Arabidopsis*. It has been shown that almost all the genes including the transcriptional regulators MYB28, MYB29, and MYB34 were downregulated under S starvation [71]. S deficiency reduced the expression of glucosinolate biosynthesis genes such as *BCAT4*, *MAM1*, and *MAM3* in SLIM1-dependant manner which suggests the possibility of a negative interaction between both pathways. However, the effect on the expression of *MYB28*, *MYB29*, and *MYB76* was unclear [59]. Later, it has been shown that all the six MYB transcription factors involved in glucosinolate biosynthesis discovered to date (including MYB28, MYB29, MYB76, MYB34, MYB51, and MYB122) control biosynthesis genes involved in S assimilation such as *APK*, *APK1*, and *APK* [143]. SLIM1 was identified as a major transcriptional regulator of plant sulfur response, upstream to the MYB factors, affecting glucosinolate metabolism [60]. Another study in *Arabidopsis* [76] demonstrated that expression of *MYB28*, *MYB29*, and *MYB76* responded to S stress in a differential manner in which the expression of *MYB29* and *MYB76* was repressed, whereas that of *MYB28* was induced, which is somewhat in contrast with the previous observation [71]. A recent study revealed that the decrease in glucosinolate content under S deficiency was not coinciding with the transcript levels of these MYB regulators. Even though the glucosinolate content were declined, the transcript level of *MYB34*, *MYB51*, *MYB122*, and *MYB28* almost remained unaltered during early time points. Interestingly with time, the transcript levels of these transcription factors increased. However, there was a correlation between the transcript levels of *MYB29* and *MYB76* and glucosinolate content. They hypothesized that negative effect of SLIM1 on glucosinolate regulatory genes can be overridden by a low

glucosinolate signal inducing the transcription of MYBs in a feedback regulatory loop, up on continued S starvation (Scheme 2) [61].

In addition, there are other minerals which also affect glucosinolate biosynthesis. This includes calcium [144], molybdenum [145], zinc, boron, selenium, and cadmium. It has been shown that selenium fortification increases glucosinolate content in broccoli [146, 147]. Boron is shown to increase content of indolic glucosinolates [148]. Heavy metals like cadmium and zinc are known to enhance biosynthesis of glucosinolates in a dose-dependent manner in white cabbage plants [149]. Apart from minerals, there are other abiotic factors affecting glucosinolate metabolism [150]. These include temperature [151, 152], light conditions [153, 154], CO_2 supply [155, 156], drought [157, 158], salinity [159, 160], and soil pH [161].

3.6 Cross Talk of Glucosinolate Biosynthesis Pathway with Other Metabolic Pathways

Cross talk between glucosinolates with other metabolic pathways has been elucidated based on the mutant phenotyping studies. Indolic glucosinolate biosynthesis is established to be linked with auxin biosynthesis/phenylpropanoid pathway/shikimic acid pathway (Scheme 2). Since indolic glucosinolate and auxin biosynthesis utilizes the common amino acid precursor Trp, the link between indolic glucosinolates and auxin has been largely investigated. Loss-of-function mutations in, *BUS*/*SPS*/*CYP79F1*, *SUR1/C-S lyase1*, *SUR2*/*CYP83B1*, and *UGT74B1*, the genes involved in glucosinolate core structure synthesis results in auxin-related phenotypes [23]. In *bus*, the knockdown mutant of CYP79F1 in *Arabidopsis*, a decrease in aliphatic glucosinolate was observed, whereas indolic glucosinolate and IAA levels were elevated [162]. It has been proposed that the level of IAA is regulated by the flux of indole-3-acetaldoxime through a cytochrome P450, *CYP83B1*, which serves as a metabolic branch point of auxin and indolic glucosinolate biosynthesis in *Arabidopsis* [163]. Disruption of function of *SUR/C-S lyase1* and *UGT74B1* also resulted in high auxin phenotype possibly due to the diversion of IAOx to IAA synthesis [164]. IAOx also serves as branch point in the synthesis of phytoalexin, camalexin [165]. It was shown that inhibition of camalexin biosynthesis from IAOx results in reduced accumulation of indolic glucosinolates and increased levels of IAA in *Arabidopsis* [23].

Characterization of *ref2* mutant (*REF2* encodes CYP83A1 involved in oxidation of aliphatic aldoximes) revealed a metabolic link between the glucosinolate biosynthesis and phenylpropanoid pathway [166]. The study suggests that a block at CYP83A1 activity can lead to phenylpropanoid and glucosinolate phenotypes having decreased COMT and caffeoyl-CoA O-methyltransferase activity as a result of overaccumulation of Met-derived aldoximes and IAA. A very recent study using *ref5* (*REF5* encoding CYP83B1) mutant showed reduced levels of indolic glucosinolate and enhanced accumulation of indole-3-acetaldoxime (IAOx). The *ref5* mutants also showed an altered phenylpropanoid metabolism. Triple mutant, *cyp83b1cyp79b2cyp79b3*, showed no IAOx accumulation while showing an

enhanced sinapoylmalate, indicating that levels of IAOx or a subsequent metabolite negatively influence phenylpropanoid accumulation. Analysis of double mutants, *ref5ref2*, showed a synergistic effect on the phenylpropanoid pathway [167].

Integrating transcriptomics and metabolomics data, it has been demonstrated that MYB51 and MYB122 have a positive correlation with the proximal network genes (downstream to Trp) that are involved in indolic glucosinolate biosynthesis, camalexin, and IAA biosynthesis. MYB29 and MYB76 showed negative correlation to genes that are involved in IAA and camalexin biosynthesis. Even though very weak, MYB28 and MYB34 showed positive correlation to the proximal network genes that are involved in indolic glucosinolate biosynthesis and negative correlation to genes that are involved in IAA and camalexin biosynthesis [168]. Recently, a novel microRNA, miR10515 has been identified to enhance IAA biosynthesis through the IAOx pathway, inhibiting both indolic glucosinolate and camalexin biosynthetic pathways [169]. However, the cross talk between these molecules is highly complex and not completely understood and needs further investigation.

3.7 Regulation of Glucosinolate Transport and Degradation

Glucosinolates are synthesized in leaves and are transported through phloem to developing seeds [97, 170]. Recently, Nour-Eldin et al. [171] have identified and characterized two members of the nitrate/peptide transporter family, GTR1 and GTR2, as high-affinity proton-dependent glucosinolate-specific transporters. The *gtr1* and *gtr2* double mutant in *A. thaliana* abolished glucosinolate accumulation in seeds and showed a concomitant overaccumulation in leaves and silique walls by about ten-fold, indicating that both transporters are essential for long-distance transport of glucosinolates. They have also suggested that GTR1 and GTR2 control the loading of glucosinolates from the apoplasm into the phloem. The regulatory mechanism of glucosinolate transport is yet unknown.

Glucosinolates are inactive in their native form. During tissue damage, glucosinolates stored in S-cells [172] get mixed with β-glucosidases called myrosinases (thioglucoside glucohydrolases, TGGs) which are compartmentalized in idioblasts or myrosin cells [173]. There are six members of TGGs identified in *Arabidopsis* [174], whereas *B. napus* have more than 20 myrosinases [175]. These have distinct organ- and tissue-specific activities [176]. Hydrolysis of thioglucoside linkage of glucosinolates results in the formation of glucose and an unstable aglycone moiety [6] which can spontaneously rearrange to form isothiocyanates or catalyzed by an epithiospecifier protein (ESP) to nitriles, epithionitriles [30, 177], depending on the biochemical properties of the specifier protein and the structure of the glucosinolate side chain, pH, and cofactors such as ferrous ion [173]. For example, if the glucosinolate side chain contains a hydroxyl group at carbon 3, oxazoline-2-thione is formed. At low pH, the formation of nitrile is favored, whereas neutral or high pH favors the formation of isothiocyanates. Other proteins like thiocyanate-forming protein (TFP) and nitrile-specifier proteins (NSPs) [173, 178] are also important in determining the type of degradation products. PEN2,

another β-glucosidase, is also demonstrated to take part in indolic glucosinolate hydrolysis [101]. However, regulatory mechanism of glucosinolate degradation at molecular level is largely unexplored.

4 Conclusions and Future Prospects

We have seen that glucosinolate biosynthesis, diversity, and distribution are controlled by multiple factors which primarily include direct transcriptional regulators and polymorphism of genetic loci harboring candidate genes for chain elongation and side-chain modifications. Since glucosinolates serve as defense metabolites, the accumulation is highly affected by biotic challenges. The defense response is largely mediated through signaling molecules such as JA, SA, and ET. Since glucosinolates are S- and N-rich compounds, status of these nutrients also plays a major role in controlling glucosinolate metabolism. Availability of metabolites common to both primary metabolism and glucosinolate biosynthesis also plays a role in glucosinolate regulation leading to homoeostasis. There are abiotic factors which also influence glucosinolate metabolism in plants. There might be other possible routes of regulation such as post-transcriptional modifications and those involved in transport and regulation. All this interconnected interactions make the clear understanding of glucosinolate regulation in *Brassica* crops very complex. Integrating genomics and metabolomics data, the understanding of this complex regulatory mechanism is unraveled in *Arabidopsis* to a larger extent. However, the information from other *Brassica* crops is highly limiting. More efforts should be made in this direction as Brassicaceae contains large number of economically important crops. Fine tuning of the glucosinolate metabolism can lead to the improvement of nutritional quality of Brassicaceae crops by enhancing the beneficial glucosinolates and reducing those with anti-nutritional effects. Customizing the pathway can also improve the defense strategy of the plant against microbial and insect invaders.

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Glucosinolate and Isothiocyanate Production for Weed Control in Plasticulture Production System

6

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Abstract

Brassicaceae cover crops have been studied as biofumigant crops for weed and other soilborne pest control in plasticulture production, especially in the absence of the commercial fumigant methyl bromide (MeBr). Brassicaceae species synthesize a variety of secondary compounds known as glucosinolates (GSLs), which upon hydrolysis are converted into biologically active compounds known as isothiocyanates (ITCs). These ITCs are volatile compounds and therefore can be used for soil fumigation. However, Brassicaceae crops are rarely comparable to MeBr in terms of weed control, partially because of low and inconsistent ITC production and weed control, especially poor efficacy on nutsedge species. This chapter compares the biofumigation potential of various Brassicaceae cover crops and discusses the factors affecting the weed control from biofumigation in plasticulture production. In addition, this chapter describes the successful integration of synthetic ITCs and plastic mulches for weed control in commercial plasticulture production.

Keywords

Allelochemicals • Biofumigation • Brassicaceae cover crops • Methyl bromide alternatives • Purple nutsedge (*Cyperus rotundus* L.) • Yellow nutsedge (*Cyperus esculentus* L.) • Virtually impermeable film

Abbreviations

GSL	Glucosinolate
ITC	Isothiocyanate
LDPE	Low-density polyethylene mulch

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_9

MeBr Methyl bromide
VIF Virtually impermeable film mulch

Contents

1 Introduction

Commercial production of many vegetable and fruit crops takes place under a plasticulture production system using raised, plastic-mulched beds with drip irrigation (Fig. 1). Plasticulture production started in the early 1960s with the introduction of plastic mulches and drip irrigation system [1]. Plastic mulches help improvement

Fig. 1 Tomato production under plasticulture system

of crop microclimate, crop yield, produce quality, soil and water conservation, efficient nutrient use, pest management, and harvest operation.

Weeds are the major limiting factor in vegetable and fruit production, competing for resources and harboring harmful crop pests. Besides tolerance issues in high-value vegetable and fruit crops, public concern about herbicidal residues has led to few herbicide labels in these crops [2]. Further contributing to the limited weed control options in plasticulture production is the phase-out of the highly effective soil fumigant, methyl bromide (MeBr). The loss of MeBr has complicated weed management in plasticulture production, especially difficult-to-control weeds like purple (*Cyperus rotundus* L.) and yellow nutsedge (*Cyperus esculentus* L.).

Plants belonging to Brassicaceae family have been tested as an alternative to MeBr because they are known to exhibit allelopathic weed suppression [3–5]. Brassicaceae species synthesize a variety of glucosinolates (GSLs), which upon tissue decomposition are converted into isothiocyanates (ITCs) [6]. ITCs are biologically active compounds and have been documented to suppress weeds [7, 8]. These ITCs are volatile compounds with biocidal properties and therefore are suitable for soil biofumigation. However, because weed suppression by Brassicaceae biofumigant crops is highly variable in field conditions [4, 9, 10], producers tend to prefer chemical alternatives like synthetically produced ITCs.

Synthetically produced ITCs are gaining interest among researchers due to their lethal activity on several pests. A number of synthetic ITCs are reported to reduce the germination of several weed species including nutsedge species [11–14]. However, the high volatility of ITCs [7] make them vulnerable to volatilization losses, thereby decreasing weed suppression. One way of reducing the ITC losses and increasing weed efficacy under field conditions is the use of low-permeability plastic mulches. For example, using a virtually impermeable film (VIF) mulch, fumigant rates can be reduced without sacrificing weed control [15]. Most of the previous research work and literature has focused on evaluating synthetic ITCs for weed suppression under greenhouse or laboratory conditions. However, field research on testing the weed control efficacy of synthetic ITCs is very limited. Therefore, there is interest in testing synthetic ITCs against nutsedge and other problematic weed species, especially in polyethylene-mulched vegetable and fruit crops where MeBr has been extensively used.

In this chapter, we focus on major weed problems, weed management strategies, and recent advances in our understanding of controlling weeds using Brassicaceae crops and synthetically produced ITCs in a plasticulture production system based on previous research findings as well as our research projects conducted in the Southern USA.

2 Major Weed Problems in Plasticulture Production

Weeds compete for light, water, nutrients, and space with economically important crops. Weeds pose serious threats to vegetable and fruit production by reducing crop yield and quality and interfering with major farm operations, thereby increasing

Fig. 2 Nutsedge penetration through plastic mulch

production costs and resulting in loss of millions of dollars annually [16]. In plasticulture production, nutsedge species (mainly purple nutsedge and yellow nutsedge) are the most problematic weeds because of their perennial life cycle and ability to pierce through the plastic mulch due to their sharp pointed tip (Fig. 2) [17–19]. Yield losses of greater than 90% caused by both species in various vegetable and fruit crops have been well documented [20–24].

The aggressiveness of nutsedge species is mainly associated with their underground tubers [25, 26]. These tubers are perennating organs, which store carbohydrates and facilitate asexual reproduction [27–29]. A single parent tuber of purple nutsedge can produce 99 tubers in 12 weeks [30]. Similarly, a single tuber of yellow nutsedge can produce more than 360 tubers within 16 weeks [31]. In addition, nutsedge tubers and foliage are allelopathic, inhibiting growth of commercial crops [32]. The majority of purple nutsedge and yellow nutsedge tubers are found within the top 15 cm of soil [26, 33]. The longevity of tubers in the soil is short for both species. Neeser et al. [34] predicated the decay of 99% purple nutsedge tubers in 3.5 years, whereas over 80% of yellow nutsedge tubers decayed in less than 3 years [35]. Although tuber life is short, even a low density of surviving tubers can rapidly result in a serious weed problem. The above findings indicate that tubers are vital to the success of nutsedge species and that successful management strategies should concentrate on depleting existing tuber reserves and suppressing new tuber production [36].

3 Weed Management in Plasticulture Production

3.1 Methyl Bromide Fumigation and Phaseout

In a plasticulture production, a preplant fumigant, MeBr, has been widely used over the last five decades for effective and broad-spectrum pest management, including difficult-to-control weeds like purple and yellow nutsedge [37]. Liquid

MeBr is injected in ground or preformed beds with special knives prior to planting. It rapidly volatilizes into a gas; therefore, a plastic mulch is placed immediately over the treated soil to prevent vapor escape and ensure thorough fumigation of treated soil [38]. However, the standard mulches used are not impermeable to gases and thereby unable to stop MeBr losses into the environment [39]. Although highly effective, MeBr is listed among the ozone-depleting substances in the Montreal Protocol, an international treaty designed to protect the ozone layer by phasing out the substances responsible for ozone depletion [40]. As a result, MeBr is being phased out of USA agriculture since 2005 and is only available in limited quantity for certain commodities under critical use exemption [41, 42].

3.2 Challenges after Methyl Bromide Phaseout

The phaseout of MeBr has complicated pest problems and negatively affected the USA vegetable and small fruit production. MeBr is now available in limited quantity under critical use exemption and therefore USA growers had to significantly reduce the use of MeBr each year since 2005 [42]. In addition, the restricted availability has resulted in increased prices of MeBr, which has increased cost of crop production. With the loss of MeBr, vegetable growers are left with few options for controlling many weeds that are capable of reducing yields by more than 90% [20–24]. Purple and yellow nutsedge are among the most troublesome weeds of plasticulture production in the USA [43] and a major limitation to the adoption of MeBr alternatives because of the ineffectiveness of current MeBr alternatives on these weeds.

The currently available fumigants are metam sodium, chloropicrin, and 1,3-dichloropropene. But, these fumigant alternatives may only be short-term replacements for MeBr because of narrow spectrum, inconsistent activity especially on nutsedges, uneven distribution in soil profile, and potential issues related to crop tolerance and human and environmental safety [37, 44]. Furthermore, chloropicrin, a leading MeBr alternative, has a stimulatory effect on purple and yellow nutsedge emergence [45, 46] that could further exacerbate nutsedge problems and increase crop yield losses. A number of herbicides have been tested for control of purple and yellow nutsedge, but success is limited due to marginal translocation of herbicides to the sites of action and inconsistent control when applied at different stages of growth and under various environmental conditions [47]. Most herbicides are registered in agronomic crops, but there are very few options in vegetable crops. This is because of high value, small hectarage, and marginal herbicide tolerance of vegetable crops, which in turn increase liability on chemical companies [48]. Significant research has been conducted in various locations throughout the USA to develop suitable alternatives for MeBr. The research emphasis has included various nonchemical and chemical strategies and their suitable integration.

4 Brassicaceae Cover Crops

4.1 Brassicaceae Cover Crops for Weed Management

Weed management using cover crops has been investigated as a nonchemical method for weed control in vegetable production, especially in the absence of MeBr. Weed suppression by cover crops is attributed to (1) competition for light, nutrients, and moisture during the cover crop growing cycle and (2) physical suppression and allelopathic effect from cover crop residues left on the soil surface or incorporated into the soil [49–51].

Brassicaceae cover crops can provide physical weed suppression because of their faster canopy development and biomass accumulation early in the season [52, 53]. Besides competing for moisture and nutrients, rapid establishment of a Brassicaceae cover crop may result in a high level of nutsedge suppression since shoot growth and tuber production in nutsedge species are closely linked to available photosynthetically active radiation [54–56]. In plasticulture production, Brassicaceae cover crops can be used to reduce weed infestation before forming beds and laying plastic mulch. In addition, Brassicaceae cover crop tissues can be incorporated into soil as green manure to provide allelopathic weed suppression after laying plastic mulch.

The allelopathic compounds released by Brassicaceae crops has been increasingly studied in recent years. These allelopathic compounds, known as ITCs, have shown allelopathic potential against a number of monocotyledonous and dicotyledonous plant species [4, 13, 57–59]. These ITCs exhibit non-specific biocidal activity from interaction with various proteins [60]. As a result, these ITCs are also effective against other soilborne pests including insects [61–65], nematodes [66–69], and fungi [70–73].

4.2 Glucosinolate Hydrolysis and Isothiocyanate Production Pathway

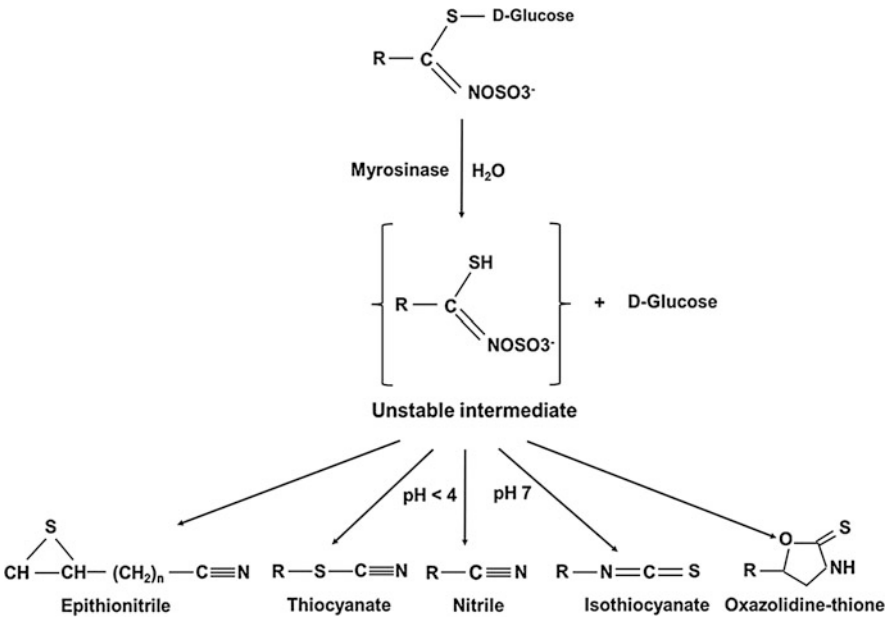
Brassicaceae crops synthesize a variety of secondary metabolites known as GSLs in their cytoplasm and store them in vacuoles. These GSLs are sulfur-rich compounds and consist of a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain [6]. Brassicaceae plants can synthesize more than 20 different types of GSLs, depending on species and growing conditions [74]. As the structure of side chain varies, the type of GSL also varies. Depending upon the type of side chain, GSLs produced by Brassicaceae crops are divided into three major groups: (1) aliphatic, (2) aromatic, and (3) indolyl (Table 1). However, only aliphatic and aromatic GSLs are converted into ITCs upon hydrolysis [75]. GSLs are hydrolyzed by the endogenous enzyme myrosinase (β-thioglucoside glucohydrolase) to produce ITCs, thiocyanates, nitriles, epithionitriles, and oxazolidine-2-thiones depending upon side-chain structure and hydrolysis conditions (Scheme 1). GSLs have limited biological activity [76], but ITCs are most phytotoxic among hydrolysis products.

Table 1 Chemical class, common name, chemical name, and variable side-chain structures of most common glucosinolates (*GSLs*) found in Brassicaceae crops[a]

Chemical class	Common name	Chemical name	Variable side-chain
Aliphatic GSL			
	Sinigrin	Allyl or 2-Propenyl GSL	CH_2CHCH_2
	Gluconapin	But-3-enyl GSL	$CH_2CHCH_2CH_2$
	Glucobrassicanapin	Pent-4-enyl GSL	$CH_2CH(CH_2)_3$
	Progoitrin	2-Hydroxybut-3-enyl GSL	$CH_2CH(OH)$ $CHCH_2$
	Gluconapoleiferin	2-Hydroxypent-4-enyl GSL	$CH_2CHCH_2(OH)$ $CHCH_2$
	Glucoiberverin	3-Methylthiopropyl GSL	$CH_3S(CH_2)_3$
	Glucoerucin	4-Methylthiobutyl GSL	$CH_3S(CH_2)_4$
	Glucoiberin	3-Methylsulfinylpropyl GSL	$CH_3SO(CH_2)_3$
	Glucoraphanin	4-Methylsulfinylbutyl GSL	$CH_3SO(CH_2)_4$
	Glucoraphenin	4-Methylsulfinylbutenyl GSL	$CH_3SOCHCH$ $(CH_2)_2$
	Glucoalyssin	5-Methylsulfinylpentyl GSL	$CH_3SO(CH_2)_5$
	Glucocheirolin	3-Methylsulfonylpropyl GSL	$CH_3SO_2(CH_2)_3$
	Glucoerysolin	4-Methylsulfonylbutyl GSL	$CH_3SO_2(CH_2)_4$
Aromatic GSL			
	Glucotropaeolin	Benzyl GSL	$C_6H_5CH_2$
	Gluconasturtiin	2-Phenylethyl GSL	$C_6H_5(CH_2)_2$
	Glucobarbarin	2-Hydroxy-2-phenylethyl GSL	$C_6H_5(OH)CHCH_2$
	Glucosinalbin	4-Hydroxybenzyl GSL	$4\text{-}OHC_6H_5CH_2$
Indole GSL			
	Glucobrassicin	Indol-3-ylmethyl GSL	$C_8H_6NCH_2$
	Neoglucobrassicin	1-Methoxyindol-3-ylmethyl GSL	$1\text{-}(CH_3O)$ $C_8H_5NCH_2$
	4-Hydroxyglucobrassicin	4-Hydroxyindol-3-ylmethyl GSL	$4\text{-}OHC_8H_5NCH_2$
	4-Methoxyglucobrassicin	4-Methoxyindol-3-ylmethyl GSL	$4\text{-}(CH_3O)$ $C_8H_5NCH_2$

[a]References [38, 74, 82, 86, 87, 92, 95, 105]

ITCs are a family of compounds composed of nitrogen (N), carbon (C), and sulfur (S), with an alkyl or aryl side chain (R) having the basic structure of R–N = C = S [6]. Plants do not release large amounts of ITCs because GSLs and myrosinase are physically separated. Therefore, large amounts of ITCs are only produced when plant cells are disrupted and GSLs come in contact with myrosinase



Scheme 1 Enzymatic hydrolysis of glucosinolates and production of isothiocyanates and other compounds

[77–79]. Brassicaceae plants utilize these ITCs as a defense mechanism against attacking herbivores, insects, and pathogens.

4.3 Brassicaceae Cover Crops for Biofumigation

Biofumigation refers to the use of plants containing biologically active volatile compounds to suppress soilborne pests in agricultural production systems [74]. The biological activity plus volatile nature of ITCs makes them suitable for biofumigation action [7]. Biofumigation has been successfully implemented in the Pacific Northwest of the USA and in Australian production systems and represents a potentially valuable weed management tool for crops [3, 80]. Biofumigation is accomplished by soil amendment with macerated Brassicaceae tissues or seed meal. Soil amendment with *Brassica* species reduced establishment of a wide range of crop and weed species from 23% to 34% and delayed their emergence by approximately 2 days [81]. Soil amendment with water extracts of *Brassica napus* shoots inhibited lettuce (*Lactuca sativa*) seed germination, while root extracts delayed seed germination [8]. Bell and Muller [78] reported that allelopathic compounds released from maceration of black mustard (*B. nigra*) inhibited the establishment of natural grasses in California. Hemp sesbania (*Sesbania herbacea*) germination and fresh weight was reduced >95% by chopped leaf tissues of five *Brassica* species [5]. White mustard (*B. hirta*)-amended soil reduced weed

emergence up to 97% in greenhouse trials and provided effective weed suppression in green pea (*Pisum sativum*) up to 1 month after incorporation in field trials [57]. Norsworthy et al. [82] reported up to 79% and 48% control of large crabgrass (*Digitaria sanguinalis*) and Palmer amaranth (*Amaranthus palmeri*), respectively, at 4 weeks after incorporation of Brassicaceae tissues. Yellow nutsedge tuber production was reduced 71% in wild radish (*Raphanus raphanistrum*)-amended soil compared with no soil amendment [83]. Limited rhizome production was observed in yellow nutsedge tubers when allowed to germinate in wild radish-amended soil [59]. Fall-planted rapeseed, incorporated in the spring, reduced weed density and biomass by as much as 85% and 96% in potato (*Solanum tuberosum*), respectively [3].

Green manuring of three Brassicaceae crops (*B. juncea*, *B. napus*, and *B. hirsuta*) reduced total weed biomass by 49% in soybean (*Glycine max*) at 6 weeks after emergence [4]. However, some of the weed species were sensitive to a particular *Brassica* species, while others were not, which indicates the selectivity of these ITCs to certain species [4]. For example, kochia (*Kochia scoparia*), shepherd's-purse (*Capsella bursa-pastoris*), and green foxtail (*Setaria viridis*) emergences were reduced by all three *Brassica* species, but redroot pigweed (*Amaranthus retroflexus*) emergence was reduced by two *Brassica* species (*B. napus* and *B. hirta*), and velvetleaf (*Abutilon theophrasti*) emergence was reduced by only one *Brassica* species (*B. hirta*). Likewise, the fresh weight of each weed species was affected by the type of *Brassica* species [4]. Therefore, testing the biofumigation efficacy of various Brassicaceae cover crops would be helpful to identify the most effective Brassicaceae species for biofumigation purposes.

4.4 Factors Affecting the Biofumigation Efficacy of Brassicaceae Cover Crops

Although Brassicaceae crops have potential for weed suppression, the efficacy of weed suppression via biofumigation is associated with the following factors: (1) biofumigation potential of Brassicaceae crops, (2) GSL to ITC conversion efficiency, (3) intrinsic toxicity of ITCs to target weed species, and (4) concentration and residence time of ITCs in the amended soil. Let us discuss these factors one by one.

4.4.1 Biofumigation Potential of Brassicaceae Crops

Biofumigation potential of any Brassicaceae crop is linked to the amount of total GSLs contributed per unit area, which is a function of GSL concentration in plant material and plant biomass per unit area [74, 82]. Glucosinolate type and concentration varies in Brassicaceae crops depending upon crop species and variety, plant parts, crop stage, soil and environmental conditions, and management practices.

GSL type and concentration differ among Brassicaceae species and varieties. For example, total GSL concentration ranged from 2.4 to 109.9 μmol g^{-1} dry material in three *Brassica* species (*B. hirta*, *B. juncea*, *and B. oleracea*) in a study conducted in

California [84]. *B. hirta* was high in sinalbin and glucotropaeolin GSLs, whereas *B. juncea* and *B. oleracea* were high in sinigrin and glucoraphanin GSL, respectively. Likewise, variation in GSL profile was reported in seed meal of three *Brassica* species (*B. napus*, *B. juncea*, and *S. alba*) and two varieties of *B. napus* (Athena vs. Sunrise) [85]. However, the GSL variation was relatively less between two varieties of *B. napus*.

Field experiments were conducted in Australia to compare the GSL profile of two varieties ("BQ Mulch," a commercial biofumigant variety, and "JR049," a canola quality variety) of mustard (*B. juncea*) and two varieties ("Nemflix," a commercial biofumigant variety, and "MaximaPlus," a fodder variety) of winter rape (*B. napus*) [86]. Results suggested contrasting GSL profiles not only between two *Brassica* species but also between two varieties within each species. GSL concentration was higher in roots than shoots of winter rape, whereas reverse was true for mustard. Biofumigant variety of winter rape species contributed around 50% of ITC-liberating GSLs in soil, whereas biofumigant variety of mustard contributed 100% of ITC-liberating GSLs in soil.

In another study, GSL production was determined in 10 different accessions of wild radish collected from eight different states of the USA and grown in same environmental conditions [87]. A total of 17 different types of GSLs were identified in wild radish accessions, but only four major GSLs (glucoerucin, glucoraphanin, glucobrassicin, and gluconasturtin) contributed more than 90% of total GSLs. Although, total biomass production was not different among wild radish accessions, total GSL concentration ranged from 107.2 μmol $plant^{-1}$ in the North Carolina accession to 618.9 μmol $plant^{-1}$ in the Mississippi accession. This indicates genetic variation in GSL production among various wild radish accessions. GSL concentration also varied among various plant parts of wild radish accessions. GSL concentration per gram of tissue was highest in primary and secondary branches followed by flowers and then by roots.

A thorough study was conducted in Australia to compare the variation in GSL production in roots and shoots of 76 entries from 13 *Brassica* species/subspecies grown in same environment and with same agronomic conditions [74]. GSL concentration (individual and total) varied four to ten times among and within *Brassica* species. The type of GSLs present in tissues also varied between species but was consistent within species. Roots, shoots, and total biomass varied among *Brassica* species. Total GSL production per unit area ranged from 0.8 to 45.3 mmol m^{-2}, depending upon total GSL concentration and biomass production of each species. Overall, GSL concentration in roots was either equivalent to or higher than that in shoot tissues. However, roots contributed an average of 23.6% of total GSLs, which was directly related to low biomass of root tissues rather than GSL concentration. In general, shoots produced high biomass than roots in most of *Brassica* crops; therefore, shoots are considered as major contributor of GSLs per unit area. GSL analysis also showed that shoot tissues were dominant in aliphatic GSLs, whereas root tissues were dominant in aromatic GSLs. If we compare the *Brassica* species, all *B. napus*, *B. campestris*, and *B. oleracea* species contained only 50% of ITC producing GSLs, whereas *B. carinata*, *B. nigra*, *B. juncea*, *B. fruticulosa*,

B. tournefortii, *S. alba*, *S. arvensis*, *Sisymbrium orientale*, and *Diplotaxis tenuifolia* species predominantly contained ITC-liberating GSLs [74]. Therefore, the huge diversity in GSL profiles and GSL contribution per unit area among *Brassica* species/subspecies/varieties/accessions indicates the importance of careful selection of a *Brassica* crop for biofumigation purpose.

GSL concentration in Brassicaceae crops changes with plant growth stage. For example, GSL concentration in *Brassica* species is generally maximum around flowering and is declined after flowering and lowest at maturity [75, 87, 88]. Bioassay study conducted in Australia revealed that biofumigation efficacy of *B. rapa/B. napus* mixture against soil pathogen increased as plant developmental stage progressed, with maximum efficacy at flowering stage [89]. This stage is also important for practical purposes because of high aboveground biomass production and ease of termination of vegetative tissues and to prevent the *Brassica* crops from seeding and becoming weeds in following economically important crop.

The difference in GSL concentrations in Brassicaceae crops is also affected by the environmental conditions. For example, spring-planted *Brassica* species have produced double the amount of GSLs produced from fall-planted *Brassica* species [90], which could be associated with differences in day length, temperature, rainfall, and light intensity during growing seasons [91]. Similarly, glucosinolate concentration varies between autumn-planted versus spring-planted Brassicaceae crops in the Southern USA [92]. However, the type of GSLs present did not vary due to planting dates. In another study conducted in the Southern USA,, total GSL concentrations in a mixture of *B. juncea*/*S. alba* varied considerably among three different locations [10]. In addition to environmental conditions, soil conditions such as soil moisture, nutrient status, and biotic factors (insect and diseases) are known to affect the GSL concentration in Brassicaceae plants [10, 58, 90]. Nutrient deficiency, moisture stress near flowering stage, and biotic stress like insect/disease attack can increase GSL concentrations in Brassicaceae crops [91]. However, these stresses can also affect the above- and belowground biomass production in Brassicaceae crops, which is directly linked to biofumigation potential of any Brassicaceae crop.

Crop management practices can also affect the GSL concentration in tissues and GSL contribution per unit area from a Brassicaceae crop. Plant spacing has shown mixed results on GSL concentrations. According to one study, narrow spacing would increase GSL concentration in plant tissues by changing the morphology [93]. In contrast, an opposite effect of plant spacing was observed on GSL concentration in a mixture of *B. juncea*/*S. alba* [10]. But, narrow spacing (19-cm wide rows) has produced significantly high biomass production than wide spacing (46-cm wide rows) [10]. Fertilization is also important for GSL production in Brassicaceae crops. Since GSLs are sulfur-rich compounds, sulfur fertilization would increase the GSL content in Brassicaceae tissues. In contrast, nitrogen fertilization at high rates has decreased GSL levels in Brassicaceae crops [91]. However, nitrogen fertilization is important for high biomass production and in turn high GSL contribution per unit area. Crop protection practices have not shown any effect on the GSL content of Brassicaceae crops [91], but they can definitely help in maintaining healthy plants and maximum biomass production. Therefore, it is important to select management

practices to maintain a balance between GSL concentration in plant tissues and biomass production to optimize the biofumigation potential of a Brassicaceae crop.

4.4.2 GSL to ITC Conversion Efficiency

Understanding the GSL profile and GSL content per unit area of a Brassicaceae crop can be helpful in predicting the potential ITCs released by Brassicaceae incorporation in the soil [74]. Therefore, high GSL- and biomass-producing Brassicaceae cover crops are expected to produce higher amounts of ITCs in the soil. Since, the actual activity is linked to ITCs [11, 12], it is critical to measure the GSL to ITC conversion efficiency in the Brassicaceae-amended soil. Several of previous studies have concentrated on measuring GSL profile and content of Brassicaceae crops, but few studies have attempted assessment of GSL to ITC conversion efficiency. In a laboratory study, the amount and diversity of ITCs released from *B. rapa/B. napus* tissues into soil was less than the potential amount available in biomass [89]. When the same test was conducted in the field, no measurable levels of ITCs were detected in amended soil [89]. In a field study in Australia, GSL to ITC conversion efficiency after incorporation of rape (*B. napus*) and Indian mustard (*B. juncea*) ranged from 26% to 56% [86]. Low conversion efficiency is believed to be due to incomplete hydrolysis of GSLs to ITCs after cover crop incorporation. In same study, un-hydrolyzed GSLs were detected in the soil after Indian mustard incorporation, which constituted up to 13% of total incorporated GSLs. In addition, non-ITC producing GSLs (indolyl GSLs) were also detected in the soil [86]. It is also important to remember that not all GSL types present in Brassicaceae crops release ITCs [75]. For example, some of the Brassicaceae species contained mainly non-ITC producing GSLs, while other species predominantly contained ITC-liberating GSLs [74]. Also, GSLs can be converted to nitriles instead of ITCs under low pH conditions [94]. In addition, low soil temperatures can slow down myrosinase enzyme activity and in turn GSL hydrolysis to ITCs [95].

In another field experiment, only 1% or less of the potential GSLs present in Indian mustard (*B. juncea*) and rapeseed (*B. napus*) were converted to ITCs [96]. This is because *Brassica* crops were incorporated into soil without pulverization. GSLs are physically separated from myrosinase enzyme in a healthy plant cell. Upon cell disruption, GSLs come in contact with myrosinase and release ITCs in the presence of water. Therefore, complete cell disruption and water are important to maximize the hydrolysis of GSLs to ITCs. For example, cell disruption by freezing and thawing Indian mustard (*B. juncea*) leaves increased ITC release efficiency from 0.03 to 13.7% [96]. In the same study, ITC production was 1.9-fold higher in amended soil that was waterlogged compared to soil with a water content of −32 kPa [96]. In the field, ITC release can be improved by using a flail mower which can provide thorough maceration of plant tissues before incorporation. In addition, the timing of incorporation is also critical to maximize ITC release in soil. ITC concentration in *B. napus*-amended soil peaked at 30 h after incorporation and decreased later on, which could be due to losses of released ITCs with time [95]. In a bioassay study, the concentration of released ITCs peaked at 4 h after *Brassica* incorporation and then declined sharply after 24 h [89]. In a field study in Australia,

GSL to ITC conversion efficiency was maximum immediately after incorporation (56% in *B. juncea* and 26% in *B. napus* after 0.5 h) and decreased with time (23% in *B. juncea* and 10% in *B. napus* after 6 h) [86]. Similarly, maximum ITC release in the soil was reported 2 h after incorporation of *Brassica* tissues in another trial [96]. Hence, the GSL to ITC conversion efficiency in field conditions is affected by incorporation method and timing, soil conditions, and moisture regime.

4.4.3 Intrinsic Toxicity of ITCs to Target Species

The mode of action of ITCs is not clear to present date, but it is believed that ITCs interact with glycolysis- and respiration-related enzymes during the germination process and thereby prevent or delay seed germination [79]. In spite of their broad-spectrum activity, the degree of toxicity of ITCs varies due to variation in side chain. For example, germination test demonstrated that aromatic ITCs (benzyl and 2-phenylethyl ITC) were the most suppressive ITCs [13]. Among alkyl ITCs, herbicidal activity decreased with increased molecular mass [13]. Wolf et al. [97] demonstrated germination inhibition of velvetleaf from benzyl ITC. Another aromatic ITC, 2-phenylethyl ITCs, inhibited wheat (*Triticum aestivum*) germination up to 100% [98]. In contrast, other research suggested that aliphatic ITCs are more effective than aromatic ITCs [72]. Vaughn and Boydston [5] found aliphatic ITC (methyl, allyl, and 3-butenyl) were effective in suppressing germination of several species, while aromatic ITC (benzyl ITC and 2-phenylethyl) were less inhibitory. Sarwar et al. [72] and Matthiessen and Shackleton [99] concluded that aliphatic ITCs are more volatile and more active in vapor phase while the aromatic ITCs have more contact toxicity and are more toxic in liquid phase. Petersen et al. [13] reported that butyl ITC (aliphatic) was more effective on weeds in vapor phase than liquid phase, while 2-phenylethyl ITC (aromatic) showed opposite effect. Norsworthy and Meehan [12] suggested that aromatic ITCs were more effective than aliphatic ITCs on dicot weeds when applied in liquid phase. Regardless of side-chain structure, shorter-chained ITCs are generally more effective than longer-chained ITCs [13, 72, 75, 99]. However, the efficacy of ITCs can change dramatically dependent upon soil and environmental conditions as well as agronomic practices. Therefore, it is important to select ITC-liberating Brassicaceae crops appropriate to a production system and target species.

In addition, the sensitivity of a target species to a specific ITC varies. For example, some of the weed species were sensitive to a particular *Brassica* species, while others were not, which indicates the selectively of these ITCs to certain species [4]. Norsworthy and Meehan [11, 12] tested eight ITCs on six weed species in a greenhouse and concluded that the effectiveness of ITCs was species specific. In another greenhouse study, it was concluded that purple nutsedge is more sensitive than yellow nutsedge to ITCs [14]. This suggests the importance of considering GSL profile while selecting a Brassicaceae crop which can deliver ITCs effective on target species. Moreover, seed size of a target species can affect their sensitivity to ITC. For example, smaller seeds tended to be more susceptible than larger seeds to methyl ITC [13]. Yellow nutsedge tubers were more tolerant to ITCs than pitted morningglory (*Ipomoea lacunosa*) than Palmar amaranth seeds [12]. Likewise,

sicklepod (*Senna obtusifolia*) seeds were more tolerant than large crabgrass and Texas panicum (*Panicum texanum*) [11]. This could be attributed to difference in size of seeds as well as hardness of seed coat among three weed species.

One limitation of Brassicaceae soil amendment could be the phytotoxicity in following economically important crops, especially small-seeded crops. For example, lettuce seed germination was inhibited by 75% when planted within 5 weeks of soil amendment with 3% seed meal of *S. alba* in a growth chamber study [85]. In field conditions, lettuce emergence was inhibited by planting after 4 weeks of soil amendment with seed meal of *B. napus*, *B. juncea*, *and S. alba* [85]. In contrast, wheat growth was not reduced when planted 4 weeks after Brassicaceae incorporation [100]. In Washington, no injury was observed in potatoes planted around 4 weeks after rapeseed (*B. napus*) green manure [3]. Therefore, there is some species selectivity. Transplanted crops have shown better tolerance than seeded crops to ITCs. For example, bell pepper (*Capsicum annum*) injury was less than 5% when planted 3 days after soil amended with Brassicaceae crops [82]. In plasticulture production, crop injury could be enhanced due to greater retention of toxic ITCs in the amended soil. Therefore, it is important to establish tolerance level and safer plant-back interval for each production system. For example, no phytotoxicity was observed in polyethylene-mulched strawberry when transplanted at least 8 weeks after incorporation of two Brassicaceae (*B. juncea* and *Eruca sativa*) crops [101].

4.4.4 Concentration and Residence Time of ITCs in the Amended Soil

Lethal concentration of ITC is needed for effective weed control. High ITC concentrations can completely inhibit seed germination through irreversible reaction with seed enzymes [79]. Large crabgrass seed consistently killed at high concentrations of methyl ITC [102]. Norsworthy and Meehan [11, 12] tested eight different ITCs at five different concentrations (0, 10, 100, 1000, and 10,000 nmole g^{-1} of soil) on six weed species in a greenhouse trial. Results indicated that weed emergence was reduced with increase in ITC concentration and the dose–response relationship was depicted by linear, quadratic, and sigmoidal models depending upon ITC type and weed species [11, 12]. In contrary, low ITC concentrations will not be effective in killing weed seeds, but low concentration can delay germination by inducing secondary dormancy in treated seeds. In a greenhouse trial, low ITC concentrations delayed germination of smooth pigweed (*Amaranthus hybridus*), but ungerminated seeds were viable [13]. In a field trial, low methyl ITC concentrations delayed the emergence of natural weed population by more than 5 weeks [102]. Soil amendment with *Brassica* species delayed emergence of a wide range of crop and weed species by approximately 2 days [81]. However, very low concentrations can sometimes stimulate seed germination by breaking dormancy [102]. In a greenhouse trial, emergence of sicklepod, large crabgrass, and Texas panicum was stimulated at sublethal ITC concentrations [11].

In addition to lethal concentration, length of exposure to target species is also critical for weed suppression. Longer length of exposure would increase interaction time between ITC and seed enzymes and thereby would improve the ITC efficacy [12]. One way to increase the length of exposure is to reduce the dissipation of

released ITCs from the treated soil. In general, ITCs have a short life span and ITCs dissipate in environment via volatilization, degradation, and sorption. For example, 90% of ITCs dissipated within 24 h of soil treatment [7]. However, the main route of dissipation is volatilization, especially for short-chained aliphatic ITCs [99]. For example, the time for 50% dissipation from soil is 16 h for 2-phenylethyl ITC (aromatic ITC), while it is less than 1 h for n-butyl ITC (aliphatic ITC) [13]. In laboratory study, 50% of methyl ITC dissipated in 1–2 days with total loss in 5–7 days [102]. Within each ITC group (aliphatic or aromatic), volatility generally decreases with increase in size of side chain [72, 103]. Hence, low pest efficacy after cover crop incorporation can been attributed to volatilization losses of ITCs from amended soil [104]. Volatilization losses of ITCs can be reduced by water sealing the soil through irrigation or covering the amended soil with low-permeability plastic mulch.

Besides volatilization losses, soil sorption can reduce active ITC concentrations in soil possibly due to reaction of ITCs with nucleophilic groups in organic particles [105]. For example, the half-life of allyl ITC ranged from 20 to 60 h in different soil types, with enhanced dissipation of allyl ITC from soils with high organic carbon content [94]. Likewise, the biological activity of various ITCs decreased in the presence of soil compared to in vitro, with the highest reduction in peat soil followed by loam followed by sandy soil [99]. However, long-chained ITCs are more affected by soil clay and organic matter compared short-chain ITCs [99]. Microbial degradation also plays an important role in ITC dissipation from treated soil. For example, allyl ITC concentration was threefold higher in an autoclaved soil compared to non-autoclaved soil [106]. Therefore, repeated use of ITCs in same field could reduce biological activity of ITCs [107, 108]. Hence, several factors can affect biofumigation efficacy of Brassicaceae crops in amended soil. Although some of these factors are in the grower's control (crop selection, agronomic practices, incorporation method), several other factors related to soil and environmental conditions are difficult to control. Therefore, it would be a challenging task to understand the complex interaction among these factors to achieve optimum biofumigation efficacy in a field situation.

4.5 Testing Biofumigation Efficacy of Brassicaceae Cover Crops in Plasticulture Production System

Previous studies have tested biofumigation efficacy of Brassicaceae crop either in greenhouse or field studies in bare ground production system. However, very few of the previous research have estimated the biofumigation efficacy of Brassicaceae crops for weed control in plasticulture production systems. It is expected that the biofumigation efficacy of Brassicaceae crops can further be improved in a plasticulture system where volatile ITCs are trapped beneath the tarp.

Laboratory and field research were conducted in Arkansas and South Carolina to test the biofumigation efficacy of seven Brassicaceae cover crops in plasticulture tomato (*Lycopersicon esculentum*) and bell pepper production system [92]. Seven

different Brassicaceae cover crops including Indian mustard (*B. juncea*) "Fumus F-E75" and "Fumus F-L71," oilseed rape (*B. napus*) "Humus," brown mustard (*B. juncea*) "Southern curled giant," turnip (*B. rapa*) "Seventop," herb cress (*Lepidium sativum*), and a blend of brown (*B. juncea*) and white (*S. alba*) mustard "Caliente" were evaluated for their GSL content and composition, ITC released efficiency, and weed efficacy mainly yellow nutsedge. These Brassicaceae species/varieties were selected because of their proven biofumigant action or high GSL content or ease of availability. All the cover crops were drill-seeded in fall of previous year (2006) or early spring of same year (2007). At mid to late flowering stage in May, all the cover crops were flail mowed (to maximize maceration) and incorporated into the top 7.5 cm of the soil profile using a rototiller (Fig. 3). Immediately after soil incorporation, raised beds were formed and covered with plastic mulch followed by tomato and bell pepper transplanting 1 week later (Fig. 3). GSL analysis demonstrated considerable variation in GSL content and composition, depending upon Brassicaceae species/variety, plant parts (shoot vs. roots), and production year/location (Table 2). Six different GSLs were detected in Brassicaceae shoots and roots including three aliphatic GSLs [(2R)-hydroxybut-3-enyl, 2-propenyl, and but-3-enyl] and three aromatic GSLs (*p*-hydroxybenzyl, benzyl, and 2-phenylethyl). Total GSLs contributed to the soil by incorporation of Brassicaceae cover crop tissues were predicted between 47 and 452 nmol g^{-1} soil and were mainly contributed by the shoots because of a higher proportion of shoot biomass (83 to 93%) than root biomass in all cover crops. From the soil samples, five different ITCs were detected including phenyl, 2-propenyl, propyl, benzyl, and 2-phenylethyl ITC. Although the soil samples were taken 3 h to 14 days after incorporation, the highest ITC concentration (1.6 to 28.4 nmol g^{-1} soil) was detected at 3 h after incorporation, and ITC concentration decreased at later timings. Similar to previous research, GSL to ITC conversion efficiency was low and ranged from 1% to 39%, with variation among cover crops and between years (Table 3). Hence, despite of using proper agronomic practices and termination method (flail mower followed by rototiller), ITC release efficiency was low in this trial. Tomato and bell pepper transplanted 1 week after Brassicaceae incorporation appeared tolerant to ITCs released in beds [92]. However, soil amended with these Brassicaceae cover crops provided marginal early-season weed (yellow nutsedge) control in plasticulture tomato and bell pepper (Table 4), which could be attributed to the low soil ITC concentration following *Brassica* incorporation. For example, in the above research, Brassicaceae cover crops can potentially contribute a maximum of 452 nmol g^{-1} soil total GSLs to the soil. Also, a maximum of 39% conversion of 2-propenyl GSL to respective ITC was achieved in the present study. Assuming a conversion efficiency of 39% for total GSLs, it was estimated that a maximum of 176 nmol g^{-1} of total ITCs was released in the soil by incorporation of Brassicaceae cover crops. When applied at a label application rate of 358 kg ha^{-1} with an incorporation depth of 7.5 cm, synthetic fumigant metam sodium can produce up to 2250 nmol g^{-1} of methyl ITC (assuming 90% conversion and soil bulk density of 1.48 g cm^{-3}), which is 13 times the molar equivalent of the maximum ITCs produced by Brassicaceae crops in the present study

Fig. 3 Brassicaceae cover crop mowing and incorporation followed by plastic laying and transplanting

[92]. Hence, the amount of ITCs produced by Brassicaceae was not sufficient to provide effective weed control.

In recent years, we have conducted several studies in the Southern USA to test crop safety and weed efficacy of various biofumigant Brassicaceae crops either alone or in combination with herbicides against nutsedge and other weeds in plasticulture tomato and bell pepper production [109–112]. Although there was no crop injury, weed

Table 2 Type and concentration of glucosinolates detected in the shoot and root tissues of Brassicaceae crops before incorporation in soil in 2006 and 2007[a, b, c]

Year and glucosinolate	Brown/white mustard blend		Herb cress		Indian mustard (Fumus F-L71)		Indian mustard (Fumus F-E75)		Turnip		Brown mustard		Oilseed rape	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
	µmol g^{-1} tissue													
2006														
(*2R*)-2-hydroxybut-3-enyl	nd	nd	nd	Nd	nd	nd	nd	nd	2.8 (0.3)	1.2 (01)	nd	nd	18.5 (0.5)	9.4 (2.6)
2-propenyl	72.3 (11.0)	5.1 (1.1)	nd	Nd	5.8 (0.1)	6.3 (0.2)	12.8 (0.1)	0.5 (0.1)	nd	nd	38.0 (1.0)	5.6 (0.1)	nd	nd
p-hydroxybenzyl	2.4 (0.2)	nd	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
But-3-enyl	nd	nd	nd	Nd	nd	nd	6.6 (0.2)	nd	24.6 (0.8)	nd	nd	nd	nd	nd
Benzyl	nd	nd	131.9 (3.3)	50.6 (1.0)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-phenylethyl	4.0 (0.2)	22.8 (0.5)	nd	Nd	0.7 (0.1)	8.9 (0.1)	1.4 (0.1)	3.5 (0.1)	2.2 (0.6)	19.5 (0.3)	4.3 (0.5)	13.8 (1.9)	2.7 (0.9)	24.7 (0.5)
Total	78.7 (10.7)	27.9 (0.4)	132.0 (3.3)	50.6 (1.0)	6.5 (0.2)	15.2 (0.2)	20.7 (0.2)	4.0 (0.1)	29.6 (1.4)	20.8 (0.3)	42.4 (0.5)	19.3 (1.9)	21.1 (0.6)	34.1 (2.1)

2007														
(*2R*)-2-hydroxybut-3-enyl	nd	nd	nd	nd	nd	nd	nd	nd	2.0 (0.1)	2.7 (0.1)	nd	nd	11.7 (0.1)	14.4 (0.2)
2-propenyl	12.7 (0.1)	nd	nd	nd	31.6 (1.5)	11.2 (0.1)	13.6 (0.4)	nd	nd	nd	59.4 (1.1)	nd	nd	nd
p-hydroxybenzyl	67.9 (0.3)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
But-3-enyl	nd	nd	nd	nd	13.2 (0.3)	nd	23.0 (0.4)	nd	21.4 (11.3)	4.7 (0.2)	nd	nd	3.6 (0.1)	2.9 (0.1)
Benzyl	4.7 (0.3)	nd	131.1 (3.0)	12.7 (0.3)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-phenylethyl	1.9 (0.4)	7.1 (0.5)	nd	nd	5.8 (0.2)	60.4 (2.5)	6.1 (0.2)	3.4 (0.5)	2.3 (0.2)	35.3 (1.9)	5.1 (1.1)	0.5 (0.1)	1.1 (0.1)	51.6 (2.6)
Total	87.1 (0.4)	7.1 (0.5)	131.2 (2.9)	12.7 (0.3)	50.7 (1.5)	71.6 (2.5)	42.7 (1.1)	3.5 (0.5)	25.7 (11.3)	42.7 (2.2)	64.5 (2.0)	0.5 (0.1)	16.4 (0.1)	68.9 (2.7)

[a]Standard error of each mean is included in parenthesis
[b]*nd* non-detected
[c]Reference [92]

Table 3 Isothiocyanate conversion efficiency of potential isothiocyanate-releasing glucosinolates at 3 h after incorporation of Brassicaceae crop tissues in 2006 and 2007[a, b, c]

Year and isothiocyanate	Brown/white mustard blend	Herb cress	Indian mustard (Fumus F-L71)	Indian mustard (Fumus F-E75)	Turnip	Brown mustard	Oilseed rape
	%						
2006							
2-propenyl	4	nd	39	25	nd	13	nd
Benzyl	nd	11	nd	nd	nd	nd	nd
2-phenylethyl	3	nd	11	1	nd	9	7
2007							
2-propenyl	14	nd	nd	nd	nd	3	nd
Benzyl	7	1	nd	nd	nd	nd	nd
2-phenylethyl	10	nd	1	2	3	1	nd

[a]Isothiocyanate conversion efficiency was calculated by using following Eq.: ITC conversion efficiency $= (Y_i/X_i) \times 100$, where Y_i represents concentration of a specific ITC in soil (nmol g^{-1} soil) and X_i is the concentration of the specific precursor GSL in plant tissue incorporated in soil (nmol g^{-1} soil)
[b]*nd* non-detected
[c]Reference [92]

Table 4 Effect of soil amendment with Brassicaceae crops on yellow nutsedge control at 2 and 4 wk after transplanting (*WATP*) tomato and bell pepper in 2006 and 2007[a, b]

	2006				2007			
	2 WATP		4 WATP		2 WATP		4 WATP	
Brassicaceae crop	%							
Brown/white mustard blend	35	b	7	bc	15	bc	1	d
Herb cress	14	c	0	c	8	c	0	d
Indian mustard (Fumus F-L71)	35	b	4	bc	48	a	13	ab
Indian mustard (Fumus F-E75)	43	ab	9	b	51	a	14	a
Turnip	53	a	18	a	33	ab	4	cd
Brown mustard	35	b	4	bc	46	a	10	abc
Oilseed rape	33	b	3	bc	39	a	6	bcd

[a]Treatment means within a column followed by the same letter are not different based on Fisher's protected LSD at $\alpha = 0.05$
[b]Reference [92]

control level provided by Brassicaceae crops was not acceptable as per commercial standard. We also compared the economics of polyethylene-mulched tomato production using biofumigation versus methyl bromide for weed control [113]. Three Brassicaceae crops (*B. rapa*, *B. juncea*, and a mixture of *B. juncea/S. alba*) were tested in combination with hand-weeding. Because of low weed control from biofumigation crops, hand-weeding cost increased up to \$489 $acre^{-1}$ over that in methyl bromide and net returns relative to methyl bromide were reduced up to \$274 $acre^{-1}$.

The above field research demonstrates that biofumigation with Brassicaceae crops could not be used alone for effective weed control in commercial plasticulture production system. Even under non-plasticulture production, biofumigation may not be useful under high weed pressure and in fields with difficult to control weeds like nutsedge species. However, they may still be useful in organic and low-input production systems where weed pressure is low and weed management tools are either limited or costly. In organic bell pepper production, soil amendment with *Brassica* species provided early-season control of large crabgrass and Palmer amaranth [82]. Farmers can select high GSL-producing varieties of Brassicaceae crops as a component of an integrated weed management program where early-season weed suppression by Brassicaceae crops can be supplemented by other weed control strategies later in season. For example, wild radish cover crop supplemented large crabgrass control in sweet corn (*Zea mays*) when used in conjunction with half rates of herbicides which was equivalent to full herbicide rates in the absence of a cover crop [114]. Likewise, soil amendment with *Brassica* tissues supplemented the herbicidal control of Johnsongrass (*Sorghum halepense*) [115]. Other benefits of Brassicaceae cover crops, such as nutrient recycling, erosion control, moisture conservation, soil health improvement, and insect, nematode, and disease suppression, support a practical application of Brassicaceae cover crops in any production system [56, 116–120]. Australian wheat farmers have observed more yield followed by *Brassica* crops [121]. In Washington, greater tuber yield was observed in potatoes planted after rapeseed (*B. napus*) green manure due to factors other than weed control [3]. Similarly, bell pepper yield was increased after soil amendment with Brassicaceae cover crops in organic bell pepper production in the Southern USA [82].

Although Brassicaceae crops have potential for weed suppression, the magnitude of weed suppression is either low or variable under field conditions [4, 9, 10], which indicates that biofumigation with Brassicaceae crops alone does not have enough potential to replace MeBr. For example, biofumigation with Brassicaceae crops in disease-infected soil led to strawberry (*Fragaria* × *ananassa*) yield higher than untreated soil, but lower than MeBr fumigation [101]. The low and inconsistent weed and pest control may lead farmers to shift more toward chemical alternatives such as synthetically produced ITCs.

5 Synthetic Isothiocyanates

5.1 Synthetic Isothiocyanates for Weed Control

Synthetically produced ITCs are gaining interest as a MeBr alternative due to their broad-spectrum lethal activity, volatile nature, and shorter persistence in soil compared to other alternatives. A number of synthetic ITCs have been reported to reduce the germination of several weed species including nutsedge species (Table 5). Norsworthy and Meehan [11, 12] found effective control of yellow nutsedge and other grass and broadleaf species with eight synthetic ITCs. However, the

Table 5 Herbicidal activity of different isothiocyanates (*ITCs*) on weeds in greenhouse and laboratory trials[a]

Chemical name of ITC	Weeds inhibited
Methyl ITC	Dandelion (*Taraxacum officinale*), large crabgrass (*Digitaria sanguinalis*), smooth pigweed (*Amaranthus hybridus*), barnyard grass (*Echinochloa crus-galli*), spiny sow thistle (*Sonchus asper*), scentless mayweed (*Matricaria inodora*), black grass (*Alopecurus myosuroides*)
Ethyl ITC	Yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*)
Propyl ITC	Yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*)
Butyl ITC	Dandelion (*Taraxacum officinale*), yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*))
Allyl or 2-Propenyl ITC	Dandelion (*Taraxacum officinale*), purple nutsedge (Cyperus rotundus), yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*)
3-Methylthiopropyl ITC	Yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*)
Phenyl ITC	Purple nutsedge (Cyperus rotundus), yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*)
Benzyl ITC	Dandelion (*Taraxacum officinale*), yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*), velvetleaf (Abutilon theophrasti)
2-Phenylethyl ITC	Dandelion (*Taraxacum officinale*), yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), pitted morningglory (*Ipomoea lacunosa*), sicklepod (*Senna obtusifolia*), Texas panicum (*Panicum texanum*), large crabgrass (*Digitaria sanguinalis*)
Benzoyl ITC	Purple nutsedge (*Cyperus rotundus*), yellow nutsedge (*Cyperus esculentus*)
o-Tolyl ITC	Purple nutsedge (*Cyperus rotundus*), yellow nutsedge (*Cyperus esculentus*)

(*continued*)

Table 5 (continued)

Chemical name of ITC	Weeds inhibited
m-Tolyl ITC	Purple nutsedge (*Cyperus rotundus*), yellow nutsedge (*Cyperus esculentus*)
tert-octyl ITC	Purple nutsedge (*Cyperus rotundus*), yellow nutsedge (*Cyperus esculentus*)
3-Fluorophenyl ITC	Purple nutsedge (*Cyperus rotundus*), yellow nutsedge (*Cyperus esculentus*)

[a]References [5, 11–14, 97, 102, 130, 131]

effectiveness of ITCs was dependent upon ITC type, concentration, and weed species. ITCs reduced yellow nutsedge emergence and shoot density and biomass in purple and yellow nutsedge [12, 14]. Another notable characteristic of ITCs is their effectiveness in killing dormant weed seeds [102]. This characteristic would be advantageous under plasticulture production, particularly in depleting dormant nutsedge tuber reserves in soil. Although the above-mentioned studies were conducted under greenhouse conditions, they indicate the potential of synthetic ITCs for nutsedge suppression. Limited research has been conducted to evaluate the efficacy of synthetic ITCs, other than methyl, against weeds under field conditions. The volatile nature of ITCs makes them vulnerable to volatilization losses in the field, but aids the fumigation effect in plasticulture production. One way of minimizing the losses of ITC in field condition is by using less permeable plastic mulches.

5.2 Improved Plastic Mulches

The commonly used plastic mulch in vegetable production is low-density polyethylene (LDPE) mulch (Fig. 4a). The low cost and high tensile strength make this mulch popular among growers. However, the LDPE mulches are permeable to gases and thereby not effective in reducing fumigant losses from treated soil. This problem can be overcome by using VIF (Fig. 4b), which is 75 times less permeable to MeBr than LDPE mulch when tested under laboratory conditions [122]. This reduced permeability is associated with a polymer barrier, ethylene vinyl alcohol, or polyamide that is incorporated into the center of the VIF mulch. In previous research, VIF mulch reduced methyl bromide emission >40% over an LDPE mulch [123]. Concentration of MeBr + chloropicrin under VIF was 6 times higher than under high-density polyethylene (HDPE) mulch at 5 days after treatment [124]. Likewise, 1,3-dichloropropene and chloropicrin emissions from VIF mulch were reduced by >90% over standard mulch at 24 h after application [125].

Several experiments have been conducted over the past few years to demonstrate that fumigant-use rates can be lowered when using VIF mulch. For example, VIF mulch provided nutsedge control with one-half of the commercially used rate of MeBr + chloropicrin [124]. In another study, MeBr and 1,3-dichloropropene rate needed for effective disease suppression in strawberry was reduced by 50% under

Fig. 4 (**a**) Low-density polyethylene (LDPE) (**b**) Virtually Impermeable film (VIF) mulch

VIF compared with a standard mulch [126]. The rate of chloropicrin needed to suppress yellow nutsedge was 2–4 times lower when using VIF mulch compared to standard mulch [125]. In Florida, effective nutsedge (mixture of purple and yellow nutsedge) control was achieved in bell pepper at 25% of the labeled rate of MeBr/chloropicrin under VIF compared to a full rate under LDPE [127]. Hence, by using a VIF mulch, fumigant rates can be substantially reduced without sacrificing weed suppression, which may be applicable to volatile compounds like ITCs. In a previous laboratory experiment, losses of methyl ITC were reduced by 40% under VIF mulch compared to LDPE mulch [128]. Based on these results, it is expected that weed control efficacy of synthetic ITCs in field conditions can be enhanced by covering the ITCs treated soil with a VIF mulch.

Because volatiles are loss at a much slower rate, the retention of lethal fumigant concentrations remains in the soil for longer periods, increasing the fumigant effectiveness on target pest but at the same time can increase phytotoxicity risk to economic crops. Locascio et al. [129] reported that tomato plant dry weights were reduced 14% by VIF mulch compared with a standard black polyethylene mulch, with the reduced growth contributed to the fumigants being held in the soil longer by the VIF mulch. Therefore, testing crop safety to establish plant-back interval for synthetic ITCs is also important in plasticulture production.

5.3 Testing Synthetic Isothiocyanates for Weed Control in Plasticulture Production

Based on previous research conducted in the Southern USA [11, 12, 14], two synthetic ITCs (allyl and phenyl ITCs) were selected for lab and greenhouse experiments to determine (1) the effect of phenyl and allyl isothiocyanate

concentration and exposure period on nutsedge tuber viability and (2) to compare the retention of these ITCs in treated soil under LDPE and VIF mulches [130, 131]. Greenhouse trial results proved that high rates of phenyl ITCs in sealed environment can reduce purple nutsedge tuber viability by 97% [130]. Lab trials showed that phenyl isothiocyanate dissipation was lower in soil covered with VIF than with LDPE mulch and half-life of phenyl ITC under LDPE and VIF mulch was 6.09 and 8.88 days, respectively [130]. Similar results were observed for allyl ITC in another greenhouse study, but mulch effect was not very strong (half-life of 0.15 days under LDPE mulch vs. 0.59 days under VIF mulch) [131].

We hypothesized that using higher concentration of synthetic ITCs in combination with VIF plastic mulch can provide weed control equivalent to MeBr in field conditions. This hypothesis led to the field testing of allyl and phenyl ITCs under LDPE vs. VIF mulch for weed control (yellow nutsedge, Palmer amaranth, and large crabgrass) in plasticulture vegetable production in Arkansas and South Carolina from 2006–2009 [132–135]. This research provides some of the first information on field-level efficacy of synthetic ITCs (other than methyl ITC) for weed management in plasticulture vegetable production. ITCs were applied from 0 to 1500 kg ha^{-1} rate range and then compared with a standard rate of MeBr [MeBr/chloropicrin (67/33%) at 390 kg ha^{-1}] under LDPE mulch. Liquid ITCs were sprayed on the soil surface and immediately incorporated into the soil by using a rototiller before forming beds and covering them with plastic mulches (LDPE vs. VIF) (Fig. 5). Tomato and bell peppers were planted 3 weeks after ITCs or MeBr application. Please note that holes were punched in the plastic 48 h before transplanting to aerate the beds and reduce the crop injury from residual ITCs. Between two synthetic ITCs tested, allyl ITC was more effective on weeds and safer on vegetable transplants than phenyl ITC [132–135]. Therefore, we will focus only on allyl ITC results. It is concluded that crop injury from allyl ITC under LDPE or VIF mulch can be minimized to acceptable levels by using a plant-back interval of 3 weeks and aerating the beds for 48 h before transplanting [132, 133]. Weed control of all species increased with an increase in allyl ITC rates and this response was described by sigmoidal model (Fig. 6). However, yellow nutsedge was the most tolerant to allyl ITC among the three weed species tested in the present study (Table 6). Large crabgrass was intermediate in sensitivity to allyl ITC, whereas Palmer amaranth was least tolerant to allyl ITC among the three species. The less sensitivity of yellow nutsedge to allyl ITC could be associated with the larger size of tubers compared to the small-seeded Palmer amaranth or large crabgrass [12, 13]. Results also proved that allyl ITC can successfully reduce the viable weed propagule density in the bedded soil [132, 133], which will reduce the weed problem in succeeding crop. This will be more valuable for producers growing more than one crop on the same polyethylene-mulched beds [136].

Contrary to our hypothesis, VIF mulch did not improve the weed control from allyl or phenyl ITC over LDPE mulch, so VIF mulch had no added advantage over LDPE mulch [132–135]. This could be due to little to no differential retention of these ITCs under LDPE and VIF mulches. In contrast, we reported higher retention of phenyl and allyl ITC under VIF than LDPE mulch in previous indoor experiments

Fig. 5 ITC application and incorporation followed by plastic laying and transplanting

[130, 131]. Similar discrepancy has been reported in previous field versus indoor experiments on ITC and mulch types. For example, in a closed system, VIF mulch proved to be 40% less permeable to methyl ITC compared to LDPE mulch [128]. However, when tested under field conditions, VIF mulch failed to improve methyl ITC retention over LDPE mulch [137]. The possible difference in the field and indoor experiments could be due to variation in experimental procedures or environmental conditions. For example, in the indoor experiments [130, 131], sterilized soil was used, which might have minimized microbial degradation of

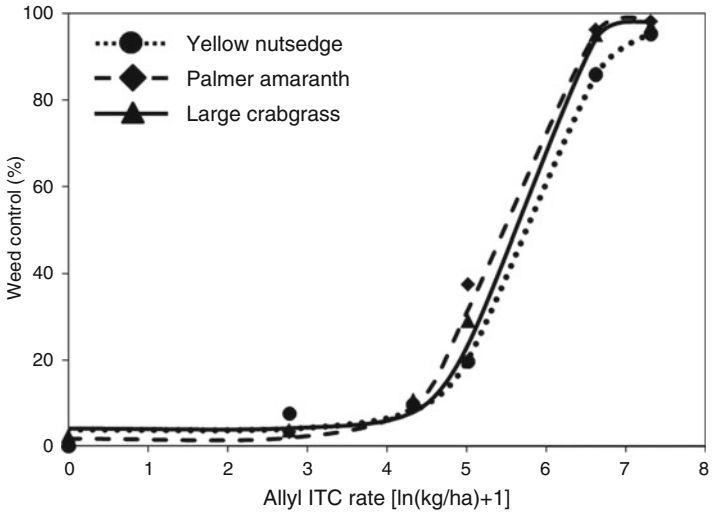


Fig. 6 Observed and predicted control of *yellow* nutsedge, Palmer amaranth, and large crabgrass at 6 weeks after transplanting as a function of allyl ITC rate. *Symbols* and *solid line* represent means and predicted response, respectively. The corresponding regression model and individual parameters are presented in Table 6 [132]

Table 6 Parameter estimates for regression model of yellow nutsedge, Palmer amaranth, and large crabgrass control at 6 wk after transplanting, averaged over mulch type (LDPE and VIF) and years (2007 and 2009), and allyl ITC rate needed to obtain weed control equivalent to methyl bromide[a, b]

	Parameters estimates (± SE)[c]				
	D	C	b	I	LR_{mb} (± SE)[d]
Weed species					Kg ha^{-1}
Yellow nutsedge	3.97 (1.28)	99.27 (3.26)	2.10 (0.21)	5.77 (0.09)	924 (74)
Palmer amaranth	1.80 (1.69)	99.90 (2.74)	2.44 (0.45)	5.36 (0.09)	888 (225)
Large crabgrass	4.13 (1.59)	99.08 (3.06)	2.65 (0.51)	5.53 (0.12)	651 (118)

[a]Abbreviations: *LDPE* low-density polyethylene mulch, *VIF* virtually impermeable film
[b]Reference [132]
[c]Regression model used to predict percent control of each weed species is represented by following Eq.: $Y = C + (D - C)/[1 + \exp\{b(\ln x - I)\}$, where Y represents the percentage control of each species, C is the upper asymptote, D is the lower asymptote, b is the slope of the response, x is the 1 + allyl ITC rate (kg ha^{-1}), and I is the natural log transformed allyl ITC rate needed to provide 50 % control. *SE* = approx. standard error of the mean
[d]LR_{mb} = allyl ITC rate required to provide percent control of a particular weed species equivalent to methyl bromide. SE = approx. standard error of the mean

ITC compared to field conditions. It is also suspected that the permeability of VIF mulches can change, depending upon changes in the physical and environmental conditions to which they are exposed [138]. VIF is less flexible than LDPE mulch [139]; therefore, excessive stretching during laying in the field can cause breakdown of the polyamide layer and increase the permeability of VIF mulch to volatile

substances. Another plausible reason of no effect of mulch type with allyl or phenyl ITC in our study [132–135] could be related to volatility of ITCs. In contrary to allyl or phenyl ITC, VIF mulch has been proved to improve MeBr efficacy over LDPE mulch even under field conditions [124, 127]. This could be attributed to lower volatility and weaker fumigant activity of allyl (vapor pressure = 0.53 kPa at 20 C) or phenyl ITC (vapor pressure = 0.19 kPa at 20 C) compared to MeBr (vapor pressure = 188 kPa at 20 C), and that is why no clear benefit of low-permeability plastic mulch (VIF) was realized in ITC field studies [132–135]. Based on our research, we concluded that regardless of mulch type, allyl ITC at high rates can provide effective, broad-spectrum weed control (Table 6, Fig. 7) and maintain fruit yield equivalent to MeBr [132, 133]. Although, allyl ITC rates are at least 2.4 times greater than current fumigants [132, 133] (Table 6), the more consistent season-long weed control provided by allyl ITC could compensate for the higher application rate. However, additional testing is suggested to refine ITC rates and application techniques not only to improve the production efficiency but also to minimize input cost and human/environmental risk.

Therefore, synthetic ITCs have shown promising results in plasticulture production system. Numerous ITCs have been identified, but the biological activity of very few ITCs has been examined under field conditions. Therefore, field testing of various synthetic ITCs would provide a better understanding for the potential fit of these compounds in a plasticulture production system. The knowledge of differential activity of ITCs in liquid or vapor phase will be critical for selection of any ITC in a production system. For example, short-chain aliphatic ITCs are more active in vapor phase [99] and therefore suitable for fumigation. Conversely, the long-chained aromatic ITCs have low fumigation potential due to their low volatile nature, but these ITCs are more toxic in liquid phase [99]. Furthermore, soil, environmental, and management factors should be considered during field testing of ITCs as they can dramatically change ITC efficacy. Moreover, to be eligible as fumigant/pesticide, any ITC needs to undergo extensive testing, including toxicology and environmental fate studies and worker exposure risk assessments.

Fig. 7 *Yellow* nutsedge control in bell pepper from allyl ITC at 6 weeks after transplanting

6 Conclusions

Weed control is challenging in plasticulture production systems in the absence of the preplant soil fumigant, MeBr. Plants belonging to Brassicaceae family have the ability to release herbicidal compounds (ITCs) in soil after tissue maceration and soil incorporation and therefore have been suggested as biofumigant crops. However, the amount of ITCs released in soil is affected by several factors like plant genotype, ontogeny, and morphology, soil and environmental conditions, and agronomic and incorporation practices. Under field conditions, the weed control provided by incorporation of Brassicaceae crops in commercial plasticulture production is not sufficient for growers to rely exclusively on biofumigation. The ineffectiveness of Brassicaceae crops in weed control can be associated with low ITC concentration in soil after incorporation. This could be mainly attributed to low production of GSLs (ITC precursors) by Brassicaceae cover crops, poor GSL to ITC conversion efficiency after tissue incorporation in soil, and loss of released ITCs in treated soil. Therefore, Brassicaceae crops can aid weed management but will not provide effective, season-long weed control required in a commercial plasticulture production. Conversely, synthetically produced ITCs at higher rates than produced by plants under natural conditions does have potential for supplying a high level of weed control. Among synthetic ITCs, allyl ITC proved to be more effective on weeds and comparatively safer than phenyl ITC in plasticulture vegetable production. However, there does not appear to be any advantage of VIF mulch over LDPE in improving weed control from allyl and phenyl ITCs in the field. Further research is needed to screen other potential synthetic ITCs and optimize their use rates and application techniques to maximize their herbicidal activity in crops produced in a plasticulture system.

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Glucosinolates and Plant Defense

7

Astha Singh

Abstract

Glucosinolates are unique secondary metabolites present in the members of family *Brassicaceae*. The major role of glucosinolates in plants is believed to be responses to external or environmental stimuli. Glucosinolates are also involved in communicating and triggering a range of information pertaining to plant defense against insects, some food bacteria, and against some fungi. Glucosinolates are hydrolyzed by the enzyme myrosinase on injury to plant to produce isothiocyanates and subsequently by PAL to toxic compounds injurious to the pathogen. In this review, the role of glucosinolates in plant defense has been discussed with possible involvement of PAL enzyme.

Keywords

Glucosinolates • Plant defense • Brassicas • Bion • SAR • Isothiocyanates • Phenylalanine ammonia lyase • Phosphonate • Broccoli

Abbreviations

HAG	High aliphatic glucosinolate
PAL	Phenylalanine ammonia lyase
SA	Salicylic acid
SAR	Systemic acquired resistance

Contents

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_8

1 Introduction

Glucosinolates are immensely unique secondary metabolites found in *Brassicaceae* family plants that are hydrolyzed by the enzyme myrosinase to produce isothiocyanates which is an intermediary compound [1]. Once hydrolysis occurs, toxic electrophilic hydrolysis products can be conjugated to glutathione and various amino acids [2]. The major role of glucosinolates in plants is believed to be responses to external or environmental stimuli. It is also involved in communicating and switching a range of information pertaining to plant defense [3–5] and to insect-attractant signals [6]. Significant research has been carried out regarding the relationship between plant resistance and glucosinolates [7], the role of insect attack [8, 9], water stress [10] on glucosinolate levels, plant age, and species [11] on glucosinolate content. However, major insights are still required to unfold the complex systems that these compounds are involved in and their fate through the course of the plant's life.

Glucosinolates are known to act as biopesticides and insect deterrents and have also been found to be beneficial to human health as antimicrobial and anticancerous compounds. Glucosinolates are considered to play a role in plant defense against insects [5, 12], some food bacteria [13], and some fungi [14, 15]. These compounds have been reported to have bio-herbicidal potential and are used as biofunigants [16, 17].

2 Chemical Structure of Glucosinolates

In relation to the chemical structure of these compounds, there is a central carbon atom which is bound via a sulfur atom. This is attached to the thioglucose group that makes a sulfated ketoxine and via a nitrogen atom to a sulfate group. An additional side group that is bound to the central atom is also involved, and the difference in glucosinolates occurs due to this side group. This side group is responsible for the variation in any activity of the glucosinolates [18, 19]. The basic structure of glucosinolates contains the side chain R, which symbolizes a variable structure that can be aliphatic or aromatic [20]. In this review, the role of glucosinolates in plant defense has been discussed with possible involvement of PAL enzyme.

3 Interaction Between Plant Defense and Phytochemicals

Plants have an array of defense mechanisms which occur soon after the pathogen attack that lead to formation of a wide range of phytochemicals and by products. These chemicals help the plant to respond to the incompatible interaction [21–23]. One of these defense responses involves phenylpropanoid metabolism in

which phenylalanine conversion to cinnamic acid and ammonia is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). The phenylpropanoid metabolism leads to various signaling pathways [24], one of which has the end product salicylic acid. Salicylic acid is a signaling molecule that activates the systemic acquired resistance (SAR) of the plant [25].

Broccoli and Indian mustard roots have been shown to accumulate high amounts of glucosinolates as a result of club root and white rust infections [26, 27]. Also, *Arabidopsis thaliana* has a defense mechanism that involves synthesis of tryptophan-derived indole glucosinolates in turn enhancing the nonhost resistance of the plant against a biotrophic fungus [28]. Research has shown a role of tryptophan-derived secondary metabolites in disease resistance of *Arabidopsis thaliana* to *Phytophthora brassicae* that induces genes encoding enzymes involved in aromatic glucosinolates biosynthesis in response to this pathogen. Therefore, disease resistance of *Arabidopsis* to *P. Brassicae* is established by aromatic glucosinolate activity [29].

The above findings suggest a role of phytochemicals in resistance, and there is a potential advantage from manipulation in the levels of the beneficial phytochemicals (in this case glucosinolates). Metabolism of glucosinolates involves precursor amino acid phenylalanine, and its deamination is catalyzed by PAL to form trans-cinnamic acid [30].

4 Biosynthetic Pathways

Understanding biosynthetic pathways of glucosinolate formation is far from clear. Significant research has been carried out relating to immunohistochemical localization of glucosinolates. In in situ localization, glucosinolates are lost to the material that was used to fix them if we follow conventional fixation and dehydration procedures. Therefore, analysis was done by using ELISA and raising antibodies against complexes of glucosinolates [31]. Another piece of work has reported the vacuolar localization of glucobrassicin and sinigrin [32] (types of aliphatic Glucosinolates).

A key regulatory enzyme in the biosynthesis of aromatic glucosinolates is PAL, the enzyme that diverts phenylalanine from primary metabolism to secondary metabolism. PAL converts phenylalanine to cinnamic acid, the basic building block for phenylpropanoids and alkaloids and the side chains of aromatic glucosinolates [33]. PAL activity increases following a range of environmental stresses, including pathogen attack [34], leading to the formation of phytoalexins, lignin, and salicylic acid, a key regulator of SAR [35]. Thus, changes in the levels of aromatic glucosinolates may result from changes in PAL activity or a switch in the balance of pathways using cinnamic acid (Fig. 1) [36]. Phenylalanine is converted to cinnamic acid via PAL enzyme and is followed by various pathways like phenylpropanoid metabolism, lignin formation, SA metabolism, and aromatic glucosinolate biosynthesis.

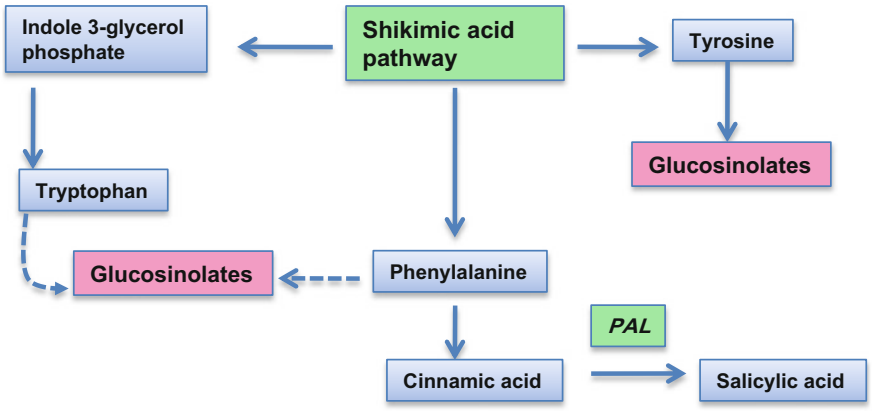

Fig. 1 Pathway of glucosinolate biosynthesis from amino acid, tryptophan. Activity of PAL and formation of cinnamic acid are also shown. Pathways with dotted line are not clearly known (Based on Dixon [21])

5 Environmental Regulation of Glucosinolate Biosynthesis

Salicylic acid is a key signal regulating plant defenses. Glucosinolate accumulation in leaves of oilseed rape plants was affected by salicylic acid applied as soil drench [37]. Byun et al. [38] recently reported that cold stress activates genes related to salicylic acid synthesis and glucosinolate synthesis in *Arabidopsis thaliana*, indicating their interdependent relationship as an early response to cold stress. Wielanek et al. [39] reported that salicylic and ethylene signaling regulates jasmonate-mediated gene expression and glucosinolate content following herbivore attack in *Arabidopsis thaliana*. Jasmonate also plays an important role in plant defense mechanisms [40].

Biosynthesis of nitrogen-containing tryptophan-derived indole glucosinolates has been reported to increase with the increased nitrogen supply. However, sulfur containing methionine-derived glucosinolates decreased with increasing nitrogen and low sulfur contents, indicating a direct relationship between biosynthesis and the availability of nitrogen and sulfur compounds [41]. Methionine-derived glucosinolates not only serve as a chemo-protective compound in plant biotic defense reactions but also exhibit strong anticarcinogenic properties beneficial to human health. In a screen for the trans-activation potential of various transcription factors toward glucosinolate biosynthetic genes, HAG1 (high aliphatic glucosinolate 1, also referred to as MYB2) was shown to act as a positive regulator of aliphatic methionine-derived glucosinolates [42, 43]. Therefore, what regulates these transcription factors and ways it is linked to environmental responses have yet to be investigated upon. Glucosinolate levels are affected by a range of environmental stimuli. PAL provides a readily monitored surrogate indicator of glucosinolate biosynthesis following pathogen attack. Therefore, monitoring PAL can be an

effective indication of glucosinolate metabolism post-pathogen attack and was brought into action by Singh et al. [27].

6 Phenylalanine Ammonia Lyase Activity

Phenylalanine is a precursor of some aromatic glucosinolates during initial biosynthesis steps [44], and the enzyme PAL is a key enzyme in phenolic metabolism of plants. PAL activity can be considered as an indirect indicator of aromatic glucosinolate synthesis; however, there are other pathways where PAL is involved, for example, SA pathway and phenylpropanoid metabolism.

PAL activity is affected by wounding of the plant; PAL accumulation was found 24 h after wounding of maize mesocotyls [45]. PAL activity has also been shown to be affected by chilling injury; the greater the injury, the more is the activity [46]. PAL activity is known to increase soon after the plant is challenged by a pathogen. As in the case of cassava following elicitation by yeast, PAL was induced after 15 h [24]. Another example of induction of PAL activity after inoculation was wheat seedlings against stripe rust [47].

The defense activator, Bion®, has also been known to affect PAL activity 6, 9, and 12 days after spraying in tomato as a defense response against *Xanthomonas vesicatoria* [48] as well as after treating wheat seedlings with Bion® as a control against stripe rust [47]. Young et al. [49] have shown that phenolic levels in conventionally grown vegetable crops and organically grown vegetable crops, which are environmentally stressed, were high. This was because of increased activity of PAL following which more phenolics are synthesized. The PAL catalyzed conversion from phenylalanine to cinnamate, and then cinnamic acid has been shown.

Phosphonate has been shown to activate defense responses in *Arabidopsis* as a result of *Phytophthora palmivora* challenge [50]. Induction of similar defense responses may explain PAL activity increases in phosphonate-treated broccoli leaves in the current research. Also phosphonate treatment has been shown to activate defense responses in *Lambertia* spp. against *Phytophthora cinnamomi*, and following which PAL activity increased 24 h after inoculation [51] (Fig. 2).

Cinnamic acid is a white crystalline acid obtained naturally from the oil of cinnamon. Cinnamic acid is also a self-inhibitor produced by fungal spores to prevent germination [52]. In relation to disease and PAL activity, research suggests that PAL activity increases significantly in all inoculated genotypes of wheat in the first few hours after inoculation with *Erysiphe* [53]. This implies that infection stimulates PAL activity that is shown in the current research. Also many studies of PAL-deficient mutants that are compromised in resistance reveal similar results [54]. These studies indicate that mutants with deficiency in PAL levels affect the phenylpropanoid pathway and the plant's ability to resist pathogens [55, 56].

Summing up the literature and various pathways, the following figure (Fig. 3) indicates that PAL activity can be monitored to derive more information about formation of phenolics (e.g., salicylic acid and some glucosinolates).

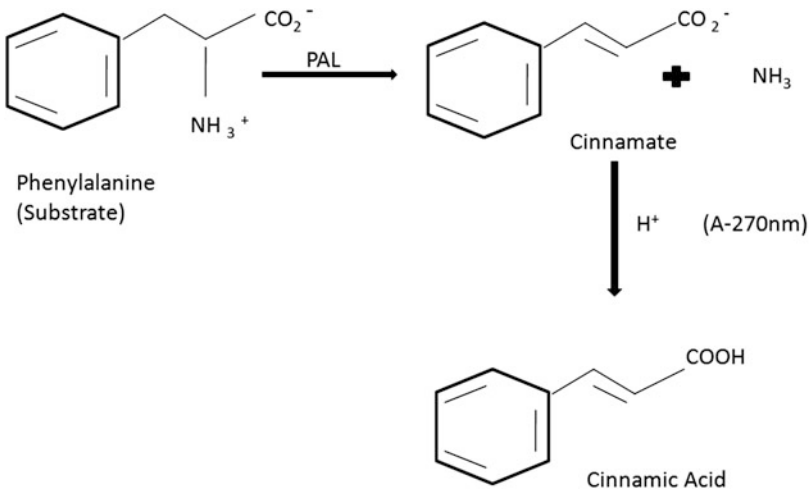

Fig. 2 Conversion of phenylalanine to cinnamic acid, catalyzed by PAL as measured at absorbance 270 nm in a UV-vis spectrophotometer (Nanodrop 2000c, thermo scientific)

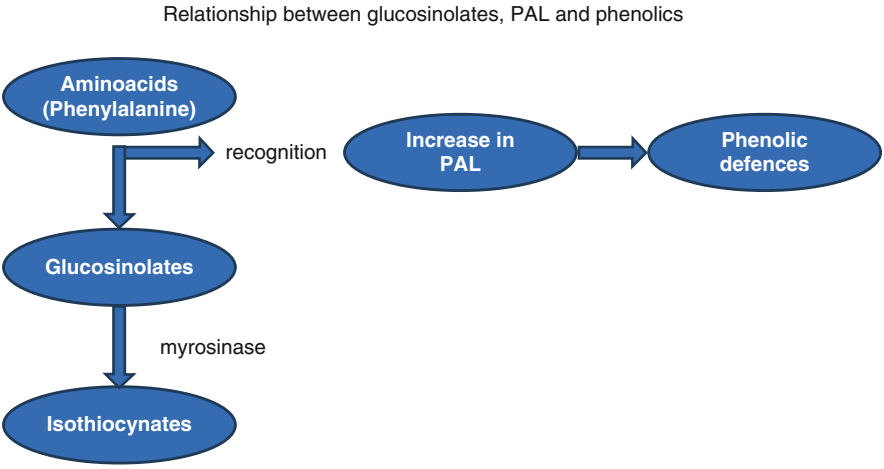

Fig. 3 Relationship between glucosinolates and PAL activity. PAL and glucosinolates are derived from the same precursor that links the two

There can be two possible circumstances, one in which there is already accumulation of glucosinolate in the crop and the other where it follows stress in the form of pathogen attack in the plant. In the first case, glucosinolates are synthesized from amino acid precursors as the normal course. These glucosinolates are in compartments within vacuoles; after injury or chewing, these compartments break, and myrosinase acts to form isothiocyanates which in turn has possible beneficial effects for human health. On the other hand, if there is stress in the plant, i.e., pathogen

infection, phenylalanine is accumulated as a result of infection in turn accumulating more glucosinolates. Increase in PAL activity was found after inoculation of *Brassica juncea* with *Albugo candida* which suggested that pathogen infection triggers the accumulation of PAL in *Brassica* crops [57]. This derivative pertains to having the potential to be beneficial to human health.

Bion® delayed the symptoms of white rust for 2 days, while phosphonate delayed symptoms for 3 days. This occured possibly due to the phenolics and phytochemicals formed after infection and defense response induction due the defense activator treatments. One of these hydrolysis compounds has been studied by measuring the PAL activity. The increase in the PAL activity after inoculation and Bion® and phosphonate treatment supports the role for PAL in plant defense in broccoli and that increase in PAL activity is a marker of plant defense [58].

PAL activity in both root and stem tissues were analyzed in chickpea variety JG-62 susceptible to *Fusarium oxysporum* causing vascular wilt. It was found that there was high PAL activity at early stages (pre-colonization stages) of the disease [34].

PAL is activated by the SAR mechanism of Bion® and defense priming function of phosphonate. This research also implies that PAL is activated by infection as one of the earliest defense responses in the plant against *Albugo candida* [27].

7 Conclusions

Glucosinolates have been known and proven for their vital role in plant defense [59, 60] as they are plant secondary metabolites that are induced by stress or infection and act as precursors to plants' defense system. Similar role is carried out by PAL activity [4, 61] in earlier stages of infection. Accumulation of certain glucosinolates, PAL activity, and hypersensitive response [62] after pathogen challenge has been regarded as indicators of expression of systemic resistance by *Brassica* plants [63–65].

Greater insight is required to determine the exact course of the compound's journey through the brassica plants and its certainty in acting as a major participant in plants' systemic resistance and defense mechanisms. This research will be highly beneficial to progress molecular and genetic studies in the area of plant defense enhancement.

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Part II

Biological Activity

Antimicrobial Activity of the Glucosinolates

8

Federica Saladino, Keliani Bordin, Fernando Bittencourt Luciano, Mónica Fernández Franzón, Jordi Mañes, and Giuseppe Meca

Abstract

The use of natural antimicrobial compounds is receiving much attention and is becoming very frequent by the importance that nowadays is given to natural resources. Natural components have been applied in several sectors such as agriculture, biomedicine and food preservation. The development of resistance to conventional antibiotic by pathogenic bacteria makes necessary to find alternative antimicrobials to eradicate these microorganisms. Many food products are perishable and require protection from spoilage to improve quality and shelf life. Numerous efforts are conducted to find safe natural alternatives to prevent microorganism growth in plants and food products, because of the consumer concern regarding synthetic pesticides and preservatives. Natural antimicrobials can be obtained from different sources including plants, animals, bacteria, algae, and fungi. Among them, glucosinolates and their derived products have been recognized for their benefits to human nutrition, plant defense, and as potent antimicrobial agents. This chapter describes the antimicrobial activity of glucosinolates and their hydrolysis products against different bacterial and fungal species, as well as the mechanism of action of these active compounds.

Keywords

Glucosinolates • Isothiocyanates • Antifungal activity • Antibacterial activity • Bioactive compounds

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_18

Abbreviations

AAM	Allylamine
AC	Allyl cyanide
AITC	Allyl isothiocyanate
ASC	Ascorbigen
ATC	Ally thiocyanate
BAM	Benzylamine
BC	Benzyl cyanide
BITC	Benzyl isothiocyanate
CEPT	1-Cyano-2,3-epithiopropane
DIM	3,3′-Di-indolylmethane
EITC	Ethyl isothiocyanate
GLS	Glucosinolate
I3C	Indole-3-carbinol
IAN	Indole-3-acetonitrile
ITC	Isothiocyanate
MAP	Modified atmosphere packaging
MCT	Medium-chain triglyceride
MITC	Methyl isothiocyanate
PAM	2-Phenylethylamine
PEC	2-Phenylethyl cyanide
PEITC	Phenylethyl isothiocyanate
PITC	Phenyl isothiocyanate
SBO	Soybean oil
SFN	Sulforaphane
TC	Thiocyanate

Contents

1 Introduction

In agreement with the current trend to value the natural and renewable resources, the interest in the use of natural antimicrobial compounds is increasing for biomedical, agricultural, and especially food applications [1, 2].

Food products are perishable by nature and can be subjected to contamination by bacteria and fungi. Many of these microorganisms can cause undesirable

reactions that deteriorate flavor, odor, color, sensory, and textural properties of foods. Some of them can also potentially cause food-borne illness. For all these reasons, food products require protection from spoilage during their preparation, storage, and distribution to give them desired shelf life. Furthermore, the dramatic rise of antibiotic-resistant microorganisms is of concern and includes food-borne pathogens that are also more tolerant to several food processing and preservation methods. The consumer concern regarding synthetic products, such as food additives and pesticides, and the necessity to overcome the emergence of antibiotic-resistant pathogens led to the research of alternative compounds with potent antimicrobial activity which can reduce the impact of synthetic products on human and animal health [1].

Natural antimicrobials can be obtained from different sources including plants, animals, bacteria, algae, and fungi. To select the appropriate biocidal product, the microorganism strain must be identified and the spectrum of antimicrobial activity of the compound considered [2–4]. Several reports have demonstrated the efficacy of plant-derived compounds, most of all in food applications. Antimicrobials derived from plants are mostly secondary metabolites that possess various benefits including antimicrobial properties against pathogenic and spoilage microbes. The structural diversity of plant-derived compounds is immense, and the impact of antimicrobial action they produce against microorganisms depends on their structural configuration [5].

Among the potent natural antimicrobials, glucosinolates (GLS) are an important class of secondary plant products found in seeds, roots, stems, and leaves of cruciferous plants including 16 families of dicotyledonous angiosperms, mainly *Brassicaceae* [6]. There are about 120 different GLS identified, derived from amino acids (alanine, leucine, isoleucine, valine, phenylalanine, tyrosine, and tryptophan) and a number of chain-elongated homologues [7]. They are classified as aliphatic, aromatic, methylthioalkyl, and heterocyclic, which have a thioglucoside component in common structure and differ at their side chains [8].

Located within vacuoles, GLS are physically separated but accompanied by β-thioglucosidase enzymes known as myrosinases [9]. Following plant tissue disruption, the enzyme and GLS come into contact, which, in the presence of water, generates a hydrolysis forming an aglycone moiety, glucose, and sulfate. The aglycone moiety is unstable and rearranges to form three main groups of substances: nitriles, thiocyanates (TCs), and isothiocyanates (ITCs) (Fig. 1) [3, 10, 11].

GLS and their enzymatic hydrolysis products are responsible for a characteristic pungent flavor [12, 13]. These compounds have shown several biological activities including plant defense (against insects and microbial infections) and benefits to human health (anticarcinogenic, antioxidant, and antimicrobial properties) and might be potential natural agents for food preservation [14]. Their response to microbial population varies according to their structural characteristics. The biocidal effect of cruciferous tissues on other microorganisms has been attributed mainly to volatile degradation products of GLS, released from their plants. Among derived

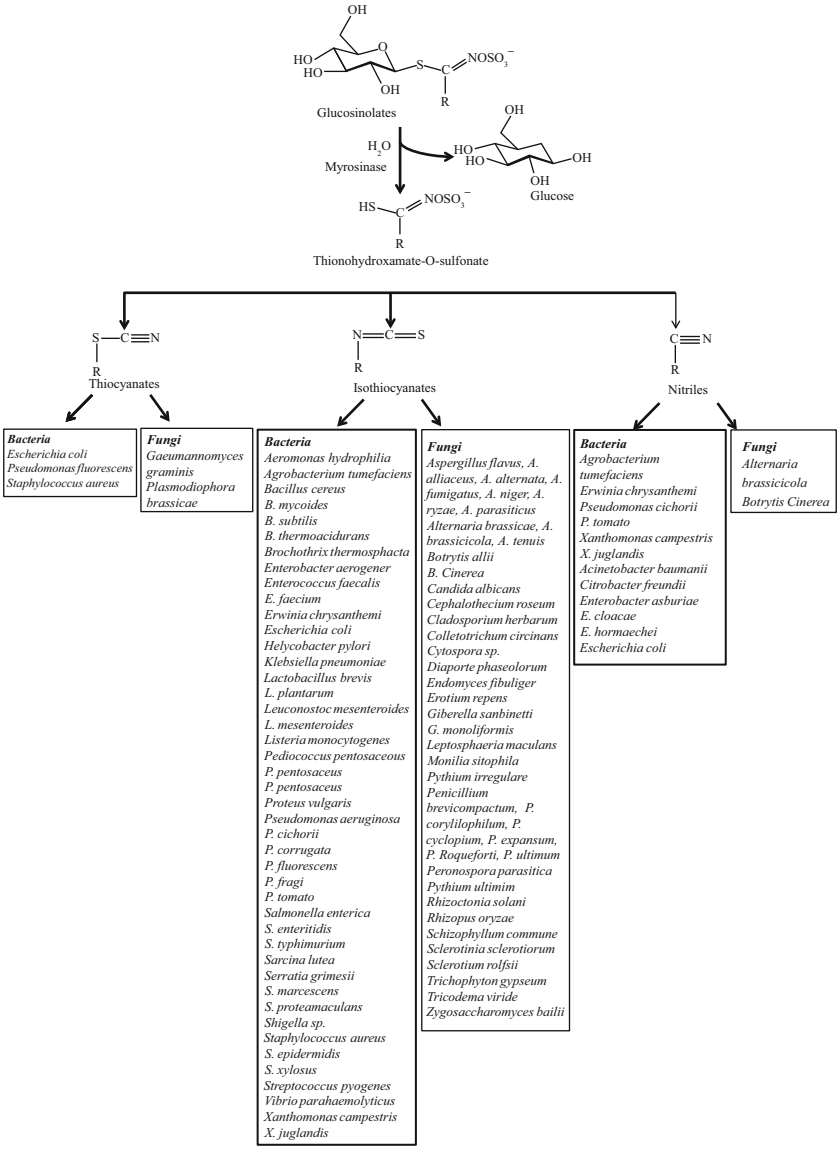

Fig. 1 Enzymatic degradation of glucosinolates and the antifungal spectrum activity of their derived products

products, ITCs are the major inhibitors of microbial activity, and they have been studied mainly for food preservation and plant pathogen control [5, 12]. ITCs are volatile substances that display an inhibitory effect on several microorganism species at low concentrations [15].

Therefore, the objective of this chapter was to present results of studies on antimicrobial activity of GLS and their enzymatically degradation products and highlight important aspects on the application.

2 Antibacterial Activity

GLS and, above all, their hydrolysis products elicit a wide spectrum of antimicrobial activity against a variety of bacteria. The concentrations of these compounds required to inhibit microorganisms are difficult to compare given differences in methodologies, materials, and test strains employed. There are considerably more data for the ITCs, and in particular for AITC, than others GLS products. Furthermore, the mode of delivery to target microorganisms has a large impact on the antibacterial effect. Dissolution of these compounds in liquid media can result in a weak antimicrobial activity, while lower concentrations in the vapor phase are sufficient to inhibit microorganisms [10]. On Table 1 are summarized some studies which report the antibacterial potential of these compounds.

Horseradish vapors, containing GLS hydrolysis products, showed stronger antibacterial activities against several bacterial strains [16, 17]. Later, also studied and compared were the bacteriostatic and bactericidal effects of AITC, EITC, and MITC against 10 strains. MITC was the most effective, in both solution and vapor phases, followed by AITC and EITC. *Escherichia coli* and *Staphylococcus aureus* appeared to be the more resistant strains, while the least resistant were *Bacillus subtilis*, *Bacillus mycoides,* and *Serratia marcescens*.

Virtanen [18] reported the antimicrobial activity of BITC and β-phenylethyl, m-methoxybenzyl, and methoxybenzyl ITCs against *S. aureus*. The activity of these ITCs was higher than the antimicrobial activity of a series of aliphatic ITCs. Zsolnai [19] demonstrated that the same concentration of AITC and PITC, used to severely inhibit the growth of yeasts and fungi, was not effective against *Streptococcus pyogenes*, *S. aureus*, and other Gram-negative bacteria. BITC was effective against *Staphylococci* but not on other bacteria.

Kanemaru and Miyamoto [20] studied the antibacterial activity of brown mustard and its major pungent compound, AITC, on the growth of *E. coli* 3301, *S. aureus* IFO 3761, *Proteus vulgaris* IFO 3851, *Pseudomonas fragi* IFO 3458, and *Pseudomonas aeruginosa* IFO 3755. To prepare the extract of black mustard extract was prepared as 20% mustard in ethanol (70%) after myrosinase treatment. AITC was also dissolved in 70% ethanol to form an equivalent concentration. The nutrient broth in which the bacteria were cultured contained the mustard extract or AITC and was stored at 30 °C on a shaker. Turbidimetry was used to determine bacterial growth. The results obtained evidenced that the antibacterial effect of mustard was mainly due to AITC. The concentrations of mustard in the medium that inhibited bacterial growth for 24 h were 0.138%, 0.104%, 0.064%, 0.043%, and 0.089% and those of AITC were 14.5, 12.3, 6.5, 3.6, and 7.2 ppm for *S. aureus*, *E. coli*, *P. vulgaris*, *P. fragi*, and *P. aeruginosa*, respectively. A bacteriostatic effect was

Table 1 Antibacterial potential of glucosinolate-derived products against several species on food products

Glucosinolate derivative	Bacterial strain	Food product	Reference
Allyl isothiocyanate	*Bacillus thermoacidurans*	Fresh apple juice	[44]
Allyl isothiocyanate	*Bacillus subtilis* IFO-13722 *Bacillus cereus* IFO-13494 *Staphylococcus aureus* IFO-12732 *Staphylococcus epidermidis* IFO-12993 *Escherichia coli* JCM-1649 *Salmonella typhimurium* A TCC-14028 *Salmonella enteritidis* JCM-189 *Vibrio parahaemolyticus* IFO-12711 P. *Pseudomonas aeruginosa* IFO-13275	Fresh beef Cured pork Sliced raw tuna Cheese Egg sandwich Noodles Pasta	[45]
Allyl isothiocyanate Phenethyl isothiocyanate Allyl thiocyanate	*Staphylococcus aureus*	Cooked roast beef	[46]
	Escherichia coli O157:H7		
	Staphylococcus typhimurium		
	Listeria monocytogenes		
	Serratia grimesii		
	Lactobacillus sake		
Allyl isothiocyanate Phenethyl isothiocyanate Allyl thiocyanate 1-Butane isothiocyanate	*Pseudomonas* spp. *Enterobacteriaceae* Lactic acid bacteria	Precooked roast beef slices	[47]
Methyl isothiocyanate, Allyl isothiocyanate	Rifampicin-resistant strain of *Salmonella,* Montevideo streptomycin-resistant strains of *Escherichia coli* O157:H7 and *Listeria monocytogenes* Scott A	Apples, tomatoes, Iceberg lettuce	[48]
Allyl isothiocyanate	*Escherichia coli* O157:H7	Fresh ground beef	[49]
Allyl isothiocyanate	*Pediococcus pentosaceus* *Staphylococcus carnosus* *Escherichia coli* O157:H7	Dry fermented sausage	[50]
Allyl isothiocyanate	*Lactobacillus algidus* *Leuconostoc mesenteroides* *Leuconostoc carnosum* *Carnobacterium maltaromaticum* *Carnobacterium divergens* *Brochothrix thermosphacta* *Serratia proteamaculans*	Marinated pork	[51]
Allyl isothiocyanate	*Salmonella*	Fresh cantaloupe	[52]

(*continued*)

Table 1 (continued)

Glucosinolate derivative	Bacterial strain	Food product	Reference
Allyl isothiocyanate	*Leuconostoc mesenteroides* *Lactobacillus plantarum*	Kimchi	[53]
Allyl isothiocyanate	*Escherichia coli* *Listeria monocytogenes*	Fresh cut onions	[54]
Allyl isothiocyanate	*Listeria monocytogenes* *Salmonella typhimurium*	Chicken breast	[56]
4-Hydroxybenzyl isothiocyanate	*Salmonella*	Sauce with particulates	[55]

shown by mustard on *S. aureus* and *E. coli* (0.8%), while the effect was bactericidal on *P. aeruginosa* at 0.2%

Shofran et al. [21] tested the antimicrobial activity of sinigrin and four sinigrin hydrolysis products, in broth culture, against different species of bacteria. Sinigrin is a GLS that, upon injury or mechanical disruption of plant tissue, is hydrolyzed by myrosinase producing up to four distinct compounds: AITC, allyl TC (ATC), allyl cyanide (AC), and 1-cyano-2,3-epithiopropane (CEPT). Sinigrin had little effect upon the growth of microorganisms [22], but its hydrolysis products were effective in inhibition of growth. The species of bacteria studied in the experiment were *E. coli* 33625, *E. coli* NC101, *Pseudomonas fluorescens* MD13, *Aeromonas hydrophilia* 7966, *S. aureus* 4220, *B. subtilis* IS75, *Pediococcus pentosaceus* FFL48, *Leuconostoc mesenteroides* FFL44, *Lactobacillus brevis* MD42, and *Lactobacillus plantarum* MOP3. Sinigrin, AC, and CETP at 1000 ppm did not show inhibitory effects against any of the bacteria tested. ATC was inhibitory to the growth of 3 strains of Gram-negative (*E. coli* 33625, *E. coli* NC101, *P. fluorescens* MD13) and 1 strain of Gram-positive bacteria (*S. aureus* 4220) with minimum inhibitory concentration (MIC) values ranged between 200 and 400 ppm. The antimicrobial activity of ATC was due to its conversion to AITC, sinigrin hydrolysis products with the highest antibacterial activity. AITC was effective against all the bacteria tested, except *L. plantarum* MOP3. The MIC of AITC against Gram-negative and Gram-positive non-lactic acid bacteria ranged between 100 and 200 ppm, while lactic acid bacteria were more resistant with MIC between 500 and 1000 ppm. It should be highlighted that the antimicrobial activity of AITC can be different if it is used in gaseous form or dissolved in broth culture. Furthermore, a lot of factors can influence the generation of AITC from sinigrin.

Kyung and Fleming [23] tested sinigrin and its derivate products against 15 species of bacteria: *Pediococcus pentosaceus* LA3, *P. pentosaceus* LA76, *L. mesenteroides* LA10, *L. mesenteroides* LA113, *L. plantarum* LA97, *L. plantarum* LA70, *L. brevis* LA25, *L. brevis* LA200, *Listeria monocytogenes* B67, *L. monocytogenes* B70, *S. aureus* B31, *E. coli* B34, *Enterobacter aerogenes*

B146, *B. subtilis* B96, and *Salmonella typhimurium* B38. Sinigrin itself was not antimicrobial because it did not inhibit growth up to 1000 ppm and microorganisms did not degrade it to its antimicrobial aglycones. AITC is known to be antimicrobial, and the MICs found ranged from 50 to 500 ppm for bacteria, including Gram-positive, Gram-negative, pathogenic, and lactic acid bacteria.

Delaquis and Sholberg [24] evaluate the microbistatic and microbicidal properties of gaseous AITC against bacterial cells of *S. Typhimurium* (ATCC 14028), *L. monocytogenes* (strain 81–861), *E. coli* O157:H7 (ATCC 43895), and *Pseudomonas corrugata* (isolated from lettuce). *S. typhimurium*, *L. monocytogenes*, and *E. coli O157:H7* were inhibited when exposed to 1000 μg L^{-1} AITC. *P. corrugata* failed to grow in the presence of 500 μg L^{-1}. Variations at different incubation temperatures were observed. Bactericidal activities varied with strain and increased with time of exposure. The most resistant bacterium was *E. coli*.

The antibacterial properties of the GLS and their hydrolysis products became of big interest and importance also in the eradication of pathogenic microorganisms that is complicated by the development of resistance to conventional antimicrobial agents. *Helicobacter pylori* is one of the most prevalent human pathogens in the world. Gastric infections with *H. pylori* are known to cause gastritis and peptic ulcers and dramatically enhance the risk of gastric cancer. Antibiotic therapy is recommended for infected patients with gastric or duodenal ulcers or gastric mucosa-associated lymphoid tissue lymphoma, but this treatment is not universally successful. Even with the combination of two or more antibiotics, *H. pylori* is difficult to eradicate due to the development of resistance of this bacteria to these antibiotics and the persistence of organisms within gastric epithelial cells and, furthermore, due to logistic, sociologic, and economic reasons. The ITC sulforaphane (SFN) appears to overcome all of these problems. SFN is abundant in certain varieties of broccoli and broccoli sprouts in the form of its GLS precursor called glucoraphanin. It has been demonstrated that SFN is a potent bacteriostatic agent against 3 reference strains and 45 clinical isolates of *H. pylori*. The MIC for 90% of the strains is <4 μg mL^{-1}, irrespective of their resistance to conventional antibiotics. It is a potent bactericidal agent against both extra- and intracellular *H. pylori* in vitro. Further, brief exposure to SFN eliminated intracellular *H. pylori* from a human epithelial cell line (HEp-2). Although higher concentrations are required to achieve bactericidal activity for the intracellular forms, SFN accumulates intracellularly to high levels, as its glutathione conjugate. It can be safely administered to humans because it is present in high concentrations in edible cruciferous vegetables and can be directly delivered to the stomach [25].

Haristoy et al. [26] evaluated the effect of SFN in vivo against *H. pylori* by using human gastric xenografts in nude mice. *H. pylori* was completely eradicated in 8 of the 11 SFN-treated grafts, after short-term administration of SFN at a dose that can be achieved in the human diet.

Haristoy et al. [27] analyzed the activities of 12 ITCs including sulforaphane on 25 strains of *H. pylori* using an agar dilution assay. The ITCs tested were iberin, cheirolin, erucin, $_{D,L}$-SFN, $_{D}$-SFN, $_{L}$-SFN, $_{L}$-sulforaphane, erysolin, berteroin, alyssin, hirsutin, PEITC, BITC, and 4-(α-L-rhamnopyranosyloxy)benzyl ITC.

Furthermore, the bactericidal activities of the six ITCs (cheirolin, $_{L}$-sulforaphane, erysolin, berteroin, hirsutin, and 4-(α-L-rhamnopyranosyloxy)benzyl ITC) that showed the lowest MICs were determined both directly and against intracellular bacteria in cultured human epithelial cells. The MIC_{90} values for these ITCs ranged between 4 and 32 μg mL^{-1}. It has been demonstrated that, in addition to SFN, four (cheirolin, berteroin, hirsutin, and 4-(α-L-rhamnopyranosyloxy)benzyl ITC) of the most active compounds exhibited high bactericidal activity against both extra- and intracellular bacteria.

Ono et al. [28] screened, isolated, and identified antibacterial compounds occurring in some common foods for bactericidal use, against *E. coli* and *S. aureus*. Among the different foodstuffs studied, wasabi stems, banana fruits, coriander leaves, and mustard seeds showed antibacterial activity. In particular, the lower minimal bactericidal concentration was obtained for wasabi stems, so their activity was highest. The compound with the antibacterial activity was identified as the 6-methyl-sulfinylhexyl ITC. The ethyl, butyl, hexyl, and octyl homologues of this ITC were determined in some *Cruciferae* plants. The main component contained in wasabi was the hexyl homologue, whereas horseradish contained the ethyl and hexyl homologues. Broccoli, Chinese cabbage, radish, and turnip almost exclusively contained the butyl homologue, and cabbage contained only the hexyl homologue. These homologues were also active against *E. coli* and *S. aureus* with minimal bactericidal concentration ranged between 0.1 and 2.0 mg mL^{-1}.

Liu and Yang [29] studied the stability and the antimicrobial activity of AITC in two medium-chain triglyceride (MCT) and soybean oil (SBO), dispersed in an oil-in-water system during long-term storage. It has been shown that the stability and antimicrobial activity were affected by the content, type, and oxidative stability of the oil. In particular, high oil content is favorable for AITC stability in the emulsion. AITC with MCT were more effective than AITC with SBO in inhibiting Gram-negative bacteria *E. coli* O157:H7, *Salmonella enterica*, and *Vibrio parahaemolyticus* and Gram-positive bacteria *S. aureus* and *L. monocytogenes*.

Luciano and Holley [30] evaluated the antibacterial activity of AITC against *E. coli* O157:H7 at different pH values and examined the inhibitory action of this compound against two enzymes important in the metabolism of this food-borne pathogen (thioredoxin reductase and acetate kinase). AITC showed greater antimicrobial activity at low pH values (4.5 and 5.5). Decomposition products of this ITC were also studied, and they did not show antibacterial activity toward *E. coli* O157:H7. Only AITC is antimicrobial in its original form. Furthermore, it has been demonstrated that only 1 μL L^{-1} of AITC could decrease the activity of thioredoxin reductase and AITC at 10–100 μL L^{-1} was able to significantly inhibit both thioredoxin reductase and acetate kinase.

The antimicrobial properties of different GLS autolysis products of *Hornungia petraea* were investigated against two isolates of *S. aureus*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Sarcina lutea*, *E. coli*, *Shigella* sp., and *Bacillus cereus*. The tested compounds were showed to be active against all tested microorganisms, with the activity ranging from 1 to 1250 mg mL^{-1} for inhibitory and 1 to 5000 mg mL^{-1}

for microbicidal activity. In particular, the assays showed a very high antibacterial activity of the tested ITCs against *S. lutea* [31].

Olaimat and Holley [32] determined the minimum inhibitory and minimum bactericidal concentrations of AITC from mustard against five strains each of *Salmonella* and *L. monocytogenes* individually and combined. The MIC and MBC values of AITC ranged from 60 to 100 ppm and 120 to 180 ppm, respectively, at 37 °C and ranged from 10 to 40 ppm and 200 to 600 ppm, respectively, at 21 °C against both pathogens. AITC had no antimicrobial activity at low temperatures (4 °C or 10 °C) and alkaline pH over 10, but at neutral pH, *L. monocytogenes* is reduced. At acidic pH, AITC was more effective against *Salmonella*. However, AITC was more effective at combinations of 21 °C and neutral pH against *L. monocytogenes* and at combinations of higher temperature and acidic pH against *Salmonella*.

A lot of data are available about the antimicrobial activity of ITCs, but the results are difficult to compare. Accordingly, Wilson et al. [33] studied the antibacterial activity of a large number of ITCs on a wide range of microorganisms, using for all the same experimental conditions. Ten ITCs were tested, and, among them, six were investigated for the first time: SFN, iberin, AITC, BITC, MITC, PITC, PEITC-, propyl-, 3-methylthiophenyl-, and 3-methylthiopropyl-ITC. The bacteria tested were fourteen and included 8 Gram-positive species (*B. cereus* CIP 78.3, *B. subtilis* ATCC 6633, *Enterococcus faecalis* G9h, *Enterococcus faecium* ATCC 19434, *L. plantarum* DSM 9843 [299v], *L. monocytogenes* LC 10, *S. aureus* ATCC 6538, and *Staphylococcus xylosus* LC 57) and 6 Gram-negative species (*K. pneumoniae* DSM 681, *E. coli* ATCC 25922, *P. aeruginosa* DSM 1128, *S. enteritidis* LC 216, *S. typhimurium* LC 443, and *S. marcescens* LC 448). A turbidimeter was used to monitor the growth of bacteria, and the antimicrobial activity was expressed as antimicrobial efficacy index that is a function of the growth delay, the reduction in the maximum population, and the reduction in maximum specific growth rate. All the ITCs tested displayed antimicrobial activity, depending on the target bacteria and the structural features of the molecule considered. BITC showed the highest value of antimicrobial efficacy index, followed by PEITC. Different from other studies, AITC was the least active ITC, and not necessarily aromatic ITCs were more active than aliphatic compound. For example, 3-methylthiopropyl-ITC was much more active than PITC. Gram-negative bacteria were overall more sensitive to ITCs than Gram-positive bacteria, and considerable variations in sensitivity were evidenced between species even within the same Gram type.

AITC, BITC, and PEITC purified from cruciferous plants were evaluated against 15 isolates of methicillin-resistant *S. aureus* (MRSA) isolated from diabetic foot ulcer patients. In general, the AITC always presented the higher MIC values and thus lower antimicrobial activity, while BITC and PEITC presented the lowest MIC. Therefore, these ITCs showed the highest antimicrobial activity. The AITC and PEITC were essentially bacteriostatic, whereas BITC was bactericidal in 11 isolates of MRSA. Based on this, BITC is more effective in suppressing MRSA strains than PEITC. The antibacterial effectiveness of these compounds depends on the dose tested and on the chemical structure [34].

GLS and their derivate products are useful also in inhibiting the growth of pathogenic bacteria that can contaminate vegetable seeds. This contamination can occur at any point, from the field to the sprouting process and during subsequent handling of sprouts until they are consumed. Populations of *E. coli* O157:H7 have been reported to reach 10^6–10^7 cfu g^{-1} of sprouts produced from contaminated seeds. *E. coli* O157:H7 causes life-threatening hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in the young, old, and immunocompromised. The efficacy of AITC in killing *E. coli* O157:H7 on dry and wet alfalfa seeds was investigated. AITC was lethal to *E. coli* inoculated onto agar disks, but, unfortunately, the enhanced effectiveness of AITC in killing the pathogen onto alfalfa seeds is offset by a dramatic reduction in seed viability. Nevertheless, the use of AITC for the purpose of killing *E. coli* O157:H7 in other fields and, perhaps, other pathogens on alfalfa seed holds promising [35].

GLS hydrolysis products also displayed antimicrobial activity against plant pathogenic microorganisms, and this feature reinforces the potential for using them as alternatives to the traditional chemical control of phytopathogenic bacteria. Aires et al. [36] evaluated the antibacterial effects of GLS hydrolysis products against six relevant plant pathogenic Gram-negative bacteria, using a disc diffusion assay: *Agrobacterium tumefaciens*, *Erwinia chrysanthemi*, *Pseudomonas cichorii*, *Pseudomonas tomato*, *Xanthomonas campestris*, and *Xanthomonas juglandis*. The GLS hydrolysis products used in the in vitro assay were AITC, AC, SFN, BITC, benzyl cyanide (BC), PEITC, 2-phenylethyl cyanide (PEC), indole-3-acetonitrile (IAN), indole-3-carbinol (I3C), and ascorbigen (ASC). A mix of AITC, BITC, and PEITC also was tested. The strongest inhibitory effect was showed by PEITC and SFN. Among the different GLS hydrolysis products studied, the ITCs were more efficient than the other products, and the antimicrobial effects were dose-dependent.

A transgenic *Arabidopsis thaliana* that overexpressed p-hydroxybenzyl GLS was used to evaluate the capacity of GLS and their breakdown products to influence and modify the natural rhizosphere community. It was showed that the proteobacteria and also the fungal community in the rhizosphere of the transgenic plant were significantly affected. Modification of the GLS content of the plant could be an alternative to the use of pesticides [37].

Aires et al. [38] evaluated the antimicrobial activity of intact GLS and their hydrolysis products and microbial catabolites, against human pathogenic or gastrointestinal tract bacteria: the Gram-positive *E. faecalis*, *S. aureus*, and *Staphylococcus saprophyticus* and the Gram-negative *Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *E. coli* (two strains), *Hafnia alvei*, *Klebsiella oxytoca*, *K. pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *P. aeruginosa*, *S. typhi*, and *Stenotrophomonas maltophilia*. The intact GLS examined were sinigrin, glucoraphanin, glucotropaeolin, gluconasturtiin, and indole glucobrassicin, while the enzymatic hydrolysis products were AITC, SFN, BITC, PEITC, I3C, AC, BC, PEC, and 3,3′-di-indolylmethane (DIM). Allylamine (AAM), benzylamine (BAM), and 2-phenylethylamine (PAM), which are microbial metabolites of GLS, were also tested. Among the compounds tested, only ITCs were effective, but GLS, nitriles,

and amines were ineffective at all the doses used. The highest activity was shown by SFN and BITC. IAN had some inhibitory activity against the Gram-negative bacteria. I3C had some inhibitory effects against the Gram-positive bacteria but had no effect, even at the highest dose, against the Gram-negative bacteria. The compound, the concentration used, and the microorganism tested influence the antimicrobial activity of the GLS hydrolysis products. Some of these were more effective than conventional antibiotics in inhibiting the growth of pathogenic microorganisms, such as ITCs. The data reported in this study demonstrate the potential for using these natural antimicrobials as an alternative or in combination with antibiotic-based therapies for treating infectious diseases.

Some ITCs display a synergy with conventional antibiotics. Tajima et al. [39] examined different hydroxy ITCs for antimicrobial synergism with various antibiotics against *E. coli* and *S. aureus*. It was demonstrated that 2-(4-hydroxyphenyl) ethyl ITC displayed antimicrobial synergism with aminoglycosides, such as streptomycin, against *E. coli* and *S. aureus* grown in glucose-containing medium. However, small changes in the concentrations of both ITC and streptomycin affect their combined action from synergism to suppression of antimicrobial activity. The mechanism of synergism and suppression remains unclear [40].

Palaniappan et al. [41] examined the synergistic interaction between natural antimicrobials and antibiotics to which the target bacteria were resistant. Among the agents studied, AITC was effective in reducing the MIC of erythromycin when tested against *S. pyogenes*.

The antibacterial effect in vitro of PEITC and its synergistic effect with antibiotics against different *E. coli* from human and animal were demonstrated by Freitas et al. [42].

Many of the older references about the antimicrobial properties of ITCs were often related to the use of these compounds as preservatives in foods. Tressler and Joslyn [43] suggested that the Romans added large quantities of mustard seed to crushed grape for preservative purposes. The use of mustard oils to fruit juices and wines has apparently been practiced for generations in some parts of the world. Kosker et al. [44] showed the possibility of using AITC as preservative in fresh apple cider at a concentration of 20 ppm. Furthermore, it was shown that the thermal resistance of *Bacillus thermoacidurans* can be greatly reduced using AITC 10 ppm in buffer and fruit juices. Kostova et al. [9] studied the use of AITC in the disinfection of eggs. It was reported that AITC could control the growth of microorganisms on the surface of goose and hen eggs by application of the solution or as vapor. This method was not pursued because the AITC was absorbed through the shell.

The major pungent component of black mustard (*Brassica nigra*) and brown mustard (*B. juncea*), which is the same as that of wasabi (*Eutrema wasabi* Maxim.), is AITC. The antimicrobial activity of brown mustard AITC vapor and the possibility of its use as modified atmosphere packing were studied by Isshiki et al. [45]. The bacteria used were *B. subtilis* IFO-13722, *B. cereus* IFO-13494, *S. aureus* IFO-12732, *Staphylococcus epidermidis* IFO-12993, *E. coli* JCM-1649, *S. typhimurium* A TCC-14028, *S. enteritidis* JCM-1891, *V. parahaemolyticus*

IFO-12711, and *P. aeruginosa* IFO-13275. First, the antibacterial activity of AITC vapor, against each microorganism, was evaluated in Petri dishes, and then application experiments were carried out with different foods. AITC vapor inhibited the growth of all microorganisms examined in the experiments. In the application experiments, none of the tested samples were spoiled after 7 days, while the controls grew sufficiently after 2 days [45].

Ward et al. [46] evaluated the effectiveness of different concentrations of a volatile distillate extracted from fresh horseradish root against the growth of spoilage and pathogenic bacteria inoculated on agar and roast beef slices at 12 °C. The distillate was composed by about 90% AITC and 9% 2-phenethyl ITC, and the bacteria tested were *S. aureus*, *E. coli* O157:H7, *S. typhimurium*, *L. m*onocytogenes, *Serratia grimesii*, and *Lactobacillus sake. L. sake* was the most resistant: 20000 nL distillate L^{-1} air were required to completely inhibit growth on agar. On the other side 4000 nL distillate L^{-1} air completely inhibited the growth of *S. aureus*, *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes,* and *S. grimesii* on agar for 7 days in aerobic storage at 12 °C. These bacteria were more resistant when inoculated on roast beef: 20,000 nL distillate L^{-1} were required to completely inhibit the growth, and *L. sake* was weakly inhibited at this concentration.

Delaquis et al. [47] determined the effect of vaporized horseradish essential oil (HEO) on microbial growth in precooked roast beef slices contaminated with *Pseudomonas* spp. and *Enterobacteriaceae* and lactic acid bacteria. The slices were stored 28 days at 4 ± 2 °C in air or 100% N_2 with and without HEO. The results showed that 20 μL L^{-1} of HEO inhibited the growth of most spoilage bacteria and *Pseudomonas* spp. And *Enterobacteriaceae* were strongly inhibited than lactic acid bacteria that were more resistant. The chemical changes and sensory properties of precooked roast beef treated with HEO were also evaluated and revealed that the development of off-flavors and odors derived from fat oxidation products was delayed by HEO.

The bactericidal activity of AITC and MITC was tested on iceberg lettuce inoculated with a rifampicin-resistant strain of *Salmonella* Montevideo and streptomycin-resistant strains of *E. coli* O157:H7 and *L. monocytogenes* Scott A in sealed containers at 4 °C for 4 days. MITC was more active against *L. monocytogenes* than the other bacteria, while AITC showed stronger activity against *E. coli* O157:H7 and *S.* Montevideo. Furthermore in this study, the AITC was tested also on tomato stem scars and skin contaminated with *S.* Montevideo and on apple stem scars contaminated with *E. coli* O157:H7. *S.* Montevideo inoculated on tomato skin was more sensitive to AITC than that on stem scars. Treatment with vapor generated from 500 mL of AITC caused an 8-log reduction in bacteria on tomato skin but only a 5-log reduction on tomato stem scars. The bactericidal activity of AITC was weaker for *E. coli* O157:H7 on apple stem scars; only a 3-log reduction in bacteria occurred when 600 mL of AITC was used [48].

The incorporation of mustard flour (non-deheated) as an ingredient in packaged ground beef to inactivate *E. coli* O157:H7 was tested by Nadarajah et al. [49]. The results showed that it is possible to use mustard flour at levels of between 5 and 10% to eliminate *E. coli* O157:H7 from fresh ground beef. The sensory evaluation of

cooked ground beef was carried out and showed that there were no significant differences between the overall sensory acceptability of ground beef formulated with 5% and 10% mustard [49].

Four sausage batters (17.59% beef, 60.67% pork, and 17.59% pork fat) were inoculated with *P. pentosaceus* and *Staphylococcus carnosus* and a five-strain cocktail of nonpathogenic variants of *E. coli* O157:H7. Microencapsulated AITC was added to three batters at 500, 750, or 1000 ppm to determine its antimicrobial effects. *E. coli* O157:H7 was reduced by 6.5 log10 CFU g^{-1} in sausages containing 750 and 1000 ppm AITC after 21 and 16 days of processing, respectively. *E. coli* O157:H7 numbers were reduced by 4.75 log10 CFU g^{-1} after 28 days of processing in treatments with 500 ppm AITC, and the organism was not recovered from this treatment beyond 40 days [50].

The antimicrobial activity of AITC against growth of typical spoilage bacteria (*Lactobacillus algidus*, *L. mesenteroides*, *Leuconostoc carnosum*, *Carnobacterium maltaromaticum*, *Carnobacterium divergens*, *Brochothrix thermosphacta*, *Serratia proteamaculans*) from marinated pork was also investigated in vacuum-packed pork meat. MICs for AITC were difficult to determine because of the absence of gastight barrier between the wells of a single plate used in the experiment. As AITC exerts antimicrobial activity in both liquid and gas phases, the addition of AITC to one well affected bacterial growth in adjacent wells. In fact, the addition of AITC completely inhibited the growth of *S. proteamaculans* and *B. thermosphacta* even in control wells containing no AITC. To determine the MIC for AITC in liquid phase, experiments with sealed wells would have to be carried out. The ability of AITC to exert antimicrobial effects in its gas phase even at low concentrations may make it more useful for applications in modified atmosphere-packaged foods [51].

AITC was also incorporated into chitosan coatings to develop an antimicrobial application against *Salmonella* that would improve the safety and extend shelf life of whole fresh cantaloupe. It has been demonstrated that with AITC concentrations increasing from 10 to 60 μL mL^{-1}, the antibacterial effects of coating treatments against *Salmonella* increased, and no visual changes in overall appearance and color of cantaloupe rind and flesh due to coating treatments were observed [52].

AITC was encapsulated using gum Arabic and chitosan to overcome the problem of its high volatility to investigate the effect of microencapsulated AITC as a natural additive on the shelf life and quality of Kimchi, a traditional Korean fermented vegetable food. Encapsulated AITC addition to Kimchi resulted in positive changes in pH, titratable acidity, and microbial analysis compared to that of control. The number of *Leuconostoc* and *Lactobacillus* species in Kimchi decreased with an increase in the concentration of AITC. However, with regard to sensory analysis, AITC concentrations of 0.10% or lower are recommended for manufacturing Kimchi [53].

The antimicrobial effect of AITC entrapped in alpha and beta cyclodextrin inclusion complexes (IC) against different target organisms, among them *Escherichia coli* and *Listeria monocytogenes*, was determined. AITC entrapped in beta IC exhibited a significantly better antimicrobial effect compared to unentrapped AITC. The antimicrobial effect of beta IC was determined during aerobic storage of

packaged fresh-cut onions. This application of beta IC (200 mL L^{-1}) to packaged fresh-cut onions effectively decreased numbers of *L. monocytogenes* [54]. ITCs are used in food active packaging to reduce, inhibit, or retard the growth of microorganisms on food products. White mustard essential oil (WMEO) showed antimicrobial activity against *Salmonella* recovered from inoculated frozen vegetables and chicken particulates. The antibacterial property was due to the production of 4-hydroxybenzyl ITC obtained by the hydrolysis of the GLS sinalbin, present in white mustard essential oil derived from white mustard seeds (*Sinapis alba L.*) [55].

AITC in combination with modified atmosphere packaging (MAP) was tested to control the growth of *L. monocytogenes* and *S. typhimurium* on fresh chicken breasts during refrigerated storage for 21 days. On day 21, the microbial counts in the products packaged with AITC and MAP were lower than ambient air and MAP, even if AITC was less effective against *L. monocytogenes* than *S. typhimurium*. Furthermore, vapor AITC has been found to be more effective than liquid AITC [56], but its strong odor can limit its use in food systems. The use of AITC as a flavoring substance has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by the EFSA (European Food Safety Authority) Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC). This report concluded that there were no safety concerns from AITC consumption at the estimated levels of intake [57].

3 Antifungal Activity

One of the first studies that demonstrated antifungal activity of cruciferous plant was carried out in the 1930s, when these authors demonstrated in vitro toxicity of volatile compounds (AITC, PITC, MITC, EITC, ethyl TC, allyl sulfide, ethyl sulfide, and sinigrin) toward certain fungi (*Colletotrichum circinans*, *Botrytis allii*, *Aspergillus niger*, *A. alliaceus*, and *Gibberella saubinetti*) [58]. The antifungal property was corroborated by Hooker et al. [59], and after this, many others investigations were followed. In general, volatile sulfur compounds demonstrate more potent inhibitory effects toward fungi than bacteria [60].

Studies have shown that GLS did not present antimicrobial activity in their intact form, only after enzymatic hydrolysis. Therefore, sinigrin which is one of the most important GLS present in oriental mustard presented no effect against *Paecilomyces fumosoroseus* [61]. Sinigrin also did not affect *Alternaria brassicae* (causative agent of black spot) in Czapek-Dox agar medium, as well as sclerotium formation of *Sclerotinia sclerotiorum* (causative agent of stem rot) [62]. Native GLS had no fungitoxic activity, whereas their hydrolytic products, in particular glucoiberin, glucoerucin, glucoheirolin, and glucotropaeolin, inhibited growth of *Rhizoctonia solani*, *S. sclerotiorum*, *Diaporthe phaseolorum*, and *Pythium irregulare* with different inhibitory responses depending upon the chemical nature of the hydrolytic products [3, 63].

The composition of hydrolysis products from GLS varies according to substrate, pH conditions, presence of ferrous ions, and specific protein factors. The chemical

nature of the breakdown products depends mainly on the structure of the GLS, plant species, and reaction conditions [64]. They are classified as nitriles, TCs, epithionitriles, oxazolidine-2-thiones, ITCs, and epithioalkanes with different antimicrobial activity [65].

From these groups, ITC is the major inhibitor of microbial populations, and differences in the potential are related to the nature of their side chain [66]. Assays with ITCs have been conducted directly as a component of a growth medium and a model food system and (generally more antimicrobial effective) in the gaseous form [49]. Volatiles released from GLS, predominantly 2-propenyl GLS, showed toxic effects to the blackleg fungus, *Leptosphaeria maculans*, in vitro [67]. AITC gaseous at 0.1 mg L^{-1} for 4 h showed a fungistatic effect against *Botrytis cinerea* (gray mold) reducing by over 45% the incidence of the gray mold on strawberries [68]. AITC (2 ppm) inhibited the growth of *Penicillium roqueforti*, *P. corylophilum*, *Eurotium repens*, *A. flavus*, and *Endomyces fibuliger* on rye bread slices in airtight environment [69].

Sellam et al. [70] demonstrate that ITCs were effective in vitro in different development stages of *A. brassicicola* and *A. brassicae*. Moreover, antifungal activity of 57 substituted derivatives of PEITC was determined on *A. niger*, *Penicillium cyclopium*, *Rhizopus oryzae*, *A. flavus*, *A. oryzae*, *A. fumigatus*, *P. brevicompactum*, *Cladosporium herbarum*, *Trichoderma viride*, *Alternaria tenuis*, *Monilia sitophila, Cytospora* sp., *Schizophyllum commune*, *Fusarium* sp., *Cephalothecium roseum*, and *Trichophyton gypseum* in culture medium. The authors describe that several PEITC derivatives, as well as the most active natural ITC analogues, represent remarkable antifungal compounds; however, there are some differences in their antifungal potential [13].

Several studies have been conducted using glucosinolate-derived products against molds and yeast (Table 2). These examples show the efficiency of ITCs against saprophytic and parasitic fungal species, usually applied at low levels in culture medium, food products, and plant defense. In agricultural sciences, ITCs such as AITC have been effective fumigants on the control of insects and fungal species [71]. Among ITCs, allyl isothiocyanate (AITC) is one of the most studied. Beneficial biological effects have been reported including antibacterial, antifungal, anti-nematode, and anti-insect activities [72]. Its uses as natural preservative have been growing because of its food origin and low toxicity [30]. The antimicrobial activity of AITC, as well as other ITCs, is related with the concentration of the compound applied, time of exposure, strains, microbial loading, temperature, food composition, pH conditions, water activity, and on diffusion of the vapor in food packaging systems [48, 71, 73]. However, its use on food products usually is limited by the interference of organoleptic characteristics, its poor aqueous solubility, instability at high temperature, and intrinsic food compounds.

GLS-derived products have also been presenting antibiocidal potential against yeast. Kyung and Fleming [23] reported that AITC showed antifungal effects against fermentative yeasts on culture media with an MIC $\leq$ 4 ppm. *Candida albicans*, a fungus potentially pathogenic to human, was inhibited by fresh cauliflower juice (*Brassica oleracea* var. *botrytis*) [74]. In the use of BITC and 3- and

Table 2 Antifungal potential of glucosinolate-derived products against several species on food products

Glucosinolate derivative	Fungal strain	Food product	Reference
4-Hydroxybenzyl isothiocyanate	*Zygosaccharomyces bailii*	Acidified fruit drink	[75]
Yellow and oriental mustard (based on allyl isothiocyanate and p-hydroxybenzyl isothiocyanate)	*Aspergillus parasiticus CECT 2681*	Peanut, cashew, almonds, walnut, pistachio, hazelnut	[81]
Allyl isothiocyanate	*Penicillium roqueforti, P. corylophilum, Eurotium repens, A. flavus, Endomyces fibuliger*	Rye bread slices	[69]
Allyl benzyl phenyl isothiocyanates	*Gibberella moniliformis strains 2983, 5847, 5850*	Bread	[78]
Allyl isothiocyanate	*Botrytis cinérea*	Strawberries	[68]
Allyl isothiocyanate	*Aspergillus parasiticus*	Fresh pizza crust	[77]
Allyl isothiocyanate	*Aspergillus parasiticus, Fusarium poae*	Wheat flour	[80]
Benzyl isothiocyanate	*Alternaria alternata*	Tomato	[79]
Ethyl isothiocyanate	*Penicillium expansum*	Apple	[71]

4-methoxybenzyl ITCs, antifungal effects against *Aspergillus fumigatus* and *C. albicans* were revealed with MIC of 1 μg mL^{-1} (Radulovic et al., 2012). An essential oil obtained from white mustard seeds containing 25 mg L^{-1} of 4-hydroxybenzyl isothiocyanate (4HBITC)/L was able to stabilize an acidified fruit drink against acid-tolerant bacteria (*Gluconobacter* species) and preservative-resistant yeast (*Zygosaccharomyces bailii*) for 28 days at ambient temperature [75].

Thus, the ability of ITCs to reduce mycotoxigenic molds and mycotoxins was also investigated. *P. expansum* (patulin producer) was inhibited with $>$ 50 mg of AITC, whereas *A. parasiticus* (aflatoxin producer) in culture medium was sensible to doses $>$ 5 [76]. *Aspergillus parasiticus* was inactivated in fresh pizza crust after 30 days of AITC exposition and suppressed aflatoxin formation [77]. AITC, BITC, and PITC inhibited the growth of *Gibberella moniliformis* strains 2983, 5847, and 5850 and reduced 2.1–89.7% of the mycelium size. ITCs also reacted with FB_2 in bread reducing the levels by 73–100% [78]. Benzyl-ITC showed antifungal activity against *Alternaria alternata* on tomato [79] and ethyl-ITC against *P. expansum* on apple [71], both patulin producers. AITC gaseous at 0.1 μL L^{-1} was investigated to reduce aflatoxin produced by *A. parasiticus* and beauvericin and enniatins produced by *Fusarium*. The authors observed reduction of 6.9% to 23% mycotoxin levels while at 10 μL L^{-1}; AITC completely inhibited the production of mycotoxins for 30 days [80]. In a commercial packaging simulation, GLS present in yellow and oriental mustard flours reduced aflatoxin B_1, B_2, G_1, and G_2 in nuts (peanut, cashew, almonds, walnut, pistachio, and hazelnut). This reduction ranged from 83.1 to 87.2%

in the oriental mustard flour, whereas it was 27.0–32.5% in the yellow flour [81]. AITC reacted with beauvericin in solution, reducing from 20% to 100%, and in a food system, beauvericin was reduced from 10% to 65%, in a dose-dependent manner [82]. AITC, BITC, and PITC diminished fumonisin B_1 (FB_1) and B_2 (FB_2) levels in solution from 42% to 100%, and on fumigation treatment (50, 100, and 500 μL L^{-1}), ITCs were able to reduce 53–96% of FB_1 and 29–91% of FB_2 contained in corn products, with four reaction products identified through the reaction [83].

3.1 Plant Protection

GLS-derived products have been recognized as antimicrobial agents, and several studies demonstrated the ability to control soil-borne plant pathogens [84–87]. The GLS content in plant reaches about 1% (highly variable) of dry weight in some tissues of *Brassica* vegetables [88]. Plant species and age are the major determinants of GLS composition [89], but also other factors such as nutritional status of the plant, fungal infection, and insect damage have significant effect on the content in growing plants [64].

Qualitative and quantitative differences of GLS composition vary also among plant organs [89]. GLS are found mainly in seeds, siliques, and young leaves, while intermediate contents are detected in leaves, stems, and roots [90]. Indole GLS and their hydrolysis products found in large amounts in roots may be related to their higher stability in the soil than air [91]. These compounds play a role in the development of root disease, caused by *Plasmodiophora brassicae* [92]. Volatile compounds from macerated *Brassicae* root tissue inhibited the fungal pathogen of wheat, *Gaeumannomyces graminis* [87]. Nevertheless, roots of a transgenic *Arabidopsis thaliana* had altered the profile of GLS compared with non-transgenic, with influence in the microbial community on roots and active populations in the rhizosphere [37]. The rhizospheric strains of *Fusarium* showed a protective effect on *Lepidium sativum* against *Pythium ultimum*. Accumulation of ITCs in roots not only increases resistance of the plant but also gives a competitive advantage to *Fusarium* strains [93].

Degradation products of GLS showed an inhibition of *L. maculans* at concentrations greater than 40 μg mL^{-1} [94]. Cauliflower plants (*Brassica oleracea* var. *botrytis*) infected by *Peronospora parasitica* resistant to downy mildew presented higher sinigrin content than the susceptible variety. The susceptible seedlings exhibited a 12% decrease in glucobrassicin and a 25% increase in methoxyglucobrassicin when compared with healthy ones six days after treatment whereas no difference in glucobrassicin and a 10% increase in methoxyglucobrassicin were observed in healthy and inoculated resistant seedlings [95].

The disease resistance may be dependent on fungal pathogen species and the composition of GLS-derived products present in the plant [91]. *Arabidopsis thaliana* mutant extracts were investigated on *B. cinerea* and *Alternaria brassicicola* isolates. *A. brassicicola* was more affected by aliphatic GLS and ITCs, while *B. cinerea*

isolates showed variable composition-dependent sensitivity to GLS and their hydrolysis products [96]. Propenyl ITC and EITC demonstrated fungistatic potential at 0.3 μL, which inhibited mycelial growth and completely suppressed conidial and chlamydospore germination of four *Fusarium oxysporum* isolates. EITC, BITC, and PEITC were fungitoxic to *F. oxysporum* conidia and chlamydospores [97]. ITCs released from cabbage tissues were effective toward *P. parasitica*, *P. ultimum*, and *Sclerotium rolfsii* [98]. PEITC inhibited the growth of a range of fungi, oomycetes, and bacteria [99]. Pedras and Sorensen [100] observed that 5-(methylsulfamyl)-pentyl-1-ITC, 6-(methylsulfamyl)-hexyl-1-ITC, and 6-(methylsulfinyl)-hexyl-1-ITC inhibited spore germination of *Phoma lingam* virulent isolate BJ 125 at a concentration of 5×10^{-4} M. *Alternaria* infection was positively correlated with GLS content in 33 oilseed rape lines (*Brassica napus* L. ssp. *oleifera*) [101].

4 Structure Activity Relationships

The mechanism of ITC antimicrobial action is unclear, but some hypotheses have been proposed. The central electrophilic carbon of ITCs (R-N = C = S) undergoes rapid reaction with hydroxyls, amines, and thiols, generating products such as carbamates, thiourea, and thiocarbamates, respectively [102, 103]. Thereby, AITC reacted with glutathione, amino acids, proteins, water, alcohol, and sulfites [104, 105], and it was able to disintegrate the cysteine disulfide bond through an oxidative process [104, 106].

Zsolnai [19] reported that thioglycolate and cysteine could diminish the antibacterial action of ITCs. The study also describes that the antimicrobial action of ITCs may be linked to the inhibition of sulfhydryl enzymes. This finding is consistent with those observations of Luciano et al. [106], who reported that AITC was able to react with glutathione and cysteine naturally present in meat, which interfered on their antimicrobial activity. In addition, the presence of proteic substances reduced genotoxic activity of AITC, PEITC [107], and MITC [108], on which the compounds were able to cause DNA damage in *Salmonella*, *E. coli*, and human cells (Hep G2) [109].

Kojima and Ogawa [110] suggested that ITCs act by inhibiting the oxygen uptake by yeast through the uncoupler action of oxidative phosphorylation in the mitochondria of yeast, inhibiting the coupling between the electron transport and phosphorylation reactions, thus hindering the ATP synthesis. However, the levels to achieve both enzymatic and oxygen uptake inhibitions used in the study were 200 times greater than the actual MIC of the ITCs for those organisms [30].

It is not clear if AITC crosses membranes and enters the cytoplasm of prokaryotic and eukaryotic cells or if it has an effect on cell membranes. Inside a cell, AITC can react with glutathione, sulfites, amino acids, oligopeptides, proteins, and water [111]. Delaquis and Mazza [10] suggest that AITC might cause inactivation of essential intracellular enzymes through oxidative cleavage of disulfide bonds. Lin et al. [112] observed damages on the bacteria cell by exposition to AITC, creating pores on cell membranes and inducing leaking of cellular substances. AITC was able

to modify the internal structure of *L. monocytogenes* when compared to non-treated cells when analyzed by transmission electron microscopy [104]. On the other hand, Ahn et al. [113] observed no damage in cell wall or leakage of ATP when AITC was tested against *L. monocytogenes*. The reduction of ATP could be the result of inhibition of enzymes related to ATP formation or depletion of proton motive force.

The mechanism of fungal death by ITCs was investigated by Calmes et al. [114]. Exposure of AITC, PEITC, and BITC in *A. brassicicola* decreased oxygen consumption rate, intracellular accumulation of reactive oxygen species (ROS), and mitochondrial membrane depolarization. The two major regulators of the response to oxidative stress, MAP kinase AbHog1 and the transcription factor AbAP1, were activated in the presence of ITCs. Once activated by ITC-derived ROS, AbAP1 may promote the expression of different oxidative-response genes. Besides, fungal strains deficient in AbHog1 or AbAP1 were hypersensitive to ITCs, and it might be useful to understand the mechanism of fungal resistance. In other studies, the authors [13] suggest some differences on the mode of action of 57 ITCs and related compounds investigated against *A. niger*, *P. cyclopium*, *Rhizopus oryzae*, and other species. These variations occurred in compounds in which -NCS group is directly bound on the aromatic moiety compared with the bounds on aliphatic radical. Normally, aromatic ITCs are more toxic than aliphatic, and the fungal toxicity of aliphatic ITCs decreased with the increasing length of the side chain [64].

Furthermore, it may be considered that AITC degraded in aqueous solution at 37 °C, generating allyl dithiocarbamate, diallyl tetra- and pentasulfide, sulfur, and N, N'-diallylthiourea, dependent on temperature and pH conditions [104] However, there is no information relating this degradation to ITC's antimicrobial potential [30].

5 Conclusions

With the current trend, natural compounds are preferred and widely studied. Considering the data from several studies carried out, it may be observed that glucosinolates demonstrate a biocidal effect after their enzymatic hydrolysis. These breakdown products show a huge antibacterial and antifungal capacity, and they may be used on food preservation as well as plant defense. Several studies have demonstrated that the structure of glucosinolates and the microbial strain are responsible for their antimicrobial potential. Among the GLS hydrolysis products, ITCs are the main group that demonstrated an efficiency to reduce microbial growth. Allyl isothiocyanates are the most investigated ITCs against microorganisms, and its use as a fumigant agent on food preservation has been investigated.

There is not enough information regarding the mechanism behind the antimicrobial activity of GLS. Studies indicated that the central electrophilic carbon of ITCs may react with hydroxyls, amines, and thiols. However, it is not clear if ITC crosses the membrane and enters the cytoplasm or if they have an effect on cell membranes. Thus, further studies are necessary to clarify the mechanism of these active

compounds on microorganisms and evaluate the feasibility application of GLS products as food preservative through fumigation treatment.

Acknowledgments This research was supported by the Economy and Competitiveness Spanish Ministry (AGL2013-43194-P), by the pre-PhD program of the University of Valencia "Santiago Grisolia," and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Process 400896/2014-1) from Brazil.

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Neuroprotective Effects of Glucosinolates

9

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Abstract

Oxidative stress, excitotoxicity, inflammation, misfolded proteins, and neuronal loss are common characteristics of a wide range of chronic neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis. For these disorders, the current healthcare outcomes are considered inadequate; in fact these pathologies are treated after onset of the disease, frequently at near end-stages, and pessimistic prognosis considers pandemic scenario for these disorders over the next 10–20 years. Phytochemicals have been regarded as an alternative and preventive therapeutic strategy to control the occurrence and progression of neurodegenerative diseases. Recent research has shown that dietary phytochemicals have pleiotropic behaviors, exerting antioxidant, anti-inflammatory, and cytoprotective effects in neuronal and glial cells. In particular, isothiocyanates, the activated form of glucosinolates present in Brassica vegetables, have shown neuroprotective activity in several experimental paradigms due to their peculiar ability to activate the Nrf2/ARE pathway, playing a role in boosting the neuronal natural phase 2 enzyme antioxidant defense system and functioning as a powerful indirect antioxidant. This chapter summarizes the preventive glucosinolate-derived isothiocyanates effects in neurodegeneration and underscores the powerful preventive role that these compounds play in assisting the body to help fend off a variety of neurodegenerative diseases.

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_20

Keywords
Glucosinolates • Isothiocyanates • Sulforaphane • Oxidative stress • Neurodegeneration • Parkinson's disease • Alzheimer's disease • Multiple sclerosis • Amyotrophic lateral sclerosis

Abbreviations

4-IPITC	4-iodophenyl isothiocyanate
6-OHDA	6-hydroxydopamine
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AGEs	Advanced glycation end products
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
ARE	Antioxidant response element
Aβ	Amyloid-β
BBB	Blood brain barrier
BDNF	Brain-derived neurotropic factor
ChAT	Choline acetyltransferase
CNS	Central nervous system
CPK	Creatine phosphokinase
CysDA	5-S-cysteinyl-dopamine
DA	Dopaminergic
EAE	Autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinase
γGCS	γ-Glutamyl cysteine synthetase
GLRX	Glutaredoxin
GLSs	Glucosinolates
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione-S-transferase
HD	Huntington's disease
HO1	Heme oxygenase 1
Hsp27	Heat shock protein 27
HSV-1	Herpes simplex virus 1
iNOS	Inducible nitric oxide synthase
ITCs	Isothiocyanates
JNK	c-Jun N-terminal protein kinase
Keap1	Kelch-like-ECH-associated protein 1
LC3	Protein 1 light chain 3
MAO	Monoamine oxidase
MIF	Macrophage migration inhibitory factor
MNCs	Mixed neural cultures

MPTP	Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
NFTs	Neurofibrillary tangles
NGF	Nerve growth factor
NOX	NADPH oxidase
NQO1	NADPH quinone oxidoreductase 1
Nrf2	Nuclear factor NF-E2-related factor 2
PD	Parkinson's disease
PKB	Protein kinase B
ROS	Reactive oxygen species
SF	Sulforaphane
TR	Thioredoxin reductase
TX	Thioredoxin

Contents

1 Introduction

The continuous extend of human lifespan has led to a concomitant increase in the incidence of age-related disorders such as neurodegenerative diseases like Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Parkinson's disease (PD). These pathologies are progressive, devastating, and terminal and makes modern societies to face a big challenge in terms of social and financial management of this situation [1, 2]. For these disorders, the current healthcare outcomes are considered inadequate; in fact, chronic pathologies such as neurodegenerative diseases are treated after onset of the disease, frequently at near end-stages, and pessimistic prognosis considers pandemic scenario for these disorders over the next 10–20 years. Although an intensive research is currently focused on deciphering the cellular and molecular mechanisms underlying neurodegeneration, the possibility to prevent, treat, or simply slowdown these disorders remains an elusive goal. The goal is to change from interventional medicine to preventive medicine and from disease to health. In the last years, the recognition of similar and common characteristic between the different neurodegenerative diseases such as loss of proteostasis [3–5] and oxidative stress [6–8] has emerged.

AD affects approximately 10% of people over 80 years and is the most common form of dementia. AD pathophysiology is characterized by both deposition of amyloid-β (Aβ) peptides, derived from amyloid precursor protein (APP) cleavage by γ-secretase and β-secretase, and intracellular neurofibrillary tangles (NFTs). NFTs are mainly constituted by insoluble form of hyperphosphorylated tau protein [9]. Even though Aβ plaques and NFTs are the main histological markers of AD, other biochemical factors are involved in AD such as mitochondrial dysfunction, loss of calcium regulation, oxidative damage to both proteins and lipids, glycation that plays a role in both oxidative stress and protein cross-linking, and increased cytokine concentration in blood circulation [9–12].

PD is a neurodegenerative disease characterized by progressive dopaminergic (DA) neuron death in the midbrain region known as substantia nigra pars compacta. Neuronal loss in this region is not the only hallmark of PD; histological analyses have demonstrated that Lewy bodies, aggregated proteins into the neurons, are frequently present in biopsies of PD suffering brains [13]. PD exhibits high incidence and is the second most common neurodegenerative disease in aged people. Motor symptoms, such as uncontrolled tremor, postural imbalance, slowness of movement, and rigidity, develop during the disease progression and characterize patients suffering from PD [14]. The onset of PD has been related either to environmental risk factors [15, 16] or to a genetic origin [17], although the exact etiology is only beginning to be elucidated. Genetic studies suggest that familial forms of PD are mainly linked to mutations in α-synuclein protein [18], a principal component of Lewy body inclusions [19], and parkin [17, 20]. Many factors are involved in the pathogenic mechanisms that lead to DA neuron loss like oxidative stress, mitochondrial dysfunction, and environmental toxins [21]. In particular, in both idiopathic and genetic cases of PD, dopamine-dependent oxidative stress is thought to be a key mechanism that leads to cellular dysfunction and demise [14]. Dopamine itself can be responsible for oxidative stress to neurons and exerts a role in PA development. It can spontaneously undergo auto-oxidation at physiological pH resulting in the production of toxic molecules such as hydrogen peroxide, superoxide radicals, and DA-quinone species [22]. Alternatively, it can be enzymatically deaminated by monoamine oxidase (MAO) into the nontoxic metabolite 3,4-dihydroxyphenylacetic acid and hydrogen peroxide [23]. DA-quinone species have been involved in mitochondrial dysfunction [24] and α-synuclein-associated neurotoxicity in PD by covalently modifying α-synuclein monomer [25] and by stabilizing the toxic protofibrillar α-synuclein [26].

Another neurodegenerative disease whose incidence has increased from 2.1 million in 2008 to 2.3 million in 2013 is MS [27], the first cause of disability after traumatic brain injury in young individuals [28]. MS is a demyelinating, inflammatory, and progressive disorder that affects the CNS. A wide set of symptoms such as fatigue, muscle weakness, sensory impairment, vertigo, mood disorder, and cognitive disabilities characterize patients suffering from MS [29]. MS etiology is multifactorial, and neurodegenerative, neuroinflammatory, autoimmune processes are related to the disease; moreover, the immune system activation toward unrecognized "self" antigens is involved in MS onset [30].

Among neurodegenerative disorders, ALS, also called Lou Gehrig's disease, is known for affecting, selectively, upper motor neurons and lower spinal motor neurons, causing their progressive degeneration and leading to paralysis of all skeletal muscles [31]. Disease onset is generally between 50 and 65 years, prognosis is unfavorable, and death occurs 3–5 years after diagnosis due to respiratory failure [32]. Two different forms of the disease are known: familial ALS (5–10%) and sporadic ALS (90–95%); both forms share initial symptoms, muscle weakness and cramping. Even though ALS pathophysiology still has to be clearly understood, numerous mechanisms are involved in its development, including oxidative stress, inflammation, protein misfolding, protein aggregation, altered RNA splicing and signaling, defects in axonal transport, mitochondrial dysfunctions, glutamate excitotoxicity, loss of proteostasis, and primary and secondary ion channel defects [33, 34]. Although enormous efforts have been made to develop agents that could be used to counteract neurodegenerative disorders, no drugs and therapies are available to definitively halt the progression of these debilitating diseases. For these reasons, phytochemicals have been regarded as an alternative and preventive therapeutic strategy to control the occurrence and progression of neurodegenerative diseases. Recent research has shown that dietary phytochemicals have pleiotropic behaviors, exerting antioxidant, antiallergic, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic effects [35–37]. In particular, isothiocyanates (ITCs), the activated form of glucosinolates (GLSs) present in Brassica vegetables, have shown neuroprotective activity in several experimental paradigms.

This chapter focuses on the role of GLS-derived ITCs in preventing neurodegenerative diseases based on the most recent scientific literature.

2 Glucosinolates

GLSs are a large family (about 200 GLSs have been identified) of plant second metabolites, rich in sulfur, particularly found in the order of Brassicales responsible for the characteristic taste and aroma of these vegetables [38]. Chemically, GLSs (Fig. 1) are characterized by β-thioglycoside *N*-hydroxysulfates substituted with different R-groups (Table 1) and a sulfur-linked β-d-glucopyranose moiety [39].

As previously described, GLSs are mainly abundant in plants of Brassicales order: broccoli are particularly rich in glucoraphanin (4-methylsulphinylbutyl), watercress in gluconasturtiin (2-phenethyl), and cabbage in sinigrin (2-propenyl) [40, 41] (Table 1). These molecules share a common sulfur-linked b-D-glucopyranose structure, while R-groups, aliphatic or aromatic, derive from specific plant biosynthetic pathways converting amino acids in GLSs side chain [42]. Other GLSs found in vegetables are progoitrin (2-hydroxy-3-butenyl), neoglucobrassicin (1-methoxy-3-indolylmethyl), glucoiberin (3-methylsulphinylpropyl), and 4-methoxygluco-brassicin (4-methoxy-indolylmethyl), glucobrassicin (3-indolylmethyl), 4-hydroxygluco-brassicin (4-hydroxy-3-indolylmethyl), and glucobrassicanapin (4-pentenyl) [43] (Table 1).

GLSs stored inside plant cells are stable, but as soon as they are exposed to the enzyme myrosinase (EC 3.2.1.147), for example, when plant cells are damaged by

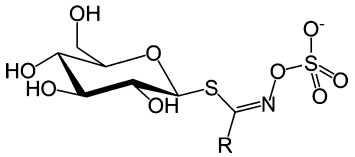

Fig. 1 Glucosinolates generic structure

Table 1 R side chains of different GLSs

GLS trivial name	R side chain
4-Hydroxygluco-brassicin	4-Hydroxyindol-3-ylmethyl
4-Methoxygluco-brassicin	4-Methoxyindol-3-ylmethyl
Epiprogoitrin	(*2S*)-2-Hydroxy-3-butenyl
Glucoalyssin	5-Methylsulfinylpentyl
Glucoarabidopsithalianain	4-Hydroxylbutyl
Glucoarabishirsuin	8-Methylthiooctyl
Glucoarabishirsutain	7-Methylthioheptyl
Glucobarbarin	(*2S*)-2-Hydroxy-2-phenethyl
Glucobrassicanapin	Pent-4-enyl
Glucobrassicin	Indol-3-ylmethyl
Glucocapparin	Methyl
Glucocheirolin	3-Methylsulfonylpropyl
Glucocleomin	2-Hydroxy-2-methylbutyl
Glucoconringiin	2-Hydroxy-2-methylpropyl
Glucoerucin	4-Methylthiobutyl
Glucoerysimumhieracifolium	3-Hydroxypropyl
Glucohesperin	6-Methylsulfinylhexyl
Glucohirsutin	8-Methylsulfinyloctyl
Glucoibarin	7-Methylsulfinylheptyl
Glucoiberin	3-Methylsulfinylpropy l
Glucoibervirin	3-Methylthiopropyl
Glucomalcomiin	3-Benzoyloxypropyl
Gluconapin	3-Butenyl
Gluconapoleiferin	2-Hydroxy-pent-4-enyl
Gluconastrutiin	2-Phenylethyl
Glucoputranjivin	1-Methylethyl
Glucoraphanin	4-Methylsulfinylbutyl
Glucoraphasatin	4-Methylthio-3-butenyl
Glucoraphenin	4-Methylsulfinyl-3-butenyl
Glucosinalbin	*p*-Hydroxybenzyl
Glucosisymbrin	2-Hydroxy-1-methylethyl
Glucosquerellin	6-Methylthiohexyl
Glucotropaeolin	Benzyl
Neoglucobrassicin	1-Methoxyindol-3-ylmethyl
Progoitrin	(*2R*)-2-Hydroxy-3-butenyl
Sinigrin	2-Propenyl

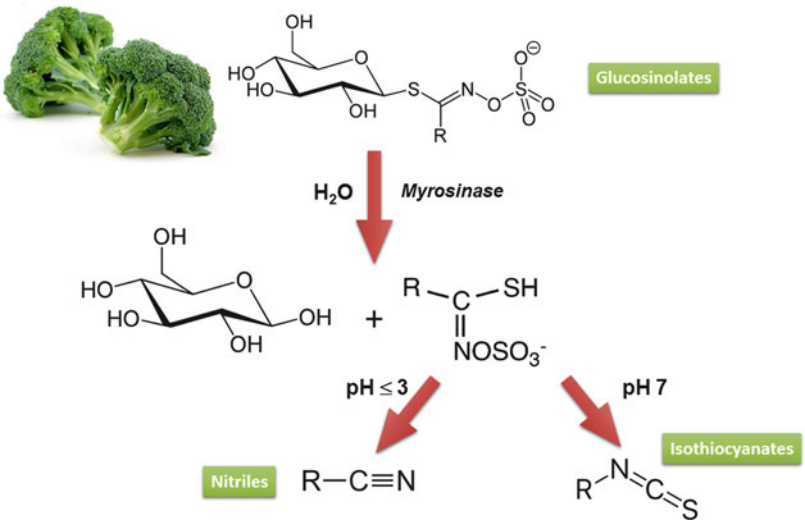

Fig. 2 Glucosinolate hydrolysis by myrosinase

cutting or chewing, GLSs are quickly hydrolyzed to glucose and aglycones (Fig. 2). Myrosinase, a β-thioglucosidase, is an enzyme involved in the plant defense system that catalyzes the initial step of a series of reactions responsible for plant defense [44]. Briefly, myrosinase hydrolyzes β-thioglucoside bond yielding to glucose and an aglycone that spontaneously loses a sulfate group. Later the aglycone is converted, according to different physiological conditions, in ITCs at neutral pH or in nitriles at pH<3 [45].

Although myrosinases are exclusively a family of plant enzymes, GLSs hydrolysis partially occurs also in humans, and it is catalyzed by thioglucosidase enzymes present in the gut microbiota [46]. Even though GLSs do not exert any significant biological properties, compounds produced by their hydrolysis are responsible for almost all of the known health-related properties of Brassicales plants. Among these compounds, the ITC sulforaphane (1-isothiocyanato-4-methylsulphinylbutane) (SF), derived from the hydrolysis of the GLS glucoraphanin, has attracted great attention for its health benefits (Fig. 3).

Absorption of ITCs and the other products of glucosinolate hydrolysis depends on plant myrosinase residual activity at gut level and on the gut microbiota thioglucosidase activity [47]. ITCs are lipophilic compounds often characterized by low molecular weight, thanks to their physical chemical characteristics ITCs can diffuse through the plasma membrane into the enterocytes [48, 49]. Once into the cell, ITCs are quickly conjugated to glutathione (ITC-GSH) by the cytosolic enzyme glutathione-S-transferase (GST), driving their passive absorption into the cell [50]. Moreover, ITCs can be converted one into the other, for example, in vivo SF is partially reduced to erucin (4-methylthiobutyl isothiocyanate) [51]. Whether or not the conversion of SF to erucin can play a role in the health-promoting effects of

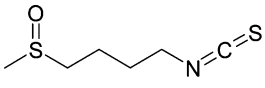

Fig. 3 Sulforaphane chemical structure

GLSs is still debated, and some scientists suggest the possibility that these two ITCs exert different activities [52–54].

Once absorbed, ITCs are extensively metabolized through the mercapturic-acid pathway leading to the production of hydrophilic conjugates such as ITC-*N*-acetylcysteine, ITC-cysteine, and ITC-cysteinylglycine that are excreted in the urine [55].

Both animal and human studies have investigated the pharmacokinetics of these compounds, demonstrating that ITCs reach their plasma peak concentration between 0.5 and 3 h after administration [56–61]; moreover, they are still detectable in plasma after 240 h in rats and 90 h in mice [62].

To act as neuroprotective agents and to maintain brain function, protective compounds need to cross the blood brain barrier (BBB) and to concentrate in the central nervous system (CNS). ITCs distribution in different tissues has been studied, demonstrating that ITCs are primarily accumulated in the liver, kidneys, and spleen and to a lesser extent in the brain [63–66].

In summary, although GLSs do not show health-promoting effects, they exert interesting biological activities as soon as they are hydrolyzed by myrosinase which represents the key step leading to ITCs formation. ITCs are bioavailable, absorbed and conjugated by the enterocytes, metabolized, distributed to different tissues including the brain, and finally excreted by urines.

3 Role of GLS-Derived ITCs in Counteracting Oxidative Stress

The brain is particularly exposed to oxidative stress because it requires high amount of oxygen for its metabolism, has an elevated content of polyunsatured fatty acids that are prone to oxidation, and has low antioxidant defenses [67–69]. Oxidative stress occurs from an impairment in the balance between free radical production and scavenging that leads to an abnormal generation of reactive oxygen species (ROS). The involvement of oxidative stress in neurodegeneration has been widely demonstrated and is a common characteristic of many neurodegenerative diseases [70].

Several potential sources of oxidative stress are present in AD brain. Trace elements like iron, aluminum, mercury, and copper are increased and enhance free radical generation [71]. Neuroinflammation, a typical characteristic of AD, provides a significant source of oxidative stress [72, 73]. Inducible nitric oxide synthase (iNOS) expression levels are elevated [74], resulting in excess release of nitric oxide and nitrative stress [75]. NADPH oxidase (NOX) subunit induction may trigger the production of superoxide and the formation of hydrogen peroxide [76]. MS is characterized by an increase in genes and proteins involved in ROS

production such as NADPH oxidase subunits [77], iNOS [78], and myeloperoxidase [79]. MS brain tissue is characterized by abundant mitochondrial defects, which represents another pathogenic process contributing to oxidative stress [80]. In PD, the primary source of ROS is the mitochondrial complex I together with elevated free iron levels [81, 82]. Mitochondria from ALS patients exhibit impaired Ca^{2+} homeostasis and an increased production of ROS [83]; moreover, the presence of mutant SOD has been linked to an increased production of free radicals such as peroxynitrite, hydrogen peroxide, and hydroxyl radicals in ALS [84].

To counteract the deleterious effects of ROS and control the delicate redox balance in the brain, cells are endowed with an efficient antioxidant defense system consisting of antioxidants and antioxidant enzymes. This system is regulated at the transcriptional level by a common mechanism that involves two proteins: the nuclear factor NF-E2-related factor 2 (Nrf2) and Kelch-like-ECH-associated protein 1 (Keap1) [85, 86].

Nrf2 is a member of cap“n”collar family of basic leucine zipper transcription factor whose functional activity is determined by the level of expression, degradation by the proteasome, and distribution between the nucleus and the cytoplasm [87]. Under homeostatic conditions, Nrf2 activity is inhibited by its negative regulator Keap1 [88]. In response to oxidative stress, Nrf2 dissociates from cytosolic Keap1, translocates to the nucleus where it binds to the antioxidant response element (ARE) [89, 90] in the promoter regions of many antioxidant genes inducing their transcription [91]. In the last years, the ability of various natural and synthetic isothiocyanates to activate Nrf2 has been investigated, and many reports describe the beneficial effects of Nrf2 activation in experimental models of neurodegeneration.

Keap1 possesses many reactive cysteine residues that are a sort of sensors of the intracellular redox state, and it has been reported that ITCs activate Nrf2 by direct reaction with these cysteine groups [92]. In particular, SF binds to 25 of the 27 cysteines of human Keap1 in a concentration-dependent manner [93]. The role of most of these bindings remains unknown, even if it has been reported that Cys273 and Cys288, but not Cys77, Cys171, Cys257, and Cys297, are required for Keap1-dependent Nrf2 ubiquitination, whereas Cys151 is required for the escape of Nrf2 from Keap1-mediated ubiquitination [94, 95].

Several reports confirm the ability of SF and other ITCs to upregulate many Nrf2-dependent antioxidant enzymes in different cell systems [96–100], like glutathione reductase (GR), glutaredoxin (GLRX), glutathione peroxidase (GPX), thioredoxin (TX), thioredoxin reductase (TR), heme oxygenase 1 (HO1), and NADPH quinone oxidoreductase 1 (NQO1) (Fig. 4).

In vitro studies demonstrate that SF can reduce oxidative stress in BV2 cells, a microglial-like cell line, by activating the Nrf2 antioxidant pathways [101] ; SF confers neuroprotection to mixed neural cultures (MNCs) consisting of neurons, astrocytes, and microglia exposed to herpes simplex virus 1 (HSV-1) by activating the Nrf2/ARE-dependent transcription of antioxidant enzymes [102]; in Neuro2A cells, SF increased the expression of Nrf2 and of downstream targets HO1 and

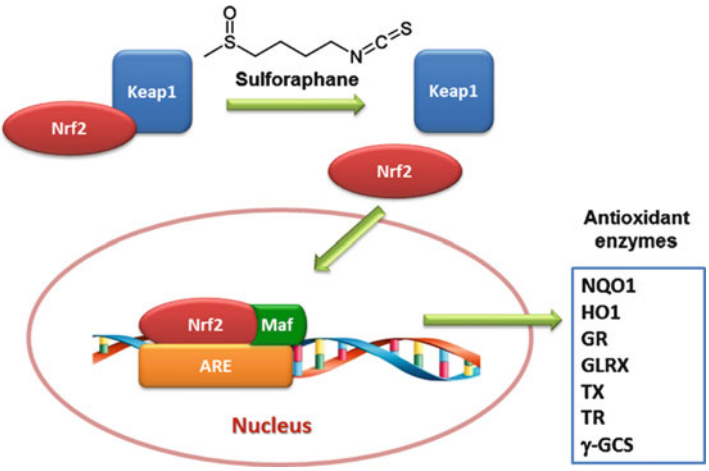

Fig. 4 Nrf2 pathway activation by sulforaphane

NQO1 [103]; SF counteracted ROS production induced by antipsychotic drugs in human neuroblastoma SK-N-SH cells, increasing GSH levels and inducing NQO1 activity [104]; in rat striatal cultures, SF and 6-(methylsulfinyl) hexyl isothiocyanate upregulated γ-glutamyl cysteine synthetase (γGCS), the rate-limiting enzyme in GSH synthesis [105]; and SF protected PC12 cells against 6-hydroxydopamine (6-OHDA)-induced damage, activating Nrf2 and increasing HO1 expression [106].

Jazwa et al. [107] investigated the relevance of Nrf2 activation on brain protection elicited by SF against the Parkinsonian toxin methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a mice model of PD. SF increased Nrf2 protein levels in the basal ganglia and led to the upregulation of HO1 and NQO1. In wild-type mice, but not in Nrf2-knockout mice, SF counteracted MPTP-induced death of nigral dopaminergic neurons.

In conclusion ITCs have been demonstrated to be potent indirect antioxidants thanks to their ability to induce the antioxidant defense system activating the Nrf2/ARE pathway. On these basis, ITCs can be considered a natural and effective therapeutic strategy in counteracting oxidative stress that characterizes neurodegeneration and many other chronic diseases.

4 Role of GLS-Derived ITCs in Loss of Proteostasis

In the last years, many researches have been focused on the mechanisms behind the loss of proteostasis in neurons and its association with neurodegeneration. The impairment of the protein degradation system leads to an abnormal accumulation of toxic protein oligomers, which are considered the starting material for the development of neurodegenerative proteinopathy. The spectrum of CNS linked to

proteinopathies is particularly broad and includes AD, PD, Lewy body dementia, Pick disease, frontotemporal dementia, Huntington's disease (HD), ALS, and many others [82].

Unwanted proteins are safely eliminated via two major mechanisms: autophagic degradation in the lysosome or by targeted breakdown in the proteasome. Even if autophagy seems to be a less selective process compared to the proteasome, in recent years, the role of autophagy impairment in neurodegenerative disease has been widely demonstrated [108, 109]. Autophagy pathway involves the de novo synthesis of vesicles called autophagosomes, which can embed organelles, protein aggregates, and invading pathogens. The autophagosomes fuse with endosomal compartments to form amphisomes before fusing with the lysosome, where their contents are degraded and the resulting metabolites are recycled back to the cytoplasm [108].

On the other hand, proteasomal degradation in neurons is under the control of the peptide ubiquitin. Ubiquitin covalently binds to the target proteins in a process called conjugation, sealing the fate of proteins that are digested by the proteasome [110]. The amino acids obtained by protein degradation are then reused by the cell.

Kwak et al. [111] observed that SF increases the expression of the catalytic core subunits of the proteasome, leading to enhanced proteasome activity in Neuro2A cells. They suggested that the protective effect of SF against H_2O_2-induced damage is, in part, mediated by proteasome induction as challenging the cells with proteasome inhibitors led to a significant attenuation of SF neuroprotection. Moreover, the overexpression of the proteasome subunit PSMB5 by gene transfection increased resistance of the cells to H_2O_2-induced damage and protein oxidation. Both proteasome activation and induction of PSMB5 have been linked to the antioxidant response under the control of the Keap1-Nrf2 pathway [111, 112]. In a subsequent paper, the same authors demonstrated that SF protects neurons from Aβ1-42 toxicity in a proteasome-dependent manner [113]. They also observed that Aβ digestion can be mediated by isolated proteasome fraction, implying a potential role of the proteasome in the proteolysis of Aβ. A more recent study reported that SF modulates the proteasome activity via the upregulation of heat shock protein 27 (Hsp27) and that the phosphorylation of Hsp27 is irrelevant to SF-induced proteasome activation [114]. Interestingly, unlike SF, benzyl ITC and phenethyl ITC did not induce Hsp27 protein accumulation or proteasome activation [115]. The ability of SF to enhance proteasome activity has also been demonstrated in vivo in a transgenic mouse model of HD [116].

Jo et al. [117] reported that SF induces autophagy in primary cortical neurons. In particular SF reduces the levels of phosphorylated tau by activating the autophagy adaptor protein NDP52. Another study demonstrated that SF induces autophagy via extracellular signal-regulated kinase (ERK) activation, independent of Nrf2 activity in neuronal cells [118]. In vivo data obtained in a mouse model of HD indicated that, in the brain, SF is able to enhance autophagy, increasing both microtubule-associated protein 1 light chain 3 (LC3)-I and LC3-II levels [116].

The effect of SF on the ubiquitin-proteasome system and autophagy has been summarized in Fig. 5.

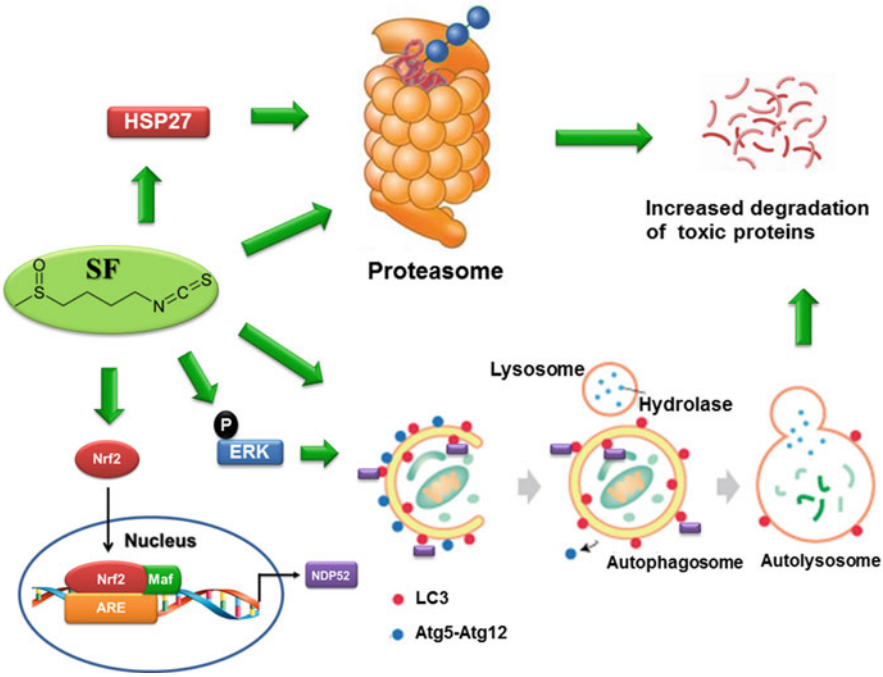

Fig. 5 Schematic representation of the effect of SF on the proteasome and the autophagic systems

5 Protective Effects of GLS-Derived ITCs Against Neurodegenerative Diseases

5.1 Alzheimer's Disease

In an attempt to characterize the etiopathogenesis of AD and identify effective therapies, an abundance of research has been carried out, primarily using transgenic animal models. Beside them, use of nontransgenic animal models of AD has been widely employed, like iron overload [119] combined administration of D-galactose and aluminum [120], injection of Aβ-peptides, and scopolamine-induced memory impairment [121] models. However to date, no single animal model has been able to exactly replicate neuropathological and behavioral symptoms of human AD [122], so the health benefits observed cannot be immediately translated to human.

The potential neuroprotective effects of SF against Aβ-induced cell death in vivo have been initially investigated in mice that underwent single intracerebroventricular injections of Aβ aggregates [123]. A 6 days SF treatment ameliorated cognitive dysfunction induced by Aβ exposure. In particular, SF improved spatial working

memory and contextual memory in Aβ treated mice. In control mice, SF did not improve or worsen animal behavior, suggesting that the compound doesn't influence cognition/memory in healthy subjects [123]. One hypothesized mechanism by which GLS-derived ITCs could counteract Aβ peptide-induced toxicity in the brain is their direct reaction with Aβ peptides. SF binds covalently and specifically at position 16 and 28 to the free NH_2 group of N-terminal aspartic acid and the ε-amino group of lysine and reduces the inclination of Aβ to aggregate [82]. The neuroprotective effect of SF has also been investigated in an AD mice model, in which AD-like lesions were induced by combined administration of aluminum and D-galactose [124]. SF improved cognitive impairment by reducing cholinergic neuron loss in the medial septal and hippocampal CA1 regions through a hypothesized mechanism associated with neurogenesis and aluminum load reduction. In a scopolamine-induced memory impairment model, SF significantly ameliorated the cholinergic system reactivity, increasing acetylcholine (ACh) level, decreasing acetylcholinesterase (AChE) activity, and increasing choline acetyltransferase (ChAT) expression in the hippocampus and frontal cortex of mice [125]. The ability of SF to positively affect the cholinergic system was also shown in vitro in scopolamine-treated primary cortical neurons [125]. These findings are not in agreement with the results of Zhang et al. [126] that observed that SF treatment did not affect acetylcholine level or the activity of choline acetyltransferase or acetylcholinesterase in the cerebral cortex. Also the ITC kaempferol has been demonstrated to be effective in counteracting Aβ peptide-induced neurotoxicity in mice. Kaempferol significantly protected against Aβ peptide-induced neurotoxicity, reducing lipid peroxidation in brain tissues and ameliorating impairments in short-term memory [127].

An extract of *M. oleifera*, rich in ITCs, improved cognitive performance of rats treated with colchicine, as a model of AD, counteracting both the reduction of brain monoamine levels and the impairment in electrical activity [128]. SF exhibited neuroprotective properties also in an iron-induced model of memory impairment by counteracting mitochondrial and synaptic alterations. In particular, SF was able to increase both the mitochondrial fission protein, DNM1L, and synaptophysin in the hippocampus leading to a recovery of recognition memory impairment induced by iron [129]. Advanced glycation end products (AGEs) are emerging as important players in the onset and progression of AD. AGEs are complex, heterogeneous molecules formed by the reaction of glucose molecules or toxic aldehydes, like methylglyoxal, with amino groups in proteins and cause extensive protein cross-linking and oxidative stress [130, 131, 132]. Their pathological role in AD has been suggested because they accumulate in the aging brain [133] and in the brains of AD individuals [134, 135]. Recently, it has been observed that SF protects SH-SY5Y cells from cell death induced by methylglyoxal by different mechanisms: modulating ERK1/2, p38 MAPK, c-Jun N-terminal protein kinase (JNK), and brain-derived neurotropic factor (BDNF)-mediated pathways, upregulating glyoxalase 1, the main methylglyoxal detoxifying enzyme, and reverting the reduction of glucose uptake induced by methylglyoxal [130].

5.2 Parkinson's Disease

As previously reported, dopamine itself can spontaneously undergo auto-oxidation leading to the formation of toxic DA-quinone species, superoxide radicals, and hydrogen peroxide [22]. For these reasons, many studies investigating PD have been carried out using oxidized DA derivatives like the MPTP and the 6-OHDA both in vivo and in vitro [136, 137].

The ITC SF demonstrated protective effects in different animal models of PD. SF significantly counteracted nigrostriatal neurodegeneration induced by MPTP in mice thanks to its ability to enhance the endogenous antioxidant defense system and to counteract neuroinflammation [107]. In particular, SF activated the Nrf2-mediated pathway inducing the overexpression of HO1 and NQO1 and reduced astrogliosis, microglia activation, and the release of two pro-inflammatory cytokines such as IL-6 and TNF-α [107]. In a 6-OHDA mouse model of PD, SF ameliorated behavioral impairments such as motor coordination and rotational behavior and counteracted 6-OHDA-induced apoptosis via blocking DNA fragmentation and caspase-3 activation [138]. The authors hypothesized that SF neuroprotection may be wholly or in part due to its ability to enhance GSH and its related enzymes and also to counteract ERK1/2 activation induced by 6-OHDA. SF neuroprotective activity has been also proved in α-synuclein-expressing flies and Drosophila parkin mutants [139]. SF protective ability to counteract different deleterious molecular mechanisms characteristic of PD has also been investigated in dopaminergic cell lines. In particular, SF was able to protect CATH.a and SK-N-BE(2)C cells, as well as mesencephalic dopaminergic neurons exposed to 6-OHDA and BH_4 [140], by increasing NQO1 mRNA level and activity, reducing quinone-modified proteins, and attenuating BH_4-induced ROS production, DNA fragmentation, and membrane breakage. Analogous results have been obtained by other authors. Siebert et al. [141] demonstrated that SF protects organotypic nigrostriatal co-cultures against 6-OHDA-induced damage by an increase in antioxidant activity mediated by Nrf2 upregulation. SF counteracts cell death of dopaminergic-like neuroblastoma SH-SY5Y cells, induced by H_2O_2 and 6-OHDA, increasing endogenous GSH levels, upregulating enzymes involved in GSH metabolism including GST and GR, and normalizing the intracellular redox status [99]. SF protective mechanism against 6-OHDA-induced cytotoxicity was further characterized by Deng et al. that confirmed SF ability to increase Nrf2 nuclear translocation [142] and upregulate HO1 expression in a PI3K/Akt-dependent manner [106]. Moreover, SF protects cortical neurons against 5-S-cysteinyl-dopamine (CysDA) [143], a neurotoxin produced in vivo by the reaction of DA-quinone with cellular thiols [144–146]. In particular, SF enhanced the cellular antioxidant defense system-activating Nrf2 pathway and upregulating a battery of antioxidant/detoxifying phase II enzymes such as GST, GR, TR, and NQO1. Moreover, a key role of ERK1/2 and Akt/protein kinase B (PKB) in SF modulation of the Nrf-2 pathway has been suggested [143].

Other isothiocyanates demonstrated neuroprotective activities against PD both in vitro and in vivo. In a 6-OHDA mouse model of PD, 6-(methylsulfinyl)hexyl isothiocyanate, one of the major bioactive component in wasabi roots, led to a

significant decrease in oxidative stress and apoptotic cell death, improving behavioral impairments, in particular motor deficits [138, 147]. A new formulation of glucoraphanin, (RS)-4-methylsulfinylbutyl glucosinolate, mainly present in Tuscan black kale seeds and hydrolyzed with myrosinase, demonstrated protective effect in a MPTP mice model of PD ameliorating motor deficits such as movement coordination and tremors [148]. It has been suggested that the molecular mechanisms behind this protection are related to the reduction of dopamine transporter degradation, tyrosine hydroxylase expression, and oxidative stress by Nrf2-mediated pathway activation, the inhibition of apoptotic cell death, and the increased level of neurotrophic factors, like BDNF, GAP-43, and nerve growth factor (NGF). The treatment of SH-SY5Y cells with erucin, produced by bioconversion of SF, prevented oxidative stress induced by 6-OHDA, reducing intracellular ROS production and loss of mitochondrial membrane potential [100].

These promising results obtained with natural ITCs in different model of PD led to the production of synthetic ITCs to be investigated in this context of neurodegeneration. Lee et al. [149] reported the synthesis of three novel ITCs (ITC-1, ITC-2, and ITC- 3) and investigated their protective effects both in vitro, using CATH.a neuronal cells and microglial BV-2 cells, and in vivo in an animal model of PD. ITC-3 demonstrated the highest performance thanks to its low cytotoxicity compared to ITC-1, ITC-2, and SF. ITC-3 exerted its neuroprotective activity through the modulation of Nrf2 pathway, enhancing the antioxidant defense system, inhibiting pro-inflammatory mediators in activated microglia, and alleviating PD-associated motor deficits. Another synthetic ITC is 4-iodophenyl isothiocyanate (4-IPITC) that elicited neuroprotective and neurotrophic activities both in vitro and in vivo [150]. 4-IPITC protected cortical neurons from different damages such as excessive glutamate exposure, oxygen-glucose deprivation, oxidative stress, and 1-methyl-phenylpyridinium; in a MPTP mouse model of PD, 4-IPITC reduced MPTP toxicity. Interestingly, 4-IPITC binds to both monoamino oxidases A and B and to the dopamine transporter.

5.3 Multiple Sclerosis

The protective effect of ITCs on MS have been mainly investigated using the experimental model of autoimmune encephalomyelitis (EAE) that reproduces many clinical and pathological characteristic of human MS, such as paralysis, weight loss, demyelination, CNS inflammation, and BBB disruption [151]. Based on this experimental model, Giacoppo et al. [152] demonstrated that one of the two enantiomers of SF (R-SF) attenuates the development of EAE. In particular they observed that R-SF modulates inflammatory pathway during EAE by inhibiting the IkB-α degradation as well as the NF-kB activation, leading to a reduction of IL-1β, JNK expression, and apoptosis. These results have been confirmed and expanded by the data of Li et al. [153] that suggested that SF may inhibit the development and severity of EAE by its potent antioxidant and anti-inflammatory activity. SF treatment reduced the loss of the BBB integrity induced by inflammation, accompanied

by reducing inflammatory infiltrates and demyelination in mice. Furthermore, SF treatment reduced oxidative stress by activating the Nrf2 pathway and upregulating HO1 and NQO1 expression, reducing antigen-specific Th17 responses, and enhancing IL-10 responses. Another suggested mechanism by which SF could counteract MS is its ability to modulate the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) that is strongly involved in MS [154], in fact its levels are elevated in the cerebrospinal fluid of MS patients during relapses [155]. Chemical inhibition of MIF tautomerase activity slows the progression of EAE, reducing migration of leukocytes into the brain and spinal cord of treated animals [154]. Interestingly, ITCs [156–158] are able to inhibit MIF tautomerase activity. Moreover, SF not only inhibits MIF tautomerase activity but induces MIF protein levels, possibly increasing its protective oxidoreductase activity [159].

Other ITCs, in addition to SF, have been investigated in relation to MS. In a mouse model of EAE, 4-(α-l-rhamnosyloxy) benzyl isothiocyanate, an ITC obtained by myrosinase hydrolysis of the GLS glucomoringin, counteracted the severity of EAE [160]. This ITC inhibited the inflammatory cascade that underlies the processes leading to severe MS, in particular, reducing TNF-α, inhibiting the activation of ERK1/2 signaling pathway, downregulating iNOS expression, and counteracting ROS and apoptosis. The synthetic 4-IPITC, thanks to its ability to modulate the antioxidant defense system by activating Nrf2 pathway, delayed EAE onset and decreased clinical score [150].

Taken together, these studies add new interesting properties and applicability of ITCs, suggesting their potential role in the treatment or prevention of MS, at least in association with current conventional therapy.

5.4 Amyotrophic Lateral Sclerosis

The effects of GLS-derived ITCs in ALS prevention/counteraction have been investigated both in vivo and in vitro. Galuppo et al. [161] treated rats overexpressing the mutated human gene $SOD1^{G93A}$, which represents a transgenic model of ALS, once a day with 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate via intraperitoneal (i.p.) injection for two weeks. Their data showed that rats treated with isothiocyanate had better performance in the open field test and showed a stronger grip capability and a higher muscle strength in the Hanging Wire Test than untreated rats.

A phenotypic characteristic of ALS is the atrophy of skeletal muscle fibers which become denervated as their corresponding motor neurons degenerate [162]. Malaguti et al. [163] demonstrated that SF treatment is able to counteract muscle damage induced by acute exercise in rats through the modulation of a scope of detoxifying phase II enzymes. In particular, SF reduced plasma LDH and creatine phosphokinase (CPK) activities, two well-known biomarkers of tissue damage, and counteracted muscle oxidative stress through the upregulation of GR, GST, and NQO1 expression and activity.

In an in vitro study, Duan et al. [164] investigated the protective effect of the ITC SF against the toxic effects of wild-type and mutant TDP-43 protein on NSC-34 cell

line. Mutant TDP-43 has been identified in familial ALS patients [165], and TDP-43 inclusions are now recognized as a common characteristic of most ALS patients [166]. SF protected mutant TDP-43 cells counteracting mitochondria dysfunction and oxidative injury. Interestingly, this protection was not afforded by the expected modulation of the Nrf2-ARE pathway but by mechanisms that must be still elucidated. Chan et al. [167] investigated the effect of SF against motor neuron degeneration in organotypic spinal cord cultures exposed to threohydroxyaspartate to induce glutamate excitotoxicity. SF was able to increase the protective activity of riluzole, the only drug proven to slow the disease process in humans and approved for management of ALS against excitotoxicity. SF enhanced riluzole efficacy by promoting the activation of the Nrf2 signaling pathway, inducing NQO1 and HO protein expression, and reducing glutamate accumulation in the extracellular space.

6 Conclusions

Researches described above suggest that GLS-derived ITCs exhibit a wide pleiotropic activity against the onset and progression of several neurodegenerative diseases such as AD, PD, MS, and ALS. ITCs show both common mechanisms of action and specific activities peculiar to the single neurodegenerative disorder. Besides the well-characterized ITC mechanism of action as inducers of cytoprotective genes through the activation of the Nrf2 pathway, the ITC ability to enhance the endogenous degradation protein systems has been emerging as a key protective mechanism to fight the abnormal accumulation of toxic protein oligomers that characterized most of the neurodegenerative diseases. Because at present there are no adequate therapies to counteract the onset and the inexorable progression of these disorders whose incidences are exponentially growing, the possibility to use ITCs, safe and cheap compounds, to counteract neurodegeneration appears as a promising avenue that absolutely need to be explored.

Acknowledgments This work was supported by MIUR-FIRB (project RBAP11HSZS) and "Fondazione del Monte di Bologna e Ravenna (Italy)."

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Antileukemic Activity of Sulforaphane

10

Elena Catanzaro and Carmela Fimognari

Abstract

The biological complexity of cancer offers numerous targets for antineoplastic therapies. Diet is a common source of biologically active compounds. Many studies demonstrated an inverse relation between the consumption of vegetables and the risk of cancer. In this context, the interest is currently focused toward the study of certain diet-derived molecules to prevent, delay, or treat cancer by acting simultaneously on different critical pathways. Among phytochemicals, sulforaphane, an isothiocyanate derived from the hydrolysis of the glucosinolate glucoraphanin mainly present in *Brassica* vegetables, is one of the most promising diet-derived molecules. In this chapter, we will present the antileukemic activity of sulforaphane and discuss its therapeutic potential in terms of mechanisms of action, safety, and limits.

Keywords

Leukemia • Sulforaphane • *Brassica* vegetables • Dietary phytochemicals • Apoptosis • Cell cycle

Abbreviations

AKT	Plasmatic protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
B-ALL	Acute B-lymphoblastic leukemia
CDK	Cyclin-dependent kinase

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_14

CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
CYC	Cyclin
CYP450	Cytochrome P450
DSB	Double-strand break
GCLC	Glutamate-cysteine ligase catalytic
GCLM	Glutamate-cysteine ligase modifier
GLS	Glucosinolate
GSH	Glutathione
HRR	Homologous recombination repair
ITC	Isothiocyanate
mTOR	Mammalian target of rapamycin
PARP	Poly (ADP-ribose) polymerase
PIP3	Phosphatidylinositol 3-kinase
QR NAD(P)H	Oxidoreductase
ROS	Reactive oxygen species
SFN	Sulforaphane
STAT	Signal transducers and activators of transcription
T-ALL	Acute T-lymphoblastic leukemia

Contents

1 Introduction

Cancer is the second leading cause of death among chronic diseases. During his entire life, one out of two people will be diagnosed with some types of cancer [1], and about 1% of blown cases will occur on children between 0 and 14 years old [2]. In 2015 leukemia counts about 54,000 new cases, and despite all the improvements in the disease understanding and treatment strategies, it persists to be fatal in 45% adults and 30% of children so far [2].

Cancer pathogenesis is a multistep process that involves a progressive transformation of healthy cells to neoplastic ones, due to subsequent molecular alterations in critical genes. These modifications are expressed by the acquisition of specific

common biological properties, which allow cancer cells to sustain proliferative signaling, resist cell death, and evade growth suppressors [3].

Carcinogenesis is divided into three sequential stages: initiation, promotion, and progression. Initiation is the first and irreversible step where genetic or epigenetic events affect cellular genome, producing the potential for tumor development. Genetic errors at molecular DNA structure can lead to mutations [4]. In particular, mutations involve one or more genes that control key regulatory cell pathways and target heritable DNA sequences. These alterations can arise spontaneously or can be induced by the exposure to a carcinogen [5]. Some xenobiotics are toxic in a direct way, but often carcinogens are not active per se. Most of them need metabolic activation to acquire their initiating potential. The activation is catalyzed by phase I enzymes, mostly belonging to the cytochrome P450 (CYP450) family. Phase I enzymes enhance the polarity of a xenobiotic through the reactions of oxidation, reductions, or hydrolysis [6]. Reactive intermediates such as electrophiles, nucleophiles, reactive oxygen species (ROS), or redox intermediates are generated, and the activated species, called ultimate carcinogens, interact with DNA, thus increasing the risk of DNA damage and inducing cancer initiation. Phase II enzymes catalyze the detoxification of carcinogens through the conjugation of the metabolites produced by phase I enzymes. In order to deactivate the reactive metabolites produced by phase I enzymes, conjugating enzymes, such as NAD(P)H-quinone oxidoreductase (QR) or glutathione S-transferases, allow the binding of more polar functional groups, such as glutathione, sulfate, or glucuronate, to the metabolite [6].

The initiated cell can persist in its silent potential state for long time, until chemical signals or other events promote its expansion into a clone of neoplastic cells during the phase of promotion [7].

Promotion is the second step of carcinogenesis. It is a reversible process that does not involve the genome structure, but rather its expression.

The last phase of carcinogenesis is the progression, where cells develop the malignant phenotype. Cells acquire aggressive characteristics, such as the ability to invade local tissues, metastasize, and promote angiogenesis. The tumor size increases and cells may accumulate further mutations that lead to a heterogeneous cell population [4].

Leukemia is a diversified group of hematological malignancies and includes numerous different biological subgroups with distinctive characteristics, but connected by the same origin: the hematopoietic precursor cells. The bone marrow is the groundwork of all leukemias, and during the carcinogenesis abnormally proliferating cancer cells displace bone marrow stem cells. The subsequent spread of a large number of cancer cells in the bloodstream makes the disease full blown [8]. Commonly, leukemia is classified as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), T-cell acute lymphoblastic leukemia (T-ALL), B-cell acute lymphoblastic leukemia (B-ALL), and chronic lymphoblastic leukemia (CLL) [9]. Today, chemotherapy remains the most effective way to induce the remission of hematological malignancies. For example, the gold standard therapy

for AML is based on the association of cytarabine and anthracyclines. However, the highest remission rate is 60% [10].

The most significant issues in leukemia pharmacological treatment are the low therapeutic index of most anticancer drugs, the frequent development of chemoresistance, and subsequent relapses [11]. The latter remains the most common cause of death [12]. Taking into account the problems associated with the use of most anticancer drugs, new therapeutic strategies have to be explored.

Diet is a natural source of many bioactive compounds. For more than 20 years, numerous epidemiological studies have shown that a regular consumption of fruit and vegetables is related with a lower cancer incidence [13]. Chemopreventive and even therapeutic effects are exerted by many phytochemicals present in fruit and vegetables [14]. Of note, many dietary compounds are characterized by a low toxicity [15].

The biological complexity of cancer pathogenesis offers a number of "druggable" targets. The interest in the identification and characterization of dietary compounds, capable of reversing, retarding, or inhibiting the carcinogenetic process, is rapidly expanding [16]. More and more interest is currently focused toward the investigation of the ability of diet-derived molecules to prevent or delay cancer outbreak by acting simultaneously on different critical pathways. In particular, the most fascinating clinical properties investigated are the abilities to interfere with tumor promotion and progression stages [16].

Among all natural products containing active molecules, isothiocyanates (ITCs) from *Brassica* family are particularly promising. A diet rich in *Brassica* vegetables, which include broccoli, cabbage, and cauliflower (Fig. 1), has been demonstrated to exhibit chemopreventive effects in laboratory animals and to decrease the risk of malignancies [7].

In this chapter, we present the antileukemic activity of the ITC sulforaphane (SFN), the different mechanisms involved in its antileukemic effects, and its therapeutic potential.

Fig. 1 *Brassica* vegetables

2 Sulforaphane

ITCs, known also as mustard oils, are the phytochemicals responsible for the pungent and acrid odor and flavor of *Brassica* vegetables. They are accumulated in different parts of the plant as inactive precursors, called glucosinolates (GLSs) [17]. They are released when tissues are broken [18]. The conversion in the ITC form is catalyzed by myrosinase, a thioglucoside glucohydrolase. The enzyme and GLSs are stored in the same tissue, but in different cell compartments. In this way, the conversion to ITCs occurs when the plant is damaged, for instance, by chewing or chopping. This traumatic event permits the contact between the GLS and enzyme and makes the reaction feasible [19]. Alternatively, gut microflora hydrolyzes GLSs into ITCs [19]. GLSs are constituted by a beta-D-thioglucose, a sulfonate oxime and a side amino acidic chain derived from tryptophane, phenylalanine, or branched amino acids, which is distinctive for each GLS. Aliphatic, aromatic, or heterocycle groups constitute the variable part of GLSs and determine the biological activity of the related ITCs [7].

In most broccoli varieties, the prevalent GLS is glucoraphanin. SFN originates from the hydrolysis of glucoraphanin (Fig. 2). SFN is a hydrophobic and extremely reactive molecule, able to penetrate cells and alter different cellular pathways involved in cancer development, such as apoptosis, cell cycle, carcinogen activation, and inflammation [20, 21].

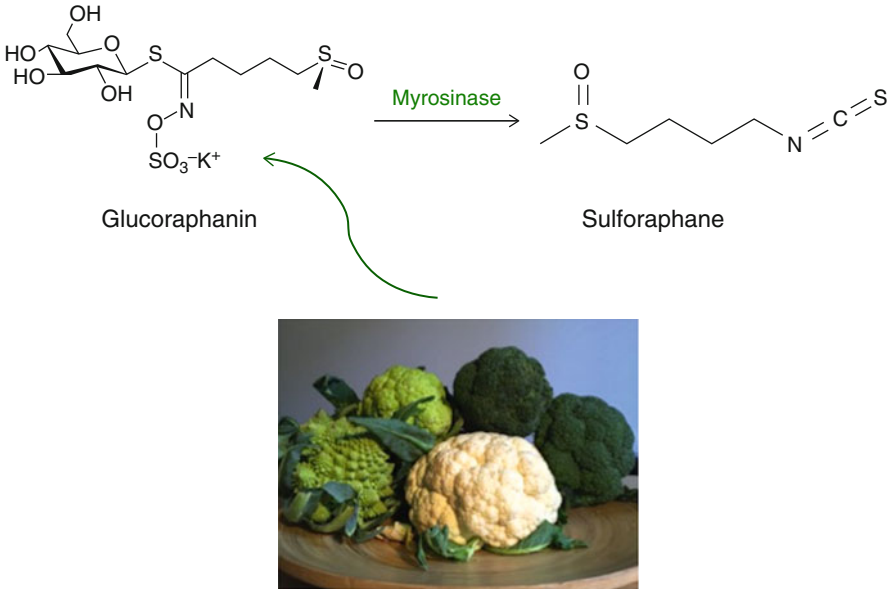

Fig. 2 Hydrolysis of glucoraphanin by myrosinase and formation of sulforaphane

3 Sulforaphane and Leukemia

The chemopreventive activity of SFN has been investigated for decades. More recently, its role as a therapeutic agent in hematological malignancies has been explored, and very interesting results have been obtained.

The antileukemic activity of SFN can be exploited in two different ways, depending on the treatment doses: at nontoxic concentrations (concentrations that don't induce a decrease in cell viability), it acts as a chemopreventive agent able to block cancer formation; at higher doses, SFN exhibits a therapeutic activity [22]. In this context, SFN has been tested as a single drug or in combination with different anticancer drugs.

4 Antileukemic Mechanisms of SFN

The antileukemic activity of SFN has been tested by different experimental approaches, such as a number of leukemia cell lines, animal models, and blast from leukemia patients.

4.1 Modulation of Phase I and Phase II Enzymes

As previously described, the bioactivation of chemical carcinogens is catalyzed by phase I enzymes, in particular the CYP450 family. The regulation of these proteins in terms of expression and function is crucial to determine the activity of a chemical carcinogen. SFN modulates the gene expression and function of specific CYP450 isoforms [23]. Several studies conducted in rats or humans showed its ability to interact with CYP2B, CYP3A, CYP1A2, and CYP2E1 [24, 25]. CYP3A catalyzes the conversion of aflatoxin B into its epoxide active form, which interacts with DNA creating the mutagenic adduct [26]. In rats, SFN decreases the synthesis or increases the degradation of CYP2B and CYP3A [24]. As well, SFN reduces mRNA levels of CYP3A4 in humans' hepatocytes [25]. CYP1A2 catalyzes the activation of heterocyclic amines [27], such as the conversion of 2-amino-3-methylimidazo-[4,5-f] quinoline to its mutagenic intermediates. Although SFN induces no alteration in CYP1A2 hepatic levels in rats, it inhibits its metabolic activity and makes the enzyme not catalytic competent [24]. At last, SFN inhibits the genotoxicity of N-nitrosodimethylamine by acting as a competitive inhibitor of CYP2E1 in acetone-treated liver rat microsomes [28]. On the whole, the above-presented studies demonstrate that, although in animal models, SFN interferes with the activation of many carcinogens.

Phase II enzymes inactivate carcinogenic intermediates before their interaction with nucleic acids. Nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) is a cytoplasmatic transcription factor expressed by nearly all cell types. Its function is blocked by the binding with its suppressor, i.e., kelch-like ECH-associated protein 1, which prevents Nrf2 nuclear translocation and activation of Nrf2-target genes

through binding to the antioxidant response element. Activated Nrf2 translocates to the nucleus and upregulates target genes such as glutamate-cysteine ligase catalytic (GCLC), modifier (GCLM) subunits, and NAD(P)H-quinone oxidoreductase (QR) [22].

In humans, oral administration of a dietary extract of broccoli sprouts increases the mRNA levels of Nrf2-dependent enzymes, such as glutathione transferase, versus a placebo-controlled group [29]. In addition, differentiated human promyelocytic cells (HL-60) used as a model of neutrophils and treated with 5 μM SFN for 16 h show an increase in glutathione (GSH) levels [30]. This is an interesting finding because GSH plays a pivotal role in preventing the uncontrolled formation of free radicals and ROS. The same study reports an increased expression of GCLC and GCLM and a better intracellular GSH/oxidized glutathione ratio [30]. Glutamate-cysteine ligase is constituted by a catalytic subunit (GCLC) and a regulatory subunit (GCLM) and represents the rate-limiting enzyme in the GSH biosynthesis pathway. Very similar effects were found on human lymphoblasts, where SFN promotes the nuclear translocation of Nrf2 through the release from kelch-like ECH-associated protein 1. In the nucleus, Nrf2 activates the antioxidant response element transcription system, which in turn increases the expression of the γ-glutamylcysteine synthetase. This enzyme catalyzes the reaction between L-glutamate and cysteine to synthesize glutamylcysteine, which is involved in GSH synthesis [31]. On the whole, the above-reported studies suggest that SFN is able to restore the intracellular redox state.

One of the most representative phase II enzymes is QR. QR blocks the production of ROS by starting from quinones and the subsequent depletion of GSH levels [16]. SFN induces QR activity in lymphoblastic cells at very low concentrations compared to those required to decrease cell viability (0.35 μM versus 1 μM) [22].

The combination of the above-described activities of SFN leads to the detoxification of carcinogens and thus improves the cellular defense mechanisms from potential mutagenic events [7].

Of note, the ability of SFN of modulating metabolic enzymes can be reached by the consumption of 200 μM of SFN-containing broccoli sprouts, which lead to plasma concentrations less than 3 μM [32].

4.2 Apoptosis Induction

Apoptosis, also called programmed cell death, is a mechanism involved in cancer development [33]. Healthy cells are able to detect and integrate intra- and extracellular signals and induce, when necessary, apoptosis through the intrinsic or the extrinsic pathway [33]. The two pathways have the same downstream effectors and initiate a proteolytic pathway involving effector caspases that directly promote apoptosis. As an example, high levels of mitochondrial calcium and ROS damage the mitochondrial membrane and allow the release of cytochrome c that initiates the caspases' cascade and triggers the intrinsic apoptotic pathway [34]. Several proteins are target of caspases and used as markers of apoptosis. PARP [poly (ADP-ribose)

polymerase] is the substrate of caspase 3 and high levels of cleaved PARP are used as marker of apoptosis [35].

Cancer cells develop numerous strategies to limit or evade apoptosis, mostly acting on key actors involved in the programmed cell death control, such as the tumor suppressor gene p53 [36]. p53 detects DNA damage and triggers apoptosis through the upregulation of BH3-only proteins, such as Bax, or by an unbalance between pro- and anti-apoptotic proteins such as Bax and Bcl-2 [33]. Bcl-2 is an anti-apoptotic protein; it acts by inhibiting proapoptotic proteins, such as Bax and Bak. Bcl-2 is located on the cytoplasmic surface of the mitochondrial outer membrane, the endoplasmic reticulum, and the nuclear envelope. It detects the damages of these organelles and modulates their activity by modifying proteins' and molecules' flux. When located on the cytoplasmic mitochondrial membrane, Bcl-2 forms heterodimers with proapoptotic proteins such as Bax and inhibits their activity. It blocks apoptosis by inhibiting both the formation of mitochondrial transition pores and the release of cytochrome c, with subsequent inactivation of the caspases' cascade [37]. When Bax is not bound, it breaks the membrane allowing the release of proapoptotic proteins. Among these, the most important one is cytochrome c, which directly activates the intrinsic apoptotic pathway through the caspases' cascade [33].

The mechanisms responsible for the proapoptotic activity of SFN are complex. ROS production is a key event in SFN-induced apoptosis. In promonocytic leukemia cells (U937), SFN causes an increase in ROS levels that triggers the loss of the mitochondrial membrane potential and activates the intrinsic apoptotic pathway [38, 39]. The rise levels of caspase 3, 8, and 9 and the cleaved PARP observed after SFN treatment on T- and B-leukemia cells [20] confirm these results.

On leukemia T cells, SFN modulates the expression of some proteins that play a critical role in the control of cell life or death: p53, Bax, and Bcl-2. p53 is a tumor suppressor gene that detects DNA damage and arrests the cell cycle at the G_1/S phase. In this way, DNA damage can be repaired before DNA replication [36]. If the damage cannot be repaired, p53 induces apoptosis. SFN increases Bax and p53 protein levels, while it doesn't affect Bcl-2 protein levels in leukemia T-cell lines [19]. Although there isn't a modulation of Bcl-2, the proapoptotic effect of SFN results from a perturbed Bax/Bcl-2 ratio. This is due to the stimulation of Bax gene expression caused by the increased p53 protein levels induced by SFN [40].

SFN induces apoptosis in each phase of the cell cycle, but with different intensities. The highest proapoptotic activity is exerted on the G_1-phase cells, followed by those that are in the S phase, and lastly by those in the G_2/M phase. This different activity is probably due to the different expressions of p53 in the different phases of the cell cycle. The highest expression of p53 is reported during the G_1 phase, whereas it decreases from the S throughout the subsequent cell-cycle phases. These data are consistent with the notion that p53 seems to play a role in the SFN proapoptotic activity [41].

SFN causes a dose- and time-dependent reduction of viability on several leukemia cell lines such as REH (ALL cell line), HL-60 (promyelocytic leukemia), and Jurkat (T-ALL) [8]. Depending on experimental conditions, it induces apoptosis or

necrosis. After prolonged treatment with high concentrations (30 μM) of SFN, necrosis of T-cell leukemia becomes the predominant mechanism of cell death [19]. These results suggest that very high levels of this molecule can be toxic. However, a 30 μM concentration of SFN cannot be reached by dietary ingestion of *Brassica* vegetables. At concentrations between 3 and 10 μM, apoptosis is the main mechanism of cellular death induced by SFN in all tested cell lines [19, 20].

To mimic the influence of tumor microenvironment and explore the SFN effects in a more representative model of the in vivo condition, some studies have been performed on ALL cell lines cultured in hypoxia. Hypoxic areas develop in many solid tumors and the bone marrow environment [8]. In hypoxia, cell metabolism changes as well as the characteristics of the microenvironment such as pH. These factors influence the cancer response to the antineoplastic drugs. After SFN treatment in hypoxic conditions, ROS production was confirmed, as well as its proapoptotic potential, even though less marked if compared to normoxic operating conditions. The pathways triggered by SFN in hypoxic and normoxic conditions are probably the same. For example, after prolonged treatment times, necrosis represents the predominant mechanism of cell death induced by SFN in hypoxic conditions. On the while, these data suggest that the SFN efficacy is comparatively conserved in hypoxia [8].

Ex vivo models are highly predictive of patients' responses and clinical outcomes, as compared to in vitro cell lines. This is mainly due to the ability of ex vivo models in maintaining the heterogeneity of in vivo tumors, as well as the pharmacological determinants and the growth kinetic factors. Cellular populations obtained from patients are largely characterized by the same in vivo environment. These features mimic the biological complexity of tumors in clinical practice, with the advantage of controlled conditions [42]. Ex vivo experiments performed on blasts from leukemia patients show an interesting proapoptotic potential of SFN in patients suffering from AML, pre-B-ALL, B-ALL, and T-ALL, but not in those suffering from CLL [8]. The IC_{50} (concentration able to decrease cell viability by 50% following one cell-cycle exposure) on patient lymphoblasts was in the 7–13 μM range [20].

Taken together, SFN controls the spread of leukemic cells, but apparently with different sensitivities for the diverse types of leukemia.

4.3 Cell-Cycle Modulation

Cell cycle is a finely regulated process that allows to control cell growth and maintain a balance between cell death and proliferation [43]. It is divided into four subsequent phases: G_1, S, G_2, and M phase. During the G_1 phase, quiescent cells (G_0 phase) start synthesizing mRNA and proteins necessary for DNA synthesis (S phase). After DNA synthesis, cells go through the G_2 phase where more biosynthetic activity occurs and finally reach M phase where cell division takes place [44]. Cyclin (cyc) and cyclin-dependent kinases (cdk) regulate each phase of this process [43]. Kinase complexes are formed by a catalytic unit (cdk) and a regulatory

subunit (cyc). Cyc_s are substrate and phase specific. They modulate cdk activity and their levels oscillate during the different phases of the cell cycle. Cdk/cyclin is regulated, both negatively and positively, by reversible phosphorylation or by inhibitory molecules such as p21 [45].

SFN induces a block of the cell cycle on T-ALL and pre-B-ALL cells, which contributes to its ability to inhibit leukemia cell growth [20]. The inhibition was time dependent and mediated by an accumulation of cells in the G_2/M phase. The block was evident after treatment with SFN at concentrations of 10 and 30 μM [19]. In the same experimental conditions, SFN induced apoptosis of proliferating cells. Taken together, these data show that the block of the cell cycle and apoptosis are two independent events induced by SFN and that apoptosis is not a consequence of cells' inability to proceed through the cell cycle and overcome growth arrest [19].

The antiproliferative effects of SFN are imputable to its capability of modulating cell-cycle regulatory proteins [20]. The G_2/M block was due to the inactivation of cdc-2, operated by a phosphatase that removes the inhibitory phosphates on the cyc/cdk complex, and to the increase in p21 protein levels. The overexpression of p21 seems to have an important role. This protein modulates cell cycle and other biological activities mainly by binding and inhibiting cdk_s such as cdc-2 and leading to growth arrest at specific cell-cycle stages. SFN inactivates the maturation-promoting factor cdc-2/cyclin B1 and blocks the cell cycle in the G_2/M phase by the inhibitory phosphorylation of cdc-2 [20]. Cyclin B1 was unchanged after SFN treatment [20]. Cdc-2/cyclin B1 complex modulates the cell transition from the G_2 to the M phase. When cdc-2 is phosphorylated, it cannot activate cyclin B and cdc-25C, responsible for the phosphorylation/activation of cdc-2 itself. In this way, cell cycle cannot proceed through the M phase [46, 47].

Another mechanism involved in the antiproliferative activity of SFN is the inhibition of plasmatic protein kinase B (AKT)/mammalian target of rapamycin (mTOR). Phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR is a pathway involved in cellular proliferation. Among various functions, it controls cell-cycle progression and is constitutively activated in different kinds of tumors. In normal cells, when activated, PI3K phosphorylates and activates AKT, which starts the signaling cascade. In turn, AKT activates mTOR that induces the transcription of many proteins, such as p70, a protein that promotes cell proliferation [48]. About 30% of all pre-B-ALL is characterized by the constitutive activation of PI3K/AKT/mTOR, which induces cancer cell survival and permanent cell growth state. Taken together, these findings support the role of PI3K/AKT/mTOR as a potential target to develop new antileukemic therapeutic strategies [49]. It has been demonstrated that SFN decreases the levels of both total and phosphorylated AKT and mTOR [20, 50]. mTOR, in turn, leads to the block of cells in the G_1 phase [50]. These results can be very useful if translated to the clinical practice, where SFN could be effective for the treatment of ALL with activated AKT.

A role for DNA damage in the antiproliferative effects of SFN was excluded. SFN did not induce double-strand breaks (DSBs) on pre-B leukocytes [20].

4.4 Other Mechanisms Involved in the Antileukemic Activity of SFN

Despite the promising antileukemic activity of SFN, the molecular mechanisms underlying its effects are not completely defined. Recent studies have found a role of SFN in the modulation of the signal transducers and activators of transcription (STAT). STAT is a group of cytoplasmic silent transcription factors. They have a critical role in different biologic processes such as cellular growth, apoptosis, differentiation, and proliferation and are finely regulated in normal cells. Hematological malignancies including leukemias are characterized by constitutively active forms of some STAT transduction pathways [51]. For example, CML is characterized by an aberrant STAT5 pathway [51]. SFN has been shown to inhibit STAT5 and thus its target genes in a mouse IL-3-dependent pre-B-cell line and in a human CML cell line [52].

Another important molecular target of SFN is NF-kB. It has a central role in the survival and differentiation pathways of B cells. It protects cells from the damage induced by chemotherapy or ionizing radiations and blocks apoptosis [53]. SFN blocks the DNA-binding activity of NF-kB through its interaction with the essential cysteine residues of NF-kB subunits [54].

5 Bioavailability and Safety Issues of SFN

An in vivo study in a mouse model demonstrated the SFN efficacy in controlling human leukemic cell expansion. After 4 days of oral administration of 4 mg/die of SFN, a significant reduction of tumor growth was observed when SFN was administrated in subcutaneous xenograft tumor models. However, the oral administration of SFN just reduced leukemia progression in the first 2 weeks, but it did not change the survival rate [20]. This incomplete response is probably due to the inability of SFN to reach pharmacologically relevant concentrations in some tissues, like the spine or bone marrow, where leukemia cells start their expansion since the very initial phases [20]. Furthermore, the metabolism of SFN generates some species, including SFN-cysteine, SFN-glutathione, and SFN-N-acetylcysteine, that are much less effective on ALL cells than SFN [20]. This suggests a limited toxic potential of SFN and a time-limited action. Therefore, further studies need to be done in order to find an administration way that allows maintaining the therapeutic concentrations of SFN in vivo.

The bioavailability of SFN depends on many factors. Its pharmacokinetics has been elucidated in some human studies. In particular, dietary SFN bioavailability depends on the vegetal source, the food preparation process, the pH during the conversion of GLS to ITCs, and many other variables. For example, 150 g of broccoli contain between 56 and 112 mg of SFN that correspond to an intake between 0.75 and 1.5 mg/kg b.w. [55]. After oral administration, ITCs are absorbed, then conjugated to GSH, and finally metabolized to mercapturic acid and eliminated

through the urine. Some in vivo studies demonstrated that the peak plasma concentration of SFN after oral administration is reached in about 1 h. The lipophilic nature and small size of the molecule favor its rapid absorption [55]. Likewise, some studies in humans showed that SFN is rapidly absorbed, reaching the peak plasma concentration after 1 h after broccoli sprouts or broccoli soup consumption [32]. Furthermore, it was demonstrated in humans that the peak plasma concentration can range from 3.4% (cooked broccoli) to 37% (raw broccoli) of the ingested dose [56]. Therefore, taking into account that broccoli sprouts contain 200 μM SFN and that SFN half life is about 1.75 h, after 1 h the blood concentration will range between 0.943 and 2.27 μM/L, implying a potential chemopreventive significance [32]. These results indicate that a dietary consumption of *Brassica* vegetables is not enough to lead to an antileukemic activity or to reach inhibitory cell growth concentrations.

Clinical studies on prostate cancer patients have been performed to test the toxicity of a daily consumption of a 200 μM lyophilized SFN prepared starting from an aqueous extract. No adverse effects were reported [57].

An ideal anticancer agent should be selective for neoplastic cells. This underlines the need to know its effects on healthy non-transformed cells. Since SFN induces apoptosis, decreases cell viability, and blocks the cell cycle of T-ALL cells, its activity has been investigated on non-transformed T lymphocytes as a normal counterpart of leukemia cells. One study showed that 72 h treatment with 30 μM SFN inhibits cell cycle and increases apoptosis in a dose-dependent manner in normal T cells. As well, it induces an increased expression of some proapoptotic proteins such as p53 and Bax. However, although SFN is not completely selective for cancer cells, the activity was less pronounced than in T-ALL cells [58]. Other studies have shown different outcomes on different cell types: SFN did not induce any significant cytotoxic or proapoptotic effect on normal peripheral blood mononuclear cells, thus suggesting a selective activity for transformed cells [20].

Of note, the above-presented studies were performed in vitro. In vivo studies have to be done in order to better evaluate its toxicological profile. In this context, some recent studies explored the genotoxicity of SFN. Phospho-H_2AX is the principal biomarker of DNA DSBs. SFN was able to induce DSBs in adenocarcinoma cells, although DNA repair is achieved through the homologous recombination repair pathway (HRR) [59]. HRR repairs DSBs through the exchange of the damaged DNA strand with a similar or identical nucleotide sequence; this information is retrieved from undamaged sequences of homologue chromosomes or sister chromatids [60]. The key effector of this pathway is Rad51 protein, which promotes strand exchange with a regular nucleotide filament and facilitates the homologous pairing [61]. In particular, it was demonstrated that DSBs induced by SFN were associated with Rad51 foci. These data confirm the hypothesis that DSBs repair is mediated by HRR. Moreover, HRR activation is dependent on the checkpoint kinase 1 (Chk-1), a kinase that controls a checkpoint in the G_2 phase of the cell cycle and induces Rad51 activation. Therefore, cell-cycle block induced by SFN in the G_2/M phase corroborates the hypothesis that SFN induces DSBs that are repairable through the HRR pathway [59, 62]. In 2012, Suppipat et al. [20] demonstrated that, compared to a frank genotoxic compound like etoposide, 7.5 μM SFN did not induce a significant

increase in phosphorylated H_2AX in human pre-B cells and T-lymphoblastoid cells. Similar results were reported on human lymphocyte, where SFN did not increase the number of micronuclei, a biomarker of DNA mutations [63]. Taken together, the above-reported studies suggest that SFN is able to induce genotoxic lesions, which are repairable and do not evolve in a frank mutagenic effect.

6 Conclusions

Carcinogenesis is a multistep process in which normal cells accumulate sequential alterations in critical genes through which cells gain the peculiar tumorigenic phenotype. These new developed biological capabilities, called "hallmarks of cancer" by Hanahan and Weinberg, allow the transformed cells to survive, proliferate, and differentiate [3]. The multifactorial and complex nature of carcinogenesis led to focus on multi-target or pleiotropic therapy able to affect different pathways simultaneously.

Diet is a common source of biologically active compounds. Anticancer activity has been reported for many natural compounds, such as curcumin, resveratrol, genistein, etc. SFN is an ITC stored in edible *Brassica* vegetables in the form of inactive precursors (GLS). SFN exhibits chemopreventive and anticancer activities on many different tumors, imputable to its capacity to interfere with different steps of the carcinogenetic process [64]. It inhibits phase I enzymes involved in the activation of xenobiotics and stimulates phase II enzymes, thus promoting the inactivation and elimination of carcinogens. Furthermore, it modulates different critical pathways for the neoplastic process, such as apoptosis and cell cycle [41].

The antileukemic activity of SFN was not completely elucidated, but recent studies evidenced a very interesting profile [20]. SFN exerts antileukemic effects in different leukemia cell lines and blasts from pediatric and adult ALL, T-ALL, B-ALL, and AML patients [8, 20]. SFN acts mostly by inducing apoptosis and cell-cycle arrest and modulating diverse signaling pathways. On human leukemias, SFN-induced apoptosis is triggered by ROS, which trigger the activation of cancer cell growth signaling pathways. Apoptosis develops typically through p53- and Bcl-2-dependent pathways. Caspases 3, 8, and 9 are implicated as well. SFN blocks the cell cycle in the G_2/M phase, and AKT/mTOR pathway, a B-ALL leukemia preferential target, is implicated.

Besides the promising pharmacological profile, SFN shows an equally favorable toxicological profile. Currently, one of the most important problems of antineoplastic therapy is its high toxicity that often limits the efficacy of the treatment [7]. The dietary origin of SFN lets presume a good tolerability. Several studies have confirmed the safety in humans and in vitro studies have excluded a genotoxic effect [63].

Drug resistance is the second most limiting effect of antineoplastic chemotherapy. SFN could be used on resistant cancer cells. As an example, SFN increased the efficacy of anthracyclines in cell models characterized by p53 dysregulations. Since p53 mutations can be detected in many types of leukemia, such as T-ALL, B-cell

precursor-ALL, and AML [65], the above-reported evidence would suggest its activity in a variety of hematological malignancies resistant to conventional chemotherapy. SFN restored chemosensitivity of doxorubicin-resistant cells and increased its potency. In particular, co-treatment with SFN decreased the effective dose of doxorubicin required to achieve the therapeutic effect within the range tolerated in clinical practice [66].

In conclusion, many studies suggest a role of SFN as candidate for the treatment of many leukemia types. Further efforts will be made in order to better define its pharmacotoxicological profile.

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Sulforaphane and Atherosclerosis

11

Pon Velayutham Anandh Babu, Chrissa Petersen, and Zhenquan Jia

Abstract

Epidemiological studies have found associations between cruciferous vegetable consumption and reduced risk of chronic conditions, such as atherosclerosis, diabetes, and cancer. Sulforaphane, a molecule found in its precursor state in cruciferous and other vegetables, is the focus of much current nutritional interest. Others and we have recently reported the beneficial cardiovascular effects of sulforaphane and the possible molecular mechanisms involved. Sulforaphane improved cardiovascular complications such as vascular inflammation, hypertension, and atherosclerosis in animal models. Evidence shows that sulforaphane may exert the beneficial cardiovascular effects by acting on multiple targets such as (i) activating Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor [erythroid-derived 2]-like 2 (Nrf2) signaling pathway, (ii) inhibiting inflammatory pathways, and (iii) regulating lipid metabolism. However, human studies related to the vascular effects of sulforaphane and cruciferous vegetables are lacking. Hence, well-designed human trials may be needed to evaluate the cardiovascular effects of sulforaphane and cruciferous vegetables and to recommend cruciferous vegetables, to improve cardiovascular health. This chapter provides an overview of recent developments toward the understanding of cardioprotective effects of sulforaphane and the molecular mechanisms involved.

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_11

Keywords
Sulforaphane • Vascular disease • Nrf2 • Atherosclerosis • Cruciferous vegetables • Endothelium • Cardioprotective

Abbreviations

ARE	Antioxidant response element
C/EBP	CCAAT/enhancer-binding protein
CVD	Cardiovascular disease
GCL	Glutamate-cysteine ligase
GCS	γ-Glutamyl cysteine synthetase
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione-S-transferase
HDL	High-density lipoprotein
HO-1	Heme oxygenase-1
ICAM-1	Intracellular adhesion molecule-1
IκBα	Inhibitor of NFκB
IκKβ	IκB kinase
IL-8	Interleukin-8
Keap1	Kelch-like ECH-associated protein 1
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
NFκB	Nuclear factor κB
NQO1	NADPH quinone oxidoreductase
Nrf2	Nuclear factor [erythroid-derived 2]-like 2
PPARγ	Peroxisome proliferator-activated receptor γ
ROS	Reactive oxygen species
SHRs	Spontaneously hypertensive rats
SHRSP	Spontaneously hypertensive stroke-prone
SMC	Smooth muscle cells
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VSMCs	Vascular smooth muscle cells
WKY rats	Wistar Kyoto rats

Contents

1 Introduction

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the United States. Each year approximately 1.5 million American adults have a heart attack or stroke [1]. In 2010 alone, CVD accounted for 31.9% of all deaths, or about one in every three deaths [2]. Lifestyle factors such as smoking, lack of physical activity, and a nutrient-poor diet play a major role in the development of CVD, and it is therefore mostly preventable [3]. Most of the time, CVD is initiated through a pathological state called atherosclerosis. Atherosclerosis is the buildup of plaques within the arterial walls that narrows the arterial lumen and restricts the amount of blood that flows through it. The plaques are formed between the tunica intima and tunica media and are comprised of an accumulation of lipids, cells, debris, and fibrous tissues [4]. In severe cases, the plaques become weak and rupture, attracting clotting factors in the blood to be attracted to and stick to the rupture site [4]. With limited blood flow through these clogged arteries, atherosclerosis leads to major complications like heart attack and stroke [4].

Evidence from epidemiological and clinical studies support that a diet containing a variety of fruits and vegetables can prevent the development of CVD [5–9]. Sulforaphane, which belongs to a group of molecules called isothiocyanates, is the focus of much current nutritional interest. Glucoraphanin is the precursor for sulforaphane and is widely present in cruciferous vegetables such as broccoli, cabbage, Brussels sprouts, and non-cruciferous vegetables in the mustard family such as radishes [10]. Epidemiological studies have found associations between cruciferous vegetable consumption and reduced risk of chronic conditions, such as atherosclerosis, diabetes, and cancer [10–13]. This chapter provides an overview of recent developments toward the understanding of cardioprotective effects of sulforaphane and the molecular mechanisms involved. This chapter will specifically focus on the effects of sulforaphane in the vasculature.

2 Pathophysiology of Atherosclerosis

The endothelium is a monolayer of endothelial cells that line the interior walls of the entire vascular system. Endothelium is essential for maintaining vascular homeostasis [14]. It forms a unique barrier for regulating the exchange of biomolecules between blood and tissues and preventing infiltration by leukocytes or other cells into the vessel wall [15]. Indeed, a healthy vascular system is maintained by the

proper functioning of the endothelium, and endothelial dysfunction contributes significantly to vascular dysfunction [16]. Endothelial dysfunction is one of the warning signs of CVD and is involved in disease states where the normal function of endothelium is severely affected such as atherosclerosis and hypertension [15, 16]. Extensive studies have confirmed that atherosclerosis is initiated due the effects of endothelial dysfunction [14–16].

Cardiovascular risk factors induce endothelial dysfunctional which can be characterized by the enhanced expression of adhesion molecules, binding of blood monocytes to endothelium, and increased endothelial permeability and accumulation of circulating lipids [14, 17–21]. Accumulating evidence has shown that leukocyte binding, facilitated by adhesion molecules and chemokines, is a key step in the development of atherosclerosis [14, 17–20]. The normal endothelial monolayer resists prolonged contact and adhesion of circulating blood monocytes. However, when endothelial cells undergo inflammatory activation, they increase the expression of leukocyte adhesion molecules [22]. Various pro-inflammatory mediators and cell adhesion molecules have been found to be secreted by injured endothelial cells, which include thrombin, tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-8 (IL-8), vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin [17–20]. Increased production of these inflammatory molecules serves as a molecular marker for endothelial inflammation. The adhesion molecules facilitate the recruitment of blood monocytes from the circulation to the endothelium followed by transmigration of the leukocytes to the subendothelium [21–23]. Furthermore, when blood monocytes migrate to the innermost layer of the arterial wall, they take up lipids and lipoprotein particles and they differentiate to macrophage foam cells [22]. These foam cells secrete large amounts of cytokines such as TNF-α, IL-8, and interleukin-1 (IL-1), which in turn amplify the inflammatory response and enhance vascular complications [22, 23].

3 Sulforaphane: Chemistry, Dietary Sources, and Bioavailability

Sulforaphane [1-isothiocyanate-(4R)-(methylsulfinyl)butane] is a small lipophilic member of the isothiocyanate family [10]. The isothiocyanates are a group of naturally occurring compounds that are usually formed by the hydrolysis of glucosinolates. Sulforaphane is formed from the precursor glucoraphanin which is a glycoside [10]. Sulforaphane is produced from glucoraphanin when cruciferous vegetables such as broccoli, cabbage, Brussels sprouts, or some non-cruciferous vegetables in the mustard family such as radishes are mechanically broken down, commonly by being chewed. These plants contain the precursor (glucoraphanin) and the enzyme (myrosinase) to perform the hydrolysis reaction. Glucoraphanin is stable and inert and the plants sequester glucoraphanin from the enzyme myrosinase. Once the barrier is broken down, myrosinase hydrolizes the glucosinolate into sulforaphane [10] (Fig. 1).

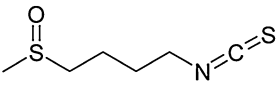

Fig. 1 Structure of sulforaphane

The amount available in different sources is highly variable. Broccoli is the most commonly studied source with a sulforaphane content of around 44–171 mg/100 g dry weight. Broccoli sprouts contain the most concentrated sulforaphane at 1,153 mg/100 g dry weight [24]. The content of sulforaphane in broccoli is greatly affected by processing. Raw broccoli has about ten times the concentration of frozen. Frozen loses the activity of myrosinase because of the flash steaming prior to freezing. It is found that sulforaphane availability is increased somewhat with mild steaming for less than 5 min. This is due to decreasing less stable intermediates which compete and are made at the expense of sulforaphane. Boiling broccoli for over a minute reduces sulforaphane to almost none [25].

Another important variable in the bioavailability of sulforaphane is digestion, absorption, and processing at the level of the cell. Sulforaphane has a high rate of absorption of around 74%. It is mostly absorbed in the jejunum, though, when supplemented or taken as glucoraphanin, can be processed into sulforaphane by bacterial myrosinase and taken up in the large intestines to a small degree [24, 25]. Sulforaphane serum concentrations in humans from raw broccoli peak at an average of 1.6 h and at 6 h when cooked broccoli is consumed [24]. This is thought to be a difference in whether the available sulforaphane is made by plant or by gut bacterial myrosinase. Once in circulation, sulforaphane is readily taken up and widely distributed in cells [24]. It is conjugated with glutathione once taken up by cells. A common polymorphism in the gene coding for glutathione-S-transferase – GSTM1 null – is shown to dramatically decrease the available sulforaphane in cells [26]. Excretion is almost complete within 24 h and for some organs it is within 4 h.

4 Cardiovascular Beneficial Effects of Sulforaphane

Recent studies support the beneficial cardiovascular effects of sulforaphane, and sulforaphane was reported to improve cardiovascular complications such as atherosclerosis and hypertension. Sulforaphane administration (10 μM/kg body weight by gavage for 4 months) significantly reduced the blood pressure and improved pathological abnormality associated with hypertension in spontaneously hypertensive stroke-prone (SHRSP) rats, a widely used animal model for hypertension [27]. Sulforaphane also prevented vascular remodeling in SHRSP rats as shown by corrected renal resistant artery wall/lumen ratio [27]. Another study investigated the vascular effect of dried broccoli sprouts that contained glucoraphanin, a precursor for sulforaphane [28]. Dietary supplementation of dried broccoli sprout for 14 weeks improved endothelial-dependent aortic vessel relaxation and reduced blood pressure in SHRSP rats [28]. In addition, broccoli-supplemented SHRSP rats exhibited reduced infiltrating macrophages indicating sulforaphane may suppress the

development of atherosclerosis. The effect of sulforaphane treatment on diabetic cardiomyopathy was also documented both in type 1 and type 2 diabetic animal models [29, 30]. Sulforaphane treatment for 3–6 months (0.5 mg/kg body weight) prevented diabetes-induced high blood pressure, cardiac inflammation, and cardiac hypertrophy in the type 1 diabetic animal model [29]. Cardiomyopathy induced by type 2 diabetes is associated with cardiac inflammation, oxidative stress, and remodeling [30]. Male C57BL/6J mice fed with high fat for 3 months followed by streptozotocin injection for induction of insulin resistance and hyperglycemia were used as type 2 diabetic animal model. Treatment with sulforaphane for 4 months (0.5 mg/kg body weight) significantly inhibited cardiac lipid accumulation and improved cardiac inflammation, oxidative stress, and fibrosis in this model [30]. We have recently shown that dietary supplementation of sulforaphane (300 ppm) for 8 weeks improved TNF-α-induced vascular inflammation in C57BL mice [23].

5 Molecular Mechanisms Involved in the Cardioprotective Effects of Sulforaphane

Evidence shows that sulforaphane may exert the beneficial cardiovascular effects by acting on multiple targets such as (i) activating Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor [erythroid-derived 2]-like 2 (Nrf2) signaling pathway, (ii) inhibiting inflammatory pathways, and (iii) regulating lipid metabolism (Table 1).

5.1 Activation of Nrf2/ARE Signaling Pathway

Oxidative stress, which is caused by an imbalance between reactive oxygen species (ROS) and antioxidant defense system, plays a major role in the development and progression of cardiovascular diseases. Indeed, enhanced oxidative stress is associated with various cardiovascular complications such as atherosclerosis, hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart failure [31–33]. ROS activate endothelium, which leads to the attraction of monocytes to the intimal layer of the vessel, a key initial event involved in atherosclerosis. In addition, ROS mediates the oxidative modification of low-density lipoprotein (LDL) to oxidized LDL. Oxidized LDL easily infiltrates endothelium to the subendothelial space where it mediates the formation of macrophage-derived foam cells. Further, oxidative stress plays a major role in the progression from endothelial dysfunction to atherosclerosis [32]. Thus, reducing oxidative stress is one of the key strategies for the prevention of atherosclerosis.

Cells have antioxidant mechanisms to protect against oxidative stress. Many antioxidant enzymes in a cell are induced and activated when the insult by ROS reaches levels beyond the ability of the cell to neutralize them. Many of the antioxidant enzymes are regulated at the genetic level, and there are three important cellular components involved in this adaptive signaling mechanism: Kelch-like

Table 1 Mechanisms involved in the vascular effects of sulforaphane

Model	Treatment	Vascular effects and mechanisms	Ref.
Spontaneously hypertensive stroke-prone rats	Sulforaphane (10 μM/kg body weight) for 4 months	↓ Blood pressure	[27]
		Prevented vascular remodeling	
Spontaneously hypertensive stroke-prone rats	Dietary broccoli sprouts for 14 weeks	↓ Blood pressure	[28]
		↓ Infiltrating macrophages	
		↓ Oxidized glutathione	
		↓ Protein nitrosylation	
		↑ Vessel relaxation	
		↑ Glutathione, GR, GPX	
Type 1 diabetic mice	Sulforaphane (0.5 mg/kg body weight) for 3–6 months	↓ Blood pressure	[29]
		↓ Cardiac inflammation	
		↓ Cardiac hypertrophy	
Type 2 diabetic mice	Sulforaphane (0.5 mg/kg body weight) for 4 months	↓ Cardiac inflammation	[30]
		↓ Oxidative stress	
		↓ Lipid accumulation	
C57BL/6 treated with TNF-α	Dietary supplementation of sulforaphane (300 ppm) for 8 weeks	↓ Monocyte adhesion to vessel	[23]
		↓ Circulating adhesion molecules and chemokines	
		↓ F4/80-positive macrophages	
Human umbilical vein endothelial cells exposed to oxidized LDL	Sulforaphane (2.5 μM–10 μM)	↑ GCLC, GCLM, glutathione, HO-1	[46]
		↑ ARE activity	
		↓ NFκB nuclear translocation	
		↓ NFκB activity	
		↓ Adhesion molecules	
		↓ Binding of monocytes to endothelial cells	
Vascular smooth muscle cells	Sulforaphane (5 μM)	↑ HO-1	[47]
		↑ Peroxiredoxin	
		↑ Glutathione	
Smooth muscle cells from spontaneously hypertensive rats	Sulforaphane (0.05–1 μM)	↑ HO-1	[48]
		↑ GPX	
		↑ Glutathione	
Spontaneously hypertensive stroke-prone rats	Sulforaphane	↑ GCLC	[27]
		↓ Nitrated proteins	

(*continued*)

Table 1 (continued)

Model	Treatment	Vascular effects and mechanisms	Ref.
Type 1 diabetic mice	Sulforaphane (0.5 mg/kg body weight) for 3 months	↓ SOD1, NQO1, HO-1	[37]
		↑ Nrf2	
Mesenteric arteries and smooth muscle cells from spontaneously hypertensive stroke-prone rats	Sulforaphane (5 μM)	↑ Vessel relaxation	[50]
		↑ SOD1, catalase, GPX	
		↓ ROS	
ECV304 endothelial cells exposed to lipopolysaccharide	Sulforaphane (5–20 μM)	↓ NFκB nuclear translocation	[62]
		↓ ICAM-1, VCAM-1	
		Suppressed TLR-4 signaling	
ECV304 endothelial cells exposed with TNF-α	Sulforaphane (2.5–10 μM)	↓ ICAM-1	[63]
		↓ IL-1β, IL-6, and IL-8	
		↓ NFκB nuclear translocation	
		↓ ICAM-1, VCAM-1	
Human aortic endothelial cells treated with TNF-α	Sulforaphane (1–4 μM)	↓ ICAM-1, MCP-1	[64]
		Inhibition of p38 MAPK	
Human umbilical vein endothelial cells exposed to TNF-α	Sulforaphane (0.5–2.5 μM)	↓ ICAM-1, E-selectin, MCP-1	[23]
		↓ NFκB nuclear translocation	
		↓ NFκB transcriptional activity	
Mouse vascular smooth muscle cells exposed to TNF-α	Sulforaphane (1–5 μg/ml)	↓ ROS, VCAM-1	[65]
		↓ IκKβ activation	
		↓ Degradation of IκBα	
		↓ NFκB nuclear translocation	
		Inhibition of MAPK and activator protein-1 signaling pathways	
C57BL/6 mice treated with lipopolysaccharide	Sulforaphane (5 mg/kg body weight)	↓ Endothelial activation	[45]
		↓ VCAM-1	
		Activation of Nrf2 signaling	
		Inactivation of p38 MAPK signaling	
3T3-L1 adipocytes	Sulforaphane (5–20 μM)	↓ Proliferation of adipocytes	[67]
		↓ Expression of C/EBPβ	

(*continued*)

Table 1 (continued)

Model	Treatment	Vascular effects and mechanisms	Ref.
C57BL/6 N mice fed with high-fat diet	0.1% Sulforaphane in diet for 6 weeks	↓ Expressions of PPARγ, C/EBPα	[68]
		↓ Fat accumulation	
		↓ Serum cholesterol, triglycerides	
		↓ Lipogenesis	
Human umbilical vein endothelial cells exposed to TNF-α	Sulforaphane (10 μM)	↓ Endothelial lipase	[69]
		↓ IκKβ phosphorylation	
		↓ IκBα phosphorylation	

↑Increase, ↓decrease, *ARE* antioxidant response element, *C/EBP* CCAAT/enhancer-binding protein, *GCL* glutamate-cysteine ligase, *GCLC* glutamate-cysteine ligase catalytic subunit, *GCLM* glutamate-cysteine ligase modifier subunit, *GPX* glutathione peroxidase, *GR* glutathione reductase, *HO-1* heme oxygenase-1, *ICAM-1* intracellular adhesion molecule-1, *IκBα* inhibitor of NFκB, IκK*β* IκB kinase, *LDL* low-density lipoprotein, *MAPK* mitogen-activated protein kinase, *MCP-1* monocyte chemotactic protein-1, *NFκB* nuclear factor κB, *NQO1* NADPH quinone oxidoreductase, *Nrf2* nuclear factor [erythroid-derived 2]-like 2, *PPARγ* peroxisome proliferator-activated receptor-γ, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *TLR* Toll-like receptor, *TNF-α* tumor necrosis factor-α, *VCAM-1* vascular cell adhesion molecule-1

ECH-associated protein 1 (Keap1), nuclear factor [erythroid-derived 2]-like 2 (Nrf2), and antioxidant response element (ARE) [34, 35]. Nrf2 is a transcription factor that plays a major role in regulating the antioxidant response and maintaining the redox homeostasis [36]. Keap1 is a cysteine-rich negative regulator of Nrf2 that regulates Nrf2 by controlling its subcellular location. Under physiological conditions, the Nrf2 pathway is suppressed by the sequestration of Nrf2 in the cytoplasm which is mediated through the binding of Nrf2 to the inhibitor Keap1. Further, Keap1 interaction with Nrf2 induces rapid ubiquitination and rapid degradation of Nrf2 by proteasome leading to low expression of ARE-driven genes [37]. Upon exposure of cells to oxidative stress or electrophilic compounds, a signal that involves phosphorylation and/or modification interferes with the Keap1-Nrf2 complex leading to dissociation of Nrf2 and translocation to the nucleus and binding to the ARE. ARE is a cis-acting regulatory region of the genome with the consensus sequence 5′-TGACnnnGC-3′ that regulates the basal and inducible expression of more than 200 genes [37]. Binding of Nrf2 to ARE induces the transcriptional activation of ARE-mediated genes that play a role in detoxification, antioxidant, and anti-inflammatory activities [37].

ARE is important in cellular protection from oxidative species, and it regulates both direct antioxidant enzymes (such as superoxide dismutase) and indirect antioxidant enzymes (such as rate-limiting enzymes of glutathione synthesis and xenobiotic-detoxifying enzymes) [38]. This includes heme oxygenase-1 (HO-1), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase,

γ-glutamyl cysteine synthetase (GCS), and NADPH quinone oxidoreductase (NQO1) [37]. Glutathione is the most abundant endogenous antioxidants used for the day-to-day protection of cells against oxidative stress. Glutathione is a tripeptide made up of the amino acids glutamate, glycine, and cysteine. The rate-limiting enzyme in the production of glutathione is glutamate-cysteine ligase (GCL). GCL has two subunits, glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM). Activation of the Nrf/ARE pathway substantially increases the concentration of active glutathione by producing enzymes involved in the production as well as the restoration of glutathione [39]. Thus, the Keap1-Nrf2-ARE signaling pathway is responsible for turning on an adaptive response against oxidative stress and maintaining the redox homeostasis [36].

ARE activators induce a conformation rearrangement of Keap1 which facilitates Nrf2 nuclear translocation and subsequent activation of ARE-mediated transcription of protective genes [40]. Evidence indicate that Nrf2 activators such as isothiocyanates alter Keap1 conformation by interacting with the thiol groups of the specific cysteine residues within Keap1 and this will prevent the ubiquitination and degradation of Nrf2. This will also facilitate Nrf2 nuclear translocation and subsequent binding of Nrf2 to ARE [40]. Once bound, they initiate transcription of many genes that are regulated by ARE. The sulforaphane activation of Nrf2 is through the interaction of the thiol groups of Keap1 followed by nuclear translocation of Nrf2 [41]. Indeed, a study showed a 1.4× increase in the amount of Nrf2 upon sulforaphane exposure at 10 μM concentration [42]. The partial as well as the full release of Nrf2 by sulforaphane was proposed for the long-lasting and consequential actions of sulforaphane to protect against cardiovascular disease [36, 43–45].

The protective effect of sulforaphane on oxidized LDL-induced endothelial dysfunction was reported recently. In this study, sulforaphane (2.5–10 μM) suppressed oxidized LDL-induced ROS production in human umbilical vein endothelial cells (HUVEC) by inducing dose-dependent increases in the expression of GCL catalytic and modifier subunits, intracellular glutathione level, HO-1 levels, and ARE activity [46]. Sulforaphane treatment (5 μM for 12 and 24 h) was also shown to increase the expressions of antioxidant enzymes (HO-1 and peroxiredoxin) and glutathione content in vascular smooth muscle cells (VSMCs) [47]. This study suggests that sulforaphane may protect against vascular SMC dysfunction in age-related cardiovascular disease.

Recent animal studies also support that the vascular protective effect of sulforaphane may be mediated through the activation of Nrf2 signaling. The glutathione system consists of glutathione, GCS, GST, glutathione peroxidase (GPX), and glutathione reductase (GR) [48]. An impaired glutathione system is associated with hypertension [48]. Smooth muscle cells (SMC) from spontaneously hypertensive rats (SHRs) exhibited enhanced oxidative stress that is associated with lower basal levels of glutathione as compared to the SMC from control Wistar Kyoto (WKY) rats [48]. In this study, sulforaphane (0.05–1 μM) induced a dose-dependent increase in cellular glutathione levels, HO-1 protein content, and GPX activity in both the strains. In addition, sulforaphane upregulated the impaired glutathione

system in vascular smooth muscle cells from SHR [48]. This could be due to the activation of GCL, as sulforaphane is also a potent inducer of GCL [49]. Oxidative stress in kidneys mediates hypertension in SHRSP rats [27]. Sulforaphane administration improved blood pressure in SHRSP rats, which is associated with significant increase in GCLC in SHRSP rat kidneys [27]. Further, this study showed a reduction in nitrated proteins with sulforaphane treatment, which is indirect evidence for decreased oxidative stress.

Treatment with sulforaphane (0.5 mg/kg for 3 months) prevented diabetes-induced vascular inflammation and aortic damage in streptozotocin-induced type 1 diabetic mice [37]. This was associated with the upregulation of Nrf2 and its downstream antioxidants such as SOD1, NQO1, and HO-1 in the aorta of diabetic mice [37]. The effect of sulforaphane on Nrf2-induced redox signaling in small arteries and smooth cells from SHRSP rats was also reported [50]. In this study, sulforaphane improved vascular function as shown by improved contractility and endothelial-dependent vessel relaxation, which was mediated through activation of Nrf2-mediated redox signaling. There was an increased ROS production and reduced Nr2 activity in arteries and VSMC in SHRSP rats. In addition, the expressions of antioxidant enzymes such as SOD1, catalase, and GPX were reduced in SHRSP rats [50]. Sulforaphane treatment suppressed ROS production and increased these antioxidant enzymes in SHRSP rats. Sulforaphane also blocked angiotensin II-induced ROS production in SHRSP rats [50].

In another study, dietary supplementation of broccoli sprouts improved endothelial-dependent aortic vessel relaxation and reduced blood pressure in SHRSP rats [28]. This was associated with improved oxidative stress as shown by increased glutathione, decreased oxidized glutathione, decreased protein nitrosylation, and increased GR and GPX activities [28]. In this study the authors didn't measure the expression of Nrf2 but the effect is possibly mediated through sulforaphane-mediated upregulation of Nrf2.

Together, these studies indicate that sulforaphane exerts vascular protective effects by activating Nrf2/ARE signaling. By activating Nrf2, sulforaphane induces the expression of antioxidant enzymes, maintains the cell glutathione status, and reduces the oxidative stress that ultimately improves the vascular complications and dysfunction.

5.2 Inhibition of Inflammatory Pathways

Atherosclerosis is an inflammatory disease, and endothelial inflammation-induced endothelial dysfunction plays a pivotal role in the development of atherosclerosis. Many of the risk factors for cardiovascular disease such as diabetes, hypertension, and smoking are potential activators of an inflammatory response in endothelium that induces the adhesion of monocytes to the vascular endothelial cells, an initial event in the development of atherosclerosis [51–53]. Oxidative stress can trigger several intracellular signaling events that ultimately induce vascular inflammation by upregulating the expression of a number of pro-inflammatory molecules such as

chemokines and adhesion molecules [54]. This includes IL-8, MCP-1, TNF-α, ICAM-1, VCAM-1, and E-selectin [17, 54, 55]. These inflammatory molecules then induce the binding of monocytes to the endothelial cells, and their subsequent transendothelial migration into the vessels, leading to the pathogenesis of atherosclerosis [54, 56].

Nuclear factor κB (NFκB) is a pro-inflammatory transcription factor that plays a major role in upregulating inflammatory molecules such as chemokines and adhesion molecules [57]. p50/p65 is the most abundant form of NFκB. Under homeostatic conditions, NFκB is made inactive by sequestration by inhibitor of NFκB (IκBα). Inflammatory conditions activate IκB kinase (IκKβ), a multienzyme kinase complex, which phosphorylates IκBα [58]. Phosphorylated IκBα is degraded by a ubiquitination process targeting IκBα to proteasomal degradation [58]. This leads to the nuclear translocation of p50/p65 [59]. In the nucleus, p50/p65 dimers bind to the promoters of NFκB-dependent inflammatory genes and regulate inflammatory genes involved in atherosclerosis [60, 61]. IκKβ has an ETGE-binding motif which is the stronger of two Keap1-binding motifs [35, 58]. When Nrf2 is released from Keap1 and the complementary motif is exposed, Keap1 will sequester IκKβ decreasing IκBα phosphorylation and degradation and decreasing the translocation of NFκB [35]. Hence Nrf2 negatively regulates the activation of the NFκB pathway.

Others and we have reported the protective effects of sulforaphane on endothelial inflammation and the possible molecular mechanisms involved in the vascular effects of sulforaphane. A recent study reported that sulforaphane inhibited the lipopolysaccharide (LPS)-induced expression of ICAM-1 and VCAM-1 in ECV304 endothelial cells by inhibiting LPS-induced nuclear translocation of NFκB [62]. In this study, sulforaphane suppressed Toll-like receptor (TLR)-4 (TLR-4, a membrane receptor of LPS), MyD88 (an effector downstream of TLR signaling), and downstream factors such as p38 mitogen-activated protein kinase (MAPK) and JNK that ultimately blocked NFκB translocation and the subsequent expression of adhesion molecules [62]. The anti-inflammatory effect of sulforaphane was also reported in oxidized LDL-induced HUVEC [46]. Treatment with sulforaphane dose-dependently suppressed NFκB nuclear translocation, NFκB activity, adhesion molecules, and binding of monocytes to EC.

TNF-α is an inflammatory cytokine that amplifies several signaling pathways that trigger the secretion of various inflammatory mediators in endothelium leading to vascular inflammation [63]. Sulforaphane treatment (2.5–10 μM) attenuated TNF-α-induced expression of ICAM-1 in ECV304 endothelial cells [63]. In addition, sulforaphane dose-dependently inhibited the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8. TNF-α activates the NFκB pathway in endothelial cells as shown by increased NFκB DNA-binding activity and increased phosphorylation of Iκκβ and IκBα [63]. Sulforaphane inhibited NFκB DNA-binding activity and downregulated RhoA/Rho-associated protein kinase and NFκB inflammatory signaling pathways in endothelial cells [63]. In another study, sulforaphane (1–4 μM) pretreatment for 1 h suppressed TNF-α-induced enhanced MCP-1 and ICAM-1 expressions in human aortic endothelial cells. This study suggests the effect

of sulforaphane on endothelial inflammation may be mediated through inhibition of p38 MAPK [64].

We have recently shown that sulforaphane, at a physiological relevant concentration, prevents TNF-α-induced endothelial inflammation in human umbilical vein endothelial cells (HUVEC) and improved TNF-α-induced vascular inflammation in C57BL/6 mice [23]. In our study, sulforaphane at a very low concentration (0.5 μM) significantly inhibited TNF-α-induced binding of monocytes to HUVEC both in static and flow conditions. In addition, sulforaphane suppressed TNF-α-induced production of inflammatory molecules such as MCP-1, VCAM-1, and E-selectin. In our animal study, dietary supplementation of sulforaphane (300 ppm) for 8 weeks significantly abolished TNF-α increased ex vivo monocyte adhesion and circulating adhesion molecules and chemokines in C57BL/6 mice [23]. We also showed that dietary sulforaphane prevented the eruption of endothelial lining in the intimal layer, preserved elastin fibers' delicate organization, and reduced monocyte-derived F4/80-positive macrophages in the aorta of TNF-α-treated C57BL/6 mice. Further, we investigated the underlying mechanisms involved in the vascular protective effects of sulforaphane [23]. Sulforaphane inhibited TNF-α-induced NFκB transcriptional activity and NFκB p65 nuclear translocation in HUVEC indicating the effect of sulforaphane may be associated with suppression of NFκB signaling.

Enhanced ICAM-1 and VCAM-1 are also associated with increased VSMC proliferation leading to increased neointima and atherosclerotic lesion formation [65]. In a recent study, the protective effect of sulforaphane (1–5 μg/mL for 2 h) on mouse VSMC exposed to TNF-α was reported [65]. Sulforaphane treatment suppressed TNF-α-induced increased production of intracellular ROS and VCAM-1 protein expression in VSMC, and this protective effect was mediated through inhibition of MAPK and activator protein-1 signaling pathway [65]. Further, sulforaphane inhibited TNF-α-induced IκKβ activation, prevented the degradation of IκBα, and reduced the nuclear translocation of p65 in VSMC indicating the anti-inflammatory effects of sulforaphane. This study suggests that sulforaphane may inhibit the adhesive capacity of VSMC and suppress inflammation within the atherosclerotic lesion.

Vascular inflammation and atherosclerosis develop predominantly in the sites of the arterial tree that are exposed to nonuniform blood flow, e.g., near the branches and bends of the arterial tree, which exerts relatively low shear stress on vascular endothelial cells activating inflammatory signals [45]. Unidirectional high shear stress suppresses vascular inflammation, whereas low shear stress activates inflammatory signals. C57BL/6 mice were treated with sulforaphane (5 mg/kg body weight by intraperitoneal injection) followed by treatment with lipopolysaccharide [45]. In this study sulforaphane treatment suppressed endothelial activation which is mediated through activation of Nrf2 and inactivating p38 MAPK and suppressing VCAM-1 signaling at the susceptible site in the murine aorta [45].

Collectively, the anti-inflammatory effect of sulforaphane is well documented in in vitro and animal studies. Sulforaphane targets multiple inflammatory signals in the vasculature to exert its anti-inflammatory effects.

5.3 Modulation of Lipid Metabolism

Dyslipidemia is one of the major risk factors for cardiovascular disease. Dyslipidemia that includes low levels of high-density lipoprotein (HDL) cholesterol and increased levels of LDL cholesterol, total cholesterol, and triglycerides contribute significantly to vascular disease [66]. Indeed, increased levels of plasma total cholesterol, triglycerides, and non-HDL cholesterol are recognized risk factors for cardiovascular disease and are involved in the pathogenesis of atherosclerosis [66]. Hyperlipidemia contributes significantly to vascular disease by activating endothelium, reducing the synthesis or bioavailability of endothelial nitric oxide, and enhancing the formation of foam cells [66].

Obesity is one of the known risk factors for the development of cardiovascular complications. Adipocyte differentiation plays a key role in the development of obesity. Sulforaphane was shown to decrease the accumulation of lipid and inhibit the triglycerides in the adipocytes [67]. Sulforaphane exerts this effect by acting on the key transcription factors required for the adipocyte differentiation that includes peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding protein (C/EBP) [67]. Sulforaphane dose-dependently (5–20 μM) decreased the expression of C/EBPβ in 3T3-L1 adipocytes. Sulforaphane treatment (20 μM for 24 and 48 h) also suppressed the proliferation of adipocytes. This study showed that sulforaphane inhibit early-stage adipocyte differentiation which could be due to the upregulation of p27 expression that leads to cell cycle arrest at G90)/G(1) phase [67].

Sulforaphane treatment (0.1% sulforaphane in diet for 6 weeks) suppressed high-fat diet-induced fat accumulation in liver and reduced serum total cholesterol and triglyceride levels in C57BL/6N mice [68]. Sulforaphane treatment also reduced visceral adiposity and adipocyte hypertrophy [68]. In addition, sulforaphane decreased the expressions of PPARγ, C/EBPα, and leptin in the adipose tissue of high-fat-treated mice, whereas it increased adiponectin expression. This study suggested that sulforaphane inhibits adipogenesis through downregulation of PPARγ and C/EBPα and suppresses lipogenesis through activation of adenosine monophosphate kinase pathway [68].

Endothelial lipase, which belongs to triacylglycerol lipase family, has been shown to decrease the level of HDL cholesterol and increase the risk of atherosclerosis [69]. A human study also supported that endothelial lipase is upregulated by inflammation [70]. Sulforaphane (10 μM) was shown to inhibit TNF-α-induced endothelial lipase expression in HUVEC, and this was mediated through inhibition of NFκB. This may have a beneficial effect on HDL cholesterol levels [69].

Together, these studies indicate that sulforaphane can positively regulate lipid metabolism that leads to suppression of lipogenesis and reduced endothelial lipase. Though there is limited research in this area, this is an emerging and promising area of investigation.

6 Conclusions

Epidemiological studies have found associations between cruciferous vegetable consumption and reduced risk of chronic conditions, such as atherosclerosis, diabetes, and cancer [10–13]. In addition, as discussed above, in vitro and animal studies support the beneficial cardiovascular effects of sulforaphane, a molecule found in its precursor state in cruciferous and other vegetables. Sulforaphane acts on multiple targets in the vascular system, including activating Nrf2/ARE signaling, improving inflammatory pathways, and positively regulating lipid metabolism. However, human studies related to the vascular effects of sulforaphane and cruciferous vegetables are lacking. In addition, studies on the structure-activity relationships of sulforaphane are needed to understand how sulforaphane interacts with various biomolecules in the cardiovascular system to exert its beneficial cardiovascular effects. The consumption of sulforaphane-containing foods is safe, but there is some research that suggests that too much might increase the risk of some types of cancer [25]. Hence, well-designed human trials may be needed to evaluate the cardiovascular effects of sulforaphane and cruciferous vegetables and to recommend cruciferous vegetables, such as broccoli, to improve cardiovascular health.

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Therapeutic Paradigm Underscoring Glucosinolate Sulforaphane in Chemo- and Radiosensitization of Cancer: Preclinical and Clinical Perspective

12

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Abstract

Fruits and vegetables harbor innocuous bioactive compounds which after absorption and distribution tend to have an effect on general defense mechanism of the body including cancer prevention and therapeutic effects. Emerging knowledge from clinical and laboratory studies reveal an important insight regarding their mechanism of action orchestrating therapeutic paradigm with conventional cancer treatment modalities to enhance the curative index of cancer treatment. However, unlike conventional cancer therapeutics, natural bioactive compounds rarely develop resistance undermining their chemopreventive actions. One such bioactive natural compound – sulforaphane, a cognate isothiocyanate limited mostly to vegetables of *Brassica* family and enriched in broccoli – is considered a promising chemopreventive agent against cancer. Sulforaphane is released from hydrolysis of glucoraphanin isothiocyanate by action of myrosinase enzyme which is also found localized inside vegetal tissues. Overwhelming evidence points to sulforaphane's multitargeted actions operationally targeting core cell survival signaling pathways in tumor cells and enzyme induction mediated by the nuclear factor erythroid 2-related factor 2 (Nrf2)-regulated transcriptions of genes encoding carcinogen detoxification, antioxidant enzymes, and other effects including reversal of resistance and reduction in the systemic toxicity of drug. This chapter presents a broad perspective on the role of sulforaphane in augmenting multimodal cancer therapy including putative mechanism complementing the efficacy of chemo- and radiotherapy with presumptive notion

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_19

of its future use in clinics in fight against cancer and patient's benefit. Clinical trials have also been reviewed to ensure clinical safety and efficacy of sulforaphane in patients diagnosed with cancer.

Keywords

Glucosinolate • Isothiocyanates • Brassicaceae • Glucoraphanin • Sulforaphane • Chemosensitization • Chemoresistance • Radiosensitization

Abbreviations

ALDH-1	Aldehyde dehydrogenase 1
ARE	Antioxidant response element
BCl_2	B-cell lymphoma 2
COX-2	Cycloxygenase-2
GSC	Genomic standards consortium
GTC	Green tea catechins
HCG	Human chorionic gonadotropin
HNF-3β	Hepatocyte nuclear factor 3β
HRR	Homologous recombination repair
IL-1β	Interleukin-1β
Keap-1	Kelch-like ECH-associated protein 1
MAPK	Mitogen-activated protein kinases
NF-κB	Nuclear factor- kappa B
NHEJ	Nonhomologous end joining
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
Nrf2	Nuclear factor erythroid 2-related factor 2
Oct 2/3	Octamer transcription factor-2/3
OTX-2	Orthodenticle homeobox 2
PDX-1	Pancreatic and duodenal homeobox 1
PET	Positron emission tomography
TP63	Tumor protein p63
UV	Ultraviolet
VEGFR-2	Vascular endothelial growth factor receptor-2

Contents

1 Introduction

Cancer is a disease that seems to be preexisting since the time of Egyptian legend – Pharaohs, with anecdotal imagination by the Greek physician Hippocrates (460–370 BC) emulating giant crab of Greek mythology-Karkinos, symbolizing present-day carcinomatous tumors. It is a major public health problem in the USA and other countries of the world with severe health consequences. Recent report compiled by the American Cancer Society projects approximately 1,685,210 new cancer cases in 2016, with a likelihood of 595,690 people succumbing to this disease in the USA [1]. According to the World Health Organization [WHO; http://www.who.int/cancer/en/], cancer is uncontrolled growth of cells beyond their usual boundaries invading into tissues and metastasizing to distant sites of the body, causing significant morbidity and, if untreated, death of the host. With advancement in knowledge about this disease over the years, additional critical hallmarks associated with its progression have been recognized which has been compiled and concisely published by Hanahan and Weinberg recently [2]. Standard treatment options for the majority of cancers include surgery and/or chemo- and radiotherapy to enhance therapeutic efficacy depending on tumor stage. This have undoubtedly helped in increasing cancer survival, but still the high mortality rate associated with this disease underscores current therapeutic interventions in curtailing this disease-related death incidence. Alongside advancements relating to understanding pathobiology and improvements in cancer therapy over the past few decades, meta-analysis of epidemiological studies emerged in parallel in literature highlighting intake of fruits and green vegetables is inversely associated with cancer incidence and mortality [3–6]. This prompted research efforts to isolate and gain insight into mechanism and efficacy of bioactive compounds present in edible fruits and vegetables that may effectively intervene and modulate the ontogeny of carcinogenesis and influence our knowledge relating to prevention of cancer. Contextually, laboratory findings till date provide a strong rationale and perspective on several food-derived bioactive compounds multi-targeting core regulatory pathways in etiology and pathobiology of the tumorigenic process and, in addition, demonstrate putative therapeutic advantage as potential drugs since the molecular mechanisms are common to both chemoprevention and cancer therapy [7, 8].

Food crops of the genus *Brassica* which include cruciferous vegetables such as broccoli, cabbage, cauliflower, kale, watercress, etc. are largely cultivated in both tropical and temperate regions of the world and constitute an important serving of different ethnic cuisines including raw intake. In context of cancer epidemiology,

Verhoeven et al. summarizing the results of 7 cohort and 87 case-control studies concluded an inverse association between intake of some types of *Brassicaceae* family vegetable and cancer risk [9]. Recently, in another large series of studies, additional evidence of favorable effect of cruciferous vegetables on several common cancers has emerged [10]. Cruciferous vegetables, like other vegetables, are also enriched in nutrients including folate, fibers, carotenoids, and chlorophyll compounds but in addition contain well-characterized sulfur-rich secondary metabolite compounds specific to this crop of vegetables that impart a characteristic pungent astringent flavor; the generation and retention of this distinctive class of metabolic product ostensibly wards off plant pathogens and defend against pests and grazing predators facilitating their survival in nature. Additional chemical characterization of the compounds present in Brassicaceae family vegetables led to their identity as belonging to a distinct class of chemicals called glucosinolates (β-thioglucoside-N-hydroxy-sulfates), whose degradation products include biologically active compounds: the isothiocyanates, indoles, and nitriles; all these presumptively offer chemoprotection against human cancer risk and impede tumor growth with accompanying therapeutic benefits as well [11]. Till date, over 150 glucosinolates have been identified, all sharing a common sulfur-linked β-D-glucopyranose structure differing in side chain [12]. Noteworthy, glucosinolate composition fluctuates among different cruciferous vegetables, e.g., the predominant glucosinolate present in broccoli is glucoraphanin (the precursor to sulforaphane), whereas sinigrin is predominant in cabbage, and watercresses are high in gluconasturtiin. Correspondingly, in intact plant tissues, an endogenous plant enzyme called myrosinase is physically separated in membranous vesicles that preempt its hydrolytic action on glucosinolates. Upon chewing, the released myrosinase enzyme action hydrolyzes glucosinolates to highly unstable aglycone intermediate; the latter undergoes rearrangement to form nitriles, isothiocyanates, or indole depending on the structure of the aglycone, temperature, and pH; without hydrolysis, glucosinolates per se are inert compounds and lack any valuable biological and therapeutic activity. The most extensively studied cognate isothiocyanates (ITCs) derived from metabolism of glucosinolates are allyl isothiocyanates (from sinigrin), benzyl isothiocyanate and phenethyl isothiocyanate (from gluconasturtiin), and sulforaphane (from glucoraphanin). With emerging recognition that chemopreventive bioactive phytochemicals may also overcome conventional drug resistance, this chapter embodies a succinct overview on therapeutic efficacy of one promising glucosinolate – sulforaphane, the cognate isothiocyanate of glucoraphanin, specifically in combination with chemotherapy (chemosensitization) and radiosensitization of different site-specific cancers reported to date.

2 Sulforaphane: Its Bioavailability and Mechanism of Action

Sulforaphane (SFN; 1-isothiocynato-4-(methyl-sulfinyl) butane) is generally found in high concentrations in broccoli sprouts (0.8–21.7 μmol/g of dry weight) with levels 20–50 times than those found in mature market-stage plants (Fig. 1) [13, 14]. It has been shown in human subjects that plasma concentrations of SFN and

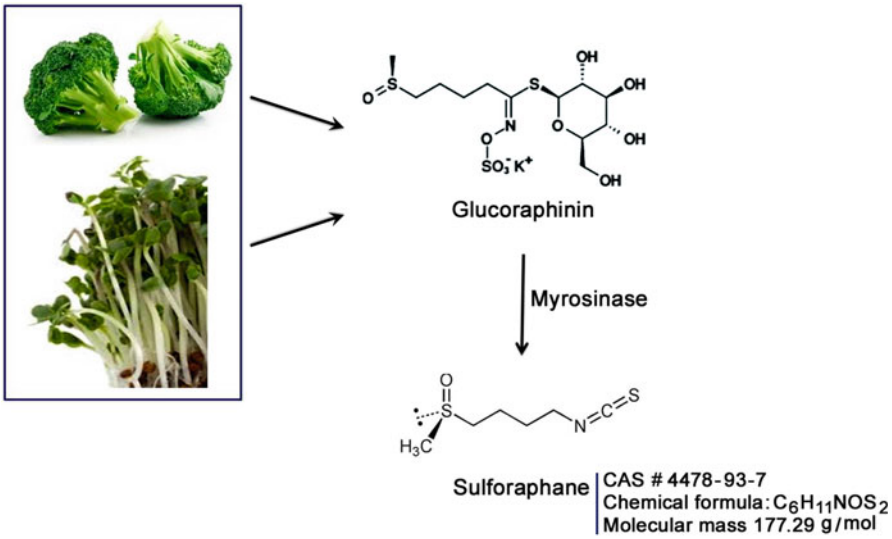

Fig. 1 Principle sources of Sulforaphane (Broccoli and brocolli sprouts), its release from plant tissues and some important information regarding its chemistry

its metabolites can reach between 2.2 and 7.3 μmol after consumption of 100 g of standard and high glucosinolate (super) broccoli, respectively [15]. Moreover, high concentrations of SFN are also achieved in tissues, and it has been reported using LC-MS that SFN and its metabolites (SFN-GSH) reach a concentration in the small intestine equivalent to roughly 3–30 μM of total SFN [16]. Strikingly, in a proportional dose-exposure relationship with increasing concentration of glucosinolates in broccoli, greater exposure to SFN has also been ascribed after its consumption. After absorption and entry into the enterocytes, SFN is predominantly metabolized via the mercapturic acid pathway with glutathione (GSH) conjugation by glutathione S-transferase followed by sequential metabolism leading to the generation of sulforaphane-cysteine (SFN-Cys) and sulforaphane-N-acetylcysteine (SFN-NAC) [17, 18]. When metabolism of glucoraphanin efficiently occurs, SFN-NAC is the primary SFN metabolite excreted in the urine. Once SFN is distributed, it accumulates in the tissues and sustains therein to achieve the antitumor actions [18]. Observations from a pilot study point that sulforaphane metabolites could be detected within measurable limits in enriched breast epithelial cells following oral dosing of broccoli sprout preparations (containing 200 μmol of sulforaphane) to healthy women undergoing reduction mammoplasty, conveying a strong rationale of its bioavailability with prospect of its protective effects in clinical trials and in women at risk for breast cancer [19]. In laboratory setting using cultured cells and preclinical animal models of cancer, SFN has received considerable attention and has been thoroughly investigated for its promising antitumor and chemopreventive activity. Several published reviews describe in-depth molecular mechanism of SFN action in tumor cells [12, 20–23]. Collectively, all mechanistic studies till date report SFN

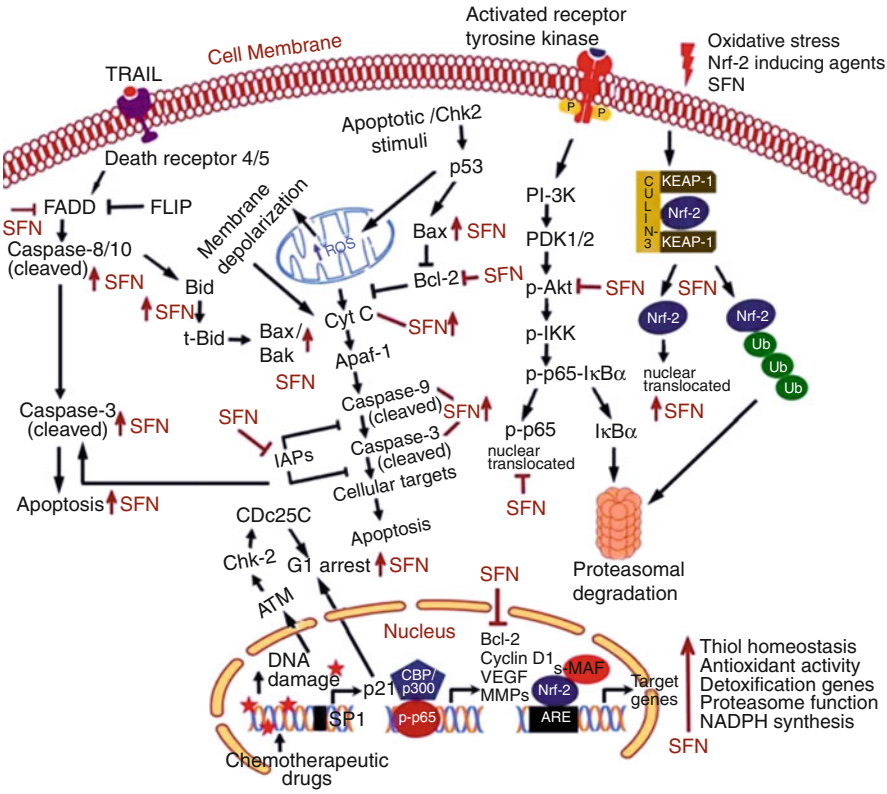

Fig. 2 Generalized schematic overview representing signaling pathways and cellular targets altered by Sulforaphane

as a promising pleiotropic bioactive molecule operating through various cellular and molecular mechanisms that interferes with multiple oncogenic pathways. It targets precancerous and cancer cells thru Nrf2/Keap1/ARE-signaling pathway considered as a major regulator of cytoprotective phase II detoxification enzymes that reduce susceptibility to carcinogens and protect against DNA adduct generation and oxidative and electrophilic stress (Fig. 2a) [24, 25]. Other studies report that SFN mediates the inhibition of cell proliferation and anti-inflammatory effect through significant downregulation of cyclooxygenase-2 (COX-2) expression by preventing the binding of NF-κB to its consensus DNA sequence and encourages apoptotic pathway by downregulation of Bcl-2 and Bcl-xL proteins rendering tumor cells to undergo apoptosis resulting in the elimination of clonal expansion of initiated and/or transformed tumor cells (Fig. 2b). SFN reportedly also modulates signal transduction pathways such as MAP kinases and CDK's mediated cell cycle progression and arrest and inhibits angiogenesis, invasion, and metastasis of

cancer [12, 21, 26]. More recently SFN has been shown to inhibit histone deacetylase (HDAC) activity leading to the reactivation of tumor suppressor genes and silencing oncogenes [27–29]. Based on accumulating evidence, SFN appears to be a promising agent for cancer therapy presumptively associated with multitargeted therapeutic effects. In line with this notion, a growing body of preclinical data on use of SF in combination with conventional therapies has been investigated and reported.

3 Sulforaphane and Chemo- and Radiosensitization of Site-Specific Cancers

In pursuit of novel therapeutic strategies to enhance the efficacy of chemotherapy, radiotherapy, and/or other conventional therapies for the treatment of human cancers, a growing body of emerging preclinical data on SFN combination points to an improvement in clinical outcome. Much of this anticipated success in therapeutic arena owes to the bioavailability of SFN needed for targeted inactivation of signaling pathways that allegedly contribute to therapeutic resistance. A number of phase I and II clinical trials have been conducted to investigate the toxicity of SFN in healthy men and women and patients with cancer. Because of the lack of evidence for toxic effects in humans alongside increased bioavailability and a growing understanding of its mechanism of action, SFN seems to be useful and holds promise when combined with conventional therapeutics in clinics. Preclinical cognizance and novel therapeutic viewpoints to improve therapeutic efficacy deem vital for cancer treatment. Interestingly, combination therapy has the advantage that each agent targets different levels and/or specific signaling pathways contributing to tumor cell survival and growth, and stalling these survival signaling targets will more effectively and efficaciously inhibit the tumor cell proliferation, tumor growth, progression, and its recurrence pragmatically with superior therapeutic outcome than individual inhibitors. Additionally, it permits reducing the therapeutic dose and thereby restricting problems of side effects of high dosages of drugs during monotherapy, alongside inducing and augmenting apoptosis of drug-resistant and tumor-initiating cells/cancer stem cells (TIC/CSC) that may have innocuously escaped the monotherapy treatment course. We summarize below current state of knowledge relating to the addition of SFN to strengthen and boost treatment armamentarium based on preclinical cognizance and clinical trials.

3.1 SFN and Breast Cancer

Breast cancer is the most common single cause of cancer-related death in women. Results from several case-controlled studies conducted in the USA, Sweden, and China came to a unanimous conclusion of significantly lower intake of cruciferous

vegetables in women diagnosed with BC than in cancer-free control group [30–32]. Oral administration of SFN inhibits mammary carcinogenesis in rats treated with 7,12-dimethylbenz[a]anthracene [13]. Sulforaphane exerts a direct chemopreventive action on animal and human mammary tissue. A single 150 μM oral dose of sulforaphane elevates cytoprotective NAD(P)H:quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1) gene transcripts in a rat mammary epithelium, which in itself provides strong evidence of a pronounced pharmacodynamic action of sulforaphane [19]. Moreover, as pointed earlier, detection of measurable quantities of sulforaphane metabolites in human breast tissue provides a strong rationale for evaluating the protective effects of a broccoli sprout preparation in clinical trials of women at risk for breast cancer. From a clinical pathologist viewpoint, breast cancer is a heterogeneous disease with distinct histologic subtypes. Approximately 75% of current diagnosed breast cancer are estrogen receptor (ER) positive, putatively implying the role of estrogen and ER contribution in expression of genes involved in the etiopathogenesis and progression of ER+ve breast cancer including women who are at elevated risk of breast cancer. Tamoxifen and its active metabolite, 4-hydroxy tamoxifen, are the first-line conventional adjuvant treatment for premenopausal BC patients with ER+ve tumors because of its ability to bind to endogenous natural ER sites depriving estrogen-mediated growth stimulus for cells to proliferate and survive. Paradoxically, tamoxifen is both an antagonist (in breast tissues) and an agonist (in the endometrium and bone) of ER, but in clinical setup, the net benefits outweigh the risk for treatment of premenopausal breast cancer patients with ER+ve tumors. However, despite the benefits, approximately 30–35% of women with ER+ve breast cancer develops de novo or intrinsic endocrine therapy resistance to tamoxifen and makes clinical management a challenge with tumor recurrence after drug therapy. Thus, to overcome this limitation, Pawlik et al. examined sensitization of ER+ve breast cancer cell lines to 4-hydroxytamoxifen by SFN. The investigators used three ER-positive BC cell lines – T47D, MCF-7, and BT-474 – as representative models to compare the sensitivity to combination therapy using SFN and 4-hydroxytamoxifen [33]. The results reveal a synergistic effect in that the combination of SFN and 4-hydroxytamoxifen inhibited cell viability more efficiently than both compounds used alone at concentrations lower than their IC50 values, and the decreased viability of cancer cells ensued from apoptosis largely due to the downregulation of anti-apoptotic proteins – Bcl-2 and survivin. Additionally, the combined treatment reduced the clonogenicity of cells as well compared to controls by about 60% (MCF-7 and BT-474 cells) and 70% (T47D cells). Further outspreading their study emulating clinical situation, T47D and MCF-7 derivatives (T47D tamR and MCF-7 tamR, respectively) which could grow in media containing 500 nM of 4-hydroxytamoxifen (tamoxifen-resistant) with retention of ER expression were established. Importantly, SFN could even sensitize these tamoxifen-resistant cells to 4-hydroxytamoxifen causing a statistically significant drop in viability of these cells as compared to the treatment with single compound. In essence, this study implies an approach that allows the use of lower doses of tamoxifen, hence decreasing the level of its toxicity and concomitantly improving the risk-benefit profile of

the drug. Moreover, such strategy might even protect against acquisition by cancer cells the drug-resistant phenotypes during therapy.

Another subtype of BC classified as HER-2-positive BC comprises about 20–25% of cases over expressing human epidermal growth factor receptor-2 (HER-2/ErbB-2/neu) associated with poor clinical outcome. HER-2 is a member of the epidermal growth factor receptor family which consists of HER-1 (EGFR/Erb-1), HER-2 (Erb-2/neu, HER-3 (Erb3), and HER-4 (Erb-4) and accepted as a potential target for anticancer therapies mainly targeted therapy. Overamplification of HER-2/neu receptor in tumor cells perpetuate constant signaling that proceeds through phosphatidylinositol-3-kinase (PI3/Akt)-mTOR-S6 kinase axis pathway and results in enhanced cell proliferation and survival of tumor cells mainly due to evasion of apoptosis, neoangiogenesis and a predilection to central nervous system metastasis making it difficult to treat the disease. Lapatinib is a relatively new specific inhibitor of EGFR and HER-2 and reportedly has some activity against brain metastasis, but its efficacy becomes limited by the emergence of lapatinib-resistant cells possibly due to hyperactivity of the signaling network downstream of Her-2 [34]. Of contextual relevance, SFN reportedly lowers the protein and phosphorylation levels of pro-survival Akt, mTOR, and S6 kinase pathway in phenotypically different breast cancer cell lines [35]. Thus, in in vitro cell culture model system with breast cancer cells overexpressing HER-2, it has been credibly shown that combination treatment of SFN and lapatinib decreases cell survival more efficiently than each of the agents used alone [36]. Possible mechanism relating to synergistic effect of the combined treatment has been reviewed by Kaczynska et al. which expectedly include the inhibition of the Akt-mTOR-S6 signaling axis [36]. More logistically, lapatinib-resistant HER-2-overexpressing breast cancer cells have been developed after treating HER2-overproducing SKBr3 cells cultured for a few months in the presence of increasing drug concentrations and mixed with isogenic lapatinib-sensitive cells in different proportions mimicking heterogeneous population of cancer cells as in tumor [37]. Simulating this as a chemoresistant model, the cells were next investigated for viability following combination treatment for 48 h with SFN (5 μM) and lapatinib (100 nM) to test the relevance of drug combination to overcome lapatinib drug resistance [37]. Additional effects of the combination of SFN and lapatinib on tumor cell migration were evaluated using transwell matrigel invasion assay. The assay results undeniably revealed that with increasing percentage of resistant cells, lapatinib alone was less effective, but a combination of lapatinib and SFN significantly and efficiently inhibited the viability and migration of the resistant cells compared to the activity of each agent alone as monotherapy [37].

Other significant discoveries with SFN as sensitization partner in breast cancer therapy have been reported in literature [38–41]. Clofarabine is a novel adenosine analogue that reactivates DNA methylation-silenced tumor suppressor genes (such as adenomatous polyposis coli (APC), phosphatase and tensin homologue (PTEN), and retinoic acid receptor beta 2 (RARβ2) as well as, surge p21 expression [38]. The apoptotic and cytotoxic effects of clofarabine have been investigated on breast cancer cell lines [42]. In breast cancer cells- MCF-7 and MDA-MB-231, SFN at physiologically relevant (10 μM) concentration leads to hypomethylation of PTEN

and RARβ2 promoters with concomitant gene upregulation [43]. It has been found that SFN alone and in combination with clofarabine epigenetically normalize the expression of DNA methylation-silenced tumor suppressor genes in invasive and noninvasive human breast cancer cells resulting in increase in cell growth arrest and apoptosis at noninvasive cancer stage and may probably contribute as a valuable therapeutic support in other solid tumors as well [43]. Sulforaphane preconditioning also sensitizes breast cancer cells to mitomycin C; the activation of NAD(P)H: quinone oxidoreductase-1(NQO1) by SFN acts as an activator of mitomycin C in tumor cells and makes them sensitive to the drug [41]. In addition, combinational treatment of SFN and gemcitabine on MCF-7 breast cancer cells shows that SFN enhances the growth inhibitory effects of gemcitabine in a synergistic manner [combination index (CI) <1] [39]. Notably, SFN induces apoptosis and anti-inflammatory effects on MCF-7 cells via downregulation of Bcl-2 and COX-2 respectively, minimizing toxicity to normal cells. Taken together, SFN might be a potent combination partner in anticancer armamentarium for breast cancer treatment.

3.2 SFN and Glioblastoma

According to the National Brain Tumor Society (http://www.braintumor.org), glioblastoma multiforme (GBM) accounts 12–15% of all intracranial tumors with a mean survival rate of ~15 months and 5-year survival rate of ~4% despite multimodal treatment strategies. Its etiology is unknown, but increasing research at molecular level points to epigenetic silencing of the MGMT (O-6-methylguanine-DNA methyltransferase) DNA repair gene by promoter methylation that compromises the DNA repair [44]. In 2005, FDA approved the alkylating agent, temozolomide (TMZ), for the treatment of adult patients with newly diagnosed and recurrent malignant gliomas based on survival benefit results and relatively low toxicity [45]. Therefore, current standard protocol adopted for GB treatment is concomitant radiotherapy combined with oral TMZ after surgery, although inherent and acquired TMZ resistance develops quickly and results in dismal prognosis. Pilot studies emphasizing SFN alone induce apoptosis and inhibits the migration and invasion of human glioblastoma malignant cells have been reported [46–48]. This has been confirmed by a multitude of assays including annexin V binding capacity with flow cytometric analysis, Wright staining, and ApopTag assay for apoptosis, while morphological scratch assay, transwell invasion assay, and microscopic observations of remarkably reduced formation of cell pseudopodia cumulatively support SFN efficacy in inhibiting the invasion and migration of glioblastoma cells [46–48]. Mechanistically, SFN increases Bax:Bcl-2 ratio and caspase-3 activity and upregulates calpain – a Ca2+-dependent cysteine protease – and subsequent release of cytochrome c from mitochondria indicate execution of apoptosis within glioblastoma cells [47]. Furthermore, the apoptosis phenomenon by SFN is favored by the downregulation of NF-κB and an upsurge in inhibitor of NF-κB-IκBα expression complimenting the process of apoptosis [47]. SFN inhibits the invasion

of glioblastoma U251MG cells via the upregulation of E-cadherin and other invasion-related proteins – phosphorylated ERK1/2 and CD44v6, and down-regulation of MMP-2, MMP-9, and galectin-3 [46, 48].

Additional, improvements in clinical efficacy of the combination of SFN and temozolomide (TMZ)-based chemotherapy in TMZ-resistant GBM cells have been reported [49, 50]. In one such study, TMZ-resistant cell lines were generated by a stepwise 6-month exposure of parental GBM cells to TMZ. Further characterization of these cells reveals the presence of constitutively active NF-κB and increases MGMT expression with expected high IC_{50} for TMZ relative to isogenic control cell lines. Of translational interest, SFN efficiently inhibits the constitutively active NF-κB signaling and reduces MGMT expression to reverse chemoresistance to TMZ in these isogenic cells resistant to TMZ [49]. Moreover, sequential combination treatment with SFN and TMZ synergistically inhibited survival proficiency alongside escalation of apoptosis in the treated TMZ-resistant GBM cells. Of greater interest was the recapitulation of the above findings with SFN in chemoresistant xenografts subcutaneously generated in the nude mouse model by injecting TMZ-resistant GBM cells, showing that SFN remarkably suppresses tumor cell growth and enhances cell death. These observations clearly attest the fact that clinical efficacy of TMZ-based chemotherapy in TMZ-resistant GBM may be improved by combination with SFN [49]. For mechanistic understanding and to explore the mechanism of resistance, several miRNAs have recently been implicated in glioma development, such as microRNA-21 (miR-21) which has been found to function as an oncogene in cultured GBM cells. miR-21 protects human glioblastoma U87MG cells from chemotherapeutic drug TMZ-induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity [51]. Further, mechanistic viewpoint investigations for its clinical application reveal that SFN effectively enhances TMZ-induced apoptosis by inhibiting miR-21 via Wnt/β-catenin signaling in GBM cells [50]. Collectively, these findings support the contention for including SFN as a potential adjuvant therapeutic agent in treating GB patients combined with TMZ in the future to overcome TMZ resistance and to avoid resistance emergence. Further explorations for the efficacious clinical application of SFN in GBM patients are clearly warranted.

3.3 SFN and Head and Neck Cancer

Head and neck cancer is a term collectively used to describe malignant tumors that develop in the thin layer of squamous cells that lines the moist mucosal surfaces in or around the throat, larynx, nose, sinuses, and mouth and often referred to as squamous cell carcinomas of the head and neck. Oncologists further classify these tumors either as carcinoma in situ or invasive squamous cell carcinoma depending on whether cancer is limited to the squamous layer of cells or has grown beyond this cell layer and moved into the deeper tissue (www.cancer.gov/types/head-and-neck). According to the American Academy of Otolaryngology, each year more than

55,000 Americans develop cancer of the head and neck; nearly 13,000 of them will die from it. Wisdom states abstaining from tobacco use is the most preventable cause from these deaths. Treatments include surgery, radiation, and chemotherapy but nevertheless have the disadvantage of significant side effects and developing radioresistance if undergoing radiotherapy. Kotowski et al. endeavored to investigate whether SFN could act as a radiosensitizer in four investigative head and neck squamous cancer cell lines – SCC9, SCC25, CAL27, and FADU [52]. The authors found SFN and radiation in combination lead to stronger inhibition of cell proliferation and clonogenic survival than each treatment method alone, leading to the conclusion that SFN may possibly be a promising agent as radiosensitizer during head and neck radiotherapeutic strategy. Besides, several clinical trials have demonstrated the efficacy of minimally invasive photodynamic treatment therapy (PDT) in the treatment of early oropharyngeal primary and recurrent cancers, as well as in palliative treatment of refractory head and neck cancers [53]. PDT is also used as a promising therapy in association with surgery to increase tumor-free margins to increase cure rates. Photofrin is one of the most commonly used photosensitizer with major side effects being skin sensitivity lasting up to 6 weeks after infusion. It has been shown that PDT combined with SFN enhances the cytotoxic effect on AMC-HN3 human head and neck cancer cells compared to PDT alone, which results from surge of reactive oxygen species (ROS) generation leading to apoptosis. The path for cell death ensues from activation of caspases by ROS driving both intrinsic and extrinsic caspase pathways of apoptosis [54].

Cancer in the salivary glands usually classified as either adenocarcinoma, adenoid cystic carcinoma (ACC), or mucoepidermoid carcinoma is uncommon, accounting for less than 5% of all cancers of the head and neck [55]. Interestingly, in a high metastatic cell line of the salivary gland, adenoid cystic carcinoma (ACC-M), sulforaphane induces G2-M arrest and apoptosis [56]. Also, the levels of NF-κB p65 in both the cytoplasm and the nucleus have been reportedly found to be markedly suppressed by SFN in a time-dependent manner in this cell line [56]. 5-Fluorouracil [5FU] appears to be active in ACC as a single agent. Based on previous reports of SFN as a chemosensitizer, prompted researchers to test whether SFN-induced suppression of NF-κB may possibly improve the effectiveness of traditional anti-ACC drug 5FU. To this, Wang et al. pursued with the investigation and found synergistic inhibitory effect of SFN and 5-fluorouracil in high (ACC-M) and low (ACC-2) metastatic cell lines of salivary gland adenoid cystic carcinoma which are relatively resistant to 5FU chemotherapy. Treatment of these cells with SFN and 5-FU in combination led to synergistic inhibition in cell growth and a decreased expression in nuclear NF-κB p65 protein; the synergistic inhibitory effect was more significant in ACC-M cells complying with the greatly decreased expression of NF-κB p65 (almost fivefold) after the combination treatment. The findings demonstrate synergism between SFN and 5-FU at higher doses against the ACC-M and ACC-2 cells, which could be related with the decreased expression of nuclear NF-κB p65 protein [57]. Clearly, more studies are warranted before SFN translates into clinics as chemo- and radiosensitizing agent for use in head and neck cancers.

3.4 SFN and Gynecologic Cancers

3.4.1 Ovarian Cancer and SF

Among gynecological disorders, ovarian cancer ranks as single most common cause of high mortality due mainly to detection at advanced stage and development of resistance to conventional chemotherapy. Multiple studies have confirmed that SFN induces growth arrest and apoptosis in multiple ovarian cancer cells (PA-1, OVCAR-3, and SKOV-3) [58, 59]. Additional studies with SFN to define downstream molecular events at the level of cell cycle control in ovarian cancer cells revealed increased levels of tumor suppressor retinoblastoma protein (RB) and decreased levels of E2F-1 transcription factor [60]. Furthermore, ovarian cancer cells treated with cytotoxic concentrations of SFN display reduced cell migration and increased apoptotic cell death due to an increase in Bak/Bcl-2 ratio accompanied by cleavage of procaspase-9 and poly(ADP-ribose)-polymerase (PARP) [60]. Recommended chemotherapy for ovarian cancer therapy is paclitaxel alone or in combination with platinum-based drugs, but emergence of resistant phenotype poses a challenge to successful chemotherapy. Thus, any alleged beneficial effect of SFN as combination partner with paclitaxel in treating ovarian cancer was investigated using MDAH-2774 cell line as model system. It was concluded that SFN (8 μM) with paclitaxel (2 μM) for 3 days of treatment drives the tumor cells to undergo synergistic cell death resulting in ~70% cell death [60]. In another unconnected study, modulation of cisplatin sensitivity in human ovarian carcinoma A2780 and SKOV3 cell lines by sulforaphane has been reported [61]. Findings by the authors reveal a dual effect as SFN significantly potentiated cisplatin-induced DNA damage in A2780 cells, while it protected SKOV3 cells against cisplatin cross-linking to DNA preventing cell death, the difference attributed to the differential inducibility of Nrf2 by SFN in these cells [61]. Clearly, more work including robust preclinical animal tumor model study is necessary before initiation of SFN as combination partner in ovarian cancer.

3.4.2 Cervical Cancer and SFN

Cervical cancer is one of the common cancers in women worldwide except the USA and other developed countries where Pap test screening is routine. Yet over 4,000 women in the USA die from cervical cancer each year. It can be treated successfully, if detected early, and usually is associated with long survival and good quality of life (http://www.webmd.com/cancer/cervical-cancer). HPV is the most common cause of cervical cancer, and the risk can be prevented by appropriate measures including vaccination. The anticancer effect of sulforaphane is associated with its apoptosis-inducing and anti-inflammatory actions in human cervical cancer cells [62]. Additionally, the combination of SFN and gemcitabine improves the growth inhibition potential in a synergistic manner in HeLa cells compared to individual drugs. Expression analysis of genes involved in apoptosis and inflammation reveals significant downregulation of Bcl-2, COX-2, and IL-1β upon treatment with sulforaphane confirming “double-edge” actions of SFN as an anti-inflammatory and proapoptotic

mediator overlaying combination strategy benefits [62]. SFN also sensitizes HeLa cells to apoptosis induced by cisplatin [63]. It causes a time-dependent cleavage of PARP protein in HeLa cells; however, a combination of 20 μM SFN and 10 μM cisplatin resulted in a large amount of cleaved PARP relative to either of the single agent implying that SFN also synergizes with cisplatin to induce apoptosis in HeLa cells [63].

Enhancement of radiosensitivity by SFN in human cervical cancer cells has also been documented [64]. Clonogenic cell survival in HeLa cells pretreated with SFN (20 μM) for 24 h and exposed to 0–6 Gy X-rays was significantly low compared to cells treated with radiation only. Compared to the cell survival rate of 0.1 (10%) for X-ray treatment alone, the relative biological effectiveness for the combined treatment was 1.5. Mechanistically, SFN augments radiosensitivity due to interference with repair of DNA double-strand breaks (DSB) induced by radiation together with downregulation of DNA damage repair proteins (phosphorylated DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Thr2609), Ku70, Ku80 expressions) and Rad51 foci implying the impairment of the NHEJ and HRR pathways by SFN. The repair inhibition by SFN partially contributed to the enhanced apoptosis induced by the combined treatment. Interestingly, combined treatment with radiation and SFN also significantly inhibits tumor growth in vivo in a tumor xenograft model [64]. Although stand-alone radiation and SFN treatments controlled the tumor growth to a certain extent, the combined treatment exhibited more effective tumor control which clearly points the usefulness of the combined regimen for improved tumor therapy.

3.5 SFN and Nonmelanoma Cutaneous Carcinoma

Nonmelanoma skin cancer is the most common form of skin cancer, and squamous cell carcinoma accounting ~16% is the most common form of nonmelanoma skin cancers (NMSC). Higher incidence of NMSCs is reported in lighter-skinned populations, inhabitants closer to the equator, and occupations compelling greater outdoor exposure. There is also a significantly greater risk of cancer in patients with genetic disorders or mutations that lead to greater UV sensitivity. Moreover, phototherapy which is employed in the treatment of various skin diseases is also associated with increased risk of NMSC; this association is stronger with psoralen photochemotherapy than with narrowband UV-B [65]. Interestingly, in healthy human subjects, topical applications of extracts delivering the Nrf2 activator sulforaphane have been recorded to reduce the degree of solar-simulated UV radiation-induced skin erythema, a quantifiable surrogate endpoint for cutaneous damage and skin cancer risk [66].

Intriguingly, population of solid organ transplant recipients receiving long-term azathioprine therapy are associated with a profoundly increased risk (in certain cases by more than 100-fold) of developing squamous cell carcinoma of the skin [67].

Azathioprine is a prodrug that is metabolized and ultimately converted to thioguanine nucleotide and results in partial substitution of guanine with 6-thioguanine in DNA. Subsequent studies revealed that 6-thioguanine DNA and UV-A radiation from exposure to sun cause mutagenic damage to DNA and synergistically increase the susceptibility to develop squamous cell carcinoma. Contextually, it has been shown that pharmacological upregulation of the Keap1/Nrf2/ARE pathway by sulforaphane results in lower 6-thioguanine incorporation in DNA and protects 6-thioguanine-treated cells against photo-oxidative stress following exposure to UV-A radiation [66, 68, 69]. Protection by SFN is essentially afforded by the increased levels of glutathione and induction of multidrug resistance-associated protein 4, an organic anion efflux pump that exports nucleoside monophosphate analogs [69]. Concludingly, SFN carries significant therapeutic advantage in non-melanoma cutaneous cancer.

3.6 SFN and Barett's Adenocarcinoma

Barrett esophageal adenocarcinoma (BEAC) develops within precursor lesion – Barrett esophagus – in which metaplasia of the normal squamous lining of the distal esophagus with columnar epithelium has developed in response to chronic gastroesophageal reflux. The incidence of this cancer is rapidly increasing in Europe and the USA, and the recommended treatment option is to undergo esophagectomy/surgical resection and other interventions [70]. The potential use of SFN in Barrett adenocarcinoma chemoprevention and as adjuvant in chemotherapy has been investigated and reported using two BEAC cell lines (FLO-1 and OE33) [71]. Of consideration, SFN induces cell cycle arrest and apoptosis and suppresses multidrug resistance protein (MRP) directed at reducing drug efflux in treated BEAS cell lines at concentration range between 3 and 7 μM, which is lower than the concentration of SFN (10–20 μM) required to kill other cancer cell lines. Additionally, treatment with SFN was also associated with induction of caspase 8 and p21 and suppression of HSP90, a molecular chaperone required for activity of several proliferation-related proteins. Significant anticancer activity of SFN has also been recorded in subcutaneously growing tumor model in vivo induced by BEAS cell lines [72]. Of clinical interest, neoadjuvant chemotherapy inducing side effects and resistance due to overexpression of multidrug resistance proteins compromising treatment options is a well-known phenomenon. Given the drug, paclitaxel, combination of SFN and paclitaxel enhanced sensitization to the drug and lead to a significant improvement in anticancer and antiproliferative activity of treated cells in part due to apoptosis and induction of caspase-8 and p21, and suppression of HSP90 and MRP reducing drug efflux [71]. Collectively, despite availability of limited data on marked anticancer activity of SFN in BEAC, rationale for its clinical evaluation in the future can be planned with more preclinical research data forthcoming in future.

3.7 SFN and Bronchial Carcinoid

Bronchial carcinoids are an uncommon group of slow-growing neuroendocrine tumors derived from peptide- and amine-producing neuroendocrine cells residing in the bronchial mucosa. These often display an indolent clinical behavior and affect patients in their forties to sixties. In neuroendocrine tumors (NET) and carcinoids, tumor-derived 5-hydroxytryptamine (5-HT) functions as an autocrine growth factor; this and the overexpression of carbonic anhydrases (CAs) together constitute a major component of the survival mechanism of tumor cells. Conventional cytotoxic agents such as 5-fluorouracil, doxorubicin, and cyclophosphamide which are effective in the treatment of other neoplasms are ineffective against carcinoids [73]. However, inhibition of CAs which regulates intracellular and extracellular pH can severely abrogate homeostatic and neuroendocrine functions, and therefore inhibition of enzymatic activity of CAs has been studied extensively as a therapeutic strategy against this cancer. Acetazolamide (AZ) is a classic pan-carbonic anhydrase (CA) inhibitor with prior inhibitory effect established on proliferation of tumor cells [74, 75]. Moreover, SFN has been reported to downregulate the expression of 5-HT receptors [76]. Thus, conceptually SFN can potentially demonstrate an anti-tumor additive or synergistic effect with AZ in pulmonary carcinoids potentially upsetting the survival mechanism of carcinoids by multiple mechanism of action. With two lung carcinoid cell lines-well-differentiated H-727 (TC) and poorly differentiated H-720 (AC) – the effects of acetazolamide (AZ) and sulforaphane on cell viability, clonogenicity, serotonin-induced growth effect and serotonin content, and growth effect on NOD/SCID mice subcutaneous xenografts have been reported [77]. Both compounds significantly reduced cell viability and colony formation in a dose-dependent manner in H-727 and H-720 cell lines. Of significant interest is the fact that treatment of H-727 and H-720 subcutaneous xenografts in NOD/SCID mice with the combination of AZ + SFN for 2 weeks demonstrated highly significant growth inhibition and reduction of 5-HT content, compromising the invasive capability of H-727 tumor cells [77]. Further, both in vivo and in vitro models, marked reduction in secretory vesicles correlated with decrease in 5-HT content. Noticeably, the combination of AZ and SFN displayed a more marked and efficacious effect than did any single agent with therapeutic effective dose within the window of clinical range and bioavailability. From these results a new potential therapeutic strategy for the treatment of bronchial carcinoids may be expected.

3.8 SFN and Hematological Malignancies

3.8.1 Multiple Myeloma

Multiple myeloma (MM) first recognized in 1848 is characterized by the proliferation of malignant plasma cells outnumbering normal plasma cells that help to fight infections and lead to overabundance of monoclonal paraprotein (M protein) in the bone marrow. It is so-called because tumors develop inside the bone in multiple parts of the body and behold as the second most common debilitating malignancy within

spectrum of hematological malignancies. Bortezomib, a proteasome inhibitor approved by the Food and Drug Administration in 2003, is taken as a standard for treatment of patients with relapsed and resistant MM, but the drug still poses a challenge notably to drug resistance and adverse side effects [78–80]. Thus, in pursuit of novel nature derived bioactive agents for use in combination with conventional therapies to improve the clinical outcome of MM patients, Jakubikova et al. attempted exploring antitumor activity and therapeutically relevant signaling events that can allegedly be reformed by isothiocyanates including SFN in MM [81]. The authors report cytotoxic effects of SFN monotherapy against a panel of primary human MM cell lines and cells resistant to conventional and/or novel anti-MM agents (MM.1S, OPM1, RPMI-S, RPMI-Dox40, RPMI-MR20, RPMI-LR5, MM.1R, and OPM2 cells) and observed an almost similar concentration-dependent anti-MM effect. Additionally, the effect of ITC on affinity-purified $CD138^+$ primary MM cells from bone marrow samples from eight MM patients was treated for 48 h with SFN, and a dose-dependent response with IC50 values in the range of 5–72 μM was observed. Further, to rule out any toxicity due to SFN, purified $CD138^+$ cells from bone marrow of healthy donors as well as peripheral mononuclear cells from healthy donors were treated with SFN [81]. Alongside, the molecular sequelae of SFN treatment in multiple myeloma cells were reviewed by running multiplex analyses using bead arrays and Western immunoblotting and found hallmarked by the induction of apoptotic cell death, modulation of mitochondrial transmembrane potential, cleavage of PARP and caspase-3 and caspase-9, as well as downregulation of antiapoptotic proteins including Mcl-1, X-IAP, c-IAP, and survivin. SFN-induced G_2/M cell cycle arrest is accompanied by mitotic phosphorylation of histone H3. Further multiplex analysis of phosphorylation of diverse components of signaling cascades pointed to changes in MAPK activation, increased phosphorylation of c-jun and HSP27, as well as changes in the phosphorylation of Akt and GSK3α/β and p53 [81]. Additionally, SFN suppressed the proliferation of myeloma cells when cocultured with HS-5 stromal cells enhancing the in vitro anti-myeloma activity of several conventional (dexamethasone, doxorubicin, and melphalan) and novel anti-MM therapies (bortezomib and lenalidomide) in MM.1S cells treated for 48 h with combinations of SFN and the drugs; additional evidence for synergistic effects was evaluated using CalcuSyn software. From the analyses, it was apparent that SFN executes a synergistic effect when combined with bortezomib and showed slight to moderate synergism (defined according to criteria detailed by Chou-Talalay) with lenalidomide [81, 82]. All investigated doses of SFN had synergistic effects when combined with conventional drugs, including dexamethasone, doxorubicin, and melphalan. The apparent synergy upholds SFN to be combined with lower doses of other anti-MM agents perceivably decreasing the severity of side effects while maintaining potent anti-MM activity and providing preclinical framework for future clinical studies of ITC in MM.

The clinical efficacy of arsenic trioxide (ATO) in the treatment of relapsed or refractory MM patients has been reported [83–85]. However, like most drugs used in the treatment of MM, >50% of patients with refractory or relapsed disease eventually present with resistance to ATO when it is used as a single agent [86]. Thus, based on the validated SFN ability to inhibit proteasomal-mediated protein degradation,

presumptive therapeutic potential of SFN to increase sensitivity of ATO was evaluated in a panel of MM cell lines (PCNY-1, KMS-11, APR-1, MM1.S, and MM.1R cells) in vitro [87]. ATO causes modest growth inhibition in three MM cell lines (PCNY-1, KMS-11, and ARP-1) when engaged as a single agent. Similarly, concentrations up to 5 μM sulforaphane had a minimal effect on the proliferation of MM cells. However, using combination protocol, the ability of these compounds to reduce proliferative capacity was dramatically enhanced with combination index analysis revealing the relationship between 0.5 μM ATO and 3 μM sulforaphane strongly synergistic in four out of five MM cell lines examined. Intriguingly, the synergistic relationship was not observed in MM1.R cells but instead found antagonistic which points to additional mechanism rather than ER stress. MM1.R is a subclone of the MM.1 human MM cell line selected for resistance to glucocorticoid therapy and lacks glucocorticoid receptors [88]. Mechanistically, ATO and sulforaphane co-treatment augmented apoptotic induction as demonstrated by cleavage of caspase-3, caspase-4, and PARP, being dependent upon the production of reactive oxygen species (ROS) as validated by glutathione depletion and partial inhibition of the apoptotic cascade after pretreatment with the radical scavenger N-acetyl-cysteine (NAC). Furthermore, the combination regimen results in enhanced ER stress signaling and activation of the unfolded protein response (UPR) signifying perturbation in protein processing (proteostasis). Consistent with this paradigm, upregulation of HSP90 – a general marker for ER stress, and activation of the PERK (protein kinase RNA-like endoplasmic reticulum kinase) pathway, eIF2α phosphorylation, and XBP1 (X-box binding protein 1) splicing-a key component of the unfolded protein response (UPR), all tend to become enhanced upon co-treatment [87]. Of noteworthy, the concentrations used in the study are clinically achievable and deserve further investigation in clinical treatment of MM.

3.8.2 Acute Myeloid Leukemia

Since the first successful clinical application of ATO given as a single agent for treatment of adult acute promyelocytic leukemia (APL; a rare subtype of acute myeloid leukemia characterized by an accumulation of abnormal promyelocytes), clinical trials with ATO have shown a remarkable rate of remission compromising long-term survival benefits in APL patients. Unfortunately, ATO has not demonstrated significant benefit in non-APL hematological malignancies [89]. Thus, narrowing down from screening a library of over 2,000 marketed drugs and natural agents, the efficacy of SFN as ATO sensitizing agent was examined in a panel of leukemic cell lines representing a variety of different myeloid malignancies [K-562, erythroblastic chronic myelogenous leukemia; U937, promonocytic leukemia; and HL60, acute myeloid leukemia] for any promising therapeutic benefit [90]. Interestingly, sulforaphane significantly increased the effectiveness of ATO-mediated cytotoxicity through enhanced growth inhibition and increased apoptotic induction via the intrinsic mitochondrial-mediated pathway. Also when leukemic cells were treated in combination, a significant increase in the cellular level of reactive oxygen

species (ROS) levels was detectable suggestive of sulforaphane as an ATO potentiator through ROS-dependent mechanism which could be reversed upon addition of the free radical scavenger N-acetyl-l-cysteine (NAC). Importantly, this study chose to use the more conservative dose of SFN that is clinically relevant and achievable through diet alone, and the dose of ATO is also clinically feasible [91]. Of relevance, isothiocyanates failed to enhance ATO-mediated growth inhibition in NB-4 (acute promyelocytic leukemia) cells which are significantly more sensitive to ATO due to the presence of the t(15;17) translocation [92]. The leukemic cell lines used in the study described above do not contain the resulting PML-RARα fusion protein which is rapidly degraded by ATO and shown to be a key mediator in APL sensitivity to ATO. Thus, combinatorial treatment with SFN isothiocyanate and ATO might provide a promising therapeutic approach in select group of myeloid malignancies.

3.8.3 Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML; also called chronic myelogenous leukemia, chronic granulocytic leukemia, and chronic myelocytic leukemia) is a slow-growing myeloproliferative disorder that occurs during or after middle age and rarely affects children and is associated with specific genetic abnormality in the leukemia cell, called Philadelphia chromosome. This abnormal gene is produced when genetic material called ABL is displaced from chromosome 9 and replaces the normal part of chromosome 22 next to the region called BCR (reciprocal translocation). The resulting fusion gene – BCR-ABL – causes abnormal function of the ABL gene, which leads to leukemia [http://www.ucsf.health.org]. It is characterized by three phases: chronic phase, subsequently transforming into accelerated phase which progresses into blast crisis phase. Current first-line therapy for CML is BCR-ABL kinase inhibitor- imatinib mesylate (Gleevac®), which shows efficacy in most patients in controlling chronic phase of the disease [93]. $CD34^{+}/CD38^{-}$-enriched cells isolated from human leukemia KU812 cells exhibit resistance to imatinib treatment because of higher endogenous expression of β-catenin and MDR-1 [94]. Interestingly, SFN treatment increases imatinib sensitivity in $CD34+/CD38^{-}$ leukemia cells; the addition of 30 μM SFN in the presence of 1 μM imatinib led to elimination of approximately 88% of $CD34^{+}/CD38^{-}$ leukemia cells compared to cells without SFN treatment, with accompanying reduction in the expression of BCR-ABL. In addition, the level of phosphorylated CrkL-the substrate of BCR-ABL kinase - is severely suppressed by the combination treatment. Furthermore, SFN treatment decreases the expression of β-catenin and MDR-1 in imatinib-resistant $CD34+/CD38^{-}$ cells implying reduced drug resistance in these cells. Mechanistically, SFN sensitizes these imatinib-resistant cells to undergo apoptosis imposing oxidative stress by depleting intracellular glutathione. In summary, this study provides evidence that combination of imatinib and SFN could target and eliminate imatinib-resistant CML cells and SFN might be a good candidate as adjuvant therapy for refractory CML patients.

3.9 SFN and Colon Carcinoma

Colon cancer begins as a small polyp and diagnosed in more than 130,000 people in the USA alone despite screening drive accounting for a 30% drop in colon cancer rate and increasing 5-year survival rate by 65%. Current treatment options for colon cancer include combination of a variety of chemotherapeutic drugs, especially the third generation of platinum-based drug, oxaliplatin, plus 5-fluorouracil and leucovorin (referred to as FOLFOX). Other options include oxaliplatin plus capecitabine (Xelox) or oxaliplatin plus cetuximab (CAPOX). Such oxaliplatin-based chemotherapeutic regimens show improved clinical efficacy as comprehended from overall survival rate, time to tumor progression, and median overall survival in patients with metastatic colorectal cancer and offer an alternative treatment option against cisplatin-resistant tumors [95, 96]. However, further drug development and novel combination regimen with higher efficacy and minimizing unwanted side effects which can possibly significantly improve patient's outcome are warranted. Kaminski et al. touched base on the issue whether SFN pretreatment can enhance the anticarcinogenic actions of the chemotherapeutic drug, oxaliplatin, in a cell culture model of colorectal cancer cells (CaCo-2) by pretreatment with SFN [97]. Using routine standard techniques to analyze cell growth, drug interactions using combination index method, and apoptotic events further complimented by Western blot results and FACS analysis, the authors came to the conclusion that SFN and oxaliplatin co-incubation enhances the inhibition of CaCo-2 cell growth displaying alongside distinctive morphological changes such as cell surface exposure of phosphatidylserine, membrane blebbing, as well as the occurrence of cytoplasmic histone-associated DNA fragments characteristic of apoptotic phenomenon by the activation of both extrinsic and intrinsic pathways. With increasing concentrations and treatment duration, a shift from apoptotic to necrotic cell death could be observed. Collectively, the data suggest that SFN sensitizes colon cancer cells to oxaliplatin-induced cell growth inhibition thru induction of different modes of cell death [97].

Another study endorses the possibility of enhancing the cytotoxicity of bioreductive anticancer prodrugs with SFN to promote therapeutic sensitivity [40]. According to published reports, SFN preconditioning sensitizes human colon cancer cells toward bioreductive anticancer prodrug PR-104A – a metabolite of the dinitrobenzamide mustard pre-prodrug PR104 [98, 99]. The enzyme aldo-keto reductase (AKR) 1C3 – a member of the AKR enzyme superfamily – mediates the aerobic activation of the DNA cross-linking prodrug PR-104A [98, 100]. A number of clinical trials for cancer therapy using PR-104 are on record in literature [101–104], sans potential beneficial effect of SF on the efficacy especially PR-104A cytotoxicity. Because of the established effect of SFN on drug-metabolizing enzymes, proteome-wide changes in human HT-29 colon cancer cells using stable isotope labeling with amino acids in cell culture (SILAC) demonstrate AKR1C3 protein upregulation, but its activity in cells exposed to (low) concentrations of SFN is ineffective in growth inhibition [40]. Of interest, preconditioning HT-29 cells with SFN reduces the EC_{50} of PR-104A 3.6-fold which is associated to

AKR1C3 abundance and activity induced by SFN in a dose-dependent manner. The effect was reproducible in another colon cancer cell line, SW620 wherein similar to HT29, SW620 cells become sensitized to treatment with PR-104A by SFN pre-treatment, but not in other colon cancer cell lines where AKR1C3 is either absent or barely detectable and cannot be induced by SFN [40]. Moreover, when the SFN-induced AKR1C3 overexpression was downregulated using the technique of RNA interference, cytotoxicity of PR-104A was reduced indicating that SFN pre-conditioning is essential for inducing AKR1C3 abundance levels attesting previously established link between AKR1C3 abundance and PR-104A efficacy [98, 105]. Cumulatively, the results imply that susceptibility to induction of AKR1C3 basal levels and activity by SFN influence the ability of cells to sustain biotransformation-linked PR-104A therapeutic potential. Interestingly, SFN had no significant influence on PR-104A cytotoxicity in noncancerous, immortalized human colonic epithelial cell line (HCEC) as a model for noncancerous tissue [106]. Summing up, dietary bioactive food components such as SFN should be considered for their potential to alter and possibly benefit bioreductive cancer drug activity with no harm to normal tissues.

3.10 SFN and Pancreatic Cancer

Pancreatic cancer (PaCa) is among the most leading causes of cancer-related deaths with an average overall 5-year survival of <5%. Conventional chemotherapy is rarely beneficial because of extensive local invasion, early systemic dissemination, and pronounced resistance to chemo- and radiotherapy. Emerging evidence points that PaCa and other solid tumors contain a subset of a rare population of cells defined by their capability to undergo extensive self-renewal, multipotency, and high tumorigenic capacity. Due to intrinsic stem cell-like properties, these cancer stem cells (CSC) are believed to underlie resistance to chemotherapy and disease relapse encouraging search for innovative therapies directed to the elimination of CSCs. Almost all pancreatic CSC, cell lines rapidly grow upon injection into nude mice with very invasive growth pattern along with self-renewal potential, express ALDH1 (a marker associated with CSCs) activity, differentiation capability, high apoptosis resistance, and a characteristic surface expression of CSC markers ($CD44^+/CD24^-$, $EpCAM^+$, $CD133^+$, $CXCR4^+$) and no E-cadherin expression [107, 108].

With significant impetus toward identifying natural bioactive compounds with potential to sensitize CSCs, SFN has shown capability to sensitize pancreatic CSCs by downregulation of NF-κB along with inhibition of CSC properties (e.g., ALDH1 and self-renewal activity, apoptosis resistance, and growth in immunodeficient mice) [109]. An extension to this study, Rausch et al. reported a strategy showing that co-treatment with SFN and sorafenib – a multikinase inhibitor – augments the elimination of pancreatic CSC and overcomes apoptotic resistance [110]. This was experimentally apparent from the fact that a combination of sorafenib with SFN considerably reduced the clonogenicity and self-renewal potential of CSCs and intensified apoptosis in vitro. Mechanistically, SFN abolishes sorafenib-induced

NF-κB activity in CSCs despite a strong increase of DNA binding induced by sorafenib as noticed in the investigated cell lines representing low and high CSCs. Complementing the in vitro findings, in vivo results show that treatment of mice harboring CSC^{high} xenografts with sorafenib reduced the tumor growth in a dose-dependent manner without obvious side effects to mice, as the general state of health, body weight, and liver parameters were constant during and after treatment [110]. Of further interest was evidence that the reduction of pancreatic tumor xenografts was due to inhibition of proliferation and angiogenesis as concluded from Ki-67 immunostaining and reduced vascularity observed in tissue samples analyzed by immunohistochemistry and evidence indicative of apoptosis induction. Other molecular entities corresponding to EMT proteins such as Zeb-1 and Twist2 and vimentin (a mesenchymal marker) were investigated, and their expressions were found diminished after co-treatment with SFN and sorafenib. Summarily, the abovementioned abridged data substantiates that combination with an NF-κB inhibitor such as SFN may be a valuable therapeutic option to improve the therapeutic efficacy of sorafenib in pancreatic cancer.

In addition to the above study, SF combinations with other common cytotoxic chemotherapeutics – cisplatin, gemcitabine, doxorubicin, and 5-flurouracil – against PaCa have also been reported [72]. It has been found that though cisplatin, gemcitabine, doxorubicin, 5-flurouracil, or SFN effectively induces apoptosis and thwarts cell viability, a combination of these agents with SFN escalates the toxicity of chemotherapeutic agent, 5FU being most effective targeting 80% of the cell population. As gemcitabine is the standard chemotherapy for treatment of pancreatic cancer, the therapeutic potential of gemcitabine and SFN co-treatment was picked up for further evaluation in detail. Based on short- and long-term assays, the combined treatment intensified the inhibition of clonogenicity, spheroid formation, and aldehyde dehydrogenase-1 (ALDH1) activity supporting the investigators hypothesis. Furthermore, because Notch signaling and the transactivation subunit of the NF-κB transcription factor – c-Rel are considered as a key player in stem cell self-renewal, these entities were further explored from mechanistic viewpoint whether CSC characteristics are targeted. Results indicate that treatment of CSC^{high} cells for 48 h with SFN downregulated basal Notch-1 expression, while gemcitabine led to a strong induction of Notch-1, and downregulation of Notch-1 was associated with downregulation of c-Rel expression. Of therapeutic interest, the downregulation of Notch1 and c-Rel was strongest for the combination treatment indicating that CSC characteristics are targeted. Further to address whether SFN sensitizes CSC to cytotoxic therapy of gemcitabine in vivo, CSC^{high} pancreatic cancer cells were subcutaneously xenografted into nude mice. Mice were left untreated or treated with SFN, gemcitabine, or both agents together, and tumor growth was measured extending over a period of 10 days. Empirically, the group with combination treatment was found most beneficially affected from therapeutic viewpoint despite a 15% drop in body weight. Additionally, based on a modified protocol suited to address whether the combination treatment affects the tumor initiating the potential of CSC^{high} pancreatic cancer cells, it was found the combination regimen totally eradicated the growth of CSC xenografts and the tumor-initiating potential

suggesting that gemcitabine delays tumor growth by diminishing the number of differentiated tumor cells while sparing the highly resistant CSCs. In contrast, SFN targets the highly therapy-resistant CSC population and completes the therapeutic effect of gemcitabine. No pronounced side effects were observed in normal cells or general health conditions of mouse. Collectively, these data suggest that SFN increases the effectiveness of various cytotoxic drugs against CSCs without inducing additional toxicity in normal cells.

Another study relating to SFN and PaCa but directed against an essential molecular chaperone – heat shock protein 90 (Hsp90) has been described [111]. The authors contemplate that SFN potentiates the efficacy of 17-allylamino-17-demethoxygeldanamycin (17-AAG) against PaCa through enhanced abrogation of Hsp90 chaperone function. Given that the stability and maturation of a wide range of oncogenic client proteins linked with survival signaling pathways including PI3K/Akt kinase, k-Ras mutation, and overexpression or activation of mutant p53 are intimately coupled with pancreatic carcinogenesis, Hsp90 has emerged as a promising target in pancreatic cancer therapeutics. Thus, inhibition of Hsp90 by the well-known inhibitor, 17-AAG, provides a sensible strategy for the simultaneous inhibition of multiple oncogenic client proteins [112, 113]. In line with the hypothesis, SFN has been found to sensitize pancreatic cancer cells to 17-AAG by significantly enhancing the antiproliferative effect of 17-AAG by inducing apoptosis [111]. Mechanistically, at cellular level, SFN causes disruption in the Hsp90-p50^{Cdc37} interaction affecting Hsp chaperone function with oncogenic client proteins; the effect on the destabilization of some proteins relative to control was 29%, 43%, 14% and 14% for Akt, p53 mutant, Raf-1 and Cdk4, respectively. Further, the combination anticancer efficacy of sulforaphane and 17-AAG was evaluated for validation in vivo in a pancreatic cancer xenograft model [111]. Individual treatment with sulforaphane and 17-AAG suppressed the tumor growth by approximately 45% and 50%, respectively, compared to control group. In contrast, combination treatment with sulforaphane and 17-AAG led to about 70% inhibition of the tumor growth compared to control group, and the final tumor volume in the combination group was significantly reduced than that of the individual treatment groups. Meanwhile, neither sulforaphane nor 17-AAG at the administered dose regimen displayed any apparent toxicity as determined by body weight measurement. Collectively, these findings provide a rationale for further preclinical and clinical investigation of sulforaphane or broccoli/broccoli sprout preparations as a supplement to 17-AAG to sensitize pancreatic cancer eliciting a more prominent clinical response.

4 SFN and TRAIL Therapy

As a member of tumor necrosis factor (TNF) receptor superfamily, TNF-related apoptosis-inducing ligand (TRAIL) is a therapeutically promising novel ligand-based biological agent for cancer therapy. It selectively induces apoptosis in many cancer and transformed cells in vitro and in vivo with little or no toxicity to normal cells. Upregulation of DR4 and DR5, the two agonistic death receptors, enhances the

sensitivity of tumor cells to TRAIL; upon ligand binding, TRAIL transmits apoptotic signaling through the cleavage and activation of caspases and Bid following interaction. Surprisingly, an increasing number of publications report evidence of TRAIL resistance in primary human tumor cells necessitating sensitization for TRAIL-induced apoptosis [114, 115]. Possible mechanisms relating to cellular resistance to TRAIL-induced apoptosis and strategies to overcome resistance to TRAIL for clinical applications have been reviewed and published [116–119]. Interestingly, SFN apparently sensitizes the killing of tumor cells through increased expression of cell surface death receptors – DR4 and DR5 that transmit apoptotic signal upon TRAIL binding. In human osteosarcoma, the most common form of primary malignant bone tumor, SFN reportedly enhances TRAIL-induced apoptosis in osteosarcoma cells (Saos2 and MG63 cells) through induction of DR5 at mRNA and protein level in a dose-responsive manner [120]. Of greater clinical relevance is the fact that SFN sensitizes TRAIL-induced apoptosis in a p53-independent manner which is contextually important because conventional anti-osteosarcoma agents such as doxorubicin, cisplatin, and etoposide mainly induce apoptosis in p53-dependent manner [121, 122]. Logistically therefore, a combination treatment of SFN and TRAIL may be therapeutically beneficial in osteosarcoma with resistance to conventional agents caused by inactivated p53 [120].

Non-small cell lung cancer (NSCLC)-derived cells such as A549 lung adenocarcinoma cells are known to be resistant to TRAIL-induced apoptosis through activation of the PI3K/Akt pathway, and, thus, any TRAIL-based combination regimen that can sensitize TRAIL-resistant NSCLC to undergo apoptosis is important for clinical management of the disease [123]. Jin et al. demonstrated that combined nontoxic doses of SFN and TRAIL inhibit proliferation and induce apoptosis in TRAIL-resistant A549 cells [124]. Moreover, the combined treatment was confirmed to induce apoptosis by upregulation of apoptotic proteins including Bax, Bid, and caspases and PARP cleavage. Further, the expression and activation of these proteins in combination treatment group were found mediated by the downregulation of pro-survival ERK and Akt signaling in the cells [124]. Thus, in conclusion, the use of TRAIL in combination with subtoxic doses of SFN may provide an effective therapeutic strategy for safe treatment of some resistant NSCLCs.

Rhabdomyosarcoma (RMS) is pathologic soft tissue sarcoma that affects mostly children and express skeletal muscle-specific markers. Two subtypes of RMS have been identified based on histopathologic features – embryonal (ERMS) and alveolar (ARMS), the latter associated with worst prognosis. The majority of ARMS tumors are associated with t(2;13) or t(1;13) chromosomal translocations, which generate *PAX3-FKHR* and *PAX7-FKHR* fusion products, respectively [125]. SFN causes dose- and time-dependent growth inhibition and apoptosis in both ERMS and ARMS subtypes with a remarkably stronger effect in the aggressive ARMS subtype [126]. Nevertheless, in ARMS cells, it was noticed that in addition to SFN-mediated decrease in mRNA levels of highly relevant RMS-correlated oncogenes such as *PAX3-FKHR*, *MYCN*, *and MET*, the mRNA levels of p21 and TRAIL receptor DR5 (but not DR4) also become elevated in these cells. Thus, from a rationalistic

viewpoint, since SFN enhances the levels of DR5 mRNA and protein only in the aggressive alveolar subtype (ARMS), the question whether SFN specifically can restore the sensitivity to TRAIL in ARMS cells was further investigated by treating the cells with TRAIL, SFN, or their combination. Though ARMS cells (RH30 and RH4) proved to be TRAIL-resistant, SFN interestingly restores their sensitivity to TRAIL-induced cell growth inhibition leading to a robust effect seen in the combination with TRAIL. By well-designed experiments, it was confirmed that SFN-induced DR5 acts as a key regulator, being directly related to the TRAIL-induced cell growth inhibition. Further, the in vivo antitumor activity of SFN and TRAIL was evaluated in a xenograft murine model of ARMS by real-time molecular imaging using microPET during a 21-day treatment schedule. The results show that the systemic treatment (3 weeks) of mice with SFN or TRAIL as single agents only delayed tumor evolution, while the combined treatment (daily, intraperitoneal) with TRAIL (1.6 mg/kg) plus SFN (50 mg/kg) led to a progressive reduction of the tumor, culminating in its elimination which was further confirmed by the absence of any metabolic signal from tumor cells as understood from semiquantitative analysis performed on each identified tumor using the target-to-background ratio (TBR) [126].

With emerging trend of increasing incidence of hepatocellular carcinoma (HCC) and hepatoma cells being resistant to the apoptotic effects of TRAIL [127, 128], it deems essential to seek a potent sensitizer for TRAIL-induced apoptosis in TRAIL-resistant HCC cells overexpressing Bcl-xL or Bcl-2. Therefore, the ability of SFN to enhance TRAIL-mediated apoptosis in human hepatoma cells was explored by Kim et al. [129]. They report first evidence that treatment with subtoxic doses of SFN in combination with TRAIL selectively sensitizes hepatoma cells to undergo apoptosis. It was established that SFN treatment significantly upregulated mRNA and protein levels of death receptor of TRAIL-DR5, accompanied by an increase in the generation of reactive oxygen species (ROS). Of greater clinical interest is the fact that combinatorial treatment with SFN and TRAIL effectively induces irreversible cell death not only in human hepatoma cells with wild-type p53 (HepG2 cells) but also in those with mutant p53 [Huh-7 cells, Hep3B (p53 deleted), SNU-398, and SNU-449] commonly through DR5 upregulation. Based upon such unique findings, the authors contemplate that this novel combined treatment could also be useful for hepatoma cells wherein p53 function has been compromised by aflatoxin B-mediated mutations or by binding of the X-protein of hepatitis B virus. In conclusion, the use of TRAIL in combination with subtoxic doses of sulforaphane may provide an effective therapeutic strategy for safe treatment of resistant hepatomas [129].

The molecular mechanisms by which sulforaphane enhances the therapeutic potential of TRAIL in androgen-independent PC-3 cells and androgen-dependent LNCaP cells in vitro and in vivo models of prostate cancer have been described [130]. Findings indicate that sulforaphane inhibits cell viability in both PC-3 and LNCaP cells. PC-3 cells are recorded as sensitive to TRAIL, whereas LNCP cells are resistant to TRAIL. Sulforaphane enhances the apoptosis-inducing potential of TRAIL in PC-3 cells and interestingly sensitizes TRAIL-resistant LNCaP cells. The enhanced apoptosis-inducing potential of TRAIL has been reported mainly

due to multiple mechanisms which include the induction of death receptors and proapoptotic members of Bcl-2 family and inhibition of antiapoptotic Bcl-2 proteins and activation of caspase(s). Also, by sulforaphane pretreatment, enhancement of the inhibitory effect of TRAIL on PC-3 colony formation and sensitization of TRAIL-resistant LNCaP colonies convey the message that sulforaphane augments the ability of TRAIL to inhibit colony growth. Additional data suggest that upregulation of death receptors DR4 and DR5 by sulforaphane may be one of the mechanisms that reinforce the proapoptotic effects of TRAIL in PC-3 cells and sensitize TRAIL-resistant LNCaP cells. In vivo, sulforaphane inhibits the growth of PC-3 cells orthotopically implanted in nude mice by inducing apoptosis and inhibiting tumor cell proliferation. It has been recorded to inhibit the expression of proteins related to invasion, metastasis, and angiogenesis by inhibiting phosphorylation of AKT, ERK1/2, and FOXO3a and activation of NF-κB [130]. These provide strong and compelling preclinical evidence that sulforaphane either alone or in combination with TRAIL can be used to prevent and/or treat prostate cancer.

5 SFN, TRAIL, and Cancer Stem Cells

5.1 Prostate Cancer

An extension to the above report in support of SFN-sensitizing prostate cancer cells to TRAIL-induced apoptosis, apoptosis-resistant cancer stem cells (CSCs) have been identified in advanced androgen-independent prostate cancer (AIPC) that are not eliminated by currently available therapeutics. Thus, the disease relapses due to the ability of CSCs to self-renew and differentiate into multiple cell types populating with all the cellular subtypes of the original tumor. In particular, these cells overexpress CSC markers including ALDH1, CD44, CD133, and c-Met [131]. Therefore, numerous investigators pursued investigating innovative approach combining TRAIL and bioactive natural compounds aimed to eliminate CSCs. In line with such objective and utilizing advanced androgen-independent prostate cancer (AIPC) cell lines – DU145 and PC3 – with highly enriched CSC characteristics and primary patient-derived prostate CSCs, Labsch et al. revealed a synergistic action between SFN and recombinant soluble TRAIL when used in combination [132]. The authors built their conclusion from results comparing the effects of the drugs either as monotherapy or in combination using multitude of in vitro assays including NF-κB activity, self-renewal and differentiation potential, stem cell signaling via spheroid, colony-forming assays, and antibody protein array. Mechanistically, the dominance of a therapeutically relevant effect associated with the combination regimen is a result of the inhibition of TRAIL-induced NF-κB activity by SFN and reduction in the levels of Oct-3/4, HNF-3β, PDX-1, Otx2, TP63, GSC, Snail, VEGFR-2, and HCG relative to monotherapy. These proteins relate to requirement for self-renewal, differentiation, cell migration, and epithelial-mesenchymal transition (EMT) and tumorigenesis. Additionally, it has been noted that the expression of CXCR4 receptor which is involved in migration and metastasis becomes inhibited

following sulforaphane-only treatment and the combination with TRAIL further reduces the expression of this receptor. Similar results were noticed for the Notch-1 receptor and its ligand, Jagged-1, which are regulators of asymmetric and symmetric division, progression, and metastasis in prostate cancer. Correspondingly, the inhibition of SOX 2 and Nanog expression which are important regulators of self-renewal potential was much stronger in the combination group. ALDH1 activity which represents differentiation and self-renewal potential was also significantly inhibited by the combination regimen attesting strong inhibition of stem cell signaling potential. Furthermore, in an in vivo tumor engraftment model, TRAIL and SFN together inhibited tumor growth, reduced CSC marker expression, and eliminated primary prostate CSCs synergistically via the induction of apoptosis and inhibited the proliferation and CSC marker expression. Conclusively, these findings suggest that sulforaphane shifts the balance from (TRAIL-induced) survival signaling to apoptosis and explains the observed synergistic effect.

5.2 Pancreatic Cancer

To address the need to eliminate oncogenically transformed CSC in pancreatic cancer (PaCa), Kallifatidis et al. pointed that pancreatic tumors harbor tumor-initiating cells (TICs; aka CSC), wherein TRAIL-activated NF-κB signaling acts as a contributor to resistance to apoptosis induction by TRAIL [109]. The reported anticancer activity of SFN in pancreatic cancer stem cells has been detailed in the preceding section [109]. Of interest, sulforaphane allegedly prevents NF-κB binding and thus is able to arrogate resistance to chemotherapy without cytotoxicity to normal cells. In a xenograft model, sulforaphane strongly blocked tumor growth and angiogenesis, while combination with TRAIL levied an additive effect without any obvious cytotoxicity to normal cells [109]. Further, primary tumor cells isolated from tumors freshly resected from patients with pancreatic cancer expressing markers for TICs could also be sensitized by sulforaphane for TRAIL-induced cytotoxicity; together these findings provide new insights into resistance mechanisms of TICs and suggest the combination of sulforaphane with TRAIL as a promising strategy for eliminating pancreatic TICs.

6 Sulforaphane Combination with Other Natural Agents in Cancer Therapy

Against the backdrop of foregoing thought-provoking reports from laboratory investigations confirming that SFN intercedes in sensitizing different treatment modalities including TRAIL therapy, anticipations from combination of SFN and other well-characterized chemopreventive bioactive natural compounds aiming to increase the antitumor response became the focus of several reports. Based on scientific rationale that health benefits of fruits and vegetables are from additive and synergistic combination of phytochemicals, SFN when combined with other bioactive

phyto-compounds also displays promising results against various cancers. Such effect may conceivably be due mainly because of the induction of a broad spectrum of anti-cancer pathways. This concept is well illustrated in study reporting chemoprevention of familial adenomatous polyposis (FAP) by combination of sulforaphane and dibenzoylmethane in $Apc^{Min/+}$ mouse colorectal cancer model that mimics the human familial adenomatous polyposis (FAP) that progresses sporadically to colorectal cancer with early *Apc* mutation [133]. In a prior study on human colon carcinoma HT-29 cells, SFN shows growth inhibition by inducing G1 cell cycle arrest and apoptosis, probably through the regulation of mitogen-activated protein kinase (MAPK) without affecting the Akt pathway [134]. On the other hand, dibenzoylmethane (DBM) – a minor constituent of licorice, inhibits cancer cell growth by inhibiting Akt pathway activity. Thus, a study that included a combination treatment regimen of SFN and DBM at half of the single dose was designed to investigate their potential synergistic effect on colon cancer chemoprevention in $Apc^{Min/+}$ mouse model [133]. The results showed that dietary administrations of SFN and DBM combination significantly inhibits the development of intestinal adenomas and block the colon tumor development by inhibition of cell survival and growth-related cell signaling pathways (such as Akt and extracellular signal-regulated kinase) and induction of apoptosis and other related biomarkers (including arachidonic acid metabolism, proliferating cell nuclear antigen, cleaved caspases, cyclin D1, and p21) conceptually supporting the hypothesis that both SFN and DBM in combination are potent natural dietary compounds in chemoprevention of gastrointestinal cancers. Moreover, the protective ability of glucosinolates due to synergistic effect with other constituents in the diet cannot be ignored. In this direction, SFN in combination with tea polyphenol – epigallocatechin (EGCG) – has been testified in ovarian, prostate, and colon cancers [135–137]. Previous published epidemiological studies indicate that consumption of green tea and cruciferous vegetables is inversely associated with occurrence of ovarian cancer [138–141]. Therefore, the combinatorial effect and underlying mechanism of major components of green tea (epigallocatechin gallate – EGCG) and cruciferous vegetables (sulforaphane) on ovarian and colon cancer cells as basis for innovative therapeutic approach befitted as subject for further investigation [135]. Encouraging data interestingly depicts that EGCG potentiates the inhibiting effect of SFN on paclitaxel-sensitive and paclitaxel-resistant ovarian cancer cell lines in time- and dose-dependent manner. Further examination points to SFN action in arresting ovarian cancer cells in G2/M phase, while EGCG and SFN co-treatment causes cell cycle arrest in both G2/M and S phases resulting in a significant escalation in apoptosis. The combination also strongly inhibits hTERT (the main regulatory subunit of telomerase) and Bcl-2 and alters the expression of phosphorylated H2AX that promotes DNA damage response specifically in paclitaxel-resistant ovarian cancer cell lines proposing the use of these compounds for overcoming paclitaxel resistance in ovarian cancer treatment [135]. Furthermore, a synergistic combination effect between sulforaphane and (-) epigallocatechin-3-gallate in human colon carcinoma cells (HT-29 AP-1) has also been reported [136]. The same group of investigators examined in prostate of Nrf2-knockout mice and

human prostate cancer cells the combination of EGCG and SFN with evidence of regulatory cross talks between the transcription factors Nrf2 and AP-1 in prostate tumors as several key Nrf2-dependent genes were found downregulated (three- to 35-fold) after in vivo administration of the combination of EGCG (100 mg/kg) and SFN (45 mg/kg) [137]. In silico bioinformatics data revealed conserved transcription factor binding site (TFBS) signatures in the promoter regions of Nrf2 and AP-1 suggesting the combination effect of SFN+EGCG is mediated via a concerted modulation of Nrf2 and AP-1 pathways in the prostate [137].

Interestingly, SFN as a supportive partner with resveratrol and eugenol in inducing apoptosis of tumor cells has also been reported [142, 143]. In glioma cells, combination treatment with resveratrol inhibits cell proliferation and migration, reduces cell viability, induces lactate dehydrogenase release, decreases pro-survival Akt phosphorylation and increases caspase-3 activation [142]. This points the worth of combining resveratrol and sulforaphane for the treatment of glioma. However, more critical studies before translational implication of this study are warranted. In human cervical cancer cells, concurrent sublethal doses of SFN and eugenol act in a synergistic manner with gemcitabine culminating in loss of viable cells by stimulation of apoptosis elicited by downregulation in the expression of Bcl-2, COX-2, and IL-β [143].

Based on the notion that additional distinctive isothiocyanate constituents of cruciferous vegetables such as indole-3-carbinol (I3C) or its condensation product 3,3′-diindolylmethane and SFN underscore any prognostic interactive influence, their role in prostate and colon cancer models has been examined. In human colon cancer cell lines, ITCs like sulforaphane (SFN) are cytotoxic, whereas indoles including indole-3-carbinol and its condensation product 3,3′-diindolylmethane act by cytostatic mechanisms. As a result, using defined combinations of SFN and 3,3′-diindolylmethane, the investigators interestingly found that at a total drug concentration of 2.5 μM, all combinations of SFN and 3,3′-diindolylmethane were antagonistic, but consecutively with increasing concentrations, the antagonistic effect gradually turned into a synergistic interaction at the highest combined cytotoxic concentration of 40 μM [144]. Furthermore, SFN and DIM combination (at 10 μM concentrations of each) results in strong G2/M cell cycle arrest, which was not observed with either compound alone. Overall, the results suggest that at low total concentrations (below 20 μM), which is physiologically more relevant, the combined broccoli compounds display antagonistic interactions in terms of cell growth inhibition. Building on their findings, further elucidation of mechanistic interactions between bioactive food components is warranted for much better prediction of beneficial health effects. With epidemiological findings weighing in favor of high intake of cruciferous vegetables reducing the risk of prostate cancer [145–147], the effect of the combination of glucosinolate hydrolysis products such as I3C and SFN which are constituents of cruciferous vegetables seems logical and therefore was examined mainly with reference to cell proliferation in a prostate cancer cell line (PC-3) model. Cell proliferation in PC-3 prostate cancer cells was significantly inhibited by I3C and sulforaphane at media concentrations of 0.2 mmol/L and 0.02 mmol/L, respectively, which explicitly explains the observed protective

effect of cruciferous vegetables in relation to the risk of developing prostate cancer [148].

At present time, there is no strong evidence for an inverse association between consumption of cruciferous vegetables and pancreatic cancer risk, except a prospective study reporting cabbage consumption and statistically significant lower risk of pancreatic cancer (one or more servings/week versus never consumption) [149]. However, in support of the notion that combination of chemopreventive bioactive compounds may afford protection against pancreatic cancer, few studies have been conducted as presented herein. In related context, Hutzen et al. appraised and reported bioactive isothiocyanates from cruciferous vegetables such as benzyl isothiocyanate (BITC) and sulforaphane, both being capable of inhibiting cell viability and inducing apoptosis in PANC-1 by mechanistically targeting inhibition of tyrosine phosphorylation of signal transducer and activator of transcription factor-3 (STAT-3) [150]. The STAT proteins comprise a family of latent transcription factors with well-established roles in cell proliferation, growth, and survival and are constitutively active in many human cancers; thus, inactivation of STAT-3 signaling has emerged as an attractive target in cancer therapeutics. Interestingly, SFN has minimal effect on the direct inhibition of STAT-3 tyrosine phosphorylation; however, combinations of BITC and SFN inhibited cell viability and STAT-3 phosphorylation more radically than either agent alone signifying that the combination of these bioactive agents, BITC and sulforaphane, carries immense translational potential as a therapeutic and interventional agent. Moreover, overlapping with this report, in a 16-week medium-term pancreatic carcinogenesis model in hamsters, the protective effects of benzyl isothiocyanate and sulforaphane against initiation of pancreatic carcinogenesis have been acknowledged [151]. The multiplicity of combined pancreatic lesions including atypical hyperplasias and adenocarcinomas was significantly decreased by feeding a diet supplemented with 80 ppm BITC and 80 ppm SFN in the initiation but not the post-initiation stage underpinning the fact that isothiocyanates BITC and SFN together can block benzo(a)pyrene initiation of hamster pancreatic carcinogenesis. Relating to pancreatic cancer therapy, another published study in literature documents that low doses of aspirin (ASP, 1 mM), curcumin (CUR,10 μM), and sulforaphane (SFN, 5 μM) (ACS) combination statistically reduce cell viability and concurrently induce apoptosis facilitated by activation of caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage, considered as hallmark of apoptosis [152]. Further mechanistic probing reveals that ACS inhibits NF-κB DNA binding activity and promotes sustained activation of phosphor-extracellular signal-regulated kinase 1/2 (p-ERK1/2), c-Jun, p38 MAPK signaling, and p53 proteins as probable mechanism [152]. In addition, the feasibility of using solid lipid nanoparticulate system to deliver this combination chemopreventive regimen has been investigated for pancreatic cancer chemoprevention [153]. Referencing previously described concept of cancer stem cells backed by in vitro data that SFN can eliminate pancreatic CSC by inhibiting NF-κB activity alongside self-renewal capability sensitizes the cells to undergo apoptosis [72, 109, 110]. An extension to this observation, SFN, quercetin, and a natural mixture of a complete set of green tea catechins (combination of green tea-derived catechins – epicatechin-2-gallate (ECG)

and catechin gallate (CG)) have been found to complement each other in the elimination of advanced pancreatic cancer by miR-let-7 induction and K-ras inhibition [154]. Additionally, this novel combination of quercetin, sulforaphane, and GTC also inhibits the self-renewal potential, apoptosis resistance, and migratory potential of tumor cells; of significant interest is the fact that the combination of bioactive agents is more effective than each single agent despite current therapeutics do not adequately target the CSCs and novel therapeutic options are critically warranted. Additional citation in the literature opinions sulforaphane synergizing with quercetin to inhibit self-renewal capacity of pancreatic cancer stem cells by inhibiting stem cell pluripotent transcription factor Nanog which could be a novel strategy to eliminate cancer stem cell characteristics and destroy CSCs [155].

7 Sulforaphane and Clinical Trials

Given the exceptional inciting laboratory research conclusions projecting SFN as a multifaceted bioactive natural compound that targets multiple processes associated with tumor growth and survival and further outcome of experimental laboratory studies in context of combined novel therapies, clinical trials are expected to be initiated in the future to confirm therapeutic benefit of its combination with standard chemotherapeutics for chemo- and radiosensitization of cancer. The toxicity and effects of SFN in healthy men and patients with cancer have been recorded. In a phase I clinical trial, SFN from broccoli sprouts has been evaluated for safety, tolerance, and metabolism and conveyed a good safety profile [156]. In this protocol, following a 5-day acclimatization period on a crucifer-free diet, broccoli sprout extracts were administered orally at 8-h intervals for 7 days (21 doses). Most clinical trials utilize broccoli sprouts or their hot water infusion as a rich and consistent source of glucoraphanin because the young florets reportedly contain glucoraphanin levels 20–50 times more than mature market-stage broccoli [13]. Current search for sulforaphane and cancer at http://www.clinicaltrials.gov indicates a total of 13 trials either ongoing, complete, or currently recruiting (Table 1). Phase II clinical trial for treating patients with recurrent prostate cancer is currently being performed (ClinicalTrials.gov, NCT01228084). Unfortunately, contextually relating to focus of this chapter, none of the projected trials is currently inclined to sensitization aspect of cancer therapy. However, the POUDER study objective which is currently recruiting participants will provide data on the clinical feasibility and acceptability of an supportive treatment option accompanying palliative chemotherapy [NCT01879878]. This pilot study, POUDER trial, determines the feasibility of a randomized controlled trial regarding the application of freeze-dried broccoli sprouts rich in sulforaphane and quercetin in patients with advanced surgically non-resectable pancreatic ductal adenocarcinoma (PDA) under palliative chemotherapy. The patients in the experimental arm will receive 15 capsules containing a total of 90 mg of active sulforaphane during the chemotherapy treatment course as a nutrition supplement, whereas patients assigned to the placebo group will receive inactive substance (methylcellulose) with identical capsule and potion distribution.

Table 1 Clinical trials with sulforaphane in cancer (http://www.clinicaltrials.gov)

Official title	Identifier #	Study design	Status
Pilot study evaluating broccoli sprouts in advanced pancreatic cancer [POUDER trial]	NCT 01879878	Intervention	Recruiting participants
The effects of sulforaphane in patients with biochemical recurrence of prostate cancer	NCT 01228084	Interventional	Study completed
Chemoprevention of prostate cancer, HDAC inhibition, and DNA methylation	NCT 01265953	Interventional	Not known
Evaluating the effect of broccoli sprouts (sulforaphane) on cellular proliferation, an intermediate marker of breast cancer risk	NCT 00982319	Interventional	Study completed
A human dietary intervention study to investigate the effect of sulforaphane on prostate cancer interception	NCT 01950143	Interventional	Ongoing, but not recruiting
Effect of topical application of sulforaphane containing broccoli sprout extracts on radiation dermatitis during external beam radiation therapy for breast cancer	NCT 00894712	Observational	Ongoing, but not recruiting participants
Sulforaphane: a dietary histone deacetylase (HDAC) inhibitor in ductal carcinoma in situ (DCIS)	NCT 00843167	Interventional	Study completed
Broccoli sprout extracts in healthy volunteers: a pilot study of Nrf2 pathway modulation in oral mucosa	NCT 02023931	Interventional	Study completed
An intervention study to assess bioavailability of sulforaphane delivered by glucoraphanin-enriched broccoli soups in healthy subjects	NCT 02300324	Interventional	Completed
A pilot study evaluation of sulforaphane in atypical nevi-precursor lesions: assessment of STAT1 and STAT3 risk markers of melanoma	NCT 01568996	Interventional	Ongoing, but not recruiting participants
Broccoli sprouts intervention in Qidong, PR, China	NCT 01437501	Interventional	Completed
Cruciferous vegetable intake and histone status in screening colonoscopy patients	NCT 01344330	Observational	Recruiting participants
A randomized, double-blind, placebo-controlled, multiple ascending dose study to evaluate the safety, tolerance, pharmacokinetics, and pharmacodynamics of Sulforadex® in healthy male subjects following daily dosing for 7 days	NCT 02055716	Interventional	Study completed

The outcome of this study will provide data on the clinical feasibility and acceptability of supportive treatment option accompanying palliative chemotherapy. Importantly, from the outcome of the results, future clinical studies to create further awareness for therapeutic benefit of sulforaphane in combination with chemotherapeutic agents and radiotherapy in the field of oncology are anticipated.

8 Conclusions and Perspective

Based on the above overwhelming evidence supporting preclinical cognizance of SFN as an adjuvant combination partner in a spectrum of organ specific antitumor treatment modalities (including targeted therapy) currently in use, emerging perspective justifies practical clinical application of SFN on cancer patients in future. However, more robust preclinical data using SFN or its analogs using available genetically modified animal models are warranted. This will add value to combined novel therapies and additionally prove an indisputable multipronged broad research-based strategy toward stronger and efficacious therapeutic outcome in the field of cancer treatment. Secondly, it is critical to explore and synthesize novel synthetic analogs with improved bioavailability and extending their retention time in tumor tissue with added benefit of capitalizing as innovative research strategy-based prescription drug for prognostic anticancer treatment benefits.

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Part III

Analytical and Processing Methods

Changing Trends in the Methodologies of Extraction and Analysis of Hydrolytic Products of Glucosinolates: A Review

13

Rohit Arora, Sakshi Bhushan, and Saroj Arora

Abstract

The increasing resistance among various pests and pathogens to the available synthetic medicines had lead to the compulsion for exploring new and improved alternatives. The phytochemicals have arisen as effective and safer alternate. Among the many plant secondary metabolites, glucosinolates surpass in biological activity and hence have been exhaustively extracted and explored for their activity against these dreaded diseases. This augmented demand for exploring the biological properties of all the available glucosinolates, especially their hydrolytic products, has lead to the development of new methods for extraction and analysis of these metabolites. The extraction methods are designed to match the volatility of the compound without degrading its quality. These methods have been improved to choose the best extraction conditions including the extracting solvents. The extracted glucosinolate hydrolytic products are further analyzed using an array of analytical methods ranging from as simple as paper chromatography to as complex as microchip analysis. These methods are designed and developed to match the needs of accuracy, reliability, and repeatability in addition to the cost effectiveness. This review thus is a highlight and a milestone for the scientists and budding researchers working on the crucial task of extracting, analyzing, and exploring the biological properties of glucosinolate hydrolytic products.

Keywords

Glucosinolate hydrolytic products • Biosynthesis • Hydrodistillation • GC-MS • HPLC • Microchip analysis

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_13

Abbreviations	
AITC	Allyl isothiocyanate
DCM	Methylene chloride
ELISA	Enzyme linked immunosorbent assay
FID	Flame ionization detector
GC	Gas chromatography
GHPs	Glucosinolate hydrolytic products
GSLs	Glucosinolates
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HS	Head space
HSCCC	High speed counter-current chromatography
ITCs	Isothiocyanates
MS	Mass spectrometry
NIRS	Near infrared reflectance spectroscopy
NMR	Nuclear magnetic resonance
PAPS	3-phosphoadenosine-5′-phosphosufate
PC	Paper chromatography
S-GT: UDPG	Thiohydroximateglucosyl transferase
SIXCPC	Strong ion-exchange centrifugal partition chromatography
SIXCPE	Strong ion-exchange centrifugal partition extraction
TLC	Thin layer chromatography
UHPLC	Ultra high performance liquid chromatography

Contents

1 Introduction

Glucosinolates (GSLs) are class of plant secondary metabolites that contain "sulfur" and are present in a number of plant families [1]. These plant metabolites are associated with the presence of enzyme myrosinase also known as thioglucosidase. Myrosinase belong to class of ß-thioglucosidases present in myrosin cells also known as idioblasts that are scattered throughout the tissue of GSL containing plant families. Both GSLs and myrosinase are present together in the different parts of the plant, viz. root, shoot, stem, seeds, etc. [2]. The plant injury results in the activation of "GSL –MYROSINASE" system and results in the formation of "mustard oil bomb." Such response of plant tissue leads to the generation of unstable thiohydroximate-*O*-sulfate moieties. The nonenzymatic removal of the sulfate group followed by rearrangement of the left out core structure results in variety of bioactive products also known as hydrolytic products. GSLs show abundant structural diversity due to the presence of a vast variety of side chain structures [3]. These structures are usually associated with protein amino acids such as alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, and methionine (Fig. 1). GSLs are mainly known due to the immense biological properties of their hydrolytic products.

Glucosinolates are broken down into different hydrolytic products by the action of myrosinase (Scheme 1). The hydrolytic products include isothiocyanates (ITCs), nitriles, epithionitriles that depends upon the plant species, side chain substitutions, pH, and ion concentration [4]. These hydrolytic products are responsible for various biological properties of this metabolite. The breakdown products of GSL are not only responsible for the flavor and aroma of plant families but also possess biological activities/properties including action against different organisms (insect, fungi, and bacteria) along with cancer chemoprevention [5]. Among the different hydrolytic products, ITCs are known to possess anticarcinogenic properties in vitro as well as in vivo systems.

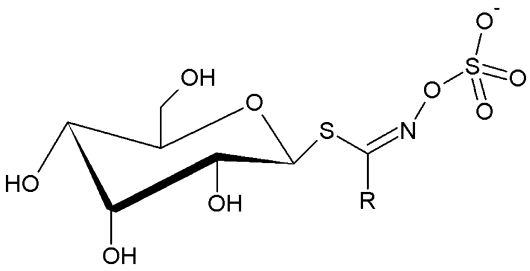
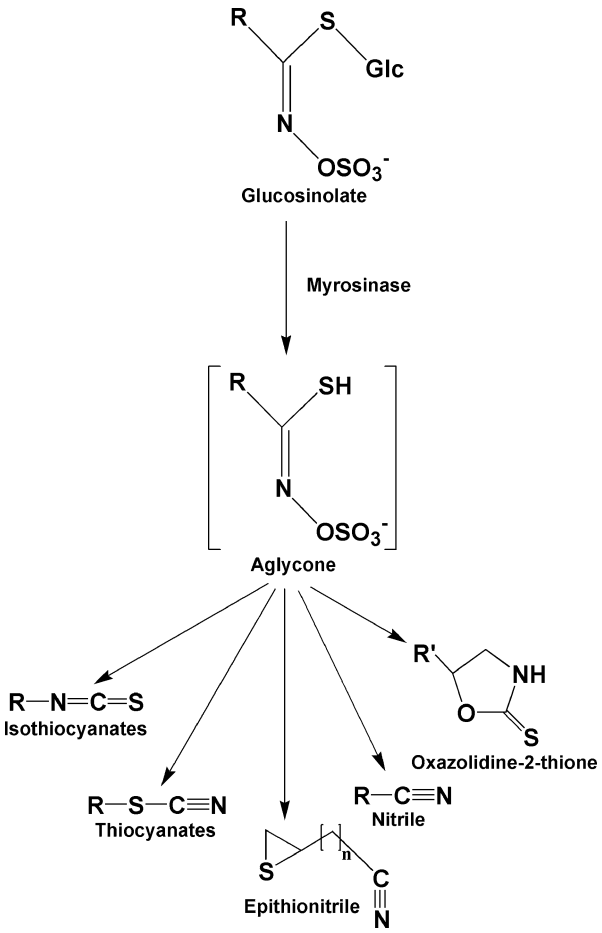


Fig. 1 Structure of glucosinolate

Scheme 1 Formation of glucosinolate hydrolytic products



2 Glucosinolates Biosynthesis

Biosynthesis of glucosinolates has been widely studied for exploring the anticarcinogenic properties of this compound. The biosynthesis of GSLs is classified on the basis of precursor amino acid. GSL synthesis is three step process, firstly amino acid chain elongation, secondly synthesis of glucon from the amino acid, and lastly chain modification (glucon addition) [6].

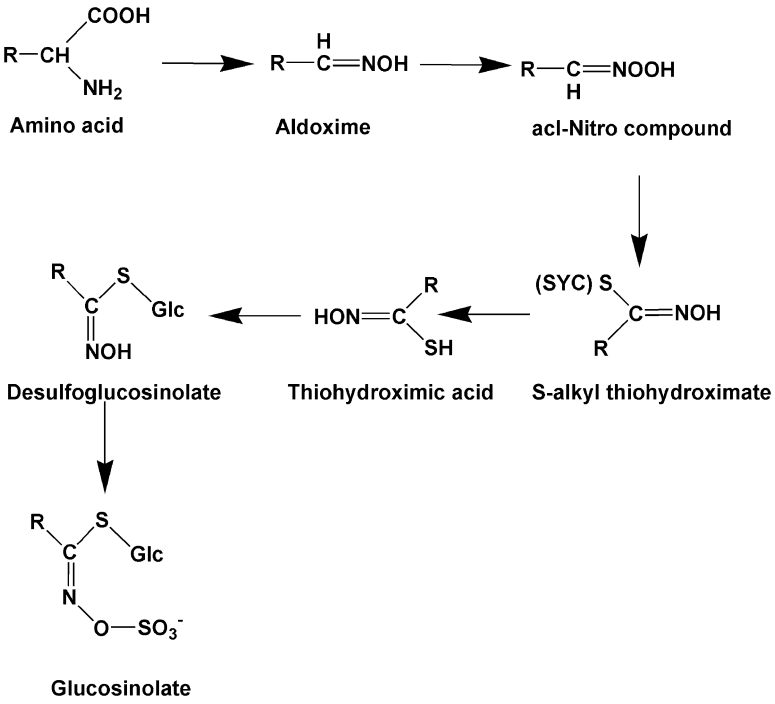
1. **Side chain elongation**
   GSLs are synthesized from chains elongated forms of phenylalanine, valine, and methionine. The synthesis of GSLs is similar to the synthesis of leucine from amino acid valine. The transamination of amino acid results in the production of α-keto acid, followed by addition/condensation of acetyl CoA, and finally by

process of isomerization involved in the shifting of the hydroxyl group and oxidation to ultimately attain elongated keto acid which is further used to obtain amino acid.

2. **Formation of glucone moiety**
   The first step in the formation of glucone moiety is the conversion of amino acid oxime. Several studies have supported that various enzyme are involved in the conversion of different amino acids that are responsible for biosynthesis of GSLs. The above studies are basically carried out on the microsomal systems. The enzymes involved in conversion of tyrosine and phenylalanine to their corresponding oxime are the best example of such conversion involving GSL synthesis. These enzymes are known to be responsible for the formation of oximes that are further required for glycoside biosynthesis. The enzymatic system involved in the chain elongation of methionine and phenylalanine is flavin monooxygenase and that of tryptophan to indole acetaldoxime is peroxidase. Above facts support the involvement of homologous enzyme system in such conversions. The intermediate moieties between the oxime and thiohydroximate have not been observed. It has been speculated that oxime is oxidized to nitro compound which conjugates with cysteine and functions as a thiol source. The above reaction is catalyzed by glutathione-S-transferase resulting in the formation of S-alkylthiohydroximate. In the final step of glucone formation, the thiohydroximate is S-glucosylated by S-GT (UDPG: thiohydroximateglucosyl transferase) to form a desulfoglucosinolate. The above formed product is further sulfated by 3-phosphoadenosine-5′-phosphosulfate (PAPS). Several studies have shown the role of S-GT in sulfonation.
3. **Side chain modifications**
   The side chain modifications of the elongated chains occur following glucone formation. The aliphatic side chains that are attached to methionine are especially modified. The pathway basically includes oxidation to methylsulfinylalkyl followed by the removal of methyl sulfinyl group and desaturation. The above step results in the formation of alkyl glucosinolates which on hydroxylation results in hydroxyl alkenyl glucosinolates. The phenomenon responsible for these modifications has been studied in case of 3-butenyl GSL that is catalyzed by cytochrome P450 hydroxylase. The benzoyloxyalkyl are formed due to the conjugation of hydroxyl alkyl GSL with benzoic acid. Interestingly, the side chains of GSLs from branched amino acid and phenylalanine can be hydroxylated. In addition to this, tryptophan amino acid chains usually adds methoxy group to result in its modification (Scheme 2).

3 Bioactivities of Glucosinolates

The formation of biologically important hydrolytic products such as isothiocyanates, nitriles, epithionitriles, oxazolidine-2-thione, and thiocyanates along with other products from glucosinolates makes this secondary metabolite an important choice for preventing and curing diseases in plants and animals.



Scheme 2 Process of biosynthesis of glucosinolates

3.1 Bactericidal/Antibacterial Activity

Glucosinolate hydrolytic products (GHPs) are known for their bactericidal activities against a wide range of bacterial strains. Among these, ITCs are effective against *Helicobactor pylori* and its related strains [7]. Allyl ITC (AITC) is often used as a preservative in industries. The aryl and alkyl GHPs have shown cytotoxic effects against *Salmonella typhimurium* [8]. Benzyl ITC has been tested for treatment against respiratory and urinary tracts [9]. The level of toxicity and activity against pathogens varies with the type of strain. It has been shown that GHPs present in *Brassica napus* have the potential to inhibit the growth of *Aphomyces euteiches* along with propionibacterium [10]. Glucoraphanine contains sulforaphane that has effective bactericidal action against *H. pylori* and related strains along with strains resistant to antibiotics [11]. Hydrolytic products like AITC, 4-hydroxybenzyl ITC, methyl ITC, 4-(Methylsulfinyl) butyl ITC, phenyl ITC, and oxazolidienethiones showed bactericidal effects against different strains of bacteria such as *Bacillus cereus* IFO-13494, *Bacillus subtilis* IFO-13722, *Salmonella enteritidis* JCM-1891, *Staphylococcus aureus* IFO-12732, *Escherichia coli* 0157:H7, *Helicobacter pylori*, and nitrifying bacteria [12–14]. Literature survey reveals antibacterial activities of most of the GHPs, but their exact mechanism of action is not known. However, the action can be attributed to the tendency

of GHPs to inactivate various intracellular enzymes, thus resulting in oxidative breakdown of sulfur bridges [15].

3.2 Antifungal Activities

The members of family Brassicaceae have the tendency to act against many fungi that are phytopathogenic in nature [16]. Presence of ITCs such as AITC and phenethyl ITC had resulted in antifungal activities of plant extracts belonging to mustard family [17]. Many fungi such as *Pernospora parasitica, Sclerotium rolfsii*, and *Pythium ultimum* were controlled using cabbage tissues due to the presence of GHPs in them [18]. GHPs in addition to this were known to possess antifungal action against *Fusarium culmorum*, *Sclerotinia sclerotiorum*, *Pythium irregular*, *Rhizoctonia solani*, and *Diaporthe phaseolorum* [19]. The fungi acting on wheat were also shown to be inhibited by various GHPs [20]. It has also been observed that *Brassica napus* c.v. inhibited fungal pathogens such as *S. sclerotium* [21]. GHPs have also shown strong fungicidal activity against many fungal species such as *Aspergillus niger, Colletotrichum circinans, Peronospora parasitica, Fusarium graminearum, Rhioctonia solani, Alternaria alternata, Penicillium glaucum*, etc. [22]. The toxicity and antifungal activity of GHPs depends on the chemical structure of ITCs [23]. The mechanism of their action depends on their tendency to enhance and trigger the defence mechanism of plants either by killing the pathogen or by providing protection by creating a plant barrier against respective fungi (pathogen) [24].

3.3 Insecticidal Activity

GHPs are bioactive agents responsible for their insecticidal properties against different pests including nematodes [25]. The ability of these compounds to biodegrade naturally makes it an excellent biofumigant [26]. Aromatic ITCs were found to be effective insecticidal agents against *Otiorhynchus sulcatus.* GHPs have also been reported to act against *Naupactus leucoloma* (weevil larvae) [27]. Inhibitory activity of AITC, benzyl ITCs, methyl ITCs, phenyl ITCs, phenylethyl ITCs, and propenyl ITC have been reported in a number of insects such as *Cyclocephala*, *Musa domestica, Rhyzopertha dominicia, Otiorhynchus sulcatus, Limonius influscatus, Naupactus leucoloma*, and *Drosophila melanogaster* [28–30].

Several reports have suggested nematode inhibitory effect of GHPs. It has been observed that rotation of crops/plants belonging to Brassicaceae family resulted in nematode control [31]. Various studies have demonstrated nematicidal activity against *Tylenchulus semipenetrans, Heterodera glycines, Meloidogyne chitwoodi, M. incognita, M. javanica*, *and Globodera rostochiensis* [32–35]. Plants belonging to Brassicaceae family have also been known to possess herbicidal properties due to the presence of various allelochemicals. Brown and Morra [36] reported the inhibition of growth of *Lactuca sativa* when grown along with *B. napus*. Such results have suggested the ability of GHPs to play important role as a biofumigant [37].

In addition to the above phenomenon, *Brassica* members have shown immense potential to act as a green manure, thus limiting the use of artificial fertilizers [38]. *Vigna unguiculata* when grown in the presence of *Brassica* showed reduction in weeds [39]. Petersen et al. [40] have demonstrated the potential of various ITCs to act against weeds. The *Brassica* species were able to suppress weeds such as *Echinochloa crus-galli* (L.), *Amaranthus hybridus* L. (smooth pigweed), *Sonchus asper* (L.) *Hill* (spiny sowthistle), *Triticum aestivum* L. (wheat), *Echinochloa crus-galli* (L.) Beauv. (barnyard grass), *Alopecurus myosuroides Huds*. (black grass), and *Matricaria inodora* L. (scentless mayweed). It has been proposed that the mechanism of GHPs as a herbicide may be due to their interference and inhibition in protein synthesis and other processes involved in plant growth [40].

3.4 Antimutagenic Activity

Literature survey has revealed that GHPs have strong antimutagenic effect. It was observed that cauliflower extract acted as an effective antimutagenic agent against quninoline induced mutagenicity [41]. It has been seen that broccoli extract administered to male BALB/C strain resulted in the reduction of mutagenicity thus supporting antimutagenic effect of GHPs [42]. Similar results have also been reported during studies involving metabolic activation involving *S. typhimurium* TA 98. A study conducted by Murugun et al. [43] reported that broccoli extracts prepared in ethanol resulted in suppression of mutagenicity caused by respective mutagens on all the strains including TA 98, TA 102, and TA 1535.

3.5 Antiproliferative Activity

GHPs have also been studied for their potential to act against carcinogens and mutagens. These phytochemicals have shown antitumor ability in tissues such as liver, colon, pancreas, bladder, etc. [44]. Hydrolytic products of methyl sulfinyl GSL have been observed to play important role in phase II enzymes induction [45]. Several studies using animal models have supported anticarcinogenic effects of cruciferous vegetables [46, 47]. Toxicity induced by administration of carcinogens in animals has also been observed to decrease following the administration of GHPs [48–51].

4 Glucosinolate Hydrolytic Products Extraction Methods

The GHPs as discussed earlier have an array of biological properties ranging from antioxidant, herbicidal, to anticancer activities. Their immense importance in pharmaceutical industries plays a key role in identifying new and improved extraction methods, which will not only increase their yield but their composition as well. The two major types of extraction methods used for this purpose are cold extraction and hot extraction.

4.1 Cold Extraction Methods

The cold extraction of GHPs is based on the solvent extraction of these important metabolites from different plant sources, viz. root, shoot, leaves, seeds, etc. [52, 53]. In this method, the plant material is washed to remove any dust particle if present. The material is then crushed and immediately added into solvent to prevent any loss of metabolite due to evaporation. The solvent depends on the type of metabolite required, since GHPs can be found in both polar as well as nonpolar phase of solvents [54]. In general, the crushed plant material is first added into hexane for defattening of the material. The time required for defattening depends on the laboratory developed method, but usually 4–24 h incubation in solvent is sufficient for this purpose. For ensuring the proper defattening process, after the initial incubation, the solvent is replaced with fresh hexane and the same is kept for 24 h. Following incubation, both the solvents are combined, filtered through 0.22 μm filter, and evaporated at 30 °C under vacuum using rotary evaporator. The defattened solvent often contains a number of GHPs and hence is also stored for further analysis. After the initial defattening, other solvents relevant to the experiment in hand are used for the extraction of other GHPs. But usually, methylene chloride (DCM) is the most commonly used solvent for the extraction of GHPs. The concentrated extracts are stored in a vial at – 40 °C until further use [55].

In another extraction method by Fahey et al. [56], triple solvents, viz., dimethylsulfoxide, dimethylformamide, and acetonitrile were used. The method involved the homogenization of the plant material followed by the addition of equal volume of the three solvents. The temperature of this mixture was kept at a low temperature of –50 °C to prevent the escaping of GHPs. The triple solvent may also be replaced with dual solvents or more according to the experimental requirements. The GHPs with the specific polarity will be absorbed in the respective solvent and can be separated using separating funnel and dried under vacuum at 30 °C to obtain the GHPs.

In another set of experiments by Arora et al. [55], the plant material was homogenized using distilled water and the extracting solvent was added to it. The extraction solvents were varied in different methods (methylene chloride, diethyl ether, and ethyl acetate). The GHPs were extracted thrice with the extraction solvents and the solvents were pooled and passed through anhydrous sodium sulfate. The extraction solvents were dried using three different drying conditions, viz. air drying, nitrogen gas drying, and rotary evaporator drying. The experiment showed that dichloromethane was the most effective extraction solvent and rotary evaporation as the most efficient drying condition.

4.2 Hot Extraction Methods

The hot extraction methods in contrast to the earlier discussed method requires a higher temperature for the extraction of GHPs from different plant materials. Since the GHPs are volatile in nature, therefore, hot extraction methods are much suitable

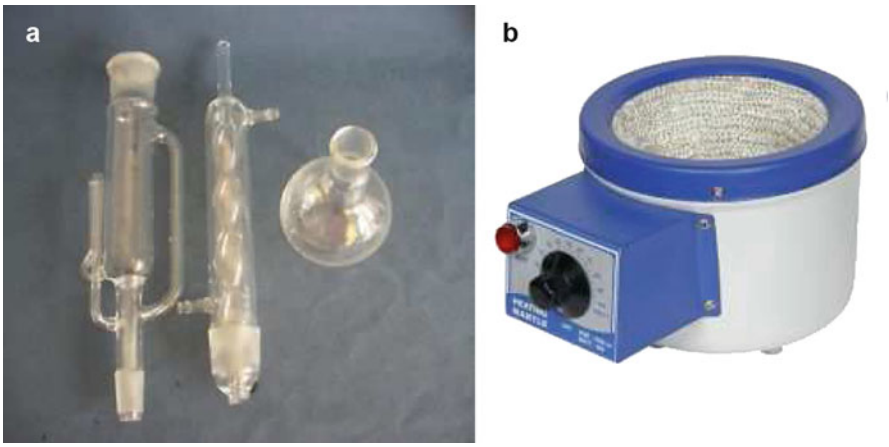


Fig. 2 Soxhlet apparatus, where (**a**) Soxhlet apparatus and (**b**) heating mantle

for the extraction of these valuable secondary metabolites. There are two basic methods for hot extraction of GHPs. The first being Soxhlet extraction in which the crushed plant material (seeds, leaves, roots, etc.) is added in the Soxhlet apparatus (Fig. 2). The desired solvent such as methylene chloride or 80% methanol is added in the round bottom flask. The flask is fitted with Soxhlet apparatus and is heated at temperature ranging from 30 °C to 50 °C using heating mantle for a time period of 3–10 h. The saturated solvent containing GHPs thus obtained is collected and concentrated under vacuum using rotary evaporator [57, 58].

In the other hot extraction method known as hydrodistillation, only one solvent is used, i.e., distilled water. The crushed plant material is added in a round bottom flask and is mixed with distilled water. The flask is fitted with a Clevenger's apparatus and the mixture is heated using heating mantle until boiling (Fig. 3). The temperature is reduced to 60 °C to continue the boiling process for 2–3 h. The condensed volatile GHPs are collected in the outer pipe. Following the completion of the protocol, the extract and water is removed and the GHPs are obtained using either methylene chloride or diethyl ether as solvent [55]. The significance of this method for isolation of GHPs leads to the development of a number of modifications for improving this method for obtaining higher yield in less time as discussed below.

Microwave assisted hydrodistillation is a modification of traditional hydrodistillation in which the heating equipment was changed from heating mantle to a microwave. As per the method given by Wei et al. [59], a microwave was modified to keep the setup including a flat bottom flask and a Clevenger's apparatus inside it (Fig. 4). The microwave was operated at a power of 577 W and for a time period of 37.5 min. The water present in the extract was removed by passing the mixture through anhydrous sodium sulfate. Although this modification provided no improvement in the yield or composition of the extract, but a significant decrease in the extraction time was observed.

Fig. 3 Clevenger's apparatus used in hydrodistillation method

4.3 Other Extraction Methods

Apart from the above mentioned extraction methods involving the conventional hot and cold extraction methods, a number of new alternatives have also been performed to obtain the improved yield and composition. Among these, supercritical fluid extraction is an advanced technique involving the use of supercritical fluids having properties of both liquid as well as gas (Fig. 5). The most common supercritical fluid used by the researchers is CO_2, owing to its low cost, nontoxicity, odorless, and colorless properties. An important aspect of using this fluid is that it can be modified to gas or liquid as per extraction requirements. A comparative extraction of GHPs using supercritical extraction by CO_2 revealed an improvement in the composition and amount [60].

The GHPs may also be extracted in an indirect way by an initial extraction of intact GSLs. This method involves the pulverization of plant material followed by the deactivation of myrosinase enzyme using liquid nitrogen or boiling water. The intact GSLs thus obtained are extracted using different solvents, viz. methanol, ethyl acetate, and methylene chloride. The intact GSLs thus obtained are desulfated in

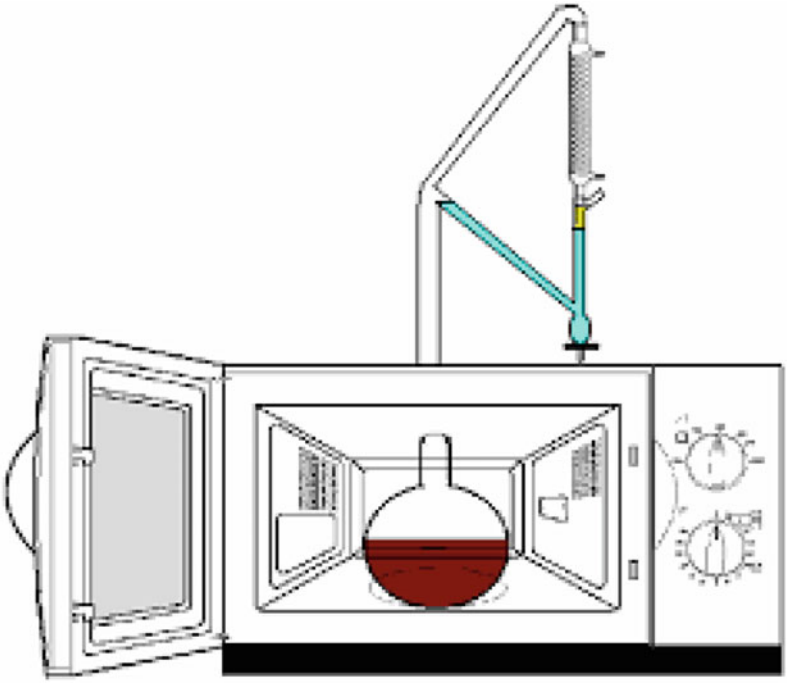


Fig. 4 Setup for microwave assisted hydrodistillation

Fig. 5 Equipment for supercritical fluid extraction

Fig. 6 Indirect extraction of glucosinolate hydrolytic products using DEAE sephadex column

DEAE sephadex column using an external supply of either artificial or natural myrosinase enzyme (Fig. 6). An important advantage of this method is that the type of GHPs required may be modified as per our requirement by changing the column conditions (acidic, basic, or neutral pH) and temperature (low, room temperature, or high) [61].

5 Glucosinolate Hydrolytic Products Analysis Methods

The GHPs extracted by the above mentioned methods can be utilized for a number of biological activities such as antifungal, antibacterial, herbicidal, and anticancer properties. Before utilizing it for any purpose, the analysis of its composition and quantity of each compound is necessary to analyze the synergistic or antagonistic effect of each compound. The analytical methods thus employed range from the most common and traditional methods like paper and thin layer chromatography to much advanced microchip analysis.

5.1 Chromatography Methods

The chromatographic methods involve the analysis of different components of the extract on the basis of polarity and size.

5.1.1 Paper Chromatography

Paper chromatography (PC) is the oldest method during early 1900s for the analysis of GHPs. The thiourea formed following the reaction amid ammonia and isothiocyanates is detected and separated by paper. While the hydrophobic components are analyzed using water saturated chloroform, the hydrophilic components on the other hand involved the mixture of butanol, toluene, and water as mobile phase. The low reproducibility of GHP analysis using this method makes it a difficult choice for analysis [62].

5.1.2 Thin Layer Chromatography

Thin layer chromatography (TLC) is much reliable and repeatable method for the analysis of GHPs. The method uses silica or cellulose gel formed on glass plate as a stationary phase, while different combination of solvents as per the polarity are used as mobile phase. TLC can be used for both qualitative as well as quantitative analysis of GHPs. The repeatability, reproducibility, and the ability to analyze a large number of samples at the same time make this method highly useful for the initial analysis of GHPs [63].

5.1.3 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is the analytical technique mostly preferred for the analysis of GHPs since late 1900s. The accurate results along with an ease of reproducibility and repeatability makes it an idle analytical method till date. The variation in the mobile phase, flow rate, and the column used helps in analyzing almost all the combination of GHPs present in different extracts (Fig. 7). The different compounds present in the extracts are analyzed using different detectors such as photodiode array and the much important mass spectrometer (MS) [64]. The MS attachment makes it easier for the identification of a compound without the presence of any standard by detecting the molecular mass of compound. New and improved versions of HPLC are regularly being formed having all the advantages of the regular HPLC, while solving its limitations. Some of these improved HPLC instruments are ultra high performance liquid chromatography (UHPLC), hydrophilic interaction liquid chromatography (HILIC), and high speed counter-current chromatography (HSCCC) [65, 66].

Fig. 7 High performance liquid chromatography equipment

5.1.4 Gas Chromatography

Gas chromatography (GC) is another commonly employed analytic technique used for analyzing volatile compounds. The volatile nature of GHPs makes GC a suitable technique for analysis. The technique utilizes solid stationary phase and gas mobile phase (Fig. 8). A high initial injection temperature allows volatilization of the solvent and sample. The type of column is often varied to suit the experiment in hand. Sometimes more than one column with varying polarity is used for eluting compounds with different polarity. The mass spectrometer (MS) detector is preferred for the gas chromatography analysis due to its ability to detect the mass spectra of unknown compounds. Other detectors commonly combined with gas chromatography are flame ionization detector (FID) and head space (HS). The use of HS along with GC provides the ability to detect the compounds present in the vapours of sample of interest [47, 67, 68].

5.1.5 Strong Ion-Exchange Centrifugal Partition Chromatography

A comparatively new analytical chromatographic method namely strong ion-exchange centrifugal partition chromatography (SIXCPC) identifies and separates purified GHPs using lipophilic entities generated by strong anion exchanger (Fig. 9). A flow rate of 2 ml/min and a productivity of 3.3 g/h/L_{VC} are attained with this instrument [69, 70]. A modification of this instrument replaces the chromatograph with an extractor and hence the name strong ion exchange centrifugal partition extraction (SIXCPE). This modification allows an astonishing increase in productivity up to 8.5-folds, i.e., 28.3 g/h/L_{VC} [70]. The increased productivity and high

Fig. 8 Gas chromatography equipment

Fig. 9 Equipment used for strong ion exchange centrifugal partition chromatography

purity of GHPs obtained using this method allows its use at industrial scale in near future.

5.2 Non-Chromatographic Methods

There are other methods for the analysis of GHPs which do not require any chromatographic technique.

5.2.1 X-Ray Spectroscopy

X-ray spectroscopic method of GHP analysis is based on the nondestructive detection of sulfur content in the compound (Fig. 10). This content is then compared with the sulfur content of the reference material. A number of factors including the reduction of moisture content and controlled heating are required for the proper analysis of GHPs [71, 72].

5.2.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy utilizes the magnetic properties of GHPs to analyze the physical and chemical properties of these compounds (Fig. 11). The structural properties of the compound are determined by using a nondestructive method. This spectroscopy is also efficient for the analysis of some of the structures which cannot be analyzed by any other method. The difficulty in interpretation, high cost, and time consumption has lead to a decreased interest for this method [73, 74].

5.2.3 Near Infrared Reflectance Spectroscopy

Near infrared reflectance spectroscopy (NIRS) is a reproducible, rapid, and nondestructive analytical method for GHPs (Fig. 12). The compound of interest is

Fig. 10 X-ray spectroscopy equipment

irradiated and the reflected light is measured for analyzing the GHP. The compound is mainly detected on the basis of the bonds present in it. There are three types of bonds detected in this method, i.e., C-H, N-H, and O-H. The spectral data received using this method is converted into chemical data by comparing with the calibration curve [72, 75].

5.2.4 Enzyme Linked Immunosorbent Assay

The enzyme linked immunosorbent assay (ELISA) is extremely specific analytic technique for the detection of GHPs (Fig. 13). Before beginning the analytical method, the GHPs needs to be conjugated with higher molecular weight immunogenic carriers owing to their smaller size. This conjugation helps in invoking the immune response and the immunogenic carrier used is either bovine serum albumin or ovalbumin. The antisera produced against this GHP-carrier conjugate leads to an increased specificity against these GHPs [76].

5.2.5 Estimation of Glucose

The estimation of glucose is an indirect method of analysis of GSLs initially developed for detecting glucose content in urine. This method is based on the reaction between glucose released from GSLs following the reaction with myrosinase enzyme and glucose oxidase impregnated on a paper. The reaction between the two leads to the formation of gluconic acid and hydrogen peroxide. The formed hydrogen peroxidase finally reacts with peroxidase thus activating and changing the color of chromagen present on the paper. This method is employed for both qualitative and quantitative analysis of GSLs [77].

Fig. 11 Nuclear magnetic resonance spectroscopic equipment

Fig. 12 Equipment for near infrared reflectance spectroscopy

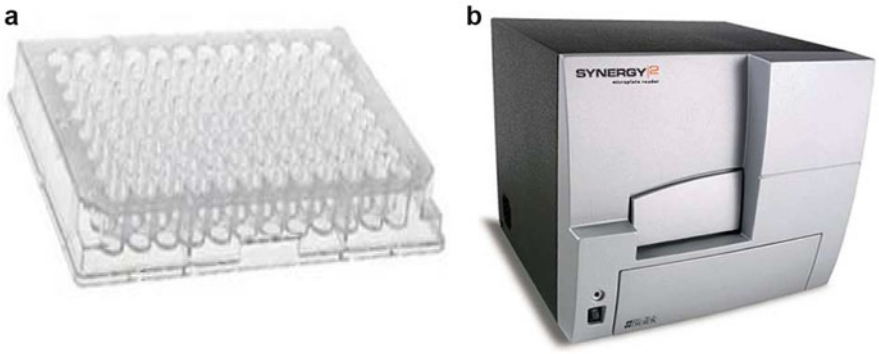


Fig. 13 Analysis of glucosinolate hydrolytic products using enzyme linked immunosorbent assay, where (**a**) 96 well plate and (**b**) biotek synergy HT ELISA reader

5.2.6 Microchip Analysis

The microchip analysis is a nondestructive and automated method employed for both quantitative and qualitative purpose with a small quantity of sample. The analysis is based on the capillary electrophoresis on a microchip. A charge transfer complex is formed between the GSLs and xanthenes dyes. It is a rapid method and requires low quantity of reagents and thus forms a successful alternate to earlier techniques [78].

6 Conclusions

Glucosinolates are important class of secondary metabolites present in a number of plant families. These metabolites are broken down into their hydrolytic products by the action of myrosinase, an enzyme present adjacent to it. The GHPs have an immense use in pharmacological industries due to their wide range of biological properties such as antifungal, antibacterial, and anticancer activity. These properties of this crucial secondary metabolite make it a significant factor for exploring a range of extraction methods, thus improving the yield, composition, time, and cost. As such both cold and hot extraction methods are used for its extraction, but due to the volatility of the compound, the most important method among these is hydrodistillation. The extracted compounds are first required to be analyzed for their content before subjecting it for biological analysis and thus a number of analytical methods were developed to suit the requirement of the researchers. These methods ranged from simple and rapid methods like paper and thin layer chromatography to complex and effective microchip analysis. But currently, the most commonly employed method is gas chromatography equipped with mass spectrometer. This method suits the volatile nature of GHPs and provides accurate results. In nutshell, the selection of extraction and analytical method depends on the problem in hand and the suitable conditions.

Acknowledgment The study was supported by the funding provided by Department of Science and Technology (DST) and University Grants Commission (UGC), New Delhi and Guru Nanak Dev University, Amritsar.

7 Cross-References

► Glucosinolates and Plant Defense
► Investigation of Glucosinolates by Mass Spectrometry

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Processing and Preparation of *Brassica* Vegetables and the Fate of Glucosinolates

14

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Abstract

The healthiness of a vegetable cannot solely be inferred from the amount of health-promoting compounds in the raw materials. *Brassica* vegetables, for example, are consumed mostly after processing to improve palatability and to extend the shelf life. However, processing also results to various changes in the content of glucosinolates which intakes are associated with a reduced risk of several cancers. The large variety in cooking practices and processing methods affect the glucosinolate content in the vegetables, particularly due to processes that allow for enzymatic hydrolysis and thermal degradation of glucosinolates, and leaching of the bioactive components. Knowledge on the effect of preparation and processing of *Brassica* vegetables is important to evaluate the healthiness of the consumed product and to investigate mechanisms to retain high glucosinolate levels at the stage of consumption and to increase the intake of health-protective compounds by the consumer. By using a mechanistic approach, the fate of glucosinolates during different processing and preparation methods and conditions can be explained. Boiling and blanching reduce the glucosinolate content significantly particularly because of the mechanisms of leaching following cell lysis and diffusion, and partly due to thermal and enzymatic degradation. Steaming, microwave processing, and stir frying either retain or only slightly reduce the glucosinolate content due to low degrees of leaching. These methods

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_10

can enhance the accessibility of glucosinolates from the plant tissue. Fermentation reduces the glucosinolate content considerably, the underlying mechanisms are not yet completely clear, but enzymatic breakdown seems to play an important role. Studying the changes of glucosinolates during processing by a mechanistic approach is shown to be valuable to redesign the processing and to reformulate the product for improving health benefits of these compounds.

Keywords
Glucosinolate • Preparation • Processing • Mechanistic approach • *Brassica* vegetable

Abbreviations

ESP	Epithiospecifier protein
GS	Glucosinolate
HPP	High pressure processing
ITC	Isothiocyanate
MW	Microwave

Contents

1 Introduction

Many representatives of the Brassicaceae family are of particular importance as vegetables for our human diet, such as *Brassica oleracea* (e.g., cabbage, cauliflower, broccoli, and Brussels sprouts), *Brassica rapa* (Chinese cabbage, pak choi, and turnip), *Brassica juncea* (Indian and Chinese mustard), *Brassica napus* (rutabaga and swede), and as seasonings and relishes (e.g., mustard and wasabi). *Brassica* vegetables are unique in that they are rich sources of glucosinolates (GSs), sulfur-containing compounds that deliver a pungent aroma and spicy or bitter taste [1–3]. Moreover, GSs are claimed to be bioactive components responsible for many of the physiological health effects proposed for *Brassica* vegetables in different types of studies, including in vitro, animal, human, and epidemiological studies [4, 5].

Most of the vegetables need some kind of treatment, either household preparation or industrial processing, in order to make them suitable and palatable for consumption. The large variety in cooking and processing methods and conditions have in common that they all affect the profile of GSs in the vegetables, particularly due to (bio)chemical and physical processes such as enzymatic hydrolysis and thermal degradation of GSs, and leaching of the bioactive components out of the plant tissue [6]. As a consequence, we can say that the healthiness of a vegetable cannot solely be inferred from the amount of the nutritional and health compounds in the raw materials. Knowledge on the effect of preparation and processing of *Brassica* vegetables is important to evaluate the healthiness of the final consumed product and to investigate ways to retain high GS levels at the stage of consumption and to increase the intake of health-protective compounds by the consumer.

2 *Brassica* Vegetable Intake and Health

Brassica vegetables, like broccoli, cauliflower, Brussels sprouts, and red cabbage, contain significant levels of health-promoting constituents, including vitamins, minerals, fibers, and numerous types of secondary plant metabolites also called phytochemicals. Many phytochemicals from vegetables contribute to the reported antioxidative, anti-inflammatory, anticarcinogenic, and cardiovascular protective effects [7]. One group of phytochemicals occurring almost exclusively in *Brassica* vegetables is the group of glucosinolates [8]. Epidemiological studies showed that the intake of *Brassica* vegetables is inversely associated with the risk of certain types of cancer, including colorectal and lung cancers [9–13].

Glucosinolates (GSs) intake is expected to play a significant role in lowering this risk of cancer. However, in epidemiologic studies, the intake of the vegetables is monitored, the real intake of protective compounds, like GSs, is often an unknown variable. It is demonstrated that many steps in the food production chain, like cultivation, storage, processing, and preparation of vegetables can dramatically affect the content and thus the intake of phytochemicals such as GSs in *Brassica* vegetables [14].

3 Glucosinolates in *Brassica* vegetables

3.1 Occurrence

Glucosinolates (GSs), a group of plant secondary metabolites, contain β-thioglucoside *N*-hydroxysulfates with a sulfur linked β-D-glucopyranose moiety and variable side group (*R*), which usually classifies the aliphatic, aromatic, and indole GSs. These three GS groups are frequently found in *Brassica* vegetable species (Table 1). Moreover, each species of the family Brassicaceae has a distinct GS profile characterized by major GSs as reviewed by Verkerk et al. [5]. Also, different species of the same genus and different cultivars of the same species can

have highly variable GS concentrations. The majority of GSs are found in every plant organ although the concentration and composition of the GSs can vary greatly and can also change during plant development [5]. Usually, a single plant species contains up to four different GSs in significant amounts, while as many as 15 different GSs can be found in lower amounts in the same plant. *Brassica* vegetables occur in different appearances: leafy (e.g., collard green and rocket salad), flowering (cauliflower and broccoli), stems (kohlrabi), roots (radish, rutabaga, and turnips), and buds (Brussels sprouts and cabbage). The content of GSs varies in these different tissues, for example, GS concentrations are higher in the florets than in the stalks of broccoli [5]. The seeds and the sprouting vegetables or cresses, such as garden cress or watercress, usually contain one specific type of GS in substantial amounts. The GS concentrations in vegetables, although often highly variable, are around 1% dry weight in some *Brassica* vegetables.

3.2 Glucosinolate/Myrosinase System

The special feature of GS-containing vegetables is the system of compartmentalization of GSs and the presence of specialized myrosin cells containing the hydrolytic

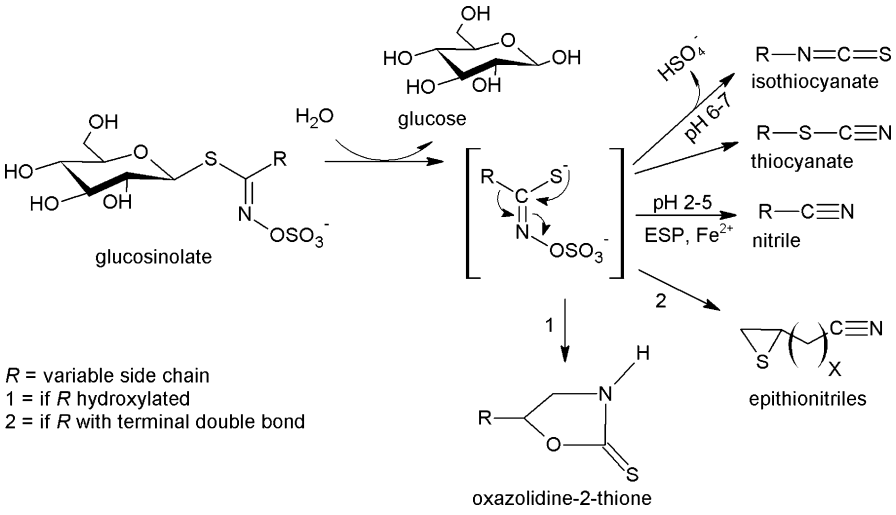
Table 1 Glucosinolates commonly found in *Brassica* vegetables [15]

Trivial name	Chemical name	Main source
Aliphatic		
Glucoibervirin	3-Methylthiopropyl-GS	Green and white cauliflowers
Glucoerucin	4-Methylthiobutyl-GS	Rocket
Glucoiberin	3-Methylsulfinylpropyl-GS	Broccoli sprouts, Savoy cabbage
Glucoraphanin	4-Methylsulfinylbutyl-GS	Broccoli(cress), Red cabbage
Sinigrin	Prop-2-enyl-GS	Brussels sprouts, White cauliflower
Gluconapin	But-3-enyl-GS	Pak choi
Glucobrassicanapin	Pent-4-enyl-GS	Chinese cabbage, Pak choi
Progoitrin	(*2R*)-2-Hydroxybut-3-enyl	Turnip, Chinese broccoli
Indole		
Glucobrassicin	Indol-3-ylmethyl-GS	Broccoli, Cauliflower, and many more
4-Hydroxy-glucobrassicin	4-Hydroxy-indol-3-ylmethyl-GS	Broccoli, Cauliflower, and many more
4-Methoxy-glucobrassicin	4-Methoxy-indol-3-ylmethyl-GS	Broccoli, Cauliflower, and many more
Neo-glucobrassicin	N-methoxyindol-3-ylmethyl-GS	Broccoli, Cauliflower, and many more
Aromatic		
Glucotropaeolin	Benzyl-GS	Garden cress
Gluconasturtiin	Phenylethyl-GS	Water cress

enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147). Upon plant's cell disruption, GSs are highly prone to degradation by myrosinase-catalyzed hydrolysis (see Scheme 1). Subsequently, the GS will degrade into glucose and an unstable aglycon intermediate. The unstable aglycon rearranges into different breakdown products, including isothiocyanates (ITCs), thiocyanates, nitriles, and epithionitriles, depending on conditions described in the Sect. 3.3 [4, 8].

Myrosinase is a relative thermo labile enzyme, which can be readily denatured at moderate to high temperatures. Especially temperatures applied during processing of *Brassica* vegetables quickly inactivate myrosinase [16–19]. The optimum temperatures for the activity of myrosinase are different between *Brassica* vegetables, in the range between 30 °C and 60 °C, and the activity is also influenced by pH, the presence of ascorbic acid, salt, and pressure [20–24]. During the various stages of storage, preparation, cooking, and processing of the vegetables, the GS–myrosinase system is affected in a complex way. Dekker et al. [25] have estimated that the concentration of GSs in *Brassica* vegetables may vary by five to tenfold at each stage.

The health-promoting effect of GSs is mainly attributed to the ITCs that are formed due to hydrolysis by myrosinase after tissue damage. Since myrosinase is mostly inactivated during processing or preparation, formation of ITCs usually does not occur in the product during mastication. However, a myrosinase-like activity is also provided by the microflora in the human's large intestine. Intake of *Brassica* products containing inactive endogenous plant myrosinase still can have benefit by the formation and absorption of bioactive breakdown products by enzymes from the gut flora. However, their bioavailability is lower than the ones with active plant myrosinase [10, 26–28].



Scheme 1 Enzymatic breakdown of glucosinolate [adapted from 15]

3.3 Breakdown Products of Glucosinolates

Although GSs can be chemically degraded at higher temperatures [29–31], the hydrolysis is mainly enzymatically driven. Previous reports have reviewed the mechanisms of GS hydrolysis [e.g., 4, 31–34], which will be briefly described here. Several products of hydrolysis of a GS can be produced, such as ITCs, thiocyanates, nitriles, epithionitriles, oxazolidine-2-thiones, or indole compounds, depending on the structure of the GS side chain, the reaction conditions (e.g., pH), presence of additional cofactors (e.g., Fe^{2+}), and proteins (e.g., epithiospecifier protein (ESP) and thiocyanate-forming protein). Most frequently, the aglycon undergoes a Lossen arrangement to produce an ITC.

At neutral pH, the major hydrolysis products are stable ITCs. For example, hydrolysis of gluconapin and sinigrin produces mainly ITCs, namely, 3-butenyl-ITC and 2-propenyl-ITC, respectively. Sulforaphane, the ITC derived from glucoraphanin, is the most widely studied as the most bioactive GS hydrolysis product. For GSs having a β-hydroxylated side chain or an indole moiety, β-hydroxy-ITCs are unstable and spontaneously cyclize to oxazolidine-2-thiones, while indole ITCs undergo breakdown producing, for example, indole-3-carbinol (I3C).

At low pH, in the presence of an ESP and ferrous ions, gluconapin and sinigrin produce cyano-epithioalkane, such as 1-cyano-3,4-epithiobutane and 1-cyano-2,3-epithiopropane, respectively, and progoitrin is hydrolyzed into an epithionitriles. Nitriles are the major degradation products under acidic conditions, which can be diminished by heating. It is formed after hydrolysis of a GS with a side chain lacking a double bond, which may involve ESP. Conversion to nitriles is also enhanced in the presence of ferrous ions. Indole GSs can form indolyl-3-acetonitrile and elemental sulfur. Moreover, ascorbigen and thiocyanate are the major products of indole GSs between pH 4 and 7 in the presence of ascorbic acid. For thiocyanates production, the mechanism from GSs is not clear yet.

4 Glucosinolate During Preparation of *Brassica* Vegetables

Brassica vegetables are mainly consumed after processing, either at a domestic or industrial level. Broccoli, cauliflower, and cabbage are boiled, steamed, stir-fried, or microwave-processed during domestic preparation to produce various dishes. Canned or fermented vegetables are also produced after industrial processing of these vegetables. Even when consumed raw, for example, in a salad, these vegetables are firstly prepared, namely, by washing, cutting, and chopping. Various products or dishes available around the globe are made based on these vegetables. For examples, many kinds of soup, steamed, and stir-fried *Brassica* produced by domestic preparation and industrial processed products, such as canned and fermented *Brassica* such as sauerkraut, or more local products as *sayur asin*, and *kimchi*. In some Asian countries, the dishes made from these vegetables are usually considered as a side dish to accompany rice or noodle [35].

Postharvest treatments of *Brassica* vegetables, such as cutting or chopping and packaging and storage, can reduce the GS content to a lower degree than the loss due to preparation itself [5, 36–38]. However, a study on storage of chopped cabbage and broccoli was reported to increase the indole GSs, which is suspected as physiological response similar to response due to insect attack [39].

The preparation methods are varied depending on the types of the vegetables, the quality attributes of intended products, and the local customs, particularly for processing at domestic level. At industrial processing level, these are more manageable and standardized. In the Southeast Asian cuisine, for example, cooking vegetables also commonly involves the addition of spices, garlic, chili, salt, sugar, etc., as ingredients for getting the optimum sensorial quality.

Preparation of vegetables is performed to increase the palatability and digestibility, change the sensorial properties (including softening of the texture, improving the appearance and taste), and minimize the risk of microbial contamination. Despite these advantages, preparation can considerably reduce the content of nutrients and phytochemicals in the vegetables including the GS, polyphenols, and ascorbic acid [40–44].

The changes of GSs due to preparation not always have negative implications to health. Although preparation can reduce the GS content, it can at the same time increase the GS accessibility of the product [45]. Depending on the decrease of the content and the increase of the accessibility, the eventual availability of GS for conversion and absorption during digestion can actually be improved by proper preparation.

4.1 Mechanisms Underlying the GS Changes

Previous studies reported a variety of results on the effects of preparation methods on GS content in *Brassica* vegetables. These lead to the complexity to interpret data directly due to the large variability of processing conditions and analytical methods that were used in the various studies. Therefore, a mechanistic approach was proposed to discuss these data by identifying the relative importance of underlying mechanisms affecting GS changes of each preparation method [6, 25].

Either sequential or simultaneous mechanisms take place during preparation (Fig. 1), depending on the processing conditions such as the temperature-time profile. These can involve physical, (bio)chemical reactions, heat, and mass transfer. These different mechanisms can be described as follows:

1. Lysis of cells and cellular compartments
   Cutting or chopping is applied prior to preparation of *Brassica* vegetable. Consequently, vegetable tissue, cells, and cellular compartments are broken. This cell lysis continues during preparation, particularly when heat is applied. During heating of the vegetable, lysis of the cell will gradually occur. Cell and cell organelle membranes will collapse and cell walls will soften. The method of processing and the type of the vegetable determine the degree of lysis.

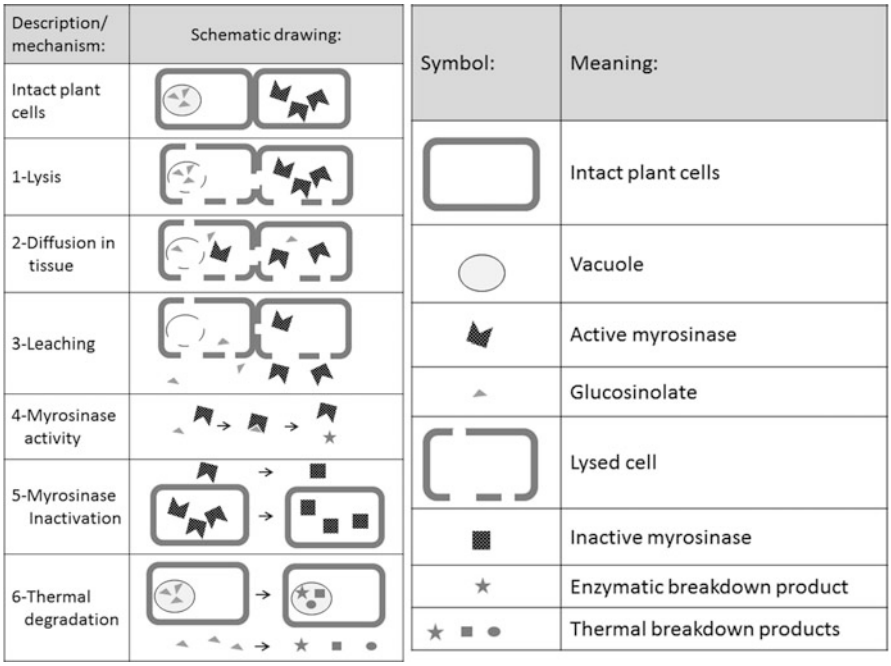


Fig. 1 *Left*: Schematic illustration of the main mechanisms responsible for the changes in glucosinolate content during *Brassica* vegetable preparation. *Right*: Legend to explain the used symbols (Taken from Nugrahedi et al. [6])

2. Diffusion of components through the lysed tissue
   Due to the disruption, components of the cells and cellular compartments, including GSs and myrosinase, will diffuse giving the opportunity for (bio) chemical reactions between these components.
3. Myrosinase-catalyzed hydrolysis of GSs
   Upon lysis and diffusion, myrosinase can have a contact to GSs and the hydrolysis of GSs occurs. The hydrolysis reaction can happen in the lysed tissue and in the cooking water, when the preparation involves water.
4. Thermal degradation of GSs
   Most preparation methods on *Brassica* vegetables apply heat. This is transferred into the plant tissue, for example, by water, steam, or cooking oil. Consequently, GSs can be chemically degraded due to the elevated temperature.
5. Inactivation of myrosinase
   Heat treatment can also cause inactivation of myrosinase, as well as inactivation of the ESP and the thiocyanate-forming protein. Also a loss can occur in the enzymatic cofactors for myrosinase, such as ascorbic acid and Fe^{2+} affecting the outcome of the hydrolysis.
6. Leaching of GSs and breakdown products

When preparation of vegetable involves water, for example, for boiling, GSs as well as the breakdown products will leach into the water, following lysis and diffusion.

To identify the underlying mechanisms involved in each preparation method, all details of preparation conditions, for example, time, temperature, size or weight of *Brassica* vegetables, and water to vegetable ratio, must be taken into account. Hence, the underlying mechanisms of GS changes in each preparation method are specific. For instance, cell lysis, diffusion in tissue, and myrosinase inactivation are identified as the main mechanisms in all preparation methods involving heat. Leaching is identified as the main mechanism affecting GS losses in boiling vegetables, but not for stir frying, short-term steaming, or microwave processing without additional water. Meanwhile, thermal degradation of GS is one of the main mechanisms involved in stir-frying, but also for other preparation methods involving heat this could play an important role depending on the conditions, such as the method (hot vs. cold start boiling), temperature, time, and the size of the vegetable parts. In Sects. 4.2 and 4.3, examples of various conditions between preparation methods will be described further.

Another benefit of using the mechanistic approach is that the GS content and all factors/conditions involved in the preparation can be predicted and optimized quantitatively by applying kinetic modeling. It is a tool to understand what is happening since the proposed mechanisms need to be confronted with experiments. These mechanisms can be subsequently formulated into mathematical equations describing the rate constant of each mechanism. The reaction rate depends on the type of GS and the plant matrix. For more detailed information, previous reports have been studied mathematical modeling of GS changes during preparation of *Brassica* vegetables [46–49].

4.2 Thermal Processing and Preparation Methods

Brassica vegetables are mainly cooked by employing high temperature. Heat treatment affects the changes of GS content mainly by the mechanisms of cell lysis and diffusion followed by thermal degradation of GSs and myrosinase inactivation. Depending on the processing conditions, such as temperature and water to vegetable ratio, other mechanisms can also play an important role, including enzymatic hydrolysis of GSs and leaching.

4.2.1 Boiling, Steaming, Blanching, and Canning

Brassica vegetables are commonly prepared either by boiling or steaming. For a longer preservative effect, canning can also be employed. Meanwhile, blanching is considered as a pretreatment prior to the core processing. In daily practice, blanching is sometimes considered as a light boiling without further cooling. Boiling is performed by immersing the vegetable into cold or already boiling water. Meanwhile, steaming is employed by exposing the vegetable to saturated steam. During

boiling, heat is transferred mainly by convection of hot water into the vegetable tissue. While for steaming, heat is transferred mainly by condensation of steam at the vegetable surface and by convection.

During boiling or water blanching, the heat of water transferred into the vegetable tissue will lead to cell lysis, which subsequently leads to diffusion of GSs and the myrosinase through the lysed tissue. Part of them will leach into the cooking water. Enzymatic breakdown of GSs can occur in the lysed tissue as well as in the cooking water as myrosinase can get in contact with GSs. This is usually expected to be limited since the temperature increases quickly and will inactivate myrosinase rapidly, depending to some extent on the stability of the specific myrosinase that is present in the vegetable. Simultaneously, inactivation of myrosinase and thermal degradation of GSs can occur due to the heat.

These preparation methods were reported to reduce considerable amount of GSs in, for example, broccoli (Table 2), white cauliflower [43], Brussels sprouts, and kale [50, 51]. Typically, leaching is the major factor of the loss of GSs during boiling followed by GS thermal breakdown. Canning will have a great impact on the loss of GSs in the products due to the more severe heat treatment. When boiling is performed at higher pressure than the normal one, a higher degree of GS loss was reported [41], although one study reported no significant difference in turnips greens [52].

Although leaching is the major factor of the loss of GSs during boiling the vegetables, the main part of leached GSs can be recovered in the cooking water. In preparation methods that use this water for consumption (e.g., soups), this leaching is not a loss. The rest of the GS loss is likely due to thermal degradation and enzymatic hydrolysis [37, 53]. Contrary to these findings, some other studies suspected that the mechanism of GS thermal breakdown is more dominant than leaching in reducing the GS content [54, 55].

A high retention of GS after boiling was reported in broccoli and Brussels sprouts [43, 56], most likely due to the large size of the vegetable parts and the short boiling time employed. Short heat treatment can result in less-intensive GS loss due to enzymatic hydrolysis, thermal breakdown, and leaching. Most of the hydrolytic enzyme myrosinase will be inactivated and there might be an increase of GS extractability during analysis. D'Antuono et al. [57] reported that the total extracted GS content was twofold higher in boiled cauliflower compared to raw.

Ranges of GS loss after blanching were also reported in broccoli (Table 2) and other *Brassica* vegetables [50, 53, 58, 59]. Higher loss of GSs than boiling can be expected [60] especially when the ratio of water to vegetable is higher, which will lead to more extensive leaching. Moreover, the differences in type of *Brassica* vegetable and blanching technique could influence the behavior of GSs during blanching. Goodrich et al. [58] have compared total GS contents in broccoli and Brussels sprouts after hot water and steam blanching techniques. The authors reported no significant losses of total GS contents in Brussels sprouts after hot water and steam blanching, but these techniques reduced total GS contents in broccoli significantly.

Table 2 Effect of boiling, steaming, and blanching on GS retention in broccoli

		GS retention (%)				
Temperature (C)	Time (min)	Aliphatic	Indole	Aromatic	Total	References
Boiling						
Boiling water	30	17.0	n.a.	50.0	19.4	[38]
	8	126.9	70.5	n.a.	95.3	[43]
	3	126.6	76.1	44.0	90.5	[56]
	5	54.2	51.7	50.0	53.5	[61]
	2; 5	70.3–85.5	71.3–97.5	n.a.	70.4–88.1	[64]
	5	58.8	40.7	n.a.	53.5	[65]
n.a.	10–15	41.5	58.1	Traces	44.2	[50]
	5	56.8	18.4	Up	25.5	[41]
Steaming						
Boiling water	15	124.3	147.0	n.a.	137.0	[43]
	3.5	111.4	104.5	Up	107.1	[41]
	2; 5	93.9–106.9	94.6–119.3	n.a.	96.8–109.6	[64]
	5	93.3	63.2	n.a.	84.5	[65]
100 (oven)	13	133.7	143.0	n.a.	138.9	[43]
Oven	n.a.	89.5	131.9	n.a.	130.8	[62]
~20–~100	2–30	91.5–136.5	44.8–135.1	n.a.	78.9–131.8	[17]
Blanching						
80	3	64.7	97.2	Traces	69.9	[50]
99	4	22.4	11.5	n.a.	17.0	[58]
Steam: 99–102	5.5	72.2	47.2	n.a.	60.1	

n.a. not available

Thus, preparation time and temperature, the ratio of vegetable to water, the preparation method, and the type and geometrical shape of the vegetable tissues are the factors strongly affecting the behavior of GS content during boiling and water blanching.

For steaming, low magnitude of GS loss is expected, since there is no direct contact between the vegetable tissue and the boiling water. The rate of cell lysis, diffusion, leaching, enzymatic breakdown, and thermal degradation are lower than the ones during boiling. Previous studies reported no significant effect of steaming on total GLS content in cabbage and broccoli, cauliflower, and Brussels sprouts [16, 26, 38, 41]. Steaming can increase the accessibility of GSs in cauliflower and broccoli [57, 61, 62]. Nugrahedi et al. [63] reported that duration of steaming affects the behavior of GS content. Total GSs in white cabbage was found to increase during steaming for 10 min followed by a decline during long-term steaming for 180 min. This can be explained by leaching of the GSs to the condensated water layer on the vegetables that is constantly refreshed by condensation and dripping.

4.2.2 Frying

There are two main methods of frying of food, namely, shallow (stir) and deep-fat frying. Stir-frying is commonly performed to prepare *Brassica* vegetable. By using a small amount of hot oil, the vegetable is stir-fried at high temperature for several minutes. This is a very common preparation method to prepare vegetables in Asian countries, usually performed by using a pressure burner to produce a powerful flame used for cooking. Small amounts of water might be added during stir-frying, depending on the local custom, type of *Brassica* vegetable, and the expected product.

Heat from the hot surface of the frying pan is transferred through a thin layer of hot oil to the vegetable. The surface temperature of the vegetable rises rapidly and a proportion of water is vaporized [66]. Compared to other thermal preparation methods, stir-frying applies high temperature of oil and shorter preparation time. Therefore, most of myrosinase is expected to be inactivated. However, the temperature of the main part the vegetable tissue will not exceed 100 °C for the short frying time usually applied since the tissue will still contain most of its water. Overall, low degrees of cell lysis and diffusion, leaching, thermal degradation of GSs, and myrosinase hydrolysis can be expected during short-time stir-frying. On the contrary, deep frying to lower water contents will reduce considerable amount of GSs due to thermal breakdown at the higher product temperatures.

Stir-frying was found to retain the GS content in green cabbage, broccoli, Brussels sprouts, and cauliflower [38, 56] and even increased the extractability of GS in Chinese cabbage [67]. However, Yuan et al. [65] observed stir-frying of broccoli reduced considerable amount of the aliphatic and indole GSs by about 55% and 67%, respectively. Possibly this is due to extensive time-temperature employed. When an amount of water is added during stir-frying, leaching does not significantly contribute to degrade GS content in broccoli as compared to the one without additional water. The authors suspected that thermal breakdown of GSs due to high temperature of stir-frying affects more than leaching [65]. In another study, the effect of various times and temperatures on the GS changes during stir-frying of Chinese cabbage and pak choy was not clearly observed [67].

4.2.3 Microwave Processing

Brassica vegetables contain dissolved ionic contents and considerable amount of water. During the absorption of microwave (MW) energy, the vegetable is heated by rotation of the dipolar water molecules and translation of the ionic components. Heat generated within is transferred throughout the tissue by conduction [68]. The mechanisms affecting the fate of GS content mainly are cell lysis and diffusion, inactivation of myrosinase, and to some extent thermal degradation of GSs.

Magnitude of GS changes is affected by processing time and applied MW power. The longer the processing time, the more the plant cell lysis and thermal degradation will occur. At moderate temperature, myrosinase activity will increase and inactivation will occur rapidly at higher temperature [45]. When considerable amount of water is added to the vegetable, leaching during MW processing can be expected.

Table 3 shows that MW processing affects to various degrees of GS retention in broccoli, depending on the output power and MW time. Fuller et al. [69] and Song and Thornalley [38] reported that MW processing can retain GS content in cabbage, Brussels sprouts, and cauliflower. Verkerk and Dekker [45] found that the combination of output powers, i.e., 180, 540, and 900 W, and various processing times, i.e., over 24 min, lead to little loss of GSs in red cabbage, while some treatments increased the extractable GS content. Therefore, higher accessibility of GS of the plant tissue can be expected during MW heating.

Loss of GSs was reported when water was added for MW processing [41, 64, 70]. Vallejo et al. [41] reported that MW processing for 5 min at 1000 W caused a loss of total GS content of broccoli florets by about 74%, but the recovery of total GSs was only about 1% in water. Although the amount of evaporated water was not reported, GS thermal degradation may play significant role during MW. When a considerable amount of water is lost from the vegetable tissue, the temperature by the MWs can easily increase to values substantially above 100 °C inducing rapid thermal breakdown. A similar microwave processing condition on broccoli, however, reduced only about 18% of total GS content and parts of this loss were recovered in the cooking water [70]. Although the amount of additional water was considerably small, the great loss of total GSs in broccoli by about 60% after microwave processing for 5 min was also reported when a high power of 1000 W was employed [65].

4.3 Other Processing Methods

4.3.1 Chilling and Freezing

Brassica vegetables can be minimally prepared by cutting or chopping followed by packaging and storing at chilling temperature at 0–5 °C. The products are ready to cook or can be prepared as salads. The combination of low temperature and modified atmosphere packaging can reduce the rate of biochemical and microbiological changes, hence extending the shelf life.

Changes of GSs can be expected due to mechanisms of cell lysis and diffusion, which will lead to enzymatic hydrolysis reaction between GSs and myrosinase. The amount of cell damage due to cutting will depend on the size of the vegetable parts, but is expected to be a low fraction of the total amount of cells in the tissue. Refrigerated storage is expected to inhibit the rate of reaction. Storage conditions at low temperature (<4 °C) and high relative humidity can maintain cellular integrity and preventing the contact of myrosinase and GSs and hence, can retard the loss of GSs [36].

Meanwhile, freezing applies temperatures below the freezing point of the cellular moisture. A proportion of water in the vegetable undergoes a change in state to form ice crystals that can penetrate the cell membranes and cell walls thereby lysing the cells. During frozen storage, this will not cause big changes, but upon thawing rapid GS hydrolysis by myrosinase can occur if the enzyme was not inactivated by blanching prior to the freezing. The extension of shelf life is acquired by a

Table 3 Effect of microwave processing on GS retention in broccoli

			GS retention (%)				
Power (W)	Time (min)	*Brassica* weight (g)	Aliphatic	Indole	Aromatic	Total	References
300	30	10 specimens (floret + stem 2.5 cm)	97.7	110.4	n.a.	102.2	[43]
500; 700; 1000	2.5; 5	150	77.4–110.5	57.6–93.8	62.4–87.1	64.3–97.7	[70]
1100	2; 5	150	83.6–100.0	86.5–118.7	n.a.	83.9–104.1	[64]
1000	5	150	15.9	27.4	Traces	25.5	[41]
1000	5	200	39.7	46.9	n.a.	41.8	[65]
900	0.5–3	20–30	n.a.	n.a.	n.a.	Not sig. loss	[38]

combination of low temperatures, reduced water activity, and pretreatment by blanching whenever applied. Compared to chilling, freezing can retain more GS content of *Brassica* vegetables.

In general, GS content can be best maintained by freezing, provided that myrosinase was inactivated prior to freezing [36]. Freezing the blanched broccoli at −18 °C within 20 min did not substantially change the total GS content but reduced myrosinase activity by 93%. Subsequently, during storage at −20 °C for 90 days, the GS content was generally unaltered [56]. Accordingly, Volden et al. [71] found no significant effects of frozen storage of cauliflower at −24 °C for 12 months on the total GS content. Another study [50] reported that prolonged freezing of blanched *Brassica* vegetables at −22 °C for 48 h did not produce any consistent changes in total GS content. Losses of total GS contents relative to the blanched vegetables were 50.7% and 4.5% in frozen Brussels sprouts and curly kale, respectively. In contrast, total extractable GS contents in frozen green cauliflower and broccoli increased by 20.9% and 28.5%, respectively [50].

The matrix structure of the vegetable tissue can also affect the magnitude of GS changes. Loose structure of the broccoli stalk and flower head are very susceptible to the leaching effects during prior blanching; hence, freezing the blanched broccoli at −20 °C retained the total GS content in the principal inflorescences but significantly decreased the total GS content in the secondary inflorescences [72].

Freezing can rupture plant cells and soften vegetables because of water crystallization in extracellular and intracellular spaces within the vegetable matrix. Freeze-thawing fracture of plant cells can disrupt the vegetable tissue to cause extensive cell lysis and diffusion. Subsequently, this will give accessibility of myrosinase to hydrolyze GSs during thawing and eventually cause significant loss of GSs. Song and Thornalley [38] reported that storage of broccoli, Brussels sprouts, cauliflower, and green cabbage at −85 °C for 2 months without prior blanching caused significant loss of GSs upon thawing.

Blanch-freezing as a treatment prior to boiling can enhance the extension of cell lysis and diffusion. This will lead to a high degree of GS loss after boiling [43]. However, Rungapamestry et al. [56] reported no significant change of aliphatic and aromatic GS contents after stir-frying of broccoli when prior blanch-freezing was applied.

4.3.2 Drying

Air drying commonly applies circulation of hot dried air on the surface of the food causing removal of some amount of moisture. High temperature during drying is expected to soften the plant tissue and induce cell lysis and diffusion of components. GS loss will be influenced by enzymatic hydrolysis and thermal degradation [73, 74].

Mrkic et al. [75] reported that different combinations of temperatures at 50 °C through 100 °C and velocities of drying air at 1.2 through 2.25 ms^{-1} affect individual GS in broccoli differently. Compared to the blanched treated only, the remaining GSs after drying are 32–90% for 4-hydroxy-glucobrassicin, 65–92% for glucobrassicin, 29–90% for 4-methoxy-glucobrassicin, and 36–92% for

neoglucobrassicin. Meanwhile, Jin et al. [76] reported that mild air drying of broccoli with the constant air temperatures at 40 °C and 50 °C can retain the glucoraphanin content.

4.3.3 Fermentation

Fermentation is depending on the growth and metabolic activity of lactic acid bacteria, either spontaneously or starter-induced. Salt is usually added to inhibit the growth of undesired microorganisms during the production of fermented products [77]. Sauerkraut is a popular fermented product of *Brassica* vegetable. Some examples of other locally produced fermented *Brassicas* especially from Asia are *Brassica kimchi* from Korea, *dakguadong* from Thailand, *nozawana*-zuke from Japan, *suan-tsai* from Taiwan, and *sayur asin* from Indonesia [21, 78–80].

Compared to the preparation methods on *Brassica* vegetables previously described, the underlying mechanisms affecting GS changes are different. Depending on the production method of fermentation, cell lysis and diffusion, enzymatic hydrolysis, and leaching can occur, which will lead to GS changes. Moreover, changes of GS content in *Brassica* during fermentation can be affected by the type and activity of bacteria, concentration of salt, pH, and fermentation substrate and temperature. Tolonen et al. [77] and Suzuki et al. [21] reported that bacteria and sodium chloride influence on the changes of GS in *Brassica* vegetables during fermentation. Moreover, a concentration of 500 mM NaCl and pH at below 5.5 inactivated myrosinase when analyzed in vitro [21].

In general, fermentation reduces the GS content significantly. In sauerkraut production and storage, there was no GS detected in the product, irrespective of cultivation season on the cabbage, type of fermentation, and concentration of salt [81–83]. The breakdown products of GS were detected, such as ITCs, cyanides, indole-3-carbinol, indole-3-acetonitrile, and ascorbigen [77, 81–83]. It is suspected that the content of the degradation products is not only influenced by the content of the native GS in raw cabbage, but also by to physicochemical properties, such as volatility, stability, and reactivity in an acidic environment, and microbiological stability [81]. Nevertheless, further studies are needed to explain the underlying mechanisms of GS changes during fermentation.

Sarvan et al. [84] and Nugrahedi et al. [85] showed that inactivation of myrosinase prior to the fermentation resulted in an increased retention of GSs in the final product. Heat treatment (i.e., blanching) was applied to the cabbage followed by fermentation (at 25 °C, 4% brine) by *Lactobacillus paracasei* LMG P22043. This treatment retained 35% (27.2 ± 2.3 μmol 100 g^{-1}) of total GSs after fermentation for 71 h as compared to the one before fermentation. Moreover, during refrigerated vacuum storage for 30 days, 23.7 ± 1.5 μmol 100 g^{-1} of GSs still retained [84]. In another fermentation study, raw Indian mustard was withered by microwave processing at 900 W for 2 min to fully inactivate myrosinase. The concentration of sinigrin, the most dominant GS in Indian mustard, can be retained at 30% of the one in the withered leaves, while common fermentation led to considerable loss of GSs. After 7 days of fermentation, about 13% of sinigrin still can be retained [85]

4.3.4 High Pressure Processing

High pressure processing can prolong the shelf life of food by destroying microorganisms. High pressures cause collapse of intracellular vacuoles and damage to cell walls and cytoplasmic membranes [66]. Besides killing microorganism, combination of high hydrostatic pressure and mild temperatures can be an alternative to thermal processing to retain the health-promoting compounds. Thus, treatment on *Brassica* vegetables by a high pressure and temperature combination gives an advantage over other conventional preparation methods [86].

Changes of GS during HPP can be expected due to mechanisms of cell lysis and diffusion, enzymatic breakdown of GS, and possibly also leaching. Nevertheless, the accessibility of GS from the matrices can be improved by this method.

Van Eylen et al. [86] reported that mild pressure processing of broccoli can induce the GS hydrolysis. Moreover, loss of 20% of GS was observed after 35 min of elevated pressure, at 200–300 MPa, and at 20 °C combined treatments. When temperature was increased at 40 °C and the range of pressure was 100–500 MPa, the GS degradation was observed after 15 min, and the greatest GS loss at 63% was obtained at 300 MPa. Thus, the parameters of the process, namely, time, temperature, and pressure, can be varied in order to obtained different amounts of health beneficial products.

5 Product and Process Design

To obtain the highest GS content possible in the processed product as well as to increase the bio-accessibility and bioavailability, the optimization of food preparation and processing methods is inevitably important. At primary production, efforts on cultivation and plant breeding have been performed to increase the level of GSs in fresh *Brassica* vegetables, such as "BroccoCress®" and "Beneforte®." However, since both culinary preparation and industrial processing considerably influence the fate of GSs in the product, there is a need to (re)design and (re)formulate the processing and preparation conditions.

To increase the GS intake in the product, as previously described in Sect. 4.3.3, Sarvan et al. [84] have been redesigned the fermentation method of sauerkraut by inactivation of myrosinase prior to fermentation. Similarly, myrosinase was inactivated prior to fermentation of Indian mustard (*Brassica juncea*) to produce *sayur asin*, a local fermented *Brassica* commonly produced in Asia. [85]. These studies indicate that enzymatic hydrolysis plays an important mechanism underlying the loss of GSs during fermentation. The reformulation strategy was employed during pasta-like production by adding broccoli powder. It was reported that the nutritional function, in terms of GS content, in the pasta and noodle can be improved by enrichment up to 20% (v/v) broccoli powder, and no negative effects on acceptability were observed [87].

To increase the bioavailability, Oliviero et al. [88] have been designed a mild air drying technique to obtain powdered broccoli containing high GSs as well as retaining active myrosinase. By optimizing temperature trajectories, broccoli

product with fully retained GSs and partially retained myrosinase can be obtained. In Sect. 4.3.4, it was shown also that HPP [86] can be considered as a promising technique to increase the bioavailability of GS breakdown products. Moreover, in order to optimize processing and preparation methods, mathematical modeling of the simultaneously occurring mechanisms that influence the fate of GSs can be a valuable tool [25].

6 Conclusions

Processing and preparation have a significant impact on the content and accessibility of GSs in *Brassica* vegetables. Different preparation methods lead to various degrees of GS changes. In general, boiling and fermentation considerably reduce the amount of GSs, while short-term steaming, microwave processing, and stir-frying can retain GS content. Moreover, these preparation methods can increase the accessibility of the compounds from the plant matrix.

A mechanistic approach is valuable to explain and describe the behavior of GSs in *Brassica* vegetables during preparation. By employing this mechanistic approach underlying the GS changes during processing alternative procedures or conditions can be redesigned to improve the retention of GSs. Moreover, reformulation can also be performed to modify the product properties in such a way that the intake of health-promoting GSs increases. Redesigning process and reformulating product can contribute to the aim of improving the diet, especially by employing mathematical modeling techniques.

In addition, by understanding the behavior of GS in Brassica vegetables during processing, a more accurate estimation of the dietary intake of GSs in prepared dishes can be performed. This estimation is important for establishing the relation between intake of phytochemicals and health effects like reducing the risk of certain diseases. However, since GSs do not have health protection effect but the breakdown products, it will be useful to further investigate the breakdown products of GSs in *Brassica* vegetables prior to consumption and the bioavailability of these compounds.

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Investigation of Glucosinolates by Mass Spectrometry

15

Giuliana Bianco, Raffaella Pascale, Filomena Lelario, Sabino A. Bufo, and Tommaso R. I. Cataldi

Abstract

There is an ever-increasing interest in the biological effects of glucosinolates (GSLs), due to their anticarcinogenic properties and potential to contribute to dietary phytonutrient consumption, thereby improving human health and well-being. Cruciferous vegetables are unique in that they are rich sources of GSLs. Several epidemiological studies have shown that a high intake of cruciferous vegetables, for example, cabbage, broccoli, or Brussels sprouts, has beneficial influence on human health. A new window of investigations has been opened by mass spectrometry (MS) and its combination with the various chromatographic techniques has proved to be highly successful to gain reliable data about the presence and abundance of GSLs in vegetables. The purpose of this chapter is focused on the methods that are currently available for their qualitative and quantitative analysis by MS and tandem MS (MS/MS). Emphasis is placed on the description and value of existing methods as well as on the many MS applications reported for GSL analysis.

Keywords

Glucosinolates • Isothiocyanate • Mass spectrometry • Tandem mass spectrometry • High resolution

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J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3_12

Abbreviations

APCI	Atmospheric pressure chemical ionization
CE	Capillary electrophoresis
CID	Collision-induced dissociation
CZE	Capillary zone electrophoresis
DART	Direct analysis in real time
ESI	Electrospray ionization
FAB	Fast atom bombardment
GC	Gas chromatography
GLC	Gas–liquid chromatography
GSLs	Glucosinolates
HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IRMPD	Infrared multiphoton dissociation
ITCs	Isothiocyanates
ITMS	Ion trap mass spectrometry
LC	Liquid chromatography
LC-ESI	Liquid chromatography–electrospray ionization
MALDI	Matrix-assisted laser desorption/ionization
MS/MS	Tandem mass spectrometry
MECC	Micellar electrokinetic capillary chromatography
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSI	Mass spectrometry imaging
NMR	Nuclear magnetic resonance
RP-HPLC	Reversed-phase high-performance liquid chromatography
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
TIC	Total ion current
TOF	Time of flight
XIC	Extracted ion chromatogram

Contents

1 Introduction

Glucosinolates (GSLs) are nitrogen- and sulfur-containing natural compounds that impart a pungent aroma and spicy/bitter taste [1]. GSLs are plant secondary metabolites, mostly common in the order Capparales, which includes the Brassicaceae family with agriculturally important crops, *Brassica* vegetables, and the model plant *Arabidopsis thaliana*. Many commonly consumed cruciferous or *Brassica* vegetables come from the *Brassica* genus, including canola, rapeseed, broccoli, Brussels sprouts, cabbage, cauliflower, collard greens, kale, kohlrabi, mustard, rutabaga, turnips, bok choy, and Chinese cabbage [2]. Rocket salad, horseradish, radish, wasabi, and watercress are also cruciferous vegetables, well recognized as rich in GSLs. The accumulating evidence of an association between disease prevention and GSLs in the diet has stimulated a great deal of scientific interest in these plant metabolites [3–6]. These vegetables have indeed engendered the interest of researchers as a potential source of substances that may find useful effects in the prevention of oncological diseases. Apparently, the antitumor properties of cruciferous edible plants are linked to the occurrence of GSLs, which are decomposed into isothiocyanates (ITCs) and indole compounds in the presence of myrosinase enzyme [7].

All glucosinolates share a common structure consisting of a β-thioglucose moiety, a sulfated oxime group, and a variable aglycone derived from an α-amino acid [8, 9] (Fig. 1). Both GSLs and their enzymatic products exhibit biological activities, which are crucial from the point of view of chemoprevention of neoplasms [10]. It is, therefore, important to identify and quantify these compounds in vegetable tissues. Several methodologies have been developed to quantify total and individual GSLs in plants, such as measurement of the GSL enzymatic breakdown products, named as desulfated GSLs, and intact GSLs [11]. The most important technique for GSL

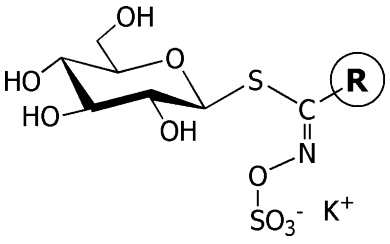

Fig. 1 General structure of GSLs as potassium salts. Every glucosinolate contains a central carbon atom, which is bound via a sulfur atom to the thioglucose group and via a nitrogen atom to an O-sulfated anomeric (*Z*)-thiohydroximate function. In addition, the central carbon is bound to a side group R; different glucosinolates have different side groups, which are responsible for the variation in the biological activities of these secondary metabolites

analysis is based on mass spectrometry (MS) [12] because of its great selectivity, high sensitivity, and the possibility to perform multicomponent and profiling of plant extracts. Detection and, in most of the cases, the identification of unknown and untargeted compounds is the main mission of MS. More than 120 different GSLs are known to occur naturally in plants, and for this reason the combination of separation techniques (e.g., liquid chromatography) with MS has tremendously expanded the chemical examination capability of complex biological samples containing highly polar compounds.

The basic information available from mass spectra is characterized by its simplicity. The spectrum displays monoisotopic masses of the ionized molecule, calculated using the sums of the masses of the component atoms. Each monoisotopic ion that is used for accurate determinations is always accompanied by additional isotopologue ions (A + 1, A + 2, A + 3, etc.). The set of these signals is called isotopic pattern. We must take care, however, to not confuse the monoisotopic mass from the molecular weight, which is the sum of average atomic weights. An example of GSL mass spectrum is shown in Fig. 2 in which the simulated mass spectrum of glucoraphanin, in its anionic form, is presented.

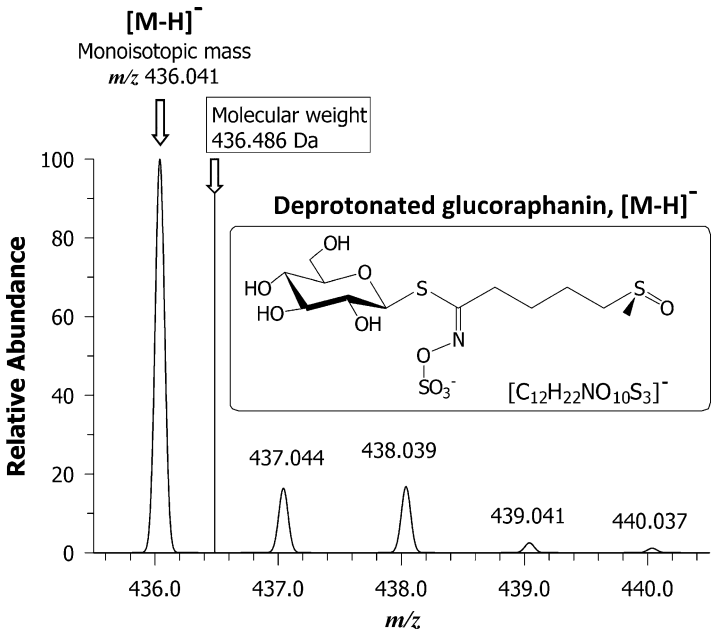

Fig. 2 Molecular weight (436.486 Da) and monoisotopic mass at *m/z* 436.041 of glucoraphanin ($[C_{12}H_{23}NO_{10}S_3 - H]^-$) taken as an example of glucosinolate in a simulated mass spectrum. The *m/z* values of isotopologue ions A + 1, A + 2, A + 3, and A + 4 at 437.044, 438.039, 439.041, and 440.037, respectively, are indicated (Note the difference between the molecular (average) mass and the monoisotopic exact mass value. The monoisotopic value is an even number due to the presence of a single nitrogen atom in the structure. Glucoraphanin is also known as 4-methylsulfinylbutylglucosinolate)

We herein describe the most common mass spectrometric methods used for GSL analyses. Our aim is to highlight recent developments with a focus on the advantages of MS as a stand-alone technique or combined with a separation system. Because sample preparation has a major impact on the results obtainable, a short discussion of GSL extraction and purification is also included.

2 Sample Preparation of GSLs

For GSL analysis, it is important to prevent analyte loss from the plant matter and to have a good extraction procedure able to take out all or the maximum number of compounds in their original state and in a quantitative manner [13]. Cutting, chewing, boiling, or fermenting destroys the cell structure of cruciferous vegetables; GSLs are hydrolyzed by myrosinase when heated at 55–65 °C [14–16]. Therefore, when GSL is to be determined, vegetables should be stored at −20 °C until analysis.

Extraction step aims to reduce myrosinase activity, dissolve a maximum number of metabolites from the plant cells, and ensure a minimal loss of the GSLs to be extracted. The myrosinase is denatured in organic solvent and heating, so most extractions are performed in ethanol/water (50/50) or methanol/water (70/30) at 70 °C [17–22]. Numerous protocols for the extraction and analysis of GSLs have been published: various parameters such as solvent composition, temperature, and number of required extraction steps were optimized [23].

As far as MS imaging (MSI), no GSLs extraction is needed and the sample preparation is very different; it consists in sample sectioning (if applicable) and matrix deposition to the sample surfaces under investigation [24].

3 Analysis of GSLs by Mass Spectrometry

The application of MS to GSL analysis has grown dramatically over the last two decades, and today MS is the sole most valuable detection mode. Especially the introduction of ESI and APCI as atmospheric pressure ionization (API) sources in the late 1980s has revolutionized the bioanalysis of small molecules. There is a great consensus that MS plays an important role in the chemical analysis of metabolites mainly because of its accessibility, versatility, and powerful technology that are best-suited to solve research and analytical problems in most fields including plant metabolites. Figure 3 illustrates the main different options available to address the GSL analysis by MS. Moreover, MS combined with a separation technique offers tremendous opportunities for analysis of complex samples because it enables the determination and identification of a large number of metabolites in a single run. For GSL analysis, two issues must be considered in connection with choosing an MS-based method: (i) scope/resolution, because the GSLs comprise a large number of compounds with distinct physicochemical properties, and (ii) sensitivity, referring mainly to very low concentrations. In the last decades, to increase the selectivity of the MS analysis, mass tandem spectrometry (MS/MS) has been successfully employed.

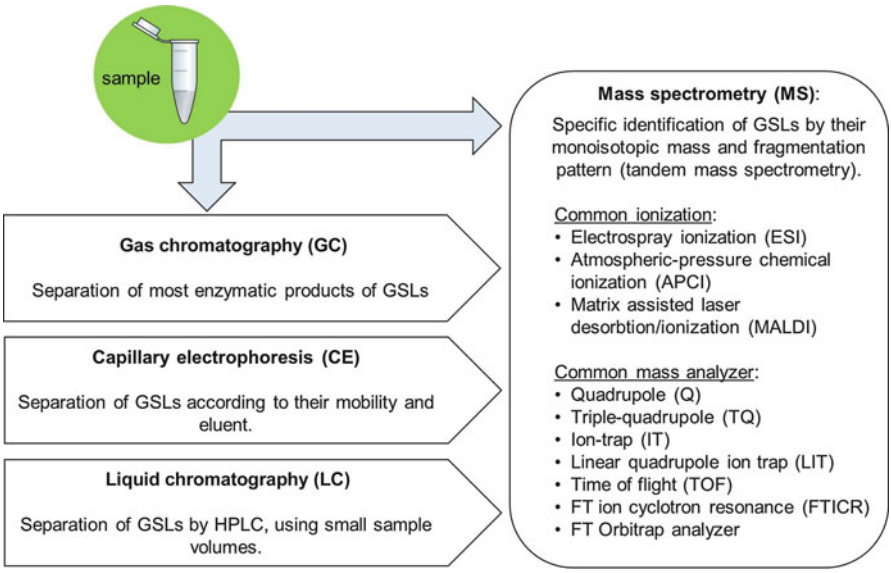

Fig. 3 Alternative ways for the most used MS analysis for GSLs. The sample can be directly analyzed by MS, or it can be first resolved with different online separation techniques

3.1 Analysis of Intact GSLs and Their Derivatives by MS

In the following, we discuss firstly the MS behavior of GSLs in MS and MS/MS investigations. Secondly, we review different hyphenated techniques that combine online separations with MS (i.e., GC/MS, CE/MS, and LC/MS), thanks to their broader applicability in GSL analyses. At the end of this section, we discuss the use of direct infusion into mass spectrometer, covering mainly fingerprinting analysis of the samples, and, finally, the application of mass spectrometry imaging for GSL mapping in plant tissues.

Nowadays, among the different analytical approaches available for determining GSLs in *Brassica* samples, analysis of intact forms or their desulfo-compounds is the most widely applied [25, 26]. However, the notorious lack of commercially available desulfo standards limits its usefulness. For both GSLs intact and desulfo-GSLs, mass spectrometry analysis can be performed either in positive or negative ionization mode. The ESI-MS of desulfo-GSLs is exemplified by a standard solution of desulfo-glucoiberin, $C_{11}H_{21}NO_7S_2$, 343 MW (Fig. 4) both in positive (panel a) as sodium adduct ($[C_{11}H_{21}NO_7S_2 + Na]^+$, *m/z* 366) and in negative ionization modes (panel b) as chloride adduct ($[C_{11}H_{21}NO_7S_2 + Cl]^-$, *m/z* 378) [27].

Taking into account the possibility to apply both positive and negative MS detections, the desulfo-GSLs are often identified in positive ion mode because their deprotonation is relatively difficult without the sulfonate moiety. Figure 5 shows the mass spectrum of desulfo-4-methoxyglucobrassicin ($C_{17}H_{22}N_2O_7S$, 398 MW); in

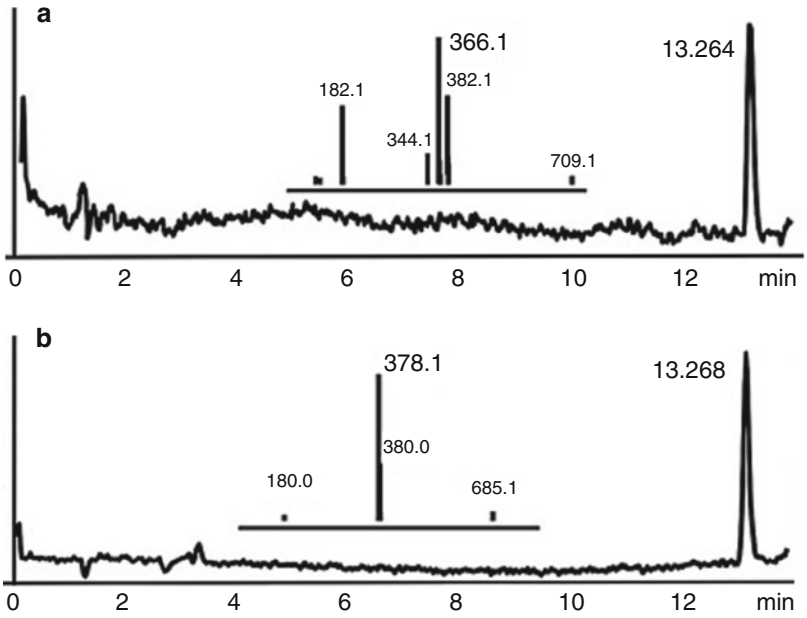

Fig. 4 LC chromatograms obtained during analyses of standard desulfo-glucoiberin by LC/ESI-MS by (**a**) MS(+) detection in positive ionization mode, i.e., desulfo-glucoiberin as Na adduct at *m/z* 366, and (**b**) MS(−) detection in negative ionization mode as Cl adduct at *m/z* 378 (Reprinted with permission from Ref. [27])

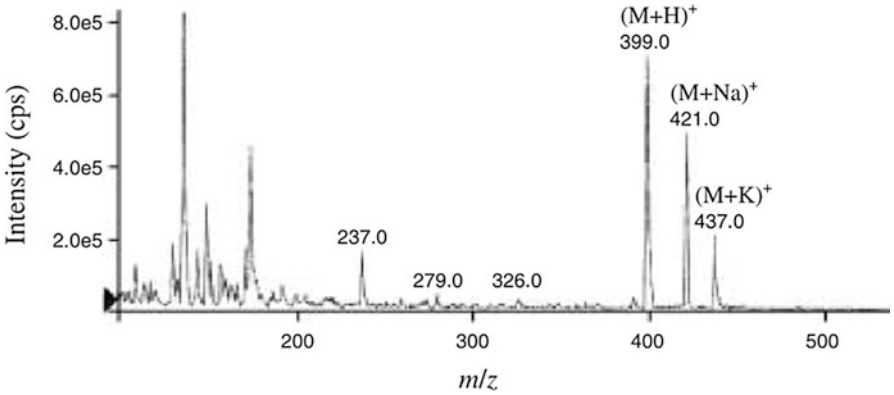

Fig. 5 Mass spectrum of desulfo-4-methoxyglucobrassicin, *m/z* 399, $[M + H]^+$ (Reprinted with permission from Ref. [28])

addition to its protonated molecule $[M + H]^+$ at *m/z* 399, sodium and potassium adducts, $[M + Na]^+$ (*m/z* 421) and $[M + K]^+$ (*m/z* 437), were observed.

Conversely, thanks to the presence of a sulfonate moiety, the intact GSLs are categorized as hydrophilic compounds, which occur in the anionic form as $[M\text{-}H]^-$

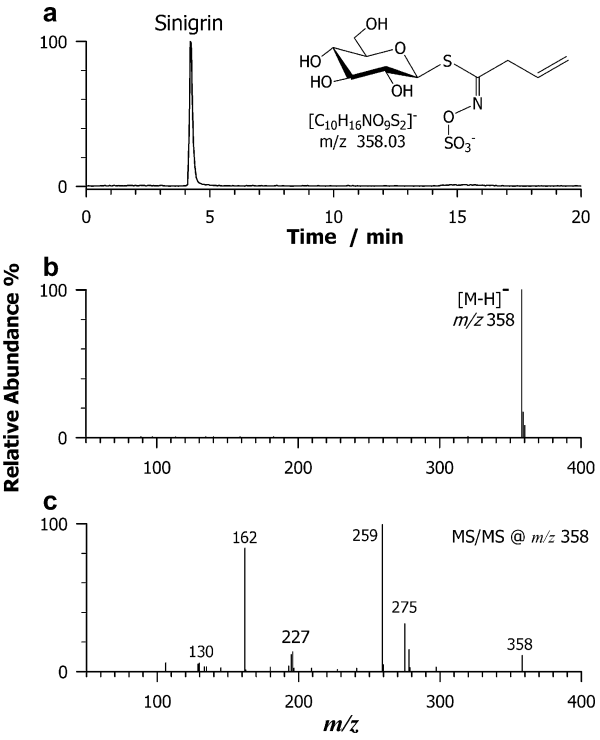

Fig. 6 (**a**) LC/ESI(−)MS of sinigrin standard solution 50 mg/L in MeOH/H_2O 70:30 (v/v). (**b**) Negative ion mass spectrum of sinigrin acquired at the peak apex. (**c**) CID-MS/MS spectrum of sinigrin (*m*/*z* 358) using a collision energy of 35%

and are readily identified in negative ion mode. Figure 6 depicts the separation and detection by LC/ESI-MS of a standard solution of sinigrin in negative ion mode (panel a) along with its mass spectrum (panel b) and tandem MS (panel c) performed at the precursor ion *m*/*z* 358 by collision-induced dissociation (vide infra) using a 3D ion trap MS analyzer.

3.2 Analysis of GSLs by Tandem Mass Spectrometry (MS/MS)

The main disadvantage of low-resolution MS is that it's impossible to distinguish isobaric GSLs featuring different chemical structures. For instance, glucoiberin (3-(methylsulfinyl)propyl glucosinolate, $[C_{11}H_{20}NO_{10}S_3]^-$ *m*/*z* 422.0255) and gluconasturtiin (phenylethyl glucosinolate, $[C_{15}H_{20}NO_9S_2]^-$ *m*/*z* 422.0585) give both the same nominal value of 422 though their monoisotopic values differ of 0.033 Da. Similar problems could be observed in high-resolution MS experiments when GSLs are structural isomers: the sinapoyl derivative of gluconasturtiin and the cinnamoyl derivative of glucobarbarin have the same molecular formula ($C_{26}H_{31}NO_{13}S_2$, 628 MW) but different positions of OH group which is a side chain's substituent in sinapoyl-gluconasturtiin and an acyl group's substituent in cinnamoyl-glucobarbarin. Under these circumstances, tandem mass spectrometry

(MS/MS) is a powerful approach for GSL characterization and identification as the fragmentation pattern helps to distinguish between isobaric and isomeric GSLs. Most theoretical and experimental aspects of collision-induced dissociation (CID) can be found in exhaustive reviews [29, 30].

As discussed above, most published works describe the MS/MS effectiveness in negative ionization mode, thanks to the hydrophilic nature of their sulfonate moiety. The negative ion ESI-MS/MS behavior of GSLs is very useful for structural examination; whereas some product ions are common to all the GSLs, others are highly specific due to the variability of the side chain (R_1) and/or of the acyl group (R_2) occurring on the thioglucose moiety (Fig. 7). The generation of a hydrogen sulfate ion, $[HSO_4]^-$ (*m/z* 97), from the precursor ion is observed as the major fragmentation pathway. Also the ions at *m/z* 139 and 129, corresponding to $[C_2H_3O_5S]^-$ and $[HO_4S_2]^-$, respectively, are very common [31]. Several additional product ions are indicative of thioglucose and sulfonated oxime moiety as the *N*-sulfate ethenimine, $[C_2H_2O_4NS]^-$ at *m/z* 136 and those at *m/z* 75 and 154, which correspond most likely to $[C_2H_3OS]^-$ and $[C_2H_4O_5NS]^-$, respectively.

Usually, identification of the intact GSLs (i.e., $R_2 = H$ in Fig. 7) is based on the detection of ions with constant neutral losses of the following *m/z* values [M-196-H]$^-$, [M-178-H]$^-$, and [M-162-H]$^-$, preceded by H-rearrangements, from the R_1 group by cleavage of the bond on either sides of the sulfur atom and with charge retention on the sugar aglycone [32, 33]. Possibly, the [M-162-H]$^-$ ion can undergo further fragmentation to give the diagnostic fragment M–162–SO_3 (−242) [34]. These fragments, depicted in Fig. 7, are very informative for the side chain of the GSLs. Frequently, the MS/MS spectrum of GSLs contains also ions corresponding to the loss of SO_3 (80 Da) from the $[M - H]^-$ ion [23, 34]. Another product ion, found at *m/z* 195, corresponds to the fragment ion of the D-thioglucose group $[C_6H_{11}O_5S]^-$. The ions at *m/z* 259 and 275 are well known as glucosinolate-specific fragments; they correspond to the neutral loss of R_1-N = C = S and R_1-N = C = O from the deprotonated molecule [M-H]$^-$. The ion at *m/z* 241 originates from ion at *m/z* 259 after loss of a water molecule. Note that all these ions at *m/z* 275, 259, and 241 are most likely formed through intramolecular rearrangements in which the sulfate group is transferred to the thioglucose moiety.

Acylated derivatives ($R_2 \neq H$) show mass spectra similar to corresponding intact GSLs ($R_2 = H$), most likely due to the identical chemical core of these compounds [21]. The product ions specific for the variable aglycone side chain exhibit unvaried *m/z* values; however, acylthioglucose sulfate ions exhibit *m/z* value increase of the acyl group mass, viz., *m/z* 176 for isoferuloyl derivative, *m/z* 130 for cinnamoyl GSL, *m/z* 146 for coumaroyl derivative, *m/z* 206 for sinapoyl GSL, and *m/z* 190 for dimethoxy-cinnamoylate derivative. For instance, the ions at *m/z* 275, 259, 241, and 195 correspond to ions at *m/z* 421 = 275 + 176, 435 = 259 + 176, 417 = 241 + 176, and 371 = 195 + 176 in isoferuloyl GSLs. Additional product ions, likely related to the acylated nature of GSLs, may also exist. Useful information can be obtained on the acyl moiety from the occurrence of ions at *m/z* 193, 147, 163, 223, and 207, corresponding to isoferuloylate ($[C_{10}H_9O_4]^-$), cinnamoylate ($[C_9H_7O_2]^-$), coumaroylate ($[C_9H_7O_3]^-$), sinapoylate ($[C_{11}H_{11}O_5]^-$), and

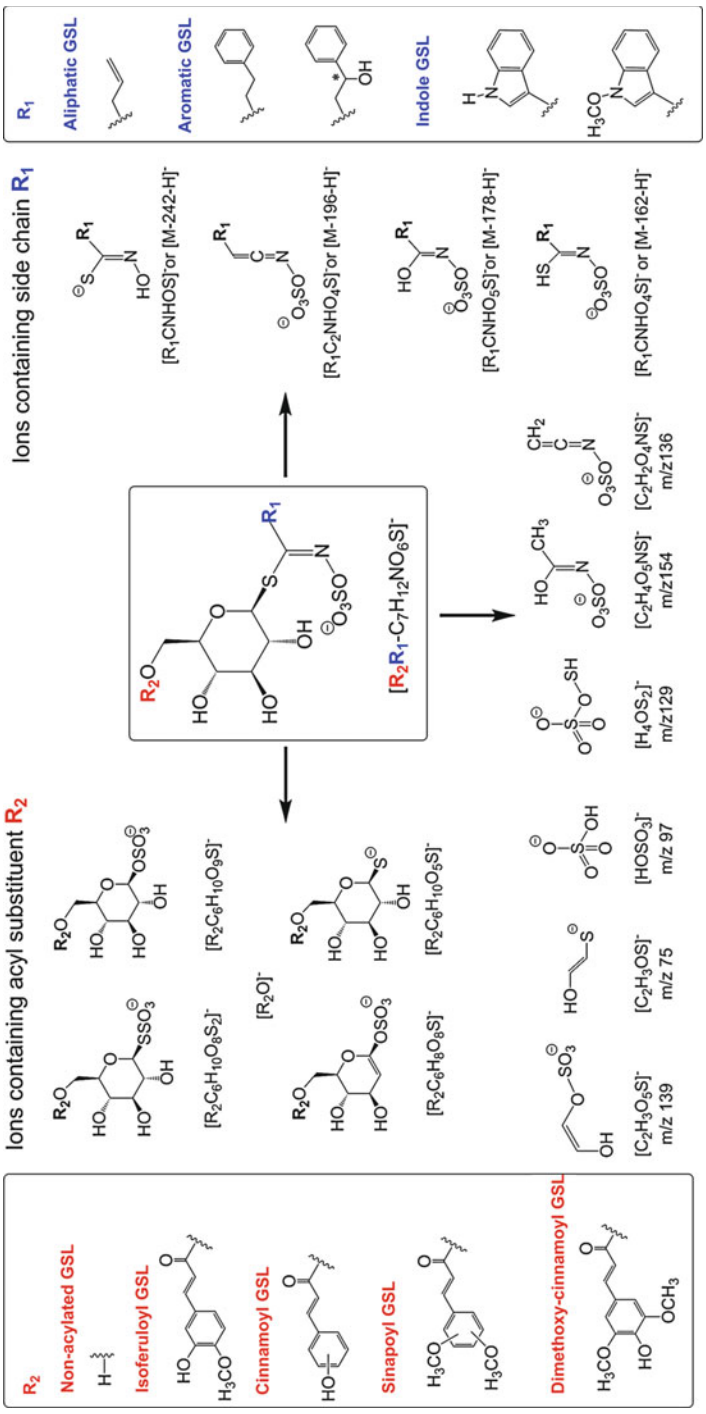

Fig. 7 Common fragments of GSL and their acylated derivatives by ESI-MS/MS. R_1 is the side chain and R_2 is the acyl group or H for intact GSLs

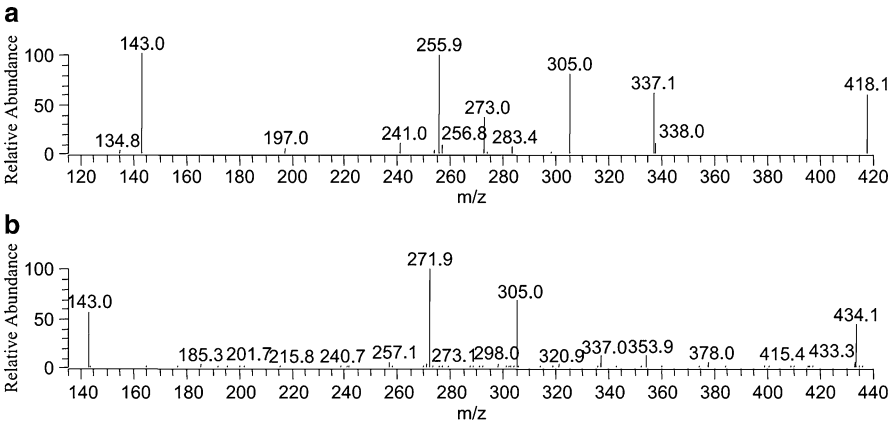

Fig. 8 ESI-MS/MS CID spectra in negative (**a**) and positive (**b**) ion modes of progoitrin/epiprogoitrin at *m/z* 388.1 $[C_{11}H_{18}NO_{10}S_2]^-$ and *m/z* 434.1 $[C_{11}H_{18}NO_{10}S_2 + 2Na]^+$, respectively (Reprinted with permission from Ref. [34])

dimethoxy-cinnamoylate ($[C_{11}H_{11}O_4]^-$) anions. The most common fragments of GSLs and their acylated derivatives are depicted in Fig. 7.

Although GSL behavior in MS/MS experiments has been investigated mainly in negative polarity, some researchers use positive ESI mode. In brief, positive product ions in ESI-MS/MS are obtained from adducts with alkali metal ions such as Na^+ and K^+, i.e., $[M + 2Na]^+$ or $[M + 2K]^+$. In Fig. 8 are reported the MS/MS spectra in both negative and positive ion modes of progoitrin/epiprogoitrin $[C_{11}H_{18}NO_{10}S_2]^-$ at *m/z* 388 and $[C_{11}H_{18}NO_{10}S_2 + 2Na]^+$ at *m/z* 434. The thioglucose product ions at *m/z* 259, 275, and 195 in ESI(−)MS detection corresponds to ions at *m/z* 305, 321, and 241 in ESI (+)MS detection mode. Besides, the aglycone ion product at *m/z* 226 ($[M\text{-}162]^-$) corresponds to ion at *m/z* 272 ($[M\text{-}162 + 2Na]^+$) [34].

In the past, the mass spectrometric behavior of GSLs has been investigated by different ionization methods, including fast atom bombardment (FAB) and thermospray (TSP) [12, 32, 33, 35–38]. However, the characterization of intact GSLs was insufficient, as no extensive fragmentation was carried out. Nowadays, the most common fragmentation method is collision-induced dissociation (CID) and infrared multiphoton dissociation (IRMPD) [18–22, 39]. The application of CID in the GSL analysis is useful if sufficient collision energy is applied; an example of mass spectra obtained at relatively low- and high-collision energy of glucoraphanin is shown in Fig. 9a, b, respectively [40].

As already mentioned, ionization techniques that primarily produce positive adducts or deprotonated molecules (i.e., ESI and APCI) are ideally suitable for coupling with tandem MS. After selection of precursor ions during preliminary LC/MS analysis, tandem MS may be used to obtain structurally definitive information. A specific precursor ion (i.e., deprotonated glucosinolate) is selected and fragmented by a suitable means giving rise to a tandem mass spectrum (MS/MS); whereas the most common fragmentation in low-resolution MS instruments is

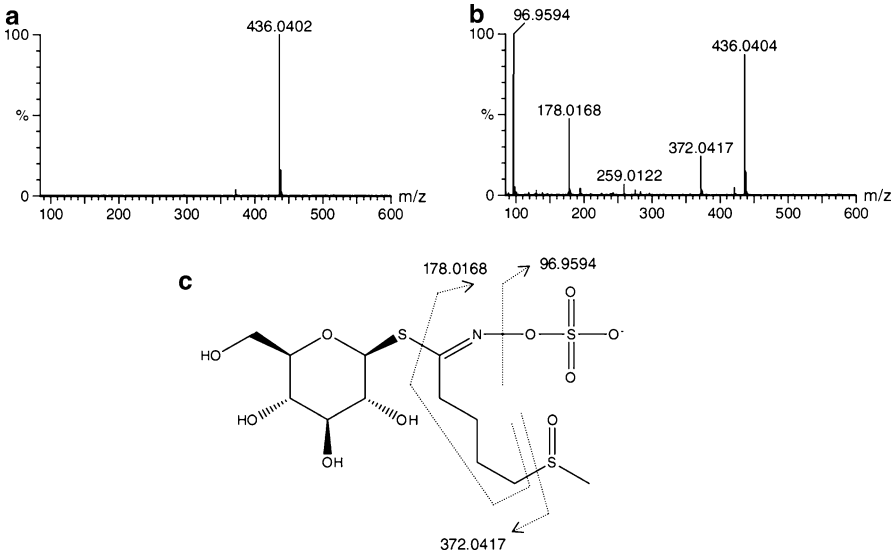

Fig. 9 High-resolution mass spectra of glucoraphanin obtained at (**a**) low-collision energy (4 eV) and (**b**) high-collision energy (25 eV). (**c**) Mass spectral fragmentation pattern for glucoraphanin (Reprinted with permission from Ref. [40])

through collision-induced dissociation using an inert gas (e.g., He, N_2), IRMPD is one of the best choices in high-resolution mass spectrometers such as Fourier transform ion cyclotron resonance (FTICR) in order to maintain the high-vacuum conditions within the trapping cell. The analytical utility of performing IRMPD on a given precursor ion population was first demonstrated on peptides and proteins by Li et al. [41]; Little et al. [42]; Laskin et al. [43]. To a great extent, the photo-fragmentation behavior of IRMPD is almost similar to CID in terms of ergodic processes whereby cleavages of the weakest bonds of the precursor ions are usually observed. Yet IRMPD is preferred over CID because no collision gas needs to be introduced into the ICR cell, thus facilitating accurate mass identification and streamlining product ion assignment with the high-resolution FTICR MS. Moreover, the main advantage in the use of IRMPD is that, as a nonresonant method, all trapped ions and all ensuing product ions are excited using IR irradiation. As a result, the formation of secondary and higher order fragments, which can provide further structural information to that obtained in a single resonant collisional activation experiment, can be observed [43]. For example, IR photo-fragmentation of glucobrassicin $[C_{16}H_{19}N_2O_9S_2]^-$ at *m/z* 447 generates additional diagnostic product ions (*m/z* 275, 269, 259, 241, 205, 171, 139, 122, 97, 75) compared to CID (*m/z* 275, 269, 259, 241, 205) and thus is more easy to assign its membership in the ample family of GSLs [20, 44].

Sometimes, identification of the major GSLs was established through the use of MS/MS mode with secondary confirmation by MS^3 acquisition [22, 44]. As an example, in Fig. 10 are shown the MS/MS spectrum of glucoalyssin

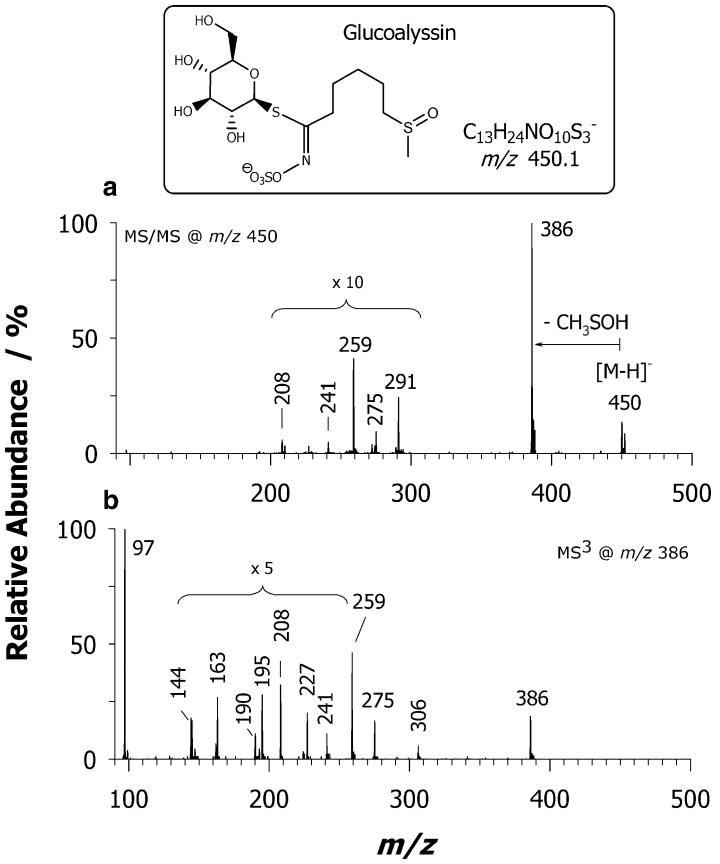

Fig. 10 (**a**) Product ion spectrum of glucoallysin, at *m/z* 450. (**b**) MS^3 spectrum of the precursor ion at *m/z* 386 selected for a further stage of fragmentation. Precursor ions were selected within the linear trap LTQ analyzer and collisionally activated to induce fragmentation. Relative collisional energy in CID equals to 24% (Reprinted with permission from Ref. [22])

($[C_{13}H_{24}NO_{13}S_3]^-$, *m/z* 450) and the MS^3 spectrum of ion at *m/z* 386 selected as the predominant ion. As previously reported, $[M\text{-}H]^-$ ions of GSLs are decomposed into specific ions when collisionally activated. The MS/MS spectrum of the deprotonated precursor ion at *m/z* 450 (Fig. 10, plot a) displays five typical peaks at *m/z* 386, 291, 275, 259, and 241 [19]. The most intense one at *m/z* 386 can be rationalized as the neutral loss of methanesulfonic acid (CH_3SOH) from the deprotonated molecule. Additional insight can be obtained by using the ion trapping and MS^3 capabilities of the LTQ MS analyzer. Such experiments on the precursor ion at *m/z* 386 (Fig. 10, plot b) reveal an interesting mass spectrum whereby the product ions at *m/z* 306, 208, 190, and 144 can be rationalized by loss of neutral molecules, i.e., 306 = $[386–80]^-$, 208 = $[386–178]^-$, 190 = $[386–196]^-$, and 144 = $[386–80–162]^-$ [22, 39].

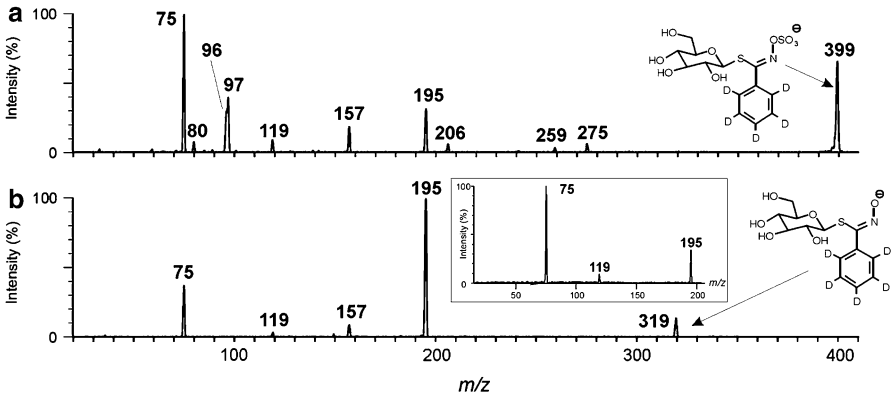

Fig. 11 (**a**) CID-MS/MS spectra of ion at *m/z* 399 derived from phenyl-$d_5$glucosinolate at collision energy setting of 30 eV and (**b**) ion at *m/z* 319 derived from phenyl-$d_5$desulfoglucosinolate at collision energy setting of 15 eV. The *inset* in **b** shows the product ion spectrum of in-source-generated *m/z* 195 ion (cone voltage 25 V) (Reprinted with permission from Ref. [31])

Until now, we described only MS/MS behavior of intact GSLs and acylated derivatives. The MS/MS experiments on their most important closely related derivatives, as isothiocyanates (ITCs) [45], desulfo-compounds [31, 46] and azolidine products [47], are also of great interest. Concerning desulfo-GSLs, Bialecki et al. [31] described a comparison of the product ion spectrum of phenyl-$d_5$glucosinolate at *m/z* 399 (Fig. 11a) with that obtained from its corresponding phenyl-$d_5$desulfoglucosinolate at *m/z* 319 (Fig. 11b), thus establishing the identity of product ions that still contain the sulfur atom from the sulfate moiety. The product ions observed at *m/z* 75, 119, 157, and 195 were common to both spectra. Because phenyl-$d_5$desulfoglucosinolate contains no sulfate residue, it is confirmed that these ions do not contain the sulfur atom from the sulfate moiety. In contrast, the product ions observed at *m/z* 80, 96, 97, 206, 259, and 275 all contain S from the sulfate moiety (Fig. 11a).

The structural characterization of an isothiocyanate has also been pursued by ESI-TOF MS/MS [45]. The mass spectrum of hirsutin, i.e., 8-methylsulphinyl octyl isothiocyanate ($C_{10}H_{19}NOS_2$, 233 MW), as protonated adduct at *m/z* 234 (see Fig. 12a), exhibits a prominent product ion at *m/z* 170 ($[M + H\text{-}CH_4SO]^+$), also appearing as an intense in-source fragment, which is a group-characteristic ion of methylsulphinyl ITCs. Another typical ion is at *m/z* 161 ($[M + H\text{-}CH_3\text{-}NCS]^+$), which is a key fragment of mustard oils along with the stable cyclic immonium ion at *m/z* 114. In Fig. 12b is reported the characteristic MS/MS fragmentation spectrum of thiazolidine-2-thione ($C_9H_9NO_2S$, 195 MW) as Na^+ adduct ($[C_9H_9NO_2S + Na]^+$, *m/z* 218) and proton adduct ($[C_9H_9NO_2S + H]^+$, *m/z* 196). For azolidine derivatives (see Fig. 12b), the neutral losses of SCO (−60 Da, peak c), H_2S (−34 Da, peak d), CH_2O (−30 Da, peak e), HOCN (−43 Da, peak f), and H_2O (−18 Da, peak g) are common in tandem mass spectrum [47].

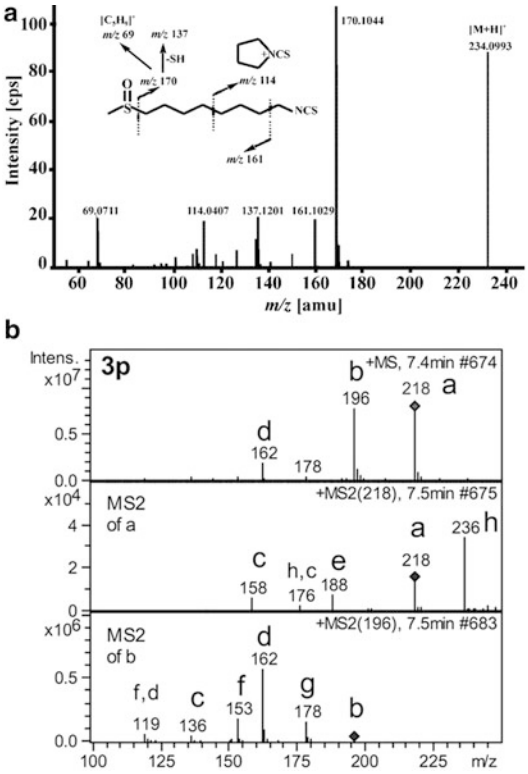

Fig. 12 (**a**) Positive ion ESI-CID-MS/MS of hirsutin at *m/z* 234 ($[C_{10}H_{19}NOS_2 + H]^+$) (Reprinted with permission from Ref. [45]). (**b**) Characteristic MS^2 fragmentation patterns of the thiazolidine-2-thione as Na^+ adduct ($[C_9H_9NO_2S + Na]^+$, *m/z* 218) and proton adduct ($[C_9H_9NO_2S + H]^+$, *m/z* 196). Their product ions are categorized according to the following letters: **c** loss of SCO (−60 Da), (**d**) loss of H_2S (−34 Da), **e** loss of CH_2O (−30 Da), **f** loss of HOCN (−43 Da), and **g** loss of H_2O (−18 Da) (Reprinted with permission from Ref. [47])

Cataldi and co-workers [19] proposed a general approach for structural elucidation of naturally occurring GSLs in crude plant extracts based on the fragmentation of both isotopologue A and A + 2 peaks. All GSLs share a common structure with at least two sulfur atoms and significant isotopic abundance of ^{34}S. Thus, the dissociation of the +2 Da isotopologue ions containing sulfur gives rise to doublets of product ions featuring a combination of ^{32}S and ^{34}S. Accordingly, their relative abundances allow one to speed up the structural recognition of GSLs with great confidence, as it produces structurally definitive ions than conventional tandem MS performed on A ions (Fig. 13). Thus, conclusive identification by MS/MS is significantly easier if the product ion also contains an atom of sulfur that provides a distinctive isotopic pattern.

4 GSLs by Hyphenated Techniques

The isolation and structural elucidation of known and unknown GSLs from plant extracts by tandem MS is a rewarding, but often time-consuming, task, since it is a major effort to isolate each compound in a pure form, even the known ones as the

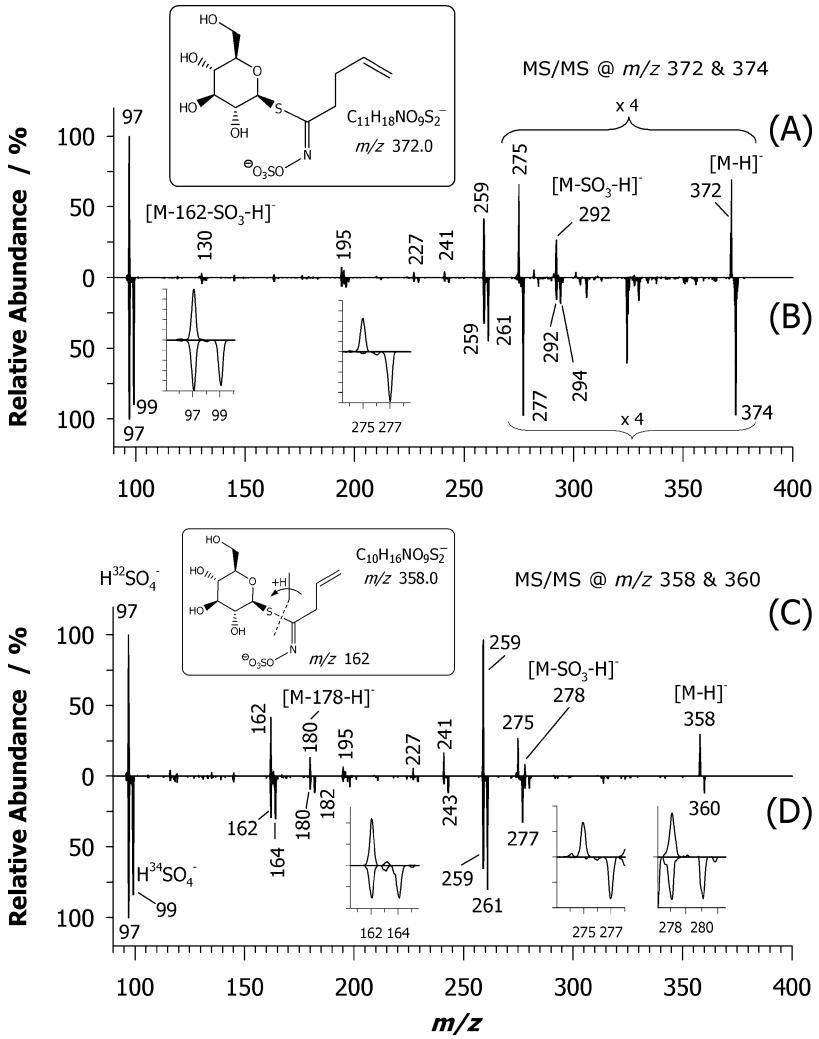

Fig. 13 Product ion spectra obtained by LC/ESI-LTQ MS/MS in negative ion mode of (**A, B**) gluconapin and (**C, D**) sinigrin. The precursor ion base peaks of gluconapin and sinigrin at *m/z* 372 (**A**) and *m/z* 358 (**c**), respectively, and the corresponding A + 2 isotope peaks at *m/z* 374 (B) and *m/z* 360 (**D**) were fragmented at 20% collision energy. The expanded views in plots (**B**) and (**D**) show as profile spectra some examples of isotopic peak doublets (Reprinted with permission from Ref. [19])

simultaneous presence of two or more components in the source region gives rise to an overlapping or mixed spectrum. Furthermore, to obtain the required milligram quantities of all GSLs, especially in the case of minor GSLs, one may need large amounts of the plant material and of expensive tools and supplies. Another challenge is to identify all GSLs because the variable side chain linked to the thiohydroximate

(see Fig. 1) and the variable acyl group linked to thioglucose afford many different structures [8]. The coupling of separation modes with MS has been recognized as a very useful approach for real-world samples analysis, in which complex mixtures are the norm. In the case of plant extracts, the hyphenation of gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) with mass spectrometry has led to several strategies that do not only permit to differentiate between known GSLs but can likewise allow the structural characterization of unknown GSLs directly from the crude extract with a minimum amount of sample.

4.1 GSL Analysis by Gas Chromatography–Mass Spectrometry (GC/MS)

Because of their ionic nature, naturally occurring GSLs are not sufficiently volatile to be separated directly on a GC column, so pre-column conversion to desulfo-GSL derivatives or conversion/hydrolysis to corresponding isothiocyanate (ITC) has to be performed. Among the myrosinase-catalyzed degradation products of GSLs, ITCs (see their formation in Fig. 14) have been generally known as the most biologically active metabolites, being reported to possess broad-spectrum biological activity against bacterial and fungal pathogens, nematodes, insects, and weeds [6, 48–53]. Also nitriles are known to be biologically active, but their effects are more restricted compared to ITCs. For these reasons the more recent applications of GC/MS for GSLs analysis are mainly focused on the monitoring of ITC and nitrile metabolites [14, 54–58].

Usually, GC/MS analysis is performed on single-quadrupole mass spectrometers, which provide nominal data of *m*/*z* values. The introduction of GC-TOF MS systems offered an attractive supplement to quadrupole instruments and provided greater mass accuracy. TOF instruments also provide high scan speeds that are compatible with fast GC separation [59] and the potential to profile complex mixtures in less time. Similarly, GC coupled to multistage MS (e.g., MS/MS and MS^n) instruments enabled the acquisition of several fragmentation data and a higher level of molecular selectivity. Nevertheless, quadrupole mass analyzers still present some extra advantages in metabolite analysis such as higher repeatability and large dynamic range for quantitative analysis. According to Nielsen et al. [60], it is possible to improve

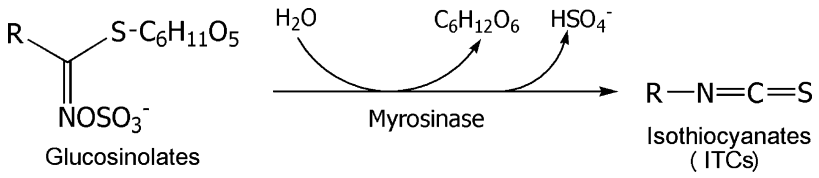

Fig. 14 Myrosinase-catalyzed glucosinolate hydrolysis (breakdown) and formation of isothiocyanates (ITCs). Sulforaphane is an ITC compound formed from glucoraphanin. When cruciferous vegetables are chopped or chewed, endogenous myrosinase can interact with glucosinolates and release ITCs from their precursors

detection sensitivity from the ng-level to the pg-level through the scanning for a selected number of characteristic ions (selected ion monitoring, SIM), which is very useful for quantitative target analysis.

4.2 GSL Analysis by Capillary Electrophoresis–Mass Spectrometry (CE/MS)

Capillary electrophoresis (CE) coupled to mass spectrometry for GSL analysis dates back to 2005 when Bringmann et al. [17] used CZE in combination with electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS). The hyphenated technique combines the advantages of capillary electrophoresis, namely, small sample volumes needed and nearly no dilution effects, with those of time-of-flight mass detectors, i.e., high sensitivity, accurate mass, and isotopic pattern. Employing an acidic electrolyte and a sheath liquid based on formic acid, the GSLs were separated and detected as anions, resulting in an excellent selectivity. Thus, the crude plant extracts could be analyzed without any interference of matrix constituents. The sensitivity together with mass accuracy and true isotopic pattern of the TOF MS allowed the identification of a broad series of glucosinolates in *Arabidopsis thaliana* seeds.

4.3 GSL Analysis by Liquid Chromatography–Mass Spectrometry (LC/MS)

GSLs and desulfo-GSLs containing sulfinyl or indolic moieties are not amenable to GC/MS analysis. Further, the preanalytical derivatization step may be avoided by employing LC/MS methods and can be applied to both GSLs and ITCs as validated by ICH guidelines [61]. Indeed, LC/MS methods have replaced not only the ISO 9167-1 method currently used but also all the HPLC–UV methods adopted for the quali-quantitative determination of ITC [8]. Currently, LC/MS is broadly used with ESI and APCI sources [18–22, 62–65]. Although ESI-MS is the most used detection tool for identifying highly polar and heat-labile compounds such as GSLs occurring as ions in solution, the direct comparison with APCI-MS demonstrates that this last is much more sensitive for aliphatic, aromatic, and indole desulfo-GSLs than ESI-MS. However, no additional information, in terms of structure determination, was obtained by APCI-MS [66].

Several modes of scanning and acquiring MS data during an LC/MS analysis are available: (i) full-scan acquisition resulting in the typical total ion current (TIC) plot, (ii) selected ion monitoring (SIM), (iii) selected reaction monitoring (SRM), or (iv) multiple reaction monitoring (MRM). The TIC and SIM modes are mainly used for GSLs profiling, whereas SRM scan is used for quantitative analysis. LC coupled with ESI(−) to a hybrid quadrupole linear ion trap and Fourier transform ion cyclotron resonance (FTICR) mass spectrometer in full-scan mode was employed for the selective and sensitive determination of intact GSLs in several crude sample extracts of broccoli

(*Brassica oleracea* L. var. italica), cauliflower (*B. oleracea* L. var. botrytis), rocket salad (*Eruca sativa* L.), and bud flowers of *Capparis spinosa* (*Capparis* species, family Capparaceae). Fragmentation induced by IRMPD was successfully applied to identify targeted and untargeted GSLs in aqueous/methanol extracts [20–22].

Notably, SIM acquisition mode is more sensitive than the full-scan experiment because the mass spectrometer can dwell for a longer time over a smaller mass range; for this reason this scan mode is employed for quantitative analysis if compounds to be quantified have different *m*/*z* values [18, 25, 63]. SRM detection provides improved sensitivity and selectivity in comparison to both full-scan and selected ion monitoring scanning modes. Tandem MS of a deprotonated GSL produces a characteristic product ion at *m*/*z* 97 ($[HSO_3]^-$) [18–22, 54, 67] with high intensity; it can be monitored in MS/MS SRM experiments. Tian et al. [68] used SRM of the transition $[M\text{-}H]^- \rightarrow$ *m*/*z* 97 to quantify all GSLs in broccoli, broccoli sprouts, Brussels sprouts, and cauliflower. The same LC/ESI-MS/MS MRM method allowed to quantify all the identified glucosinolates in different tissues of *Moringa oleifera* and in broccoli, oil seed, *Brassica napus* seeds, cabbage, Brussels sprouts, cauliflower, *Brassica oleracea* seeds, and winter cress *Barbarea verna* seeds [69–71].

5 GSL Analysis by Off-Line and Shotgun MS

Off-line HPLC followed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), or direct analysis of a sample extract by MALDI-TOF MS, through off-line vacuum deposition onto standard stainless-steel MALDI targets, are both used. The off-line approach allows the decoupling of the separation step from the MS analysis, which can be independently optimized in terms of time. To illustrate the use of MALDI-TOF MS, we discuss the work of Botting et al. [72] who demonstrated the determination of the GSL profile in different crude plant samples, viz., *Lunaria annua* seeds, cauliflower florets, rutabaga peel, and turnip. Informative mass spectra were obtained from the plant extracts with predominant ion peaks corresponding to deprotonated GSLs. In Fig. 15, the mass spectrum obtained from the *L. annua* extract clearly shows three peaks corresponding to GSLs with isopropyl, 5-methylsulfinyl, and 6-methylsulfinyl side chains. In previous work, these three GSLs were identified by LC/MS upon the analysis of myrosinase-induced breakdown products [73, 74].

Direct infusion MS or shotgun approaches are also been developed for rapid screening of GSLs in vegetable extracts. Indeed, flow injection ESI-MS has been used for the profiling of GSLs and phenolic compounds in pulp seed, seed coat, leaves, and roots of *M. oleifera* 12-day old seedlings [70]. The full-scan MS spectrum of each sample was recorded in triplicate with the aim to rapidly provide a visual and statistical evaluation of similarities and differences of secondary metabolites among tissues. The ESI-MS fingerprints of samples were very distinguishing, showing distinctive sets of polar markers for each different tissue. Considering the large amount of data set (4 tissues × 3 replicates) obtained by negative ion ESI-MS fingerprints of extracts under investigation, a chemometric

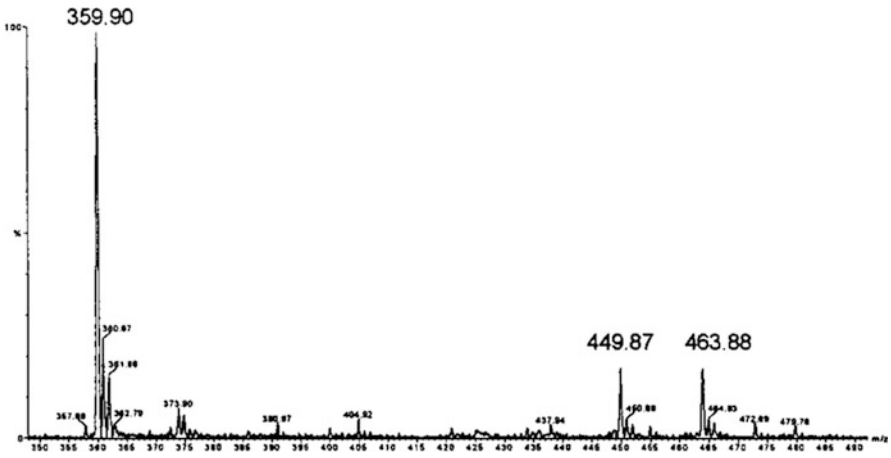

Fig. 15 MALDI-TOF mass spectra of extracts from *L. annua* seeds (Reprinted with permission from Ref. [72])

approach was performed by principal component analysis, in order to characterize the different plant's tissue and to evaluate differences in terms of metabolites. The simultaneous determination of 11 glucosinolates in different *M. oleifera* tissues was described.

The use of high-resolution mass spectrometry (HRMS) in direct analysis greatly improves the analytical performance and offers a good combination of selectivity and sensitivity. High mass resolution/accuracy along with measured relative isotopic abundance ratios are typically employed to tentatively identify elemental formulae of thousands of different compounds. An example of application of HRMS is by direct analysis in real time (DART). In DART, an electrical potential is applied to a heated gas stream (typically helium or nitrogen) to form a plasma of excited-state atoms and ions that desorb low molecular weight molecules from the surface of a sample. DART-TOF MS has been fruitfully used to examine yellow mustard seeds [75]; the presence of 4-hydroxybenzyl GSL, also known as sinalbin, was evidenced through characteristic ions at *m/z* 180.09 $[C_6H_{12}O_6 + NH_4\text{-}H_2O]^+$, 198.10 $[C_6H_{12}O_6 + NH_4]^+$, 214.08 $[C_6H_{12}O_5S + NH_4]^+$, and 296.20 $[C_4H_{17}O_8 + 3H_2O + H]^+$ (Fig. 16a). All these ions were consistent with the sinalbin standard compound (Fig. 16b). The DART-TOF MS method was optimized and validated in comparison to LC/MS.

6 Comparison of the Different MS-Based Methods for GSL Analysis

There are different issues in the determination of GSLs and their decomposition products in cruciferous vegetables. First, there are a lot of compounds with similar physical and chemical properties which may appear in the investigated samples, even

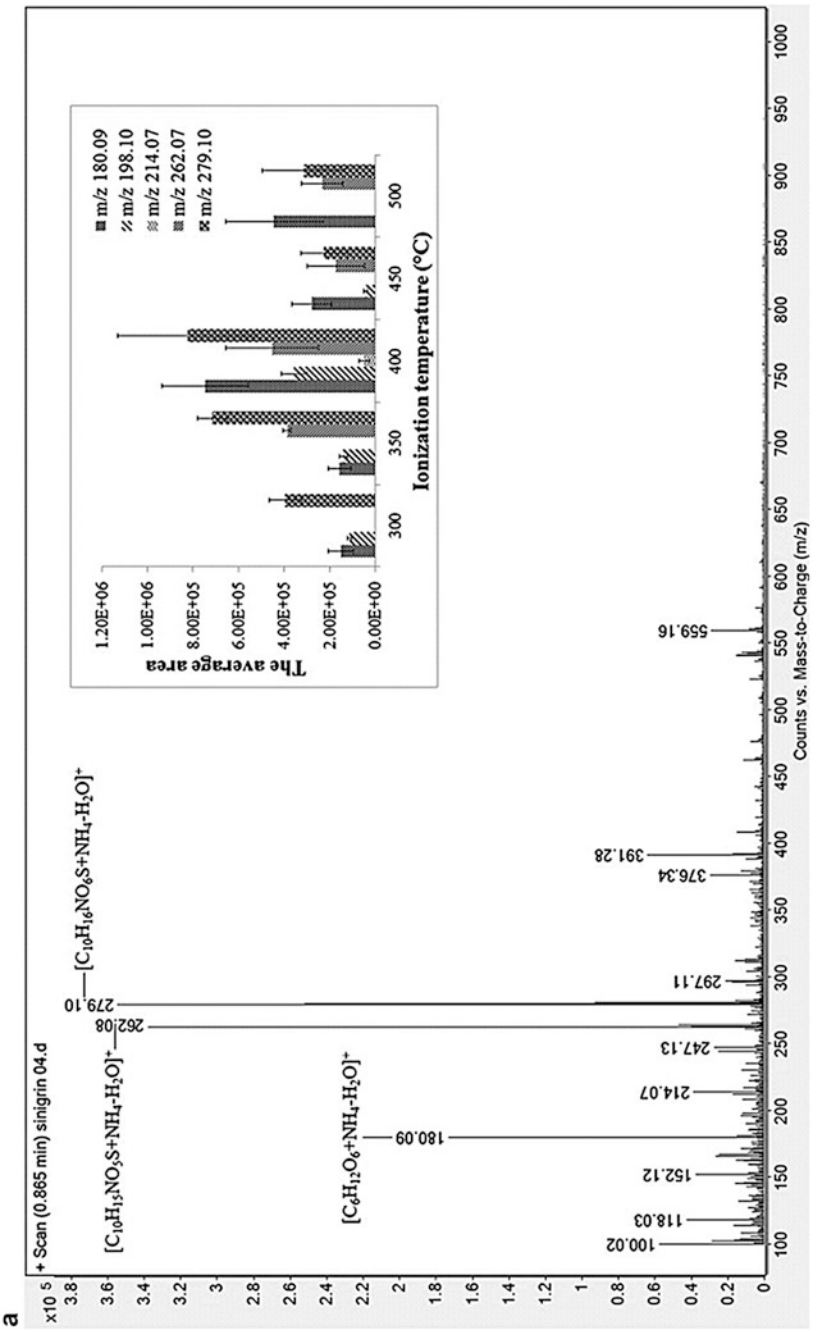

Fig. 16 (continued)

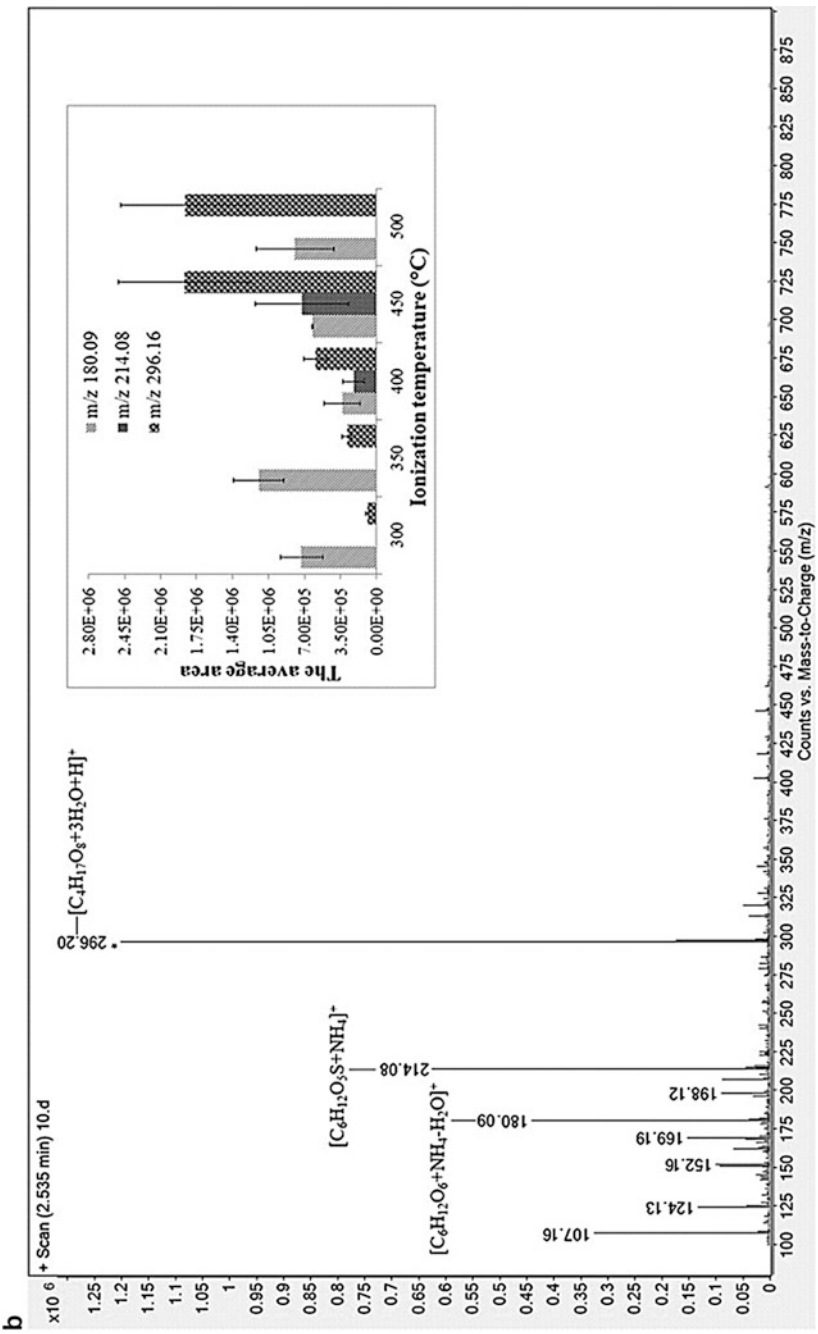

Fig. 16 MS spectrum of (**a**) yellow mustard seeds (positive mode, ionization temperature 400 °C) and (**b**) sinalbin 1 g/l (positive mode, ionization temperature 450 °C). Possible structure of fragments and dependence selected intensity of ions on temperature are shown (Reprinted with permission from Ref. [75])

at considerably higher levels than targeted metabolites. Second, reference materials are not always available, which causes serious problems in carrying out quantitative determinations. Table 1 summarizes data from the literature regarding the analytical techniques used for samples of cruciferous plants to determine the content of GSLs and their breakdown products: strengths and weaknesses of each method using MS as a selective detector are highlighted. As already mentioned LC/MS is indeed a mature technique in GSLs analysis and, as a consequence, has been largely applied as extensively described in the literature. Because of their ionic nature, GSLs cannot be investigated directly by GC but must be hydrolyzed to corresponding ITC, nitrile, etc. It has a great potential for quantitative analysis and metabolite profiling, and the

Table 1 Example of application of different MS methods for GSL analysis

Analytical technique	Application	Advantages	Disadvantages	References
GC/MS	Quantitative analysis or qualitative analysis depends on detection, the SIM, and SCAN modes, respectively	High chromatographic resolution, ideal to resolve complex samples	Sample preparation step complicated and time-consuming; unable to analyze thermo-labile GSLs	[57, 58, 60]
			Nonvolatile GSLs must be derivatized before analysis	
			Difficult to identify unknown compounds after derivatization	
LC/MS	Separation, identification, and quantification of a very broad group of metabolites. Limited potential in identification unless MS/MS techniques are used	High sensitivity; average to high chromatographic resolution; no derivatization required; enable analysis of thermo-labile GSLs	Sample preparation step labor-intensive and time-consuming; restriction on LC eluents	[18–21, 68, 70]
CE/MS	Separation, identification, and quantification of polar GSLs, using small sample volumes	Small volumes; high resolution; flexible technique	Buffer incompatibility;	[17]
			Difficulty in MS interfacing	
MS	Identification of GSLs by their characteristic *m*/*z* values. Usually applied in GSL profiling without any quantification	Rapid screening of metabolites; versatile and reproducible technique	Low sensitivity, no quantification analysis	[72, 75]

variation between those two approaches is basically performed through a switching of the detection from the SIM to the full-scan mode. However, GC/MS analysis of breakdown products possesses specific limitations as some GSL side chains (e.g., indolyl or hydroxylated side chains) produce poorly volatile or unstable breakdown products [72]. An alternative is the conversion of GSLs into their volatile desulfo-GSL derivatives with pre-column derivatization. The mandatory requirement to derivatize nonvolatile metabolites often complicates the identification of unknown compounds, increases the sample preparation variability, adds time to the analysis, causes more complex sample handling, and increases variance analysis as well. For these main reasons, LC/MS and CE/MS are more suitable for GSL analysis. Both intact GSLs and desulfo-GSLs can be separated and detected by LC/MS. Desulfation results in better separation and cleaner samples and also makes the compounds easier to analyze by traditional mass spectrometric techniques [76, 77].

In evaluating the combination CE/MS, many efforts are needed to establish optimum composition of the CE background electrolyte, which allows a satisfactory separation coupled to an efficient ionization by ESI. Although the low sample volume required for CE and the high speed would be an advantage over LC/MS, the technique still suffers from low sensitivity. Indeed, compared to LC/MS analysis, one major drawback is the relatively high concentration limit of detection because of the very low injection volume used in CE [78].

Both chromatographic and electrophoretic separations reduce ion suppression effects originating from the sample matrix. The major drawback of hyphenated MS-based techniques lies in their limitation to analyze large sets of samples to detect changes in composition in a fast and simple way. On the contrary there is a strong need for more rapid, high-throughput MS approaches for nontargeted investigations. Nowadays, direct MS analysis is the method of choice to achieve the maximum high-throughput information data from the largest possible number of samples [79]. Direct infusion ESI-MS offers several advantages since it is a very fast, versatile, reproducible, and sensitive technique, which requires little or no sample preparation [70]. Any separation step prior to MS detection is avoided and thus direct analysis of samples (previously processed or not) is carried out, making it particularly attractive when dealing with large sample sets. The analysis of multicomponent mixtures by direct infusion MS can, however, be hindered by the competitive ionization of the analytes that leads to a low sensitivity. In addition, isobaric interferences in the mass spectrum adversely affect compound identification as well as other aspects of performance. On the other hand, the high throughput and the reduction of sample preparation time are the main advantages of direct MS-based approaches over conventional hyphenated techniques [79].

7 GSL Analysis by Mass Spectrometry Imaging (MSI)

The surface localization of plant GSLs has been a controversial topic for many years. GSLs are frequently cited as key compounds for plants in deterring generalist insect herbivores and for specialist ones in choosing plants for feeding and oviposition

[7, 80–82]. Since insect behavioral choices are sometimes made after surface contact, GSLs have been sought on the surface. With the capabilities of mass spectrometry imaging (MSI) to track and map the spatial distribution of small molecules in plants, the data generated may essentially lead to improved understanding of the metabolism of plants in response to diseases and certain stimuli from the environment, including pest attack [83].

The MSI method used most frequently is MALDI-MS [84–86]. In plants, MALDI-MSI has been successfully applied to study the spatial distribution of molecules such as sugars, lipids, and secondary metabolites like GSLs, flavonoids, and alkanes in leaf tissue and tissue sections of different plants [84, 87–89]. Shroff et al. [84] performed the analysis of GSL distribution within *Arabidopsis thaliana* demonstrating a nonuniform distribution throughout the leaf tissue. The accumulation of GSLs close to the leaf margin and the middle vein strongly influences the feeding preference of *Helicoverpa armigera* larvae and might play a key role as a first barrier against chewing herbivores. The identity of these detected compounds was confirmed by CID mass spectra [84]. Sarsby et al. [86] studied flower buds, sepals, and siliques of *A. thaliana* using MALDI-MSI. More recently Shroff et al. [85] modified their procedures to quantify GSLs occurring on *A. thaliana* leaf surfaces by matrix deposition through sublimation and confirmed the previous qualitative results concerning nonuniform distribution by monitoring three different GSL compounds: 4-methylthiobutylglucosinolate (4MTB), 4-methylsulfinylbutylglucosinolate (4MSOB), and indol-3-ylmethylglucosinolate (I3M) at *m*/*z* 420, 436, and 447, respectively (Fig. 17).

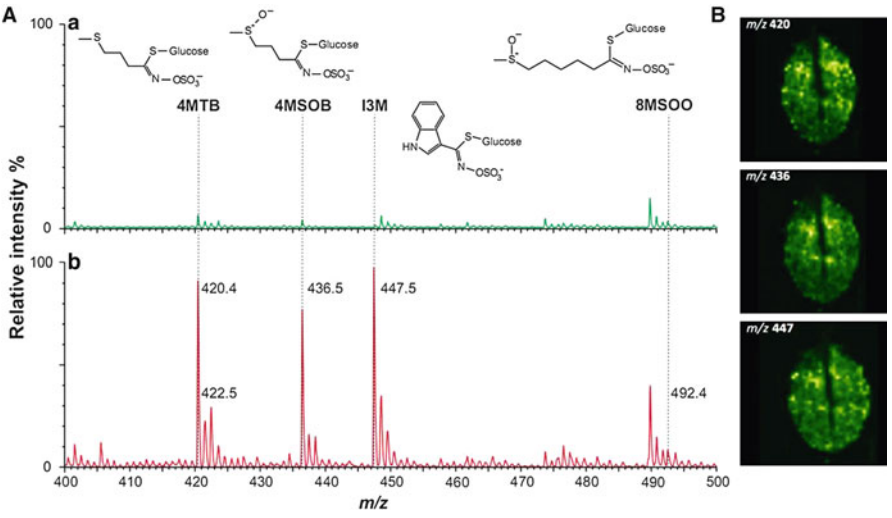

Fig. 17 (**A**) MALDI-TOF spectra and (**B**) MALDI-MSI spectra of the *Arabidopsis thaliana* leaf surface. (c) MALDI-TOF images constructed for abundance of *m*/*z* 420 (4MTB, 4-methylthiobutylglucosinolate), 436 (4MSOB, 4-methylsulfinylbutylglucosinolate), and 447 (I3M, indol-3-ylmethylglucosinolate) (Reprinted with permission from Ref. [85])

8 Conclusions

Over the past two decades, mass spectrometry has proved to be one of the most effective techniques in plant metabolite research, including GSLs. Due to their ionic character and low volatility, GSLs are perfectly suitable for determination by liquid chromatography. Thus, the combination of RP-HPLC and ESI-MS/MS has given to us a tool with high sensitivity and selectivity to establish the occurrence of GSLs in vegetable samples, also at the low levels of many minor species. We believe that the existing generation of LC/ESI-MS technology is sufficiently robust for structural identification and especially to answering some questions about the role of GSLs in human health. Currently, quantitation assays are faced by external standards and concentration/response curves. As stable isotopically labeled GSL standards become available, quantitation will be possible by isotope dilution LC/MS, thus improving precision and streamline analysis.

Acknowledgments This study was supported by the Università degli Studi di Bari Aldo Moro (Fondi di Ateneo 2012) and Università degli Studi della Basilicata.

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Index

J.-M. Mérillon, K.G. Ramawat (eds.), *Glucosinolates*, Reference Series in Phytochemistry, DOI 10.1007/978-3-319-25462-3