Case Study for Trait-Related Gene Evolution: Glucosinolates

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Abstract

Glucosinolates are a group of secondary metabolites involved in plant defense and found mainly in the Brassicales order. While the breakdown products of some glucosinolates are beneficial to human health, many glucosinolates are toxic. The recently sequenced genomes of *Brassica napus* and its parental species *Brassica rapa* and *Brassica oleracea* provided the *Brassica* scientific community with a valuable tool for systematically investigating glucosinolate biosynthesis, transport, and breakdown genes, elucidating the relationship between variation of glucosinolate profiles and the evolution of glucosinolate-related genes in *Brassica* crops. In this chapter, we summarized the variation in glucosinolate composition and content in *Brassica* crops and identified 166, 167, 191, 333 genes in *B. rapa*, *B. oleracea* var. *capitata*, *B. oleracea* var. *italica,* and *B. napus,* respectively, as orthologs of 78 glucosinolate biosynthetic, transport, and breakdown genes in *Arabidopsis thaliana*. Among these glucosinolate-related genes, transcription factor, side-chain modification, and breakdown genes experienced significant expansion in the four *Brassica* crops. Moreover, phylogenetic and expression pattern analyses of the glucosinolate-related genes *HAG1*, *MAM*, *AOP,* and *GTR* correspond with the glucosinolate profiles and total seed glucosinolate contents in *B. napus* and its parental species. These results, together with those published previously, provide a valuable resource for understanding the genetic mechanism underlying glucosinolate metabolism and transport and suggest novel approaches for improving the nutritional quality of *Brassica* crops through breeding cultivars with lower glucosinolate contents.

12.1 Introduction

Glucosinolates (GSLs) are a group of sulfur-rich, nitrogen-containing plant secondary metabolites mainly found in the Brassicales order (Fahey et al. 2001), which includes many economically and nutritionally important crops and condiments, such as oilseed rape (*Brassica napus*), broccoli (*Brassica oleracea* var. *italica*), cabbage (*B. oleracea* var. *capitata*), turnip (*Brassica rapa*), mustard (*Brassica juncea* L. *Czern*), and wasabi (*Wasabia japonica*), as well as the model

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plant *Arabidopsis thaliana*. In addition, GSLs have also been identified in the genus *Drypetes* of the family *Euphorbiaceae* (Fahey et al. 2001). GSLs share an identical core structure containing a *β*-thioglucose group linked to a sulfonated aldoxime moiety, plus a variable aglycone side chain (R) derived from one of eight amino acids (Halkier and Du 1997). Based on the amino acid precursors and the type of modification to the R group, GSLs can be divided into three major classes: aliphatic, indole, and aromatic GSLs (Halkier and Gershenzon 2006). Aliphatic GSLs are derived from alanine, leucine, isoleucine, valine, and methionine, while indole GSLs and aromatic GSLs are derived from tryptophan and phenylalanine or tyrosine, respectively. By 2000, at least 120 different GSLs were reported in 16 families of the order *Capparales*, and the Brassicaceae family alone was found to contain at least 96 of these. A more recent review elucidated and documented the discovery of additional natural GSL structures, citing around 132 unique GSLs from nature (Agerbirk and Olsen 2012). The structural diversity of these compounds is mainly caused by extensive modification of the variable side chain by elongation of the amino acid precursors and from a wide variety of side-chain modifications, including hydroxylation, oxidation, methylation, glucosylation, desaturation, and sulfation (Halkier and Gershenzon 2006).

Though most GSLs are not bioactive in their intact form, they are rapidly hydrolyzed by an endogenous family of plant enzymes called myrosinases (thioglucoside glucohydrolases (TGGs); EC 3.2.1.147), *β*-glucosidases that are compartmentalized in the vacuoles of myrosin cells, a location separate from that of GSLs. Once plant tissues are damaged by wounding, herbivore or pathogen attack, freezing, or grazing (Bones and Rossiter 2006; Fahey et al. 2001), the myrosinases are mixed with GSLs, resulting in hydrolysis of the thioglycoside bond to yield glucose and an unstable aglucone. The latter compound is either spontaneously rearranged into bioactive isothiocyanate or is converted into alternative hydrolysis products such as simple nitriles, epithionitriles, or organic thiocyanates (Wittstock and Burow 2010). The types of breakdown products of the GSL–myrosinase system depend mainly on the chemical nature of the side chain of the parent GSL, the reaction conditions, and the cofactors that are present (Fahey et al. 2001; Halkier and Gershenzon 2006).

GSLs and their degradation products have been recognized for their roles in plant defense and their distinctive effects on human health and on the flavor of cruciferous vegetables. Glucoraphanin (4-methylsulfinylbutyl, GRA), which is known to reduce the risk of aggressive prostate cancer (Halkier and Gershenzon 2006), is the most widely studied GSL. Despite the importance of certain GSLs and their metabolites to human health, most GSLs are also undesirable substances in *Brassica* crops for animal feed, due to the deleterious effects of their breakdown products on animal growth and reproductive performance. To reduce the levels of GSLs in *Brassica* crops, oilseed rape breeders have devoted much effort to developing genetically improved varieties with lower amounts of GSLs. Significant progress has been made toward this goal through classical breeding approaches, and several varieties with low levels of seed GSLs (less than 30 μmol/g in defatted meal) and erucic acid (less than 2% of the total fatty acids present in the oil) have been released in Canada and marketed under the name "canola." While processed canola meal has been widely accepted in the feed industry as a high-quality feedstuff for livestock and poultry, a number of reports have documented reduced performance in farm animals fed diets containing significant amounts of canola meal (Khajali and Slominski 2012; Leeson et al. 1987).

The completion of genome sequencing of *B. napus* and its parental species *B. rapa* and *B. oleracea* provided the *Brassica* scientific community with a valuable tool for further improving seed quality through regulating and controlling secondary metabolism pathways (Chalhoub et al. 2014; Liu et al. 2014; Parkin et al. 2014; Wang et al. 2011b). In *Arabidopsis*, the close relative of *Brassica* crops, most genes responsible for GSL biosynthesis, breakdown, and transport have

been characterized using biochemical and reverse genetics approaches (Halkier and Gershenzon 2006). Based on this research in *Arabidopsis*, orthologous genes involved in GSL metabolism and transport in *Brassica* crops have been identified, allowing for the manipulation of these genes and the development of *Brassica* vegetables with high levels of anticancer GSLs and *B. napus* varieties containing much lower levels of undesirable GSLs.

This chapter presents an overview of the genes responsible for GSL biosynthesis, transport, and breakdown in *Brassica* crops, with special emphasis on elucidating the evolutionary processes that resulted in the variation in GSL profiles of *Brassica* crops. Based on this information, we present perspectives for further research aimed at modifying and reducing different kinds of GSLs in *B. napus*.

12.2 Variation in GSL Composition and Content in *Brassica* Crops

The chemical composition of many Brassicaceae genera has been studied, with a focus on identifying variations in oil content and seed fatty acid and GSL composition (Warwick 2011). Comparative studies of GSL profiles indicate that the type of GSLs present and their concentrations vary considerably between species in the Brassicaceae family, as well as between cultivars of the same species, and within different organs or developmental stages of the same plant (Daxenbichler et al. 1991; Fahey et al. 2001). In addition, the total GSL content and the relative proportion of individual GSLs are also influenced by the genotype and by agronomic and environmental factors (such as growth stage, harvest time, soil moisture, and temperature) (Gu et al. 2012; Padilla et al. 2007; Wang et al. 2012; Yang and Quiros 2010).

Numerous studies have described the GSL contents and composition in representative *Brassica* species, and these data have been compiled in several reviews (Daxenbichler et al. 1991; Fahey et al. 2001; Ishida et al. 2014; Jeffery et al. 2003; Padilla et al. 2007). As many as 20 kinds of GSLs have been identified in commercial *Brassica* crops (Table 12.1), which possess substantially different GSL profiles, and usually only 3 or 4 predominant kinds of GSLs occur in the same plant (Rosa 1997). Comparison of the GSL profiles and concentrations in

Table 12.1 Major GSLs present in *Brassica* crops

GSL name	Trivial name	Systematic name	Abbreviation
Aliphatic 3C	Sinigrin	2-propenyl	SIN
	Glucoiberverin	3-methylthiopropyl	GIV
	Glucoiberin	3-methylsulfinylpropyl	GIB
Aliphatic 4C	Progoitrin	(2R)-2-hydroxy-3-butenyl	PRO
	Gluconapin	3-butenyl	NAP
	Glucoerucin	4-methylthiobutyl	GER
	Glucoraphanin	4-methylsulphinylbutyl	GRA
Aliphatic 5C	Gluconapoleiferin	2-hydroxy-4-pentenyl	GNL
	Glucobrassicanapin	4-pentenyl	GBN
	Glucoberteroin	5-methylthiopentyl	GBE
	Glucoalyssin	5-methylsulphinylpentyl	GAL
Indole	Glucobrassicin	3-indolylmethyl	GBS
	Neoglucobrassicin	1-methoxy-3-indolylmethyl	NGBS
	4-Hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl	4HGBS
	4-Methoxyglucobrassicin	4-methoxy-3-indolylmethyl	4MGBS
Aromatic	Gluconasturtiin	2-phenylethyl	GST

different tissues during different growth stages from four *Brassica* crops of the "triangle of U" (*Brassica carinata*, *Brassica nigra*, *B. juncea,* and *B. rapa*) revealed that sinigrin (2-propenyl, SIN) is the dominant GSL in three mustards (*B. carinata*, *B. nigra,* and *B. juncea*) (Table 12.2), where it represents more than 90% of the total GSL concentration in ripe seeds and over 50% of the total GSL concentration in green tissues (Bellostas et al. 2007). *B. carinata* contains other GSLs, including gluconapin (3-butenyl, NAP), 4-hydroxyglucobrassicin (4-hydroxyindol-3-ylmethyl, 4HGBS), gluconasturtiin (2-phenylethyl, GST), and progoitrin (2-hydroxy-3-butenyl, PRO), the last of which is ultimately decomposed into oxazolidine-2-thiones, which are considered to be goitrogenic compounds in monogastric animals (Bellostas et al. 2007; Fahey et al. 2001). The GSL profile of *B. rapa* is quite distinct from that of the aforementioned three mustards (Table 12.2). In *B. rapa*, 16 GSLs have been identified. Among these, the aliphatic GSLs, NAP, and glucobrassicanapin (4-pentenyl, GBN), and their hydroxylated forms, PRO and gluconapoleiferin (2-hydroxy-4-pentenyl, GNL), were found to be the most abundant, while the concentrations of indolic and aromatic GSLs were low and showed the fewest differences among the different varieties (Cartea and Velasco 2008). Most *B. rapa* varieties had a proportion of NAP of between 70 and 95% of the total GSL content and a proportion of GBN of below 20% of the total GSL content, while other minor GSLs, such as glucoiberin (3-methylsulfinilpropyl, GIV), PRO, glucoalyssin (5-methylsulphinylpentyl, GAL), and GST, accounted for less than 20% of the total GSL content (Padilla et al. 2007).

Diversity in the concentration and type of GSLs is much higher in *B. oleracea* than in *B. rapa* species (Ishida et al. 2014). All *B. oleracea* types and cultivars contain high concentrations of glucobrassicin (3-indolymethyl, GBS) and GIV and most contain substantial amounts of SIN. For example, SIN accounts for most of the GSLs in kale (*B. oleracea* var. *acephala*), while GBS and GIB account for most of those in cabbage (*B. oleracea* var. *capitata*) leaves (Cartea et al. 2008). The most common GSLs found in broccoli (*B. oleracea* var. *italica*) are GRA, SIN, PRO, NAP, and the indole GSLs GBS and neoglucobrassicin (1-methoxy-3-indolylmethyl, NGBS) (Kushad et al. 1999). The predominant GSL GRA (accounting for more than 50% of the total GSLs and the precursor of sulforaphane) is the most important health-promoting compound in broccoli, but only trace amounts of GRA are present in most *B. rapa*, *B. napus,* and *B. juncea* vegetables and oilseeds (Liu et al. 2012; Tian et al. 2005). GRA was not detected in several *B. oleracea* crops, including cabbage, Brussels sprouts (*B. oleracea* var. *gemmifera*), and cauliflower (*B. oleracea* var. *botrytis*). In cauliflower, SIN and GIB are the major aliphatic GSLs present (together occurring at a concentration of 0.42 μmol/g FW), and GBS (1.5 μmol/g FW) and 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl, 4MGBS, 0.4 μmol/g FW) are the major indole GSLs (Tian et al. 2005). Broccoli sprouts and Brussels sprouts contain higher amounts of total GSLs than do broccoli and cauliflower. The major GSLs detected in broccoli sprouts are 4MBGS, GRA, GER, and GIB (0.385, 1.33, 1.02, and 0.599 μmol/g FW, respectively; Tian et al. 2005; West et al. 2002). GBS (3.74 μmol/g FW) is the most abundant GSL in Brussels sprouts, while the concentration of SIN, PRO, and NAP (1.55, 1.33, and 1.08 μmol/g FW, respectively) is also relatively higher than that of other GSLs (Tian et al. 2005). In Chinese kale (*B. oleracea* var. *alboglabra*), the total and individual GSL contents varied extensively among the different edible parts, and NAP was the most abundant GSL in the edible plant parts (Sun et al. 2011).

Due to their toxic and antinutritive effect on animals, GSLs have long since been regarded as unfavorable components of *B. napus* seeds. Hence, developing a double-low *B. napus* variety with seeds lacking erucic acid and containing only low levels of GSL has been an important objective of rapeseed breeding programs, and much research examining variation in GSL composition and content in *B. napus* has been conducted (Font et al. 2005; Sang et al. 1984).

Table 12.2 Distribution of GSLs among the six *Brassica* crops in the triangle of U

Crop	Aliphatic										Indole				Aromatic
	3-carbon			4-carbon				5-carbon							
	GIV	GIB	SIN	GER	GRA	NAP	PRO	GBN	GAL	GNL	GBS	NGBS	4HGBS	4MGBS	GST
Brassica carinata[a]	–	–	*	–	–	+	+	–	–	–	+	+	+	+	+
Brassica junce [b]	–	–	*	–	–	*	+	–	–	–	+	+	+	+	+
Brassica napus															
Swede[c]	–	–	–	–	+	–	+	+	–	+	+	*	+	*	+
Leaf rape[c]	+	–	–	–	–	+	+	*	+	+	+	+	–	+	+
High GSL variety[d]		–	–	–	+	*	*	+	+	+	+	+	+	+	+
Low-GSL variety[d]	–	–	–	–	+	+	+	+	+	+	+	+	*	+	+
Brassica nigra[a]	–	–	*	–	–	+	–	–	–	–	+	–	+	+	+
Brassica oleracea[c]															
White cabbage	+	*	*	+	+	+	+	+	+	–	*	+	+	+	+
Savoy cabbage	+	*	*	–	+	+	+	+	–	–	*	+	–	+	+
Red cabbage	+	*	*	–	+	+	+	–	–	–	*	+	–	–	–
Kale	+	*	*	–	+	+	+	–	–	–	*	+	+	+	+
Collard	+	+	*	+	–	–	*	–	–	–	*	–	–	–	–
Tronchuda cabbage	+	*	*	–	+	+	+	+	+	–	*	+	+	+	+
Broccoli	–	+	+	+	*	*	*	+	+	+	*	+	+	+	+
Brussels sprouts	+	+	*	–	+	+	*	–	–	–	*	+	–	–	–
Cauliflower	+	*	*	–	+		*	–	–	–	*	+	–	–	–
Kohlrabi	+	+	*	–	+	+	+	–	+	–	*	+	+	+	–
Brassica rapa[b, c]															
Turnip	–	+	–	+	–	*	*	*	–	+	+	+	+	+	*
Turnip greens	+	+	–	+	+	*	+	*	+	+	+	+	+	–	+
Turnip tops	+	+	–	–	–	*	+	*	–	–	+	+	+	–	+
Chinese cabbage	–	+	–	–	–	*	+	*	–	*	+	+	–	+	+
Bok choy	–	–	–	–	–	*	*	+	–	+	+	+	+	–	+

GIV Glucoiberverin (3-methylthiopropyl); *GIB* glucoiberin (3-methylsulfinylpropyl); *SIN* sinigrin (2-propenyl); *GER* glucoerucin (4-methylthiobutyl); *GRA* glucoraphanin (4-methylsulphinylbutyl); *NAP* gluconapin (3-butenyl); *PRO* progoitrin (2-hydroxy-3-butenyl); *GBN* glucobrassicanapin (4-pentenyl); *GAL* glucoalyssin (5-methylsulphinylpentyl); *GNL* gluconapoleiferin (2-hydroxy-4-pentenyl); GBS: glucobrassicin (3-indolylmethyl); *NGBS* neoglucobrassicin (l-methoxy-3-indolylmethyl); *4HGBS* 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl); *4MGBS* 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl), *GST* Gluconasturtiin (2-phenylethyl). Major GSLs found in each crop are indicated with the * symbol (data sources: [a]Ishida et al. Breed Sci. 2014, 64: 48–59. [b]Yang et al. BMC Genomics 2014, 15:107. [c]Cartea and Velasco Phytochem Rev. 2008, 7:213–229. [d]Li et al. Sci Agric Sin 2005, 38: 1346–1352)

Based on the GSL content, the seeds of 499 *B. napus* accessions were divided into three types, containing high, medium, and low levels of GSLs, and the GSL components of each of these types were systemically analyzed by high-performance liquid chromatography (Li et al. 2005). In *B. napus* varieties containing high and medium levels of GSLs, but not in those containing low levels, the dominant and stable components are PRO and NAP. Although GST and 4HGBS are minor components of *B. napus* varieties containing high levels of GSL, they are major components of varieties containing low GSL levels (Li et al. 2005). Accurately measuring the GSL profiles and identifying the corresponding GSL biosynthetic, breakdown, and transport genes in different *Brassica* crops are of great importance for further improving the GSL profiles in given tissues and organs. For instance, ideal *B. napus* varieties would have high levels of GSLs in the vegetative tissues, but lack GSLs in the seeds.

12.3 Genes Involved in the Metabolism, Transport, and Regulation of GSLs

Substantial advances have recently been made in our understanding of the metabolism and regulation of GSLs in plants, particularly in *Arabidopsis*, where structural and regulatory genes involved in GSL biosynthesis, transport, and degradation pathways have been identified through in vitro biochemical assays and mutant studies (Burow et al. 2010; Radojčić Redovniković et al. 2008; Sønderby et al. 2010).

12.3.1 GSL Biosynthetic Genes

GSL biosynthesis is comprised of three independent stages: (i) amino acid chain elongation, in which additional methylene groups are inserted into the side chain of certain aliphatic and aromatic amino acids, (ii) conversion of the amino acid moiety to form the core structure of GSLs, and (iii) subsequent secondary modifications of side chains to generate chemical diversity (Grubb and Abel 2006; Halkier and Gershenzon 2006). Methionine undergoes a series of chain elongation cycles in which one methylene group is added per time prior to entering the core structure pathway. These chain elongation reactions include deamination by a branched-chain amino acid aminotransferase (BCAT), condensation with acetyl-CoA by a methylthioalkylmalate synthase (MAM), isomerization by an isopropylmalate isomerase (IPMI), and oxidative decarboxylation by an isopropylmalate dehydrogenase (IPM-DH) (Sønderby et al. 2010). The newly formed 2-oxo acid can either be transformed into the corresponding methionine derivative and enter the core GSL structure pathway or undergo another round of chain elongation (Radojčić Redovniković et al. 2008). In *A. thaliana*, three tandemly duplicated and functionally diverse MAM members were identified as being responsible for the condensation step of the chain elongation. Functional analysis demonstrated that AtMAM2 (absent in ecotype Columbia) and AtMAM1 catalyze the condensation reaction of the first and the first two elongation cycles, respectively, for the synthesis of aliphatic GSLs with short carbon chains (3C and 4C, respectively) (Benderoth et al. 2006; Kroymann et al. 2003; Textor et al. 2004), while AtMAM3 catalyzes all six additions of methylene groups and the formation of all aliphatic GSLs, especially long-chain GSLs (6C, 7C, and 8C) (Textor et al. 2007). Hence, the number and expression patterns of *MAM* genes in a plant determine variations in aliphatic GSLs during the earliest stages of GSL biosynthesis and have a fundamental impact on GSL composition and diversity in plant tissues.

The GSL core structure is formed from precursor amino acids via a series of reactions catalyzed by various cytochrome P450 (CYP) monooxygenases (Halkier and Gershenzon 2006). Briefly, the five characterized CYP79 homologs in *Arabidopsis* catalyze the conversion of amino acids to their corresponding aldoximes. *CYP79F1* and *CYP79F2* encode the enzymes that catalyze aldoxime production in the biosynthesis of the major GSLs derived from chain-elongated

methionine derivatives. *CYP79B2* and *CYP79B3* catalyze the biosynthesis of indole-3-acetaldoxime from tryptophan, whereas *CYP79A2* converts phenylalanine to phenylacetaldoxime, the precursor of benzyl GSL (Radojčić Redovniković et al. 2008). Biochemical studies identified differences in the substrate specificity of CYP79F1 and CYP79F2, showing that CYP79F1 metabolizes homomethionine and di-, tri-, tetra-, penta-, and hexahomomethionines, resulting in both short- and long-chain methionine derivatives, whereas CYP79F2 only catalyzes the production of long-chain penta- and hexahomomethionines (Chen et al. 2003; Radojčić Redovniković et al. 2008). The aldoximes are further metabolized to form *S*-alkylthiohydroximates by CYP83A1 and CYP83B1, cytochrome P450 of the CYP83 family (Bak and Feyereisen 2001). Both biochemical and transgenic lines of evidence show that CYP83A1 mainly metabolizes the aliphatic aldoximes to form aliphatic GSLs, whereas CYP83B1 mostly metabolizes indole-3-acetaldoxime and aromatic oximes to synthesize the corresponding substrates for indolic and aromatic GSLs, respectively (Bak and Feyereisen 2001; Naur et al. 2003). In a subsequent step, the resulting *S*-alkylthiohydroximates are cleaved to yield thihydroximates by a *C-S* lyase SUR1 (Mikkelsen et al. 2004). The second to last step in the formation of GSLs is the *S*-glycosylation of thihydroximates, a reaction that is catalyzed by glucosyltransferases of the UGT74 family. This reaction appears to be unique and catalyzes the formation of an *S*-glycosidic bond between glucose and the acceptor thiohydroximate, leading to the production of the corresponding desulfo-GSL (Grubb et al. 2004). The results of biochemical and genetic analyses demonstrated that UGT74C1 plays a key role in the biosynthesis of aliphatic GSLs and that UGT74B1 catalyzes the formation of aromatic GSLs (Grubb et al. 2004, 2014). Three sulfotransferase (SOT) proteins perform the final step of GSL biosynthesis. Biochemical characterization showed that SOT16 metabolizes tryptophan- and phenylalanine-derived desulfo-GSLs, whereas SOT17 and SOT18 metabolize long-chained aliphatic desulfo-GSLs (Piotrowski et al. 2004).

After parent GSL formation, a wide range of further modifications can occur on the methionine side chain and occasionally on the glucose moiety (Mikkelsen et al. 2002; Neal et al. 2010), giving rise to an enormous variety of GSL structures. These secondary modifications, which take place in an organ- and developmental stage-specific manner (Radojčić Redovniković et al. 2008; Sønderby et al. 2010), are particularly important as the structure of the side chain largely determines the nature of the products formed following GSL hydrolysis by myrosinases (Sønderby et al. 2010; Wittstock and Halkier 2002). For aliphatic GSLs, these modifications include oxidations, hydroxylations, alkenylations, and benzoylations, while for indole GSLs, they include hydroxylations and methoxylations.

The *S*-oxygenation of aliphatic GSLs is a common modification catalyzed by five flavin-monooxygenases, designated $FMO_{GS\text{-}OX1}$ to $FMO_{GS\text{-}OX5}$ (Li et al. 2008). $FMO_{GS\text{-}OX5}$ shows substrate specificity for the long-chain 8-methylthiooctyl GSLs (8MTOs), whereas $FMO_{GS\text{-}OX1}$ to $FMO_{GS\text{-}OX4}$ exhibit broad chain length specificity and catalyze the conversion from methylthioalkyl (MT) GSL to the corresponding methylsulfinylalkyl (MS) independently of chain length (Li et al. 2008), resulting in the production of the potent cancer-preventive substances sulforaphane (4-methylsulfinylbutyl isothiocyanate, 4MSB ITC), which is derived from GRA, and the 7-methylsulfinylheptyl (7MSOH) and 8-methylsulfinyloctyl (8MSOO) isothiocyanates, derived from 7-methylthioheptyl GSL (7MTH) and 8MTO, respectively (Li et al. 2008). Hence, the five $FMO_{GS\text{-}OX}$ genes could potentially be used in genetic engineering strategies to optimize the GSL profiles of *Brassica* crops. Substantial variation in *Arabidopsis* GSL profiles between different genotypes has expedited the identification of the GS-AOP locus, which encodes the two tandemly duplicated 2-oxoglutarate-dependent dioxygenases, AOP2 and AOP3 (Kliebenstein et al. 2001). AOP2 directly catalyzes the conversion of methylsulfinylalkyl GSLs to the alkenyl GSLs NAP or GBN (n = 2–3), and the GS-OH

locus can further convert NAP to PRO (Hansen et al. 2008). AOP3 controls the production of hydroxyalkyl GSLs ($n = 2$) from methylsulfinylalkyl GSLs. When both AOPs are non-functional, the plant accumulates the precursor methylsulfinyl alkyl GSLs (Liu et al. 2014). Secondary modifications of indole GSLs mainly include hydroxylation by CYP81F2, which is essential for the 4-hydroxylation of unmodified indolyl-3-methyl (I3M), and catalyzes the formation of 4-hydroxy I3M (4OH-I3M) and 4-methoxy I3M (4M-I3M) from I3M (Bednarek et al. 2009; Pfalz et al. 2009; Sønderby et al. 2010).

12.3.2 Regulatory Genes of GSL Biosynthesis

Biosynthesis of GSLs is tightly regulated by six R2R3-MYB transcription factors (TFs) belonging to subgroup 12 of the R2R3 MYB family, which has a conserved "[L/F]LN[K/R]VA" motif (Dubos et al. 2010). In *Arabidopsis*, *MYB28*, *MYB29*, and *MYB76* positively regulate the biosynthesis of aliphatic GSLs with partial functional redundancy (Hirai et al. 2007). During aliphatic GSL biosynthesis, *AtMYB28* acts as the major positive regulator and *AtMYB29* as an accessory factor in the response to methyl jasmonate signaling in the *trans*-activation of the aforementioned aliphatic GSL biosynthetic genes, i.e., *AtMAM1*, *AtMAM3*, *AtCYP79F1*, *AtCYP79F2*, *AtCYP83A1, AtAOP2*, *AtSOT17,* and *AtSOT18* (Gigolashvili et al. 2008a; Hirai et al. 2007). *Arabidopsis* mutants defective in MYB28 function had decreased amounts of both long- and short-chain aliphatic GSLs, whereas the *myb29* or *myb76* mutant contained significantly reduced levels of short-chained aliphatic GSLs, indicating that MYB28 regulates the biosynthesis of all methylsulfinyl GSLs, whereas MYB29 and MYB76 regulate the biosynthesis of short-chained GSLs (Gigolashvili et al. 2008b). The total aliphatic GSLs but not indolic GSLs were significantly increased in the leaves of plants overexpressing *AtMYB28*, *AtMYB29*, or *AtMYB76* (Gigolashvili et al. 2008b; Hirai et al. 2007). Overexpression of both *AtMYB28* and *AtMYB29* significantly repressed the expression of the indolic GSL pathway genes, indicating that a reciprocal antagonistic relationship exists between the aliphatic and indolic GSL biosynthetic pathways (Gigolashvili et al. 2008a).

Conversely, *AtMYB34*, *AtMYB51*, and *AtMYB122*, which were identified as important regulators of the indolic GSL biosynthetic pathway, significantly reduced the transcript levels of *AtCYP79B2*, *AtCYP79B3*, *AtCYP83B1*, *AtUTG74B1*, *AtSOT16,* and *3'-phosphoadenosine 5'-phosphosulphate transporter* (*PAPST1*) genes, which are involved in the indolic GSL biosynthetic pathway (Frerigmann and Gigolashvili 2014; Guo et al. 2013; Sønderby et al. 2010). The three *MYB* transcription factors exhibit both additive and epistatic interactions in the regulation of indolic GSL biosynthesis (Frerigmann and Gigolashvili 2014). Lines lacking the two main regulators of indolic GSL biosynthesis, *MYB34* and *MYB51*, exhibit a significant reduction in total indolic GSLs, demonstrating the importance of these two genes for indolic GSL biosynthesis. Previous research also showed that *MYB34* and *MYB51* have distinct roles in indolic GSL production, functioning in different tissues or under different environmental conditions. *MYB51* is the central regulator of indolic GSL biosynthesis in shoots and is activated by salicylic acid (SA) and ethylene (ET) treatments. By contrast, *MYB34* regulates indolic GSL biosynthesis mainly in the roots and functions in abscisic acid (ABA) and methyl jasmonate (MeJA) signaling. Interestingly, *MYB51* appears to regulate indolic GSL biosynthesis in roots in the *myb34* mutant. *MYB122* only plays an accessory role in indolic GSL biosynthesis and in JA/ET-induced GSL biosynthesis (Frerigmann and Gigolashvili 2014).

In addition to the MYB transcription factors, some other regulators of GSL biosynthesis have also been characterized. *Arabidopsis* CaM-binding protein IQ-DOMAIN1 (IQD1) binds calmodulin in a Ca^{2+}-dependent manner and is a positive regulator of total GSL accumulation during biotic stress responses, with a

gain-of-function *IQD1* mutation resulting in elevated levels of both indole and aliphatic GSLs and a reduction in insect herbivory and infestation (Laluk et al. 2012; Levy et al. 2005). Another CaM-binding transcription factor SIGNAL RESPONSIVE1 (AtSR1) also proved to be a key regulator of GSL levels through transcriptional regulation of several genes involved in GSL metabolism, including *AtIQD1*, *AtMYB51,* and *AtSOT16*, and is a negative regulator for herbivory tolerance in *Arabidopsis* (Laluk et al. 2012). *AtSLIM1* was identified as a central transcription factor that negatively regulates both aliphatic and indolic biosynthesis under sulfur-limiting conditions and downregulates *AtMBY34* transcription (Maruyama-Nakashita et al. 2006). Another characterized regulator of GLS biosynthesis is DNA-binding-with-one-finger (DOF) transcription factor *AtDof1.1* (also known as *AtOBP2*), which is induced by wounding and herbivore attack and MeJA treatment, and specifically upregulates *CYP83B1* expression and promotes indolic GSL accumulation (Skirycz et al. 2006). Although *AtDof1.1* does not seem to regulate the expression of *CYP79F1* and *CYP79F2*, the aliphatic GSL content was altered in *AtDof1.1* overexpression lines (Skirycz et al. 2006). Loss-of-function mutations of *Arabidopsis TERMINAL FLOWER2* (*TFL2*, also known as *LHP1* or *TU8*) significantly increased the abundance of four long-chain aliphatic GSLs in the seeds, whereas indolyl-3-methyl GSL levels were significantly reduced relative to the wild type, leading to a reduction in symptoms resulting from infection by the obligate biotrophic fungus *Plasmodiophora brassicae*, which causes clubroot disease, a damaging disease in Brassicaceae (Kim et al. 2004; Le Roux et al. 2014). In addition, TFL2 regulates heterochromatin formation and represses the expression of genes involved in flowering time, floral organ identity, meiosis, and seed maturation (Nakahigashi et al. 2005).

12.3.3 GSL Transport Genes

The GSLs are believed to be synthesized mainly in rosette leaves and silique walls and then to be relocated to embryos through phloem by specific transporters (Lu et al. 2014). In *Arabidopsis*, GSLs have successfully been eliminated from the seeds by silencing two recently identified nitrate/peptide transporter family members, *GTR1* and *GTR2*, which suggests that manipulation of these two transporters may increase the nutritional value of crops and be used in biotechnological approaches to control the allocation of GSLs to seeds in *Brassica* crops (Nour-Eldin et al. 2012). The *gtr2* single mutant exhibited a significant reduction in total GSL levels in seeds and a threefold increase in aliphatic GSLs in source tissues (i.e., senescent leaves and silique walls), but no significant changes in GSL content in the seeds (Jorgensen et al. 2015; Nour-Eldin et al. 2012). In the *gtr1 gtr2* double mutant, aliphatic and indolic GSLs were absent in the seeds, but exhibited a more than tenfold increase in source tissues, demonstrating that both plasma membrane-localized transporters are essential for long-distance GSL transport to the seeds and are responsible for loading GSLs from the apoplasm into the phloem, and finally for determining the tissue-specific distribution of GSLs in plants (Nour-Eldin et al. 2012). Identifying these two GSL transporters provides a strategy for breeding *Brassica* varieties that contain extremely low levels of total GSLs in the seeds but high levels in the green tissues by reducing functional GTR activity and blocking the translocation of GSLs.

12.3.4 GSL Breakdown Genes

Numerous studies to date have focused on the beneficial effects of GSLs and their breakdown products on human health and plant defense, and on their negative effects on animal nutrition. In

the well-studied GSL–myrosinase-specifier protein system, myrosinases hydrolyse GSLs in the presence of water, producing a series of degradation products (Wittstock and Burow 2010). The types of products of myrosinase hydrolysis depend on the structure of the parent GSLs, reaction conditions, and availability of epithiospecifier proteins (ESPs) and nitrile-specifier proteins (NSPs) (Kissen and Bones 2009).

In *Arabidopsis*, six genes (*TGG1*-*TGG6*) encoding classical myrosinases have been identified on two chromosomes (Xu et al. 2004). Among these genes, *TGG1* and *TGG2* were tandem duplicates of *TGG3*, while *TGG5* and *TGG6* were tandem duplicates of *TGG4*. These duplicated genes share the same gene structure as their parent genes. Although *TGG3* and *TGG6* are predominantly expressed in specific tissues (Xu et al. 2004), both are probably pseudogenes that encode non-functional proteins due to multiple frameshift mutations (Wang et al. 2009). *TGG1* is expressed in myrosin cells, stomatal guard cells, and phloem cells of all the aboveground organs except the seeds (Barth and Jander 2006; Xue et al. 1995). Similar to *TGG1*, *TGG2* is also highly expressed in the aboveground tissues (Xu et al. 2004), but is much less abundant in the rosette leaves than is *TGG1*, and was not detected in guard cells (Zhao et al. 2008). *TGG4* and *TGG5* are primarily expressed in the roots. Despite the distinct expression patterns and the difference in vitro myrosinase activities of TGG1 and TGG2, GSL breakdown in the crushed leaves of *TGG1* or *TGG2* single mutants is basically unchanged, indicating that the two myrosinases may have redundant functions (Barth and Jander 2006). Leaf extracts of *TGG1 TGG2* double mutants had no detectable in vitro myrosinase activity on exogenously applied aliphatic GSLs, and endogenous aliphatic GSLs were no longer broken down in disrupted leaf material of the double mutant (Barth and Jander 2006). However, myrosinase-independent breakdown of indolic GSLs still slowly proceeds, indicating the presence of a breakdown pathway for these GSLs that is independent of TGG1 and TGG2.

Several specifier proteins, such as ESPs and NSPs, myrosinase-associated proteins (MyAPs), such as EPITHIOSPECIFIER-MODIFIER1 (ESM1), MODIFIED VACUOLE PHENOTYPE1 (MVP1), and enzymes involved in further metabolism, such as nitrilases, have been shown to be involved in the generation of diversified GSL metabolic products in *Arabidopsis* (Wittstock and Burow 2010). Specifier proteins do not exhibit hydrolytic activity on GSLs, but affect the outcome of GSL hydrolysis products. In the absence of specifier proteins, ITCs are typically formed at neutral pH (Bones and Rossiter 2006). ESPs and the related thiocyanate-forming proteins (TFPs) catalyze the formation of epithionitrile, in the presence of GSLs with terminal double bonds in the side chain and ferrous ions, while the formation of thiocyanate purely depends on TFPs (Wittstock and Burow 2010). NSPs are involved in simple nitrile formation at acidic pH values, but do not catalyze epithionitrile or thiocyanate formation. The simple nitrile can be further converted by nitrilases (NITs) to a carboxylic acid in the presence of a specifier protein (Vorwerk et al. 2001; Wittstock and Burow 2010). ESP function is inhibited by ESM1, leading to decreased simple nitrile formation and increased ITC production for benzyl and alkyl GSLs, but not for alkenyl GSLs (Zhang et al. 2006). Cloning and sequence analysis of *ESM1* revealed that it encodes a putative endoplasmic reticulum (ER) binding protein and that allelic variation in this gene contributes to the variation in GSL breakdown among different *Arabidopsis* accessions (Zhang et al. 2006). *MVP1* is expressed ubiquitously and encodes another MyAP-like protein that is closely related to ESM1. The *mvp1* mutant is impaired in endomembrane protein trafficking and shows a significant increase in simple nitrile production from allyl GSLs (Agee et al. 2010). Interestingly, MVP1 interacts with TGG2 and the PYK10 complex, but not with TGG1 in vitro, suggesting that MVP1 functions in the quality control of GSL hydrolysis by contributing to the proper tonoplast localization of TGG2 and in ER body-related defense systems by regulating the PYK10 complex (Agee

et al. 2010; Nakano et al. 2012). An atypical myrosinase gene, *PEN2*, which may be limited to indole GSL hydrolysis and is required for pathogen resistance, was recently identified in *Arabidopsis* (Bednarek and Osbourn 2009).

12.4 Evolution of GSL-Related Genes in *B. Napus* and Its Parental Species

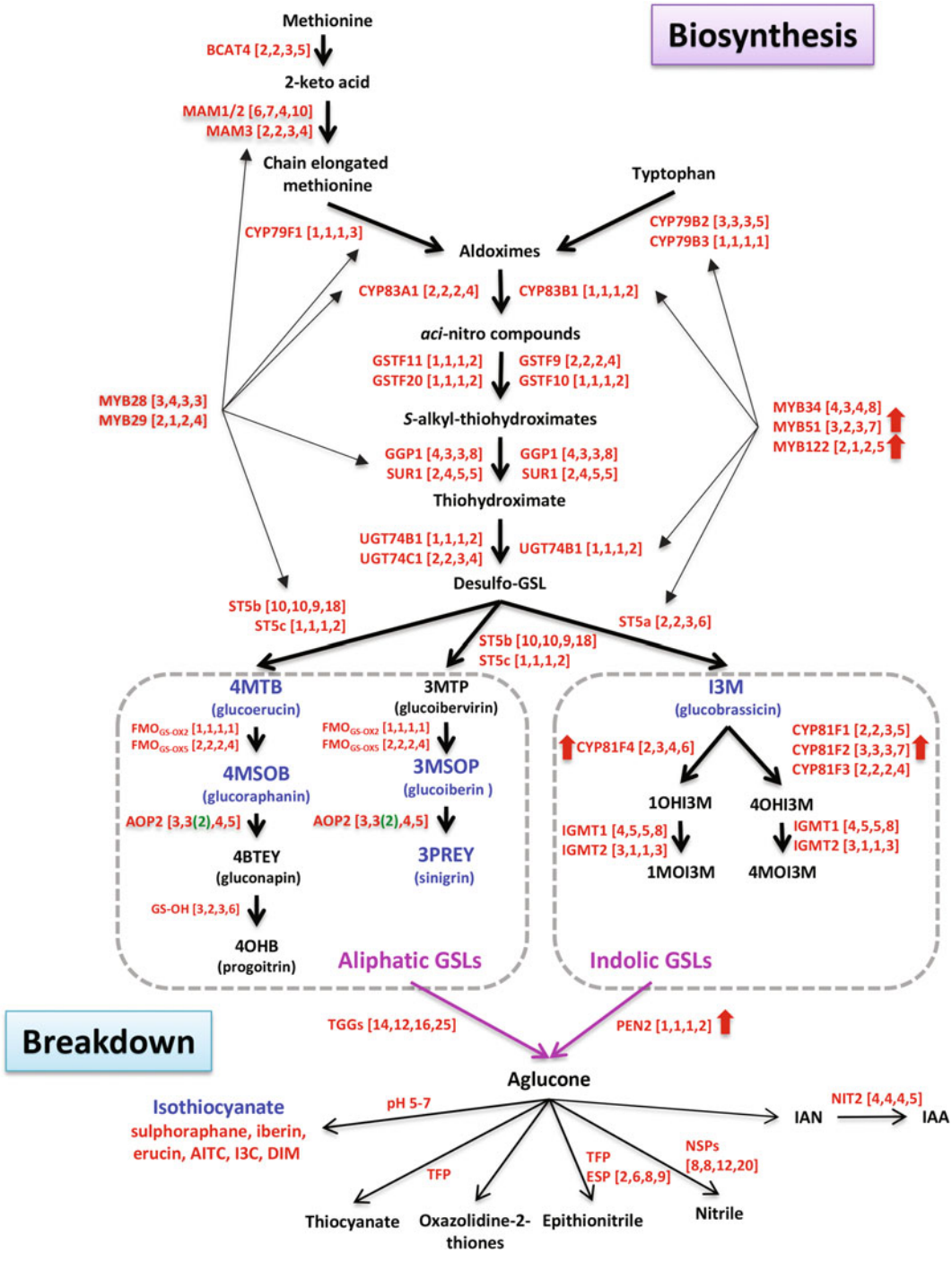
12.4.1 Identification of GSL-Related Genes from *B. Napus* and Its Parental Species

To identify GSL-related genes from *B. napus* and its parental species *B. rapa* and *B. oleracea*, we used the sequences of 58 GSL biosynthesis, 3 GSL transport, and 17 GSL breakdown genes characterized in *A. thaliana* as queries against the four publicly available genomes of *Brassica* crops based on a combination of syntenic and nonsyntenic homology analyses (Table 12.3). We identified 119, 119, 134, and 240 GSL biosynthetic genes in *B. rapa*, *B. oleracea* var. *capitata*, *B. oleracea* var. *italica,* and *B. napus* (both 120 genes in A and C subgenomes), respectively (Fig. 12.1). The fact that more GSL biosynthetic genes were identified in *B. oleracea* var. *italica* than in the other three *Brassica* crops is mainly a consequence of the expansion of genes responsible for core structure formation and side-chain modification. For three *Arabidopsis* GSL transporters, there are 8 orthologs in both *B. rapa* and two subgenomes of *B. napus*, while only 7 and 6 orthologs exist in *B. oleracea* var. *capitata* and *B. oleracea* var. *italica*, respectively. The number of GSL breakdown genes is almost identical among *B. rapa*, *B. oleracea* var. *capitata,* and two subgenomes of *B. napus*, while *B. oleracea* var. *italica* contains many more.

After the split with *Arabidopsis*, the *Brassica* progenitor species experienced a whole-genome triplication (WGT) and subsequently diverged into three diploid *Brassica* species, *B. rapa*, *B. oleracea,* and *B. nigra*. As a young allopolyploid species, *B. napus* was formed from multiple independent hybridization events between ancestors of the diploids *B. rapa* (A genome donor) and *B. oleracea* (C genome donor) (Nagaharu 1935). Hence, we found that most multi-copy genes might have originated from WGT events and that several gene families involved in GSL metabolism

Table 12.3 GSL-related genes in *Arabidopsis* and in *B. napus* and its parental species

Pathway	*Arabidopsis*	*B. rapa*	*B. oleracea* var. *capitata*	*B. oleracea* var. *italica*	A subgenome of *B. napus*	C subgenome of *B. napus*
GSL biosynthesis	58	119	119	134	120	120
Transcription factors	9	21	21	23	20	20
Side-chain elongation	10	18	20	20	20	19
Core structure formation	18	37	38	43	39	37
Side-chain modification	15	28	23	33	26	30
Co-substrate pathways	6	15	17	15	15	14
GSL transport	3	8	7	6	8	8
GSL breakdown	17	39	41	51	38	40
Total	78	166	167	191	165	168


Biosynthesis
Methionine
BCAT4 [2,2,3,5]
2-keto acid
MAM1/2 [6,7,4,10]
MAM3 [2,2,3,4]
Chain elongated methionine
Typtophan
CYP79F1 [1,1,1,3]
CYP79B2 [3,3,3,5]
CYP79B3 [1,1,1,1]
Aldoximes
CYP83A1 [2,2,2,4]
CYP83B1 [1,1,1,2]
aci-nitro compounds
GSTF11 [1,1,1,2]
GSTF20 [1,1,1,2]
GSTF9 [2,2,2,4]
GSTF10 [1,1,1,2]
S-alkyl-thiohydroximates
MYB28 [3,4,3,3]
MYB29 [2,1,2,4]
MYB34 [4,3,4,8]
MYB51 [3,2,3,7]
MYB122 [2,1,2,5]
GGP1 [4,3,3,8]
SUR1 [2,4,5,5]
GGP1 [4,3,3,8]
SUR1 [2,4,5,5]
Thiohydroximate
UGT74B1 [1,1,1,2]
UGT74C1 [2,2,3,4]
UGT74B1 [1,1,1,2]
Desulfo-GSL
ST5b [10,10,9,18]
ST5c [1,1,1,2]
ST5a [2,2,3,6]
ST5b [10,10,9,18]
ST5c [1,1,1,2]
4MTB
(glucoerucin)
3MTP
(glucoibervirin)
FMOGS-OX2 [1,1,1,1]
FMOGS-OX5 [2,2,2,4]
FMOGS-OX2 [1,1,1,1]
FMOGS-OX5 [2,2,2,4]
4MSOB
(glucoraphanin)
3MSOP
(glucoiberin)
AOP2 [3,3(2),4,5]
AOP2 [3,3(2),4,5]
4BTEY
(gluconapin)
3PREY
(sinigrin)
GS-OH [3,2,3,6]
4OHB
(progoitrin)
Aliphatic GSLs
I3M
(glucobrassicin)
CYP81F4 [2,3,4,6]
CYP81F1 [2,2,3,5]
CYP81F2 [3,3,3,7]
CYP81F3 [2,2,2,4]
1OHI3M
4OHI3M
IGMT1 [4,5,5,8]
IGMT2 [3,1,1,3]
IGMT1 [4,5,5,8]
IGMT2 [3,1,1,3]
1MOI3M
4MOI3M
Indolic GSLs
Breakdown
TGGs [14,12,16,25]
PEN2 [1,1,1,2]
Aglucone
Isothiocyanate
sulphoraphane, iberin, erucin, AITC, I3C, DIM
pH 5-7
IAN
NIT2 [4,4,4,5]
IAA
TFP
TFP
ESP [2,6,8,9]
NSPs
[8,8,12,20]
Thiocyanate
Oxazolidine-2-thiones
Epithionitrile
Nitrile

◀ **Fig. 12.1** Comparison of aliphatic and indolic glucosinolate biosynthetic and breakdown genes in *A. thaliana*, *B. rapa*, *B. oleracea* var. *capitata,* and *B. napus*. The copy number of GSL biosynthetic genes in *A. thaliana*, *B. rapa*, *B. oleracea* var. *capitata* and *B. napus* is listed in square brackets. Potential anticancer substances/precursors are highlighted in blue bold. The most important transcription factors, amino acid chain elongation and side-chain modification loci MYB28 (HAG1), MAMs, and AOP2, are highlighted in red bold, with the number in parentheses (green) representing the number of non-functional genes. 1MOI3 M: 1-methoxyindol-3-ylmethyl GSL; 1OHI3 M: 1-hydroxyindol-3-ylmethyl GSL; 3 MSOP: 3-methylsulfinylpropyl GSL; 3 MTP: 3-methylthiopropyl GSL; 3PREY: 2-Propenyl GSL; 4BTEY: 3-butenyl GSL; 4BzOB: 4-benzoyloxybutyl GSL; 4MOI3 M: 4-methoxyindol-3-ylmethyl GSL; 4OHB, 4-hydroxybutyl GSL; 4OHI3M: 4-hydroxyindol-3-ylmethyl GSL; 4MSOB: 4-methylsulfinylbutyl GSL; 4MTB, 4-methylthiobutyl GSL; AITC: allyl isothiocyanate; DIM: 3,3'-diindolylmethane; ESP: epithiospecifier protein; I3C: indole-3-carbinol; IAA: indole-3-acetaldehyde; IAN: indole-3-acetonitile; I3M: indolyl-3-methyl GSL; NSP: nitrile-specifier protein; TFP: thiocyanate-forming protein; and TGG: thioglucoside glucohydrolase (Figure reprinted, with modifications, from Liu et al. (2014) under a CC BY license (Creative Commons Attribution 4.0 International License))

or transport also experienced homeologous gene loss events after the WGT, leading to the formation of 13 conserved single-copy GSL biosynthesis genes and single copies of GSL transport (*PEN3*) and breakdown (*PEN2*) genes in *B. rapa*, *B. oleracea,* and two subgenomes of *B. napus*. The 78 GSL-related genes present in *Arabidopsis* represent 0.28% of all *Arabidopsis* genes, while the GSL-related genes in *B. rapa*, *B. oleracea* var. *capitata*, *B. oleracea* var. *italica,* and *B. napus* represent 0.40, 0.36, 0.33, and 0.33% of all predicted genes in the corresponding species, indicating that the expansion levels and total numbers of GSL-related genes in *Brassica* crops are similar to the whole-genome gene expansion levels of the corresponding crops (P-value > 0.05).

To reveal the retention status of the GSL-related genes after the WGT, we determined the ratio of single- to multi-copy paralogous genes involved in various steps of GSL metabolism (Table 12.4). The proportion of total paralogous sets with different copy numbers over the whole genome was used as background, and we found that the expansion levels of transcription factors, side-chain modification, and breakdown genes in *B. rapa* were significantly higher than those of their backgrounds ($P < 0.05$). The same trends were observed for GSL breakdown genes in two *B. oleracea* genomes and for transcription factors in *B. oleracea* var. *italica*, indicating that a specific subset of GSL-related genes was retained in *B. oleracea*. Over-retention of GSL transcription factors occurred in the C subgenome of *B. napus*, while those associated with side-chain modification and breakdown were only over-retained in the A subgenome of *B. napus*. It seems that the GSL-related genes responsible for chain elongation, core structure formation, co-substrate pathways, and transport did not experience significant expansion, since they showed no significant difference from the background (Table 12.4). However, the GSL-related genes were significantly retained in all four studied *Brassica* crops, since the ratio of single- to multi-copy paralogous genes was significantly smaller than the background (P-value < 0.05), suggesting that GSL-related genes expanded in *B. rapa* and *B. oleracea* and were retained in the two subgenomes of *B. napus*. Tandem duplication (TD) also contributed greatly to the evolution of GSL-related genes in both *Arabidopsis* and *Brassica* species. We identified 11 TD events in *Arabidopsis* GSL-related genes, including 8 and 3 events associated with GSL biosynthesis and breakdown, respectively. We found that 21 pairs of paralogous genes had undergone more recent TD events after WGT in two *B. oleracea* crops and two subgenomes of *B. napus*. For example, *SOT18* consists of 10 copies in *B. rapa*, *B. oleracea* var. *capitata,* and the C subgenome of *B. napus*, and 9 and 8 copies in *B. oleracea* var. *italica* and A subgenome of *B. napus*, respectively. At least six *SOT18* genes originated from three TD events in all of these *Brassica* species, implying that these ancient TD events might have occurred after the *Arabidopsis–Brassica* split and before divergence of *B. rapa* and *B. oleracea*.

Similar to the findings of a previous study in *B. rapa* (Wang et al. 2011a), we found that a total

Table 12.4 Number of single- and multi-copy paralogs of GSL-related genes and their ratios among *Brassica* crops

Role in GSL metabolism	*B. rapa*			*B. oleracea* var. *capitata*			*B. oleracea* var. *italica*			A subgenome of *B. napus*			C subgenome of *B. napus*		
	Single[a]	Multi[b]	*P*-value[c]	Single[a]	Multi[b]	*P*-value[c]	Single[a]	Multi[b]	*P*-value[c]	Single[a]	Multi[b]	*P*-value[c]	Single[a]	Multi[b]	*P*-value[c]
Transcription factor	0	8	0.0021**	2	6	0.1572	0	8	0.0098**	1	7	0.0811	0	8	0.0102**
Chain elongation	2	6	0.1553	2	6	0.1572	3	5	0.7359	2	6	0.3083	2	6	0.3097
Core structure formation	8	9	0.6317	8	9	0.6342	8	9	1.0000	7	10	0.8116	7	9	1.0000
Side-chain modification	1	10	0.0039**	2	8	0.0529	2	9	0.1261	1	8	0.0475*	2	8	0.2014
Co-substrate pathways	2	4	0.4245	2	4	0.4270	2	4	0.6959	2	4	0.6966	2	4	0.6973
Transport	1	2	0.5987	1	2	0.6015	1	2	1.0000	1	2	1.0000	1	2	1.0000
Breakdown	2	10	0.0165*	3	10	0.0474*	2	12	0.0282*	2	11	0.0472*	4	9	0.4070
Total	16	49	0.0000**	20	45	0.0003**	18	49	0.0029**	16	48	0.0015**	18	46	0.0078**
Background [d]	9172	7868		8977	7841		8641	10,420		8520	10,398		8460	10,401	

[a]The number of single-copy paralogs. [b]The number of multi-copy paralogs. [c] The proportion of total paralogous sets with different copy numbers over the whole genome was used as background to calculate the *P*-value using Fisher's test. The "*" and "**" indicate *P*-values of less than 0.05 and 0.01, respectively, which means the ratio of single to multiple copies of these kinds of GSL-related genes shows a significant difference from the background. [d] The background data were derived from Liu et al. (2014) and Chalhoub et al. (2014)

of 11 GSL-related genes in *Arabidopsis* have no orthologs in the studied *Brassica* genomes, including a transcription factor (*MYB76*), two amino acid side-chain elongation genes (*IPMDH3* and *IPMI SSU3*), one core structure formation gene (*CYP79F2*) for long-chain aliphatic GSL, four side-chain modification genes ($FMO_{GS\text{-}OX1}$, $FMO_{GS\text{-}OX3}$, $FMO_{GS\text{-}OX4}$ and *AOP3*), and three GSL breakdown genes (*NSP3*, *NIT1,* and *NIT3*). It seems that the loss of these genes is not indispensable for GSL biosynthesis and breakdown, as paralogs with similar functions are present in the *Brassica* species.

12.4.2 Evolution of GSL Biosynthesis Genes Influencing Variation in GSL Profiles in *B. napus* and Its Parental Species

To date, more than 20 kinds of GSLs have been identified in commercial *Brassica* crops. The diversity of GSL types and variation in GSL profiles in these *Brassica* species are largely due to the evolution of GSL-related genes. In our study, we mainly focused on the evolution of *MAM* and *AOP* gene families in the four *Brassica* crops.

The *MAM* genes encode methylthioalkylmalate synthase, which is involved in amino acid chain elongation, and gave rise to GSLs with diverse chain lengths during the biosynthesis of methionine-derived GSLs (Zhang et al. 2015a, b, b). The phylogenetic and synteny relationships of *MAM* genes from 13 sequenced Brassicaceae species indicated that the *MAM* genes taken two independent lineage-specific evolution routes after the divergence from *Aethionema arabicum*. In the lineage I species such as *A. thaliana*, the *MAM* loci evolved three tandem genes encoding enzymes responsible for the biosynthesis of aliphatic GSLs with different carbon chain lengths, while in lineage II species such as *Brassica* crops, the *MAM* loci encode enzymes responsible for the biosynthesis of short-chain aliphatic GSLs (Zhang et al. 2015). In *Arabidopsis*, the *MAM* family contains three tandemly duplicated and functionally diverse members, *MAM1*, *MAM2*, and *MAM3* (*MAM-L*). Functional analysis demonstrated that MAM2 and MAM1 catalyze the condensation of the first and the first two elongation cycles for the synthesis of short-chain Met-derived aliphatic GSLs (3C and 4C), respectively, while MAM3 catalyzes the formation of all aliphatic GSLs, especially long-chain GSLs (6C, 7C, and 8C) (Textor et al. 2007).

In *B. rapa*, *B. oleracea* var. *capitata*, and *B. oleracea* var. *italica*, *MAM1/MAM2* genes experienced independent TD after WGT to produce 6, 7, and 6 orthologs, respectively (Fig. 12.1). Due to gene loss that occurred after the formation of *B. napus* from the fusion of two parental species, only 5 and 3 orthologs were retained in the A and C subgenomes of *B. napus*. The greatest diversity of GSL side-chain structures in *Brassica* is observed within *B. oleracea*. The main GSLs in this species (i.e., PRO, NAP, GRA, and SIN) are restricted to either 3C or 4C side-chain lengths (Liu et al. 2014). In contrast to the diversity observed in *B. oleracea*, *B. nigra* and the amphidiploid *B. carinata* only have the 3C GSL and SIN, and *B. juncea* mainly has 3C and 4C GSLs (SIN and NAP). *B. rapa* and *B. napus* lack 3C GSLs and predominately possess a mixture of 4C GSLs (NAP and PRO and their hydroxylated homologs), with small amounts 5C GSL GBS. Thus, all of these *Brassica* species can be considered to have functional alleles at the *MAM1/MAM2* loci, while some variation occurred at the *MAM3* locus, which led to the existence of 5C GSL in *B. rapa* and *B. napus*. Based on our analyses of expression patterns and phylogenetic and syntenic relationships, we identified a pair of genes, *Bol017070* and *Bra013007*, which are the only orthologs with high expression in *B. oleracea* var. *capitata,* but are silenced in *B. rapa* (Liu et al. 2014). Their two descendant orthologs in *B. napus, BnaA03g39720D* and *BnaCnng21190D,* both showed weak expression in roots and silenced in siliques simultaneously, implying that *Bol017070* might greatly promote the accumulation of the 3C GSL anticancer precursor SIN in *B. oleracea*. At the *MAM3* locus, one orthologous group of genes, *Bra008532*, *Bol040636*, *BnaA02g36350D,* and *BnaC02g27590D,* showed no expression due to

pseudogenization. In another *MAM3* orthologous group, expression of *Bra018524* is much higher than that of *Bol016496*, *BnaA02g20830D*, and *BnaC02g26810D*. Expression differences of *MAM3* genes among *Brassica* crops most likely resulted in the increased biosynthesis of the 5C GSLs GBN and GNL in *B. rapa*.

In addition to *MAM* genes, *AOPs* are other crucial regulators of variation in aliphatic GSL profiles in Brassicaceae species (Hasan et al. 2008). Previous phylogenetic analyses showed that the core Brassicaceae species have retained AOP1, while AOP2 is retained by most of the lineage II species (excluding *Sisymbrium irio* and *Raphanus sativus*), and AOP3 by lineage I species. The variation in AOP2/AOP3 has led to different aliphatic GSL profiles in each lineage (Al-Shehbaz and Al-Shammary 1987). While the function of GSL-AOP1 is currently unknown, AOP2 catalyzes the conversion of methylsulfinylalkyl GSLs (GRA and GIB) to alkenyl GSLs (NAP and SIN), and the GS-OH locus can further convert NAP to PRO. AOP3 is associated with the production of hydroxyalkyl GSL, a compound not found in *Brassica* crops. When both AOPs are non-functional, the plant accumulates the methylsulfinylalkyl GSL precursor (Liu et al. 2014). Genetic variation at *AOP2* is also linked to increased GSL accumulation, since its expression promotes the transcription of most GSL biosynthetic genes and two R2R3 domain MYB transcription factors (*MYB28* and *MYB29*) of the pathway, suggesting that *AOP2* plays a role in the positive feedback loop controlling aliphatic GSL biosynthesis (Burow et al. 2015).

Phylogenetic and BLASTN analysis indicated that the genomes of *B. rapa*, *B. oleracea* var. *capitata,* and *B. napus* possess 3, 3, and 5 orthologs of *AOP2* and contain 3, 2, and 7 orthologs of *AOP1,* respectively (Fig. 12.2). Not all *Brassica* species have an ortholog of *AtAOP3*, and such species are unable to produce hydroxyalkyl GSLs. Similar to our results, a natural frameshift mutation resulting from a 2-bp deletion was identified in broccoli, which accumulates GRA by ceasing downstream biosynthesis of other 4C aliphatic GSLs (Li and Quiros 2003). In our previous study, we found that 2 non-functional *AOP2* genes contributing to the accumulation of GRA due to the presence of premature stop codons (Liu et al. 2014). Hence, it would be a useful strategy to enhance the GRA concentrations in *Brassica* crops by blocking the side-chain modification pathway downstream of GRA through silencing of all orthologs of *AOP2*. Recently, this strategy has been successfully applied in the metabolic engineering for increasing the anticancer compound GRA by suppressing *AOP2* gene family in both *B. juncea* and *B. napus* (Liu et al. 2012; Augustine and Bisht. 2015). In *B. rapa*, all three *BrAOP2* paralogs have been proved to be active but functionally diverged (Zhang et al. 2015). Expression patterns of five *AOP2* genes in *B. napus* are quite different, *BnaA09g01260D* and *BnaC09g00410D* showed the highest expression in siliques, while the rest *AOP2* paralogs showed higher expression in flower and stem (Fig. 12.2), implying that these *Bna.AOP2* genes might be functional. These results provide insight into the relationship between observed GSL profiles and the evolution of GSL biosynthesis genes and explain why anticancer compound GRA is abundant in *B. oleracea*, but not in *B. rapa* and *B. napus*. The *AOP2* genes in *B. rapa* and *B. napus* are functional, reflecting the fact that the dominant GSLs are NAP and PRO in both *B. rapa* and *B. napus*.

12.4.3 Evolution of Major Genes Controlling the Seed GSL Content in *B. napus*

Quantitative trait locus (QTL) mapping and association mapping (AM) are powerful methods for analyzing the genetic structure of quantitative traits and have been widely used to characterize the total seed GSL contents and profiles in different populations of *B. napus* (Fu et al. 2015; Hasan et al. 2008; Li et al. 2014; Uzunova et al. 1995). Recently, the orthologs of *HAG1* (*MYB28*), which controls aliphatic GSL biosynthesis in *Arabidopsis*, were suggested as candidates for major QTLs on A09, C02, C07, and C09 of *B. napus*. These QTLs were detected

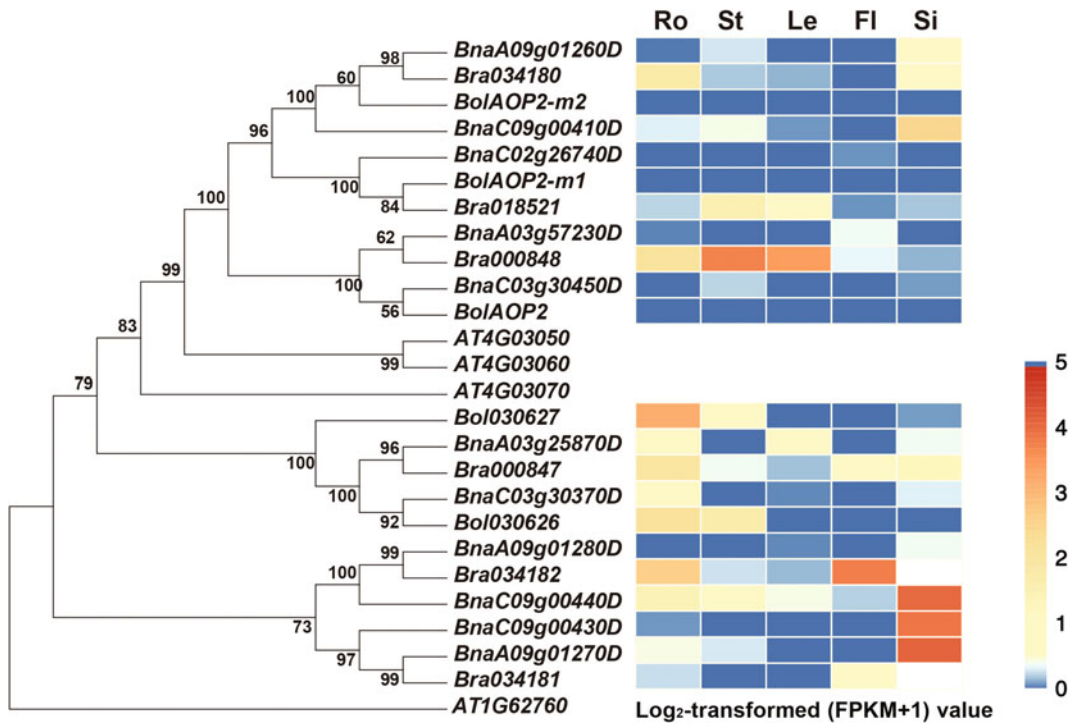


Fig. 12.2 Phylogenetic analysis of three AtAOP genes and orthologs in *B. rapa*, *B. oleracea* var. *capitata,* and *B. napus*. Full-length sequences of AOP proteins from *Arabidopsis*, *B. rapa*, *B. oleracea* var. *capitata,* and *B. napus* were aligned using ClustalW2. The phylogenetic tree (left panel) was constructed using MEGA 6.0 and the neighbor-joining method (1000 bootstrap replicates). Expression levels of *Brassica AOP* genes were derived from Tong et al. (2013) and Liu et al. (2014) and are presented as the $\log_2$-transformed (FPKM + 1) values

independently in different studies using different methods, including conventional QTL mapping, AM, and associative transcriptomic analysis (Li et al. 2014; Lu et al. 2014; Zhao and Meng 2003). Howell et al. (2003) detected four QTLs that together accounted for at least 76% of the phenotypic variation in the accumulation of GSLs in *B. napus* seeds and revealed that the QTLs on A09, C02, and C09 were homoeologous loci (Howell et al. 2003). Harper et al. (2012) revealed that the *HAG1* transcription factor gene family was a candidate in the quantitative control of GSL content of *B. napus* and that the orthologous genes on C02 and A09 had been lost from the low-GSL accessions (Harper et al. 2012). In our study, we identified three copies of *HAG1* genes (*BnaA03g40190D*, *BnaCnng43220D,* and *BnaC09g05300D*) from the genome sequence of the French homozygous *B. napus* winter line "Darmor-*bzh*," which is a double-low *B. napus* cultivar lacking detectable levels of erucic acid in the seed oil and with a low seed GSL content (Chalhoub et al. 2014). We found that the *AtHAG1* orthologs on A09 and C02 were deleted from the double-low *B. napus* cultivar "Darmor-*bzh*," leading to a reduction in seed GSL accumulation. The expression patterns of the three *Bna.HAG1* genes were investigated in an elite semi-winter double-low *B. napus* cultivar "Zhongsuang No. 11," which is widely cultivated in the Yangtze River region of China (Fig. 12.3). Among the three retained *Bna.HAG1* genes, neither *BnaA03g40190D* nor *BnaCnng 43220D* was expressed in siliques, indicating that the proteins encoded by these two genes probably lost DNA-binding activity for seed GSL accumulation. *BnaC09g05300D* exhibited the highest transcription levels in the root, followed by the stem and flower, and was expressed at very low levels in the leaf and siliques. Sequence

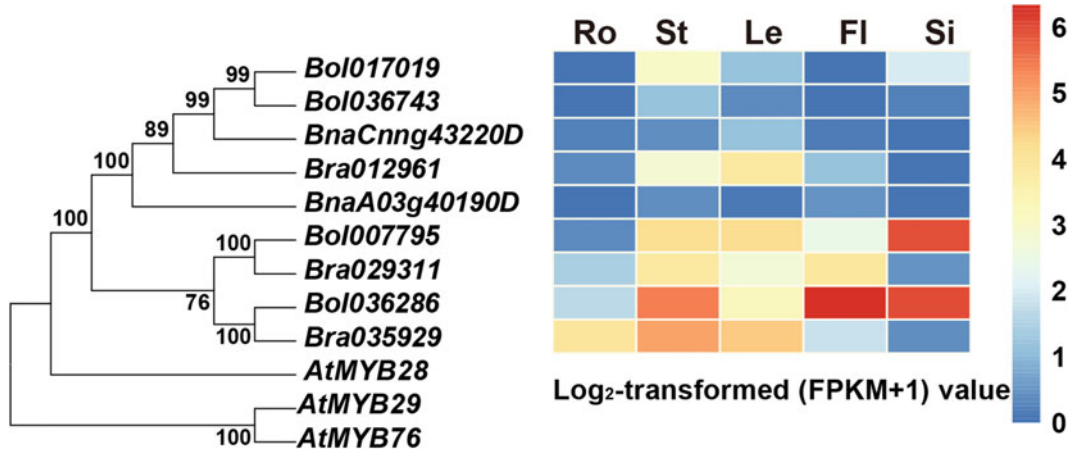


Fig. 12.3 Phylogenetic analysis of AtHAG1 and orthologs in *B. rapa*, *B. oleracea* var. *capitata,* and *B. napus*. Full-length sequences of AtHAG1 (MYB28), AtMYB29, AtMYB76, and three Bra.HAG1, four Bol.HAG1, and two Bna.HAG1 proteins were aligned using ClustalW2. The phylogenetic tree (left panel) was constructed using MEGA 6.0 and the neighbor-joining method (1000 bootstrap replicates). The BnaC09g05300D protein sequence was too short to be excluded in the phylogenetic analysis. Expression levels of *Brassica HAG1* genes were derived from Tong et al. (2013) and Liu et al. (2014) and are presented as the $\log_2$-transformed (FPKM + 1) values

alignment revealed that the *BnaC09g05300D* coding sequence is only 420 bp long, much shorter than that of *AtHAG1* and other members of the *HAG1* gene family in *Brassica* crops, but the intact MYB DNA-binding domain (PF00249) was still predicted to exist in the BnaC09g 05300D protein sequence. These data suggest that the *Bna.HAG1* gene family experienced not only gene loss due to segment deletion, but also loss of most function in the seeds during the breeding of low-GSL *B. napus*. In current low-GSL *B. napus* accessions, *BnaC09g05300D*, which controls the biosynthesis of aliphatic GSLs, might be the only functional *Bna.HAG1* gene. Therefore, it is possible to further reduce the seed GSL content in low-GSL *B. napus* lines by silencing *BnaC09g05300D* expression.

12.4.4 Evolution of GSL Transport Genes in *B. napus*

The GSLs are believed to be synthesized mainly in the roots and vegetative tissues and accumulate abundantly in the embryos, where no de novo synthesis occurs (Nour-Eldin and Halkier 2013). Therefore, there must be specific transporters that are responsible for the relocation of GSLs from source tissues to embryos through the phloem. Recently, two members of the nitrate/peptide transporter family in *Arabidopsis*, GTR1 and GTR2, were identified as high-affinity plasma membrane-localized, GSL-specific proton symporters in a screen of an in vitro library of *Arabidopsis* transporters (Nour-Eldin et al. 2012). Previous studies suggested that GTR2 is essential for loading GSLs into the phloem, while GTR1 additionally may be involved in distributing GSLs within the leaf. Importantly, GTR1 and GTR2 are essential for the long-distance transport of both aliphatic and indole GSLs to seeds, because the *gtr1 gtr2* double mutant had only trace levels of GSLs in seeds and a concomitant increase in rosettes and silique walls (Nour-Eldin et al. 2012). However, it is notable that indole GSLs are transported between rosettes and roots in the absence of GTRs, suggesting the existence of an indole glucosinolate-specific transporter besides GTR1 and GTR2 (Jorgensen et al. 2015).

We identified 32 orthologs of *AtGTR* in the four *Brassica* crops we investigated, including

15 *GTR1* and 17 *GTR2* genes. Phylogenetic analysis and tissue-specific expression detection showed that the transcription levels of most *Bna. GTR* genes are lower than those of orthologs in the parental species *B. rapa* or *B. oleracea* var. *capitata* (Fig. 12.4). For example, *Bra029248* and *Bol020699* showed higher expression than *BnaA02g33530D* and *BnaC02g42260D*. The expression of GSL-related genes was determined in the Chinese double-low *B. napus* cultivar "Zhongsuang No. 11." This analysis indicated that the expression of *Bna.GTR* genes is reduced in this cultivar, suggesting that the reduced transport of GSLs from source tissues to seeds accounts for the hypo-accumulation of GSLs in the seeds of this low-GSL content variety. For each *AtGTR* gene, we identified at least one *Bna. GTR* ortholog with high expression (Fig. 12.4). For instance, *BnaA09g06190D*, *BnaC09g 05810D*, *BnaC03g51560D,* and *BnaC03g 75950D* which might be the major GTR members responsible for the long-distance transport of GSL in the *B. napus* cultivar "Zhongsuang No. 11," were expressed at higher levels than

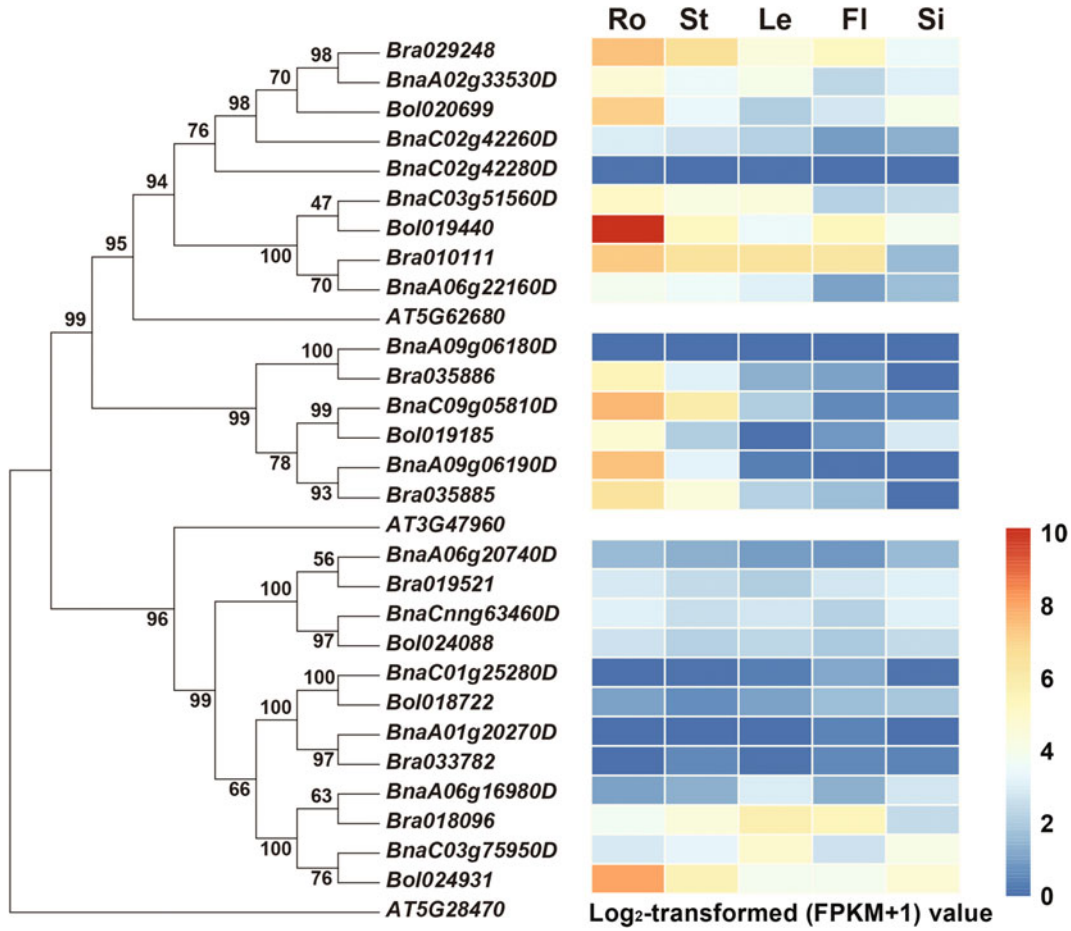


Fig. 12.4 Phylogenetic analysis of two AtGTR genes and orthologs in *B. rapa*, *B. oleracea* var. *capitata,* and *B. napus*. Full-length sequences of GTR proteins from *Arabidopsis*, *B. rapa*, *B. oleracea* var. *capitata,* and *B. napus* were aligned using ClustalW2. The phylogenetic tree (left panel) was constructed using MEGA 6.0 and the neighbor-joining method (1000 bootstrap replicates). Expression levels of *Brassica GTR* genes were derived from Tong et al. (2013) and Liu et al. (2014) and are presented as the $\log_2$-transformed (FPKM + 1) values

other members. Lu et al. (2014) reported that the transcript abundance in the leaves of the candidate gene involved in GSL transport, *BnaA. GTR2a,* located on chromosome A02, was correlated with seed GSL content, accounting for 18.8% of the phenotypic variation in seed GSL content between *B. napus* cultivars (Lu et al. 2014). Recently, we also found that *Bna.GRT2* on chromosome A09 is a candidate GSL transporter and is associated with seed GSL content based on AM analysis of seed GSL content using the 60K *Brassica* Infinium SNP array in 520 *B. napus* accessions. These results strongly suggest that transport engineering can be used to eliminate antinutritional GSLs in seeds by silencing GTR transporters in *B. napus*.

Indole GSL 4HGBS is the major GSL present in the low-GSL *B. napus* varieties. Whether the total GSL content can be further reduced by silencing all of the *Bna.GTR* genes merits further investigation. In addition, the major GSL transporter, GTR1, is multifunctional and may be involved in the transport of structurally distinct compounds, including GSLs, jasmonoyl-isoleucine, and gibberellin, and may positively regulate stamen development by mediating gibberellin transport in *Arabidopsis* (Saito et al. 2015). The *gtr1* mutants are severely impaired in filament elongation and anther dehiscence, resulting in reduced fertility, and hence, it is uncertain whether silencing of all of the *Bna.GTR* genes would produce normal *B. napus* plants that lack GSLs in the seeds. Although there are potential limitations in genetic engineering applications, the *Bna.GTR* genes represent the most promising regulation loci among the GSL-related genes and have potential applications in molecular breeding efforts to further reduce GSL levels in the seeds and increase them in the vegetative tissues and roots, where they play important roles in enhancing biotic and/or abiotic resistance in *B. napus*.

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