

Developing multifunctional crops by engineering Brassicaceae glucosinolate pathways

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<https://doi.org/10.1016/j.xplc.2023.100565>

ABSTRACT

Glucosinolates (GSLs), found mainly in species of the Brassicaceae family, are one of the most well-studied classes of secondary metabolites. Produced by the action of myrosinase on GSLs, GSL-derived hydrolysis products (GHPs) primarily defend against biotic stress *in planta*. They also significantly affect the quality of crop products, with a subset of GHPs contributing unique food flavors and multiple therapeutic benefits or causing disagreeable food odors and health risks. Here, we explore the potential of these bioactive functions, which could be exploited for future sustainable agriculture. We first summarize our accumulated understanding of GSL diversity and distribution across representative Brassicaceae species. We then systematically discuss and evaluate the potential of exploited and unutilized genes involved in GSL biosynthesis, transport, and hydrolysis as candidate GSL engineering targets. Benefiting from available information on GSL and GHP functions, we explore options for multifunctional Brassicaceae crop ideotypes to meet future demand for food diversification and sustainable crop production. An integrated roadmap is subsequently proposed to guide ideotype development, in which maximization of beneficial effects and minimization of detrimental effects of GHPs could be combined and associated with various end uses. Based on several use-case examples, we discuss advantages and limitations of available biotechnological approaches that may contribute to effective deployment and could provide novel insights for optimization of future GSL engineering.

Key words: glucosinolate, glucosinolate hydrolysis product, metabolic engineering, Brassicaceae, multifunctional crop ideotype

Qin H., King G.J., Borpatragohain P., and Zou J. (2023). Developing multifunctional crops by engineering Brassicaceae glucosinolate pathways. *Plant Comm.* 4, 100565.

INTRODUCTION

The prevalence and versatility of glucosinolates (GSLs) and GSL-derived hydrolysis products (GHPs) have underpinned the success and wide diversity of crops in the plant family Brassicaceae, as well as species in 16 other Brassicales families (Barco and Clay, 2019). Although GSLs have evolved in plants by providing strategies to manage challenging biotic and abiotic environments, they are mostly compatible with the human gut system and, with some exceptions, confer beneficial properties to crops and derived by-products (Liu et al., 2021; Sikorska-Zimny and Beneduce, 2021). The ability of humans to digest tissues from every part of Brassicaceae plant anatomy has led to domestication-related selection of diverse morphotypes distinguished by the proliferation of different organs and tissues (Cheng et al., 2016). The extent to which this has been achieved in the past few thousand years is almost unique among flowering plants (Mabry et al., 2018; Gallagher et al., 2019; Bryce et al., 2021; McAlvay et al., 2021) and is likely due

to the absence of more toxic plant secondary metabolites such as cyanogenic glycosides (Moreira et al., 2018).

This review aims to provide an overview of current knowledge and opportunities for future GSL engineering. The availability of annotated genomes, first for *Arabidopsis* and then for each of the *Brassica* crops and other Brassicaceae species, has provided a rich source of information that complements the increased resolution of chemical analysis and functional mode-of-action studies for GSLs, precursors, and GHPs (Wang et al., 2013; Guo et al., 2016; Bell et al., 2020; Harun et al., 2020; Song et al., 2021; Zhao et al., 2021). Understanding the detailed evolution of distinct GSL subclasses within different taxa, along with relevant biosynthetic and regulatory networks, now enables the

Published by the Plant Communications Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and CEMPS, CAS.

development of predictive strategies for metabolic engineering to define specifications or ideotypes. In particular, there is scope to recombine subnetworks and pathways to deliver desirable GSLs/GHPs and reduce antinutritional counterparts in a wider range of crops, which could provide both unique flavors for the human diet and specific resistance to crop pests and pathogens (Miao et al., 2021). Based on our review, we propose an integrated roadmap for generating multifunctional Brassicaceae crops to meet future demand for food diversification and sustainable crop production. Use-case examples are outlined to explore the possibilities of crop ideotype design and the availability of current biotechnological approaches, while also considering how beneficial effects of GHPs could be maximized and detrimental effects minimized in target crops.

DIVERSITY OF GSL STRUCTURES AND TAXONOMIC DISTRIBUTION

Glucosinolates (GSLs) are a large group of sulfur-rich, amino acid-derived secondary metabolites with similar chemical structures that include a β -D-thioglucose group, a sulfonated oxime group, and an amino acid-derived R group (or side chain). A series of analytical methods have been developed to identify and isolate diverse GSL structures and quantify GSL content (Blažević et al., 2020). By mid-2021, at least 90 distinct GSL structures had been satisfactorily characterized in plants, with a further ~49 structures remaining to be examined (Blažević et al., 2020; Montaut et al., 2020; Trabelcy et al., 2021). GSLs are mostly reported and studied in species of the Brassicaceae family, which includes a wide range of globally important vegetable, fodder, forage, condiment mustard, and oilseed crops and accounts for most (84 out of 90) well-characterized GSL structures. Using the comprehensive GSL number system for enumeration, these GSLs are **5, 11, 12, 15, 19, 22, 23, 24R, 24S, 28–31, 38S, 40R, 40S, 43, 45–48, 50R, 51, 54, 56–58, 61–69, 72, 73, 77, 79, 80, 82–84, 87, 88, 92, 94, 95, 101, 105, 107, 111, 112, 114, 117, 121R, 121S, 122R, 122S, 125–127, 129–143, 148, 149, 151, and 152** (Fahey et al., 2001; Blažević et al., 2020; Montaut et al., 2020). Most GSLs in Brassicaceae crops are derived biosynthetically from either methionine (Met), phenylalanine (Phe), tyrosine (Tyr), or tryptophan (Trp) (Wu et al., 2021a). Met-derived aliphatic GSLs appear to predominate in most species, both in content and number of structures (Table 1; Supplemental Table 1). Indeed, Met-derived aliphatic GSLs constitute the majority of all known GSLs (Blažević et al., 2020). Much of this diversity derives from chain elongation of the Met with consecutive CH_2 groups early in GSL biosynthesis (Figure 1A). Accordingly, the Met-derived aliphatic GSLs are often assigned to different subgroups according to the number of C atoms between the side-chain sulfur and the oxime carbon (Table 1; Supplemental Table 1). For some Met-derived aliphatic GSLs, the terminal methyl and sulfur are eliminated, and in those cases the subgroups simply reflect the number of carbon atoms in the side chain (Blažević et al., 2020). The usual GSLs in common Brassicaceae species belong to the C3–C5 subgroups, corresponding to one to three added CH_2 groups, but in some crops such as watercress (*Nasturtium officinale*) and wasabi (*Eutrema japonicum*), as well as non-domesticated species, longer side chains are found, up to the C11 subgroup (Blažević et al., 2020) (Table 1; Supplemental Table 1). Trp-

derived GSLs are usually known as indole GSLs because the indole moiety from Trp contained in these GSLs is structurally conserved in general. Only four indole GSLs are commonly encountered in crops and typically all occur (Table 1). Only three GSLs derived from Phe occur at significant levels in crops. Glucotropaeolin is predominant in garden cress (*Lepidium sativum*) seeds and contains a benzyl side chain that is directly derived from the precursor amino acid (Table 1). Gluconasturtiin is predominant in several organs of watercress and results from a single-chain elongation of Phe (Figure 1B; Table 1; Supplemental Table 1). Further hydroxylation of gluconasturtiin results in glucobarbarins that occur at high levels in some weeds, at moderate levels in watercress, and at very low levels in rapeseed (*Brassica napus*) (Blažević et al., 2020) (Table 1; Supplemental Table 1). Only one GSL derived from Tyr, sinalbin, is common in crops; it carries the same phenol group as the precursor amino acid and for this reason has numerous chemical similarities to the indole GSLs (Blažević et al., 2020). The full structural diversity of GSLs is richer than we can fully capture here and includes GSLs derived from alanine, valine (Val), leucine (Leu), isoleucine (Ile), and glutamate (Blažević et al., 2020). We solely list one Ile-derived GSL, glucocochlearin (Table 1), to represent branched-chain GSLs (derived from branched-chain amino acids Val, Leu, Ile, and their homologs). Although glucocochlearin occurs rarely in Brassicaceae crops (Agerbirk et al., 2021), it has been detected in several species: at relatively high levels in seeds of Chinese cabbage (*Brassica rapa* spp. *pekinensis*), kale (*Brassica oleracea* var. *acephala*), mustard (*Brassica juncea*), and radish (*Raphanus sativus*) and at lower levels in other organs of Chinese cabbage, kale, rapeseed, pak choi (*B. rapa* spp. *chinensis*), wasabi, and horseradish (*Armoracia rusticana*) (Table 1; Supplemental Table 1).

Beyond this structural diversity, the content, concentration, and dynamic changes of GSLs vary among plant growth stages, organs, and tissues (Bell and Wagstaff, 2017). Comparison of available GSL information between tissues and organs of several Brassicaceae species clearly shows that these variations in GSL profiles are associated with different taxa (Table 1; Supplemental Table 1). In *B. napus*, for example, progoitrin and gluconapin are the major GSLs in seeds, whereas glucobrassicinapin is predominant in leaves. Among *B. napus* oilseed cultivars, the difference between traditional high-GSL and modern selected low-GSL (canola) types is primarily due to a significant reduction in progoitrin and gluconapin, as well as a moderate reduction in 4-hydroxyglucobrassicin (March et al., 1989; Fieldsend and Milford, 1994; Li et al., 1999; Andini et al., 2019; Missinou et al., 2022). Nevertheless, progoitrin remains the most abundant GSL in the seeds of low-GSL cultivars (Velasco et al., 2008). The Phe-derived gluconasturtiin is frequently most abundant in roots, particularly in *Brassica* species (Kirkegaard and Sarwar, 1998; Bhandari et al., 2015) (Supplemental Table 1).

Over time, the ancestors of most Brassicales families have undergone β (~85 million years ago) and α (~31.8 million years ago) whole-genome duplication (WGD) events, with a series of recent gene duplications and rearrangements (Edger et al., 2015, 2018). This has provided the opportunity for divergence and specialization of genes involved in GSL biosynthesis, transport,

Precursor amino acid	Group	Trivial name	Number ^c	Semisystemic name	<i>Arabidopsis thaliana</i> ^b	<i>Brassica carinata</i>	<i>Brassica juncea</i>	<i>Brassica napus</i>	<i>Brassica nigra</i>	<i>Brassica oleracea</i> ^b	<i>Brassica rapa</i> ^b	<i>Eruca sativa</i>	<i>Lepidium sativum</i>	<i>Nasturtium officinale</i>	<i>Raphanus sativus</i>	<i>Sinapis alba</i>
					Seed ^a	Seed	Seed	Seed	Seed	Leaf	Leaf	Leaf	Seed	Leaf	Root	Seed
Met	C3	sinigrin	107	2-propenyl GSL	0	+++	+	tr,†	+++	+	0	0	0	0	0	tr
		glucoibererin	95	3-(methylthio) propyl GSL	tr	0	tr	0	0	tr	0	0	0	0	0	0
		glucoiberin	73	3-(methylsulfinyl) propyl GSL	tr	0	tr	0	0	+	0	0	0	tr	0	0
	C4	glucoerucin	84	4-(methylthio) butyl GSL	+++	0	+	tr	0	tr	tr,†	+	0	0	+,†	0
		glucoraphasatin	83	4-(methylthio)but-3-enyl GSL	0	0	0	0	0	0	0	0	0	0	+++	0
		glucoraphanin	64	4-(methylsulfinyl) butyl GSL	+	0	+	+	0	++	tr,†	++	0	0	tr	0
		glucoraphenin	63	4-(methylsulfinyl) but-3-enyl GSL	0	0	0	0	0	+	0	0	0	0	++,†	0
		gluconapin	12	3-butenyl GSL	0	tr	+++	++	0	tr	+++	0	0	0	0	tr
		progoitrin	24R	(R)-2-hydroxybut-3-enyl GSL	0	+	+	+++	0	+	++	+	0	0	0	+
		epiprogoitrin	24S	(S)-2-hydroxybut-3-enyl GSL	0	0	0	tr	0	++	0	+,†	0	0	0	0
	C5	glucobertoin	94	5-(methylthio) pentyl GSL	+	0	tr	tr	0	0	0	tr,†	0	0	0	0
		glucoalysinn	72	5-(methylsulfinyl) pentyl GSL	tr	0	tr	+	0	tr,†	+	tr	0	0	0	0
		glucobrassicinapin	101	4-pentenyl GSL	0	0	tr	+	0	tr	++	0	0	0	+,†	0
		gluconapoleiferin	38S	2-hydroxypent-4-enyl GSL	0	0	tr,†	tr	0	0	+	0	0	0	0	0
	C6–C10 ^d				+++	0	0	tr,†	0	0	0	0	0	+	0	0
Phe/Tyr		glucotropaeolin	11	benzyl GSL	0	+	tr,†	0	0	0	0	tr,†	+++	tr	0	tr
		sinalbin	23	4-hydroxybenzyl GSL	0	0	0	tr,†	0	0	0	tr,†	0	0	0	+++
		gluconasturtiin	105	2-phenylethyl GSL	tr	0	tr	tr	tr	+	tr,†	tr	0	+++	+,†	0
		glucobarbarins	40	2-hydroxy-2-phenylethyl GSLs	0	0	0	0	0	0	0	0	0	+	0	0
Trp		glucobrassicin	43	3-indolylmethyl GSL	+	tr	tr	tr	tr	++	+	tr	0	+	tr	tr
		4-hydroxyglucobrassicin	28	4-hydroxyindol-3-ylmethyl GSL	tr	tr	tr,†	+	tr	0	tr,†	+	0	tr	tr,†	0
		neoglucobrassicin	47	1-methoxyindol-3-ylmethyl GSL	0	0	tr	tr	0	tr,†	+	tr,†	0	0	0	tr

Table 1. The classification of major GSLs and their taxonomic distribution among important Brassicaceae species.

(Continued on next page)

Precursor amino acid	Group	Trivial name	Number ^c	Semisystemic name	<i>Arabidopsis thaliana</i> ^b	<i>Brassica carinata</i>	<i>Brassica juncea</i>	<i>Brassica napus</i>	<i>Brassica nigra</i>	<i>Brassica oleracea</i> ^b	<i>Brassica rapa</i> ^b	<i>Eruca sativa</i>	<i>Lepidium sativum</i>	<i>Nasturtium officinale</i>	<i>Raphanus sativus</i>	<i>Sinapis alba</i>
					Seed ^a	Seed	Seed	Seed	Seed	Leaf	Leaf	Leaf	Seed	Leaf	Root	Seed
		4-methoxyglucobrassicin	48	4-methoxyindol-3-ylmethyl GSL	0	0	tr	tr	tr	tr	tr	+	0	tr	tr	0
Ile		glucocochlearin	61	1-methylpropyl GSL	0	0	++	0	0	0	+	0	0	0	0	0

Table 1. Continued

+++, the predominant GSL, which accounts for $\geq 40\%$ of the total GSL content in an organ. ++, GSL that accounts for $\geq 20\%$ and $<40\%$ of the total GSL content in an organ. +, GSL that accounts for $\geq 1\%$ and $<20\%$ of the total GSL content in an organ. tr, GSL remains in trace amounts, accounting for $<1\%$ of the total GSL content in an organ. 0, GSL that was not detected in an organ. †, GSL that was detected in only a few cultivars but was absent in other cultivars of a species. All the GSL content provided here are dry weight data.

^aThe representative organ is selected to illustrate GSL profiles of each species.

^bGSL profiles of Col-0 ecotype, pak choi, and white cabbage are selected to represent *A. thaliana*, *B. rapa*, and *B. oleracea*, respectively.

^cThe bold GSL numbers proposed by Fahey et al. (2001) are listed here for reference in Supplemental Table 1. In particular, the number 40 is used to represent glucobarbarins, which include two epimers, glucobarbarin (40S) and epiglucobarbarin (40R).

^dLong-chain GSLs are included in the group C6–C10 and specified in Supplemental Table 1, which includes (1) 6-(methylthio)hexyl GSL (88), also glucolesquerellin; (2) 6-(methylsulfinyl)hexyl GSL (67), also glucohesperin; (3) 7-(methylthio)heptyl GSL (87); (4) 7-(methylsulfinyl)heptyl GSL (66), also glucoibarin; (5) 8-(methylthio)octyl GSL (92); (6) 8-(methylsulfinyl)octyl GSL (69), also glucohirsutin; (7) 8-(methylsulfonyl)octyl GSL (80); (8) 9-(methylsulfonyl)nonyl GSL (79); and (9) 10-(methylsulfinyl)decyl GSL (65), also glucocamelinin.

Data sources: Matthäus and Luftmann (2000); Brown et al. (2003); Boyd et al. (2006); Bellostas et al. (2007); Gill et al. (2007); Velasco et al. (2008); Pasini et al. (2012); Augustine et al. (2013b); Wiesner et al. (2013b); Chun et al. (2013); Zhu et al. (2013); Agerbirk et al. (2014); Bhandari et al. (2015); Zhang et al. (2015c); Yi et al. (2015, 2016); Giallourou et al. (2016); Taranto et al. (2016); Jeon et al. (2017, 2022); Park et al. (2017); Klopsch et al. (2018); Andini et al. (2019); Bell et al. (2021); Li et al. (2021b); Gohain et al. (2021); Wang et al. (2022b); Missinou et al. (2022).

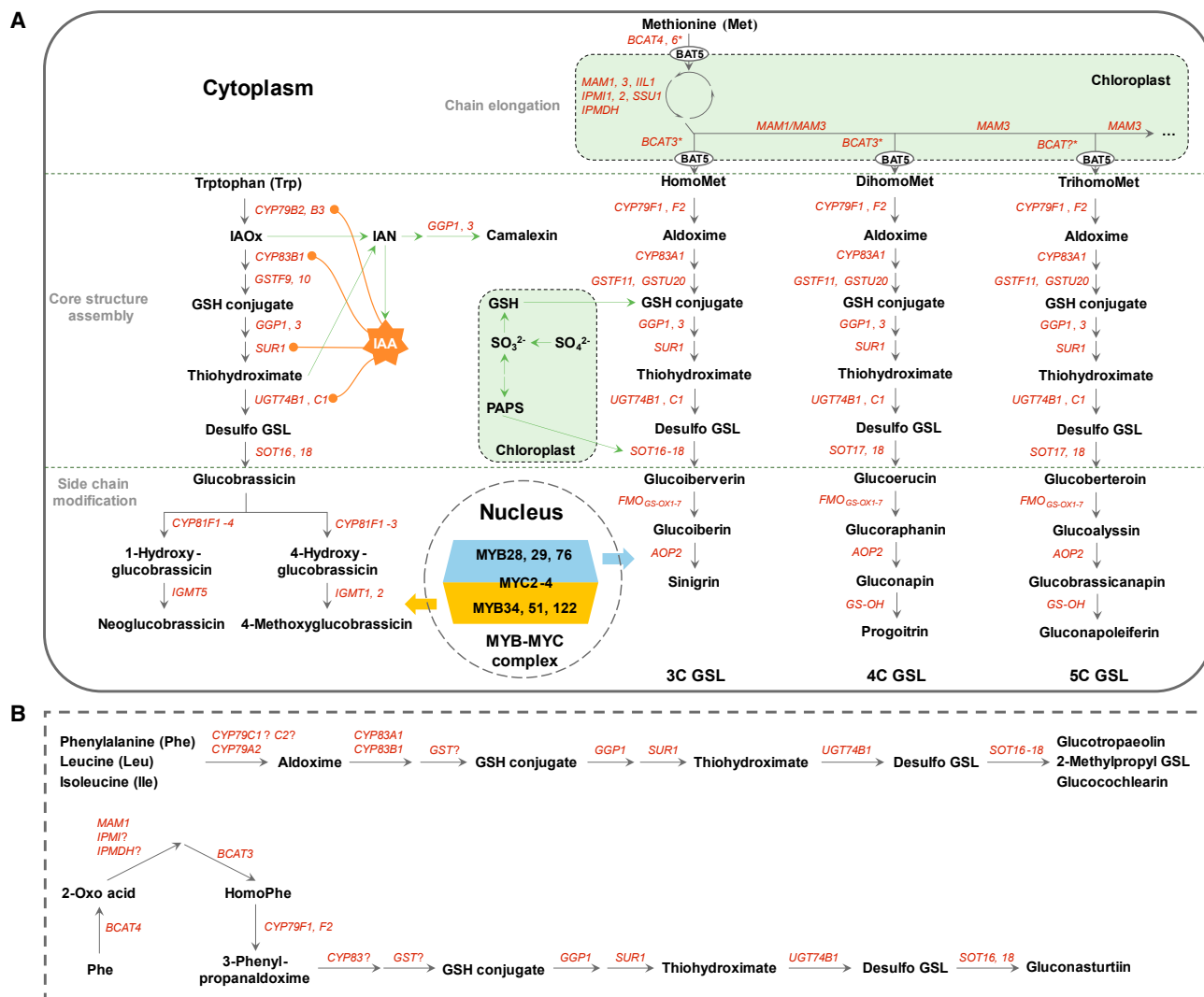


Figure 1. De novo biosynthetic pathways of GSLs in *A. thaliana*.

(A) De novo biosynthesis of Met-derived aliphatic GSLs and Trp-derived indole GSLs. BCAT3 is a chloroplast enzyme that has a specific role in the production of homoMet and dihomomet. BCAT4 and BCAT6 are both cytosolic enzymes. Catalyzed by MAMs, chain-elongated 2-keto acids can be transaminated either in chloroplasts by BCAT3 or by BCAT4/BCAT6 following export by BAT5 from the chloroplasts to the cytoplasm. This transport process is not shown in Figure 1. The negative feedback loops between IAA and GSL biosynthetic genes are indicated by orange lines. The camalexin biosynthetic pathway that intersects with GSL biosynthesis intermediates is indicated by green arrows. GSH, glutathione; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; IAOx, indole-3-acetaldoxime; IAN, indole-3-acetonitrile.

(B) The proposed biosynthetic pathways of Phe-derived glucotropaeolin, Leu-derived 2-methylpropyl GSL, and Ile-derived glucocochlearin (upper), as well as homoPhe-derived gluconasturtiin (lower). The involvement and function of genes that are followed by a question mark ("?") remain to be investigated in *A. thaliana*.

Information sources: Hull et al. (2000); Mikkelsen et al. (2000, 2004); Bak et al. (2001); Bak and Feyereisen (2001); Reintanz et al. (2001); Chen et al. (2003); Naur et al. (2003); Grubb et al. (2004, 2014); Schuster et al. (2006); Knill et al. (2008); Sønderby et al. (2010b); Geu-Flores et al. (2011); Lächler et al. (2015); Liu et al. (2016b); Petersen et al. (2019); Harun et al. (2020); Wang et al. (2020); Shang et al. (2022).

and hydrolysis (Barco and Clay, 2019). Subsequently, the evolution of diverse GSL profiles has been accelerated through domestication and artificial selection, with the accumulation of morphologically distinct subspecies and subsequent radiation into locally adapted landraces and numerous modern cultivars (Chalhoub et al., 2014; Kim et al., 2016; Branca et al., 2018; Chopra et al., 2020; Arias et al., 2021). This provides us with a broad and flexible platform that contains abundant species, genetic loci, and GSL resources and could be exploited for GSL engineering, as we discuss in detail below.

CANDIDATE TARGETS FOR CURRENT AND FUTURE GSL METABOLIC ENGINEERING

Manipulating genes involved in biosynthesis to alter GSL diversity

Over the past few decades, a number of genes involved in GSL biosynthesis have been identified and characterized in the model plant *Arabidopsis thaliana* (Harun et al., 2020) and subsequently

Locus	Strategy	Species	Plant growth and agronomic traits ^a				GSL content		Reference
			Height	Fertility	Yield	Survival	Met-derived	Indole	
MAM1	KO	<i>A. thaliana</i>					N ^c	+ ^c	Kroymann et al., (2001) Textor et al. (2007)
	OE	<i>B. rapa</i>	N	N	N	N	+ ^b	+ ^b	Zang et al. (2008)
MAM3	OE	<i>A. thaliana</i>	N	N	N	N	N ^c		Field et al. (2004); Textor et al. (2007)
	KO	<i>A. thaliana</i>					+ ^c	N ^c	Textor et al. (2007)
GS-ELONG (MAMs)	KD	<i>B. napus</i>	N	N	N	N	- ^b	N ^b	Liu et al. (2011)
BCAT4	KO	<i>A. thaliana</i>					-	+	Schuster et al. (2006); Sawada et al. (2009)
	KO	<i>A. thaliana</i>	N	N		N	-/N	+	Lächler et al. (2015)
BCAT6	KO	<i>A. thaliana</i>	N	N		N	N	N	Lächler et al. (2015)
BCAT3	KO	<i>A. thaliana</i>					+/N	-/+	Knill et al. (2009)
	KO	<i>A. thaliana</i>	N	N		N	N/+	+	Lächler et al. (2015)
BCAT3BCAT4	KO	<i>A. thaliana</i>					-	+	Knill et al. (2009)
BCAT4BCAT6	KO	<i>A. thaliana</i>	N	N		N	-	+	Lächler et al. (2015)
BCAT3BCAT4BCAT6	KO	<i>A. thaliana</i>	-		-		-	+	Lächler et al. (2015)
IPMI1	KO	<i>A. thaliana</i>	N	N		N	N	-/N	Knill et al. (2009)
	KO and KD	<i>A. thaliana</i>	N	N	N	N	N	N	He et al. (2010); Imhof et al. (2014)
IPMI2	KO	<i>A. thaliana</i>	N	N		N	N/+	N	Knill et al. (2009); Imhof et al. (2014)
	KO	<i>A. thaliana</i>					N	N	He et al. (2010)
IPMI1IPMI2	KO and KD	<i>A. thaliana</i>					- ^c	N ^c	He et al. (2010)
IPMDH	KO	<i>A. thaliana</i>	N	N	N	N	- ^c	+ ^c	He et al. (2009); Sawada et al. (2009); He et al. (2011)

Table 2. Phenotypic alterations following mutagenesis of genes involved in the GSL chain elongation step.

KO, knockout; KD, knockdown; OE, overexpression.

^aSeveral representative plant growth and agronomic traits are selected to show phenotypic alterations following gene mutagenesis. Plant height refers to the height of plants at full maturity or to putative height because dwarfism and subsequent death may occur at early stages. Fertility refers to plant male fertility rate at the flowering stage. Yield refers to per-plant seed yield at full maturity. Survival refers to the observed plant survival rate under normal growth conditions. Because the source data represent a mixture of descriptive and quantitative information, N (no changes), + (increase), and - (decrease) are used to indicate, although not precisely quantify, observed alterations in plant growth, agronomic traits, and GSL content.

^bThe available GSL data are only from plant leaves. Other available GSL data are derived from the combination of leaves and seeds. A single N, +, or - is used to indicate the overall alteration in content in leaves and seeds. A virgule ("/") is used to show distinct alterations in leaves/seeds.

^cGSL alterations are not supported by specific data but are speculated from the relevant description or calculated by indirect data evidence described in the source literature.

in other Brassicaceae crop species, particularly in the genus *Brassica* (Zhu et al., 2012; Augustine et al., 2013a; Kim et al., 2013; Zhang et al., 2015a; Seo et al., 2016; Sharma et al., 2016; Yin et al., 2017; Liu et al., 2020; Tandayu et al., 2022). Apart from biosynthesis of indole GSLs, *de novo* biosynthetic pathways of intact GSL molecules involve elongation of side chains, assembly of core structures, and secondary modification of side chains (Figure 1A). Previous attempts have been made to extend GSL diversity in various Brassicaceae species through different molecular approaches (Miao et al., 2021). However, it should be recognized that metabolic engineering is a complex undertaking, given the innate interactions among GSLs, phytohormones (Malka and Cheng, 2017), glutathione (Sonderby et al., 2010b), phytoalexins (Geu-

Flores et al., 2011), and primary metabolic pathways (Borpatragohain et al., 2016) that also affect plant growth and development. We therefore considered those genes that may affect GSL profiles and also have pleiotropic effects on normal plant growth (Tables 2 and 3; Supplemental Table 2). Although several genes have already been exploited as engineering targets in different species (Miao et al., 2021), a few that appear capable of modifying GSL profiles are yet to be explored. However, the potential for negative effects on normal plant growth and agronomic traits after molecular engineering may reduce the total number of available genes. We further collated a subset of biosynthetic genes, for some of which targeted mutagenesis has led to perturbations in normal plant growth and important agronomic traits, as well as in GSL profiles

Locus	Strategy	Species	Plant growth and agronomic traits ^a				GSL content ^a		Tissue/organ	Benefit	Reference
			Height	Fertility	Yield	Survival	Met-derived	Indole			
CYP79F1	KO	<i>A. thaliana</i>	–	–	–		–/+/–	+/N/+	leaf/root/seed		Reintanz et al. (2001)
	OE	<i>B. rapa</i>	–				+	N	leaf		Zang et al. (2008)
CYP79F2	KO	<i>A. thaliana</i>	N	N	N	N		+/N/N	leaf/root/seed		Chen et al. (2003)
	KO	<i>A. thaliana</i>	N	N	N	N	–/+/–/–	N/+/+	leaf/stem/root/ flower		Tantikanjana et al. (2004)
	OE	<i>A. thaliana</i>			–			N	leaf/root/seed		Chen et al. (2003)
CYP79F1CYP79F2	KO	<i>A. thaliana</i>	–	–	–		–	+/+–/+	leaf/stem/root/ flower		Tantikanjana et al. (2004)
CYP79B2CYP79B2	OE	<i>A. thaliana</i>	–		–		N	+	leaf	resistance to <i>Pseudomonas syringae</i> pv. <i>maculicola</i>	Hull et al. (2000); Mikkelsen et al. (2000)
	KO	<i>A. thaliana</i>	N	N	N	N					Zhao et al. (2002)
CYP79B3CYP79B3	KO	<i>A. thaliana</i>	N	N	N	N					Zhao et al. (2002)
	OE	<i>B. rapa</i>						N	root		Zang et al. (2009)
CYP79B2CYP79B3	KO	<i>A. thaliana</i>	–					–			Zhao et al. (2002)
CYP83A1	KO	<i>A. thaliana</i>	N	N	N	N		+/N	leaf/seed	resistance to <i>Erysiphe cruciferarum</i> , <i>Glovinomyces orontii</i> , and <i>G. cichoracearum</i>	Hemm et al. (2003); Weis et al. (2013), 2014; Liu et al. (2016a)
	OE	<i>A. thaliana</i>	N	N	N	N	N	N	seedling/leaf		Naur et al. (2003)
	OE	<i>B. napus</i>					+	N	leaf/seed		Zhang et al. (2015b)
CYP83B1	KO	<i>A. thaliana</i>	–		–			–	seedling		Bak et al. (2001); Bak and Feyereisen (2001)
	OE	<i>A. thaliana</i>	–		–			+	seedling		Bak and Feyereisen (2001)
	OE	<i>A. thaliana</i>			–		+	N	leaf		Naur et al. (2003)
CYP79B2CY P79B3CYP83B1	OE	<i>B. rapa</i>						N	root		Zang et al. (2009)
GSTF11	KO	<i>A. thaliana</i>	N	N	N	N	–	N	leaf/seed		Zhang et al. (2022)

Table 3. Phenotypic alterations following mutagenesis of genes involved in the GSL core structure assembly step.

(Continued on next page)

Locus	Strategy	Species	Plant growth and agronomic traits ^a				GSL content ^a		Tissue/organ	Benefit	Reference
			Height	Fertility	Yield	Survival	Met-derived	Indole			
<i>GSTU20</i>	KO	<i>A. thaliana</i>	N	N	N	N	–	N	leaf/seed		Zhang et al. (2022)
<i>GSTF11GSTU20</i>	KO	<i>A. thaliana</i>					–	N	leaf/seed		Zhang et al. (2022)
<i>GGP1GGP3</i>	KD	<i>A. thaliana</i>					–	+	leaf		Geu-Flores et al. (2011)
<i>SUR1</i>	KO	<i>A. thaliana</i>	–		–	–	–	–	shoot		Mikkelsen et al. (2004); Grubb et al. (2014)
	OE	<i>A. thaliana</i>	N	N	N	N	N	N			Mikkelsen et al. (2004)
<i>UGT74B1</i>	KO	<i>A. thaliana</i>	–	–	–		–	–	leaf/shoot		Grubb et al. (2004); Grubb et al. (2014)
	OE	<i>B. oleracea</i>	N				N	+	leaf		Zheng et al. (2021)
	OE	<i>B. napus</i>					+	+	leaf/seed	resistance to <i>Sclerotinia sclerotiorum</i> and <i>Botrytis cinerea</i>	Zhang et al. (2015b)
<i>UGT74C1</i>	KO	<i>A. thaliana</i>	N	N	N	N	N	N	shoot		Grubb et al. (2014)
<i>UGT74B1UGT74C1</i>	KO	<i>A. thaliana</i>	–	–	–	–	–	–	shoot		Grubb et al. (2014)
<i>SOT16</i>	KO	<i>A. thaliana</i>	N	N	N	N	N ^b	N ^b			Klein and Papenbrock (2009)
	OE	<i>A. thaliana</i>						+ ^b			Klein and Papenbrock (2009)

Table 3. Continued

^aThe abbreviations of mutagenesis strategies and phenotypic alterations (N, +, –) follow Table 2. A single N, +, or – is used both for GSL alteration of a single tissue/organ and a similar alteration among multiple tissues/organs. A virgule (“/”) is used to show distinct alterations in GSL content among different tissues/organs based on the order in the column “Tissue/organ.”

^bGSL alterations were not supported by specific data but are speculated from the relevant description or calculated by indirect data evidence described in the source literature.

(Tables 2 and 3; Supplemental Table 2). This enables us to explore the feasibility and potential of exploiting these genes as future GSL engineering targets.

Enzymes encoded by GSL biosynthetic genes have been engineered in different species; these include methylthioalkylmalate synthases (MAMs), cytochrome P450 enzymes (CYP79F1, CYP79Bs, CYP83B1, and CYP83A1), UDP-glycosyltransferase 74B1 (UGT74B1), flavin-containing monooxygenases (FMO_{GS-OXs}), and 2-oxoglutarate-dependent dioxygenase 2 (AOP2) (Tables 2 and 3; Supplemental Table 2). In the chain-elongation step, MAMs have been ideal engineering targets because of their dominant roles in determining the structures of Met-derived aliphatic GSLs by facilitating the chain elongation reaction of Met derivatives (Figure 1A). Apart from its role in biosynthesis of Met-derived aliphatic GSLs, MAM1 is also responsible for the elongation of Phe to generate homoPhe, the precursor of gluconasturtiin (Shang et al., 2022) (Figure 1B). Other chain-elongation genes also exhibit the ability to modify GSL profiles (Table 2), including three genes (BCAT3, BCAT4, and BCAT6) encoding branched-chain-amino-acid aminotransferases that are potential candidate engineering targets. Single- and double-knockout mutants of these genes exhibit similar alterations in GSL profiles, with elevated indole GSLs and decreased Met-derived aliphatic GSLs (Table 2). Only the triple mutant showed abnormal morphological phenotypes (Table 2). Another important GSL biosynthesis enzyme, 3-isopropylmalate dehydratase (IPMI), comprises a large subunit (IPMI LSU1) and three small subunits (IPMI SSU1–3), which are encoded by *ILL1*, *IPMI SSU1*, *IPMI1*, and *IPMI2*, respectively (Lächler et al., 2020). The phenotypic defects exhibited by *ILL1* and *IPMI SSU1* knockout mutants are associated with the important roles of these enzymes in plant primary metabolism and developmental processes, making them unsuitable for molecular engineering (Knill et al., 2009; Sureshkumar et al., 2009; Imhof et al., 2014) (Table 2). However, unlike *ILL1* and *IPMI SSU1*, a combination of *IPMI1* and *IPMI2* could represent a target for GSL engineering, although single *IPMI1* or *IPMI2* knockout/knockdown mutants showed limited alterations in GSL profiles (Table 2). Finally, the gene encoding 3-isopropylmalate dehydrogenase 1 (IPMDH) also has the potential to be engineered in more species owing to its ability to alter Met-derived aliphatic GSLs; more importantly, the knockout mutant showed an agronomic phenotype indistinguishable from that of wild-type plants (Table 2).

In the core structure assembly step, indole-3-acetaldoxime (IAOx) is a Trp-derived intermediate involved in indole GSL biosynthesis that also represents a key intersection point connecting primary and secondary metabolism as well as auxin biosynthetic pathways (Figure 1A). As an important naturally synthesized auxin, indole-3-acetic acid (IAA) dynamically regulates plant growth and development (Zhao, 2010). It is believed that numerous plant species can use aldoximes as precursors for IAA biosynthesis (Perez et al., 2021), and for *A. thaliana*, functional mutation of several GSL biosynthetic genes (*CYP79B2*, *CYP79B3*, *CYP83B1*, *SUR1*, and *UGT74B1*) has shown pleiotropic indirect effects on IAA homeostasis via effects on IAOx (Figure 1A). Functional mutation of these genes not only alters GSL profiles but also leads to abnormal IAA-related phenotypes (Table 3). Among these

genes, *SUPERROOT1* (*SUR1*) may not be suitable as a potential GSL engineering target. This is because functional loss of *SUR1* results in the elimination of almost all GSLs, a lack of seed development, and plant death, whereas overexpression of *SUR1* has no effect on GSL profiles or plant morphology (Table 3; Supplemental Table 2). Apart from these IAA-related genes, *CYP79F1* and *CYP79F2* have shown correlations with cytokinin levels, and their functional mutagenesis in *A. thaliana* and *B. rapa* leads to exaggerated plant morphology (Table 3). *CYP79A2* solely controls the conversion of Phe to phenylacetaldoxime, which is the precursor of glucotropaeolin and a naturally occurring auxin phenylacetic acid (Wittstock and Halkier, 2000). Overexpression of *CYP79A2* leads to high-auxin phenotypes with overaccumulation of glucotropaeolin and phenylacetic acid (Perez et al., 2021). If such genes are to be appropriate targets for GSL engineering, these negative phytohormone-related phenotypes will need to be overcome in the future. Given the wide range of Brassicaceae species with complex polyploid genomes that harbor multiple homologs of a high proportion of genes, there is considerable scope for selection of appropriate paralogs for mutagenesis that may reduce or even eliminate undesirable pleiotropic changes, expanding the range of GSL engineering targets.

In addition to phytohormone-related GSL biosynthetic genes, other genes within the core structure assembly step can alter GSL profiles without affecting normal plant growth. *CYP83A1* has been an ideal engineering target because knockout mutants exhibit positive effects in controlling several biotrophic fungi (Table 3; Supplemental Table 2). This suggests potential for *CYP83A1* orthologs in GSL engineering of a wider range of Brassicaceae crops as a strategy to improve specific disease resistance. *GSTF11* and *GSTU20* are two newly verified genes encoding glutathione-S-transferases. Knockout mutants did not show morphological alterations and had a significantly reduced Met-derived aliphatic GSL content, making them appropriate targets for GSL engineering (Table 3). *GGP1* and *GGP3*, which encode γ -glutamyl peptidases, connect glutathione sulfurdonating pathways with GSL biosynthetic pathways and are also involved in indole-sulfur phytoalexin biosynthetic pathways in *A. thaliana* (Geu-Flores et al., 2011; Klein and Sattely, 2017) (Figure 1A). Trp-derived phytoalexins, of which camalexin is a representative, are similar to GSLs and contribute to plant resistance against various pathogens (Pedras et al., 2011; Ahuja et al., 2012; Plaszkó et al., 2022). In the *A. thaliana* double mutant *ggp1-1ggp3-1*, indole GSL content was enhanced and camalexin content was reduced (Geu-Flores et al., 2011) (Table 3), but the effects on morphological phenotypes and plant resistance to specific pathogens remain unknown. However, accumulation of camalexin is confined to the tribe Camelinae (Bednarek et al., 2011). In *Brassica* species, different indole-sulfur phytoalexins such as brassinin occur and can undergo further reactions controlled by a series of downstream genes to form numerous derived phytoalexins (Pedras et al., 2011; Plaszkó et al., 2022). The metabolic connection between indole GSLs and brassinin biosynthesis has been established in cabbage, indicating the likely involvement of *SUR1* and GGPs in the relevant pathways (Klein and Sattely, 2017). Therefore, if GGPs and *SUR1* were selected for engineering in more Brassicaceae species, future studies should carefully examine both the possible negative

effects on plant growth and agronomic traits and also the resulting changes in plant resistance to specific pathogens. At the end of the core structure assembly step, three genes encoding cytosolic sulfotransferases (*SOT16–18*) are responsible for the sulfonation of desulfo GSLs. Although the substrate specificities of SOT enzymes for different desulfo GSLs have been elucidated in *A. thaliana* and *B. napus*, it remains unclear whether functional mutation of SOTs can significantly alter GSL profiles (Klein and Papenbrock, 2009; Hirschmann and Papenbrock, 2015). Located downstream of GSL biosynthesis, their engineering potential is probably limited, although this remains to be verified in future work.

In the side-chain secondary modification step, different gene families (*FMO_{GS-OXs}*, *AOP2*, *GS-OH*, *CYP81Fs*, and *IGMTs*) ultimately determine GSL side-chain structures, and there is no evidence that mutagenesis of these downstream genes has negative effects on normal plant growth and agronomic traits (Supplemental Table 2). This underlies their potential to be engineered in a wide range of species. As mentioned above, *FMO_{GS-OXs}* and *AOP2* have been used to modify GSLs in species besides *A. thaliana* (e.g., *B. rapa*), but other secondary modification genes have not (Supplemental Table 2). For Met-derived aliphatic GSLs, *GS-OH* encodes a 2-oxoacid-dependent dioxygenase that is solely responsible for the conversion from gluconapin to progoitrin (Figure 1A). Transgenic research in *A. thaliana* and *B. oleracea* has revealed multiple potential functions of *GS-OH* in GSL engineering: knockout or silencing may eliminate goitrogenic progoitrin, whereas overexpression may promote progoitrin accumulation to control specific generalist insects such as *Trichoplusia ni* (Hansen et al., 2008; Wu et al., 2022). For indole GSLs, four *CYP81F* genes (*CYP81F1–4*) and three genes encoding indole glucosinolate O-methyltransferases (*IGMT1*, *IGMT2*, and *IGMT5*) fine-tune the conversion among different indole GSL structures (Figure 1A). Among these genes, *CYP81F2* controls the conversion from glucobrassicin to 4-methoxyglucobrassicin (Figure 1A). The hydrolysis of 4-methoxyglucobrassicin confers plant resistance against green peach aphid (*Myzus persicae*) by post-ingestive breakdown, independent of classical myrosinases (Pfalz et al., 2009), and against fungi through the action of the atypical myrosinase PEN2 to activate the innate immune response in *A. thaliana* (Bednarek et al., 2009; Clay et al., 2009). These results indicate the great potential for *CYP81F2* (and likewise *IGMT1* and *IGMT2*) to be overexpressed in more species that overaccumulate 4-methoxyglucobrassicin to control fungal diseases, although the resulting effects on normal plant growth remain to be investigated. Finally, *IGMT5*, which is solely responsible for the conversion from 1-hydroxyglucobrassicin to neoglucobrassicin, also represents an engineering target (Figure 1A). Functional loss of *IGMT5* has been shown to enhance resistance to the root-knot nematode *Meloidogyne javanica*, indicating its potential role in *Arabidopsis* belowground defense (Pfalz et al., 2016) and inspiring us to make similar engineering efforts in other species.

Detailed understanding of GSL biosynthetic gene functions in the model plant *A. thaliana* has facilitated recent progress in the biosynthetic pathways of Phe-derived GSLs and corresponding engineering attempts in GSL-free species. Through the use of comparative genomic tools and transcriptomic data, a relatively

clear *de novo* pathway for biosynthesis of gluconasturtiin from Phe and its further hydroxylation was proposed in the wild species *Barbarea vulgaris*, in which oxidized phenylethyl GSLs predominate (Agerbirk and Olsen, 2015; Liu et al., 2016b). Subsequently, the genetic mechanisms of Phe-derived GSL biosynthesis were investigated in a genome-wide association study on *B. rapa* (Shang et al., 2022). Based on the collective results, we provide the potential biosynthetic pathways of two Phe-derived GSLs in *A. thaliana* (Figure 1B), glucotropaeolin and gluconasturtiin, whose potent health-promoting effects have been widely reported (see section “generating multifunctional crops with defined GSL profiles and novel end uses”; Table 4). Attempts to heterologously produce Phe-derived glucotropaeolin and gluconasturtiin by expressing biosynthetic genes from *A. thaliana* or co-expressing biosynthetic genes from *A. thaliana* and *B. vulgaris* have been successful in potato (*Solanum tuberosum*) and tobacco (*Nicotiana benthamiana*) (Møldrup et al., 2012; González-Romero et al., 2021; Wang et al., 2021). Although the substrate specificity of the involved enzymes was clearly revealed in the engineering studies, their *in planta* functions in Phe-derived GSL biosynthesis, especially for gluconasturtiin, remain to be clarified in *A. thaliana* (Figure 1B). The broad specificity of newly characterized CYP79C enzymes, which accept both Leu and to some extent Ile in addition to Phe (Figure 1B) and the altered specificity of CYP79C1 in the presence of chain elongating enzymes were surprising (Wang et al., 2020). Therefore, further studies are also required to fill gaps in the proposed biosynthetic pathways of GSLs derived from Leu, Ile, and additional amino acids in *A. thaliana* (Figure 1B). This would help us understand how activities of specific enzymes are orchestrated to catalyze the formation of rare and unexploited GSLs, beyond the context currently dominated by Met-derived aliphatic GSLs in *A. thaliana* and most Brassicaceae crop species. Such insights could be further exploited and applied in a wide range of engineering contexts.

In addition to biosynthetic enzymes, MYB transcription factors have a global impact on biosynthetic gene expression and total GSL content (Figure 1A), endowing them with considerable engineering potential. MYB28, MYB29, and MYB76 regulate the expression of genes related to Met-derived aliphatic GSL biosynthesis (Sønderby et al., 2010a). Owing to their relative functional conservation among species and prominent roles in determining overall Met-derived aliphatic GSL content, *MYB28* and *MYB29* have been widely used for GSL engineering of different Brassicaceae species (Miao et al., 2021; Hözl et al., 2023; Zhou et al., 2023). *MYB76* mainly plays an auxiliary role in regulating secondary modification genes (Sønderby et al., 2010a). As an engineering target, it could be combined with *MYB28*, *MYB29*, or biosynthetic genes to make a more significant impact on GSL profiles in practical applications. For indole GSLs, *MYB34*, *MYB51*, and *MYB122* directly regulate constitutive and pathogen-induced *de novo* biosynthesis of glucobrassicin and indirectly control accumulation of IAOx derivatives in *A. thaliana* (Friggmann and Gigolashvili, 2014; Friggmann et al., 2016). *MYB34* and *MYB51* are crucial for GSL biosynthesis in roots and shoots separately, whereas *MYB122* plays an accessory role but becomes particularly significant in the context of environmental stress (Friggmann and Gigolashvili, 2014).

Overexpression of *MYB34* and *MYB122* gives rise to exaggerated IAA phenotypes, whereas that of *MYB51* does not, mainly because of its role in activating core structure assembly genes both upstream and downstream of IAOx (Gigolashvili et al., 2007). Considering the importance of minimizing negative effects of target genes after mutagenesis, *MYB51* appears to be a primary choice for GSL engineering. To obtain engineering targets for a wider range of species, there is a need to identify and characterize *MYB34* and *MYB122* orthologs that lack negative effects on normal plant growth or agronomic traits.

Simultaneous manipulation of genes involved in transport and biosynthesis to modify GSL spatiotemporal distribution

GSL biosynthesis and accumulation have distinct spatiotemporal patterns (Jørgensen et al., 2015). Once assembly of intact GSL molecules is complete, they are transported into neighboring specialized sulfur-rich cells (S-cells) for storage (Schuster et al., 2006), as well as accumulating in specific tissues and organs remote from their site of synthesis (Jørgensen et al., 2015). In recent years, identification and investigation of GSL transporters (GTRs) in *A. thaliana* have provided new information on how GSLs are retained, mobilized, and allocated throughout plant growth and development (Burow and Halkier, 2017; Xu et al., 2019; Hunziker et al., 2021).

GTR proteins belong to the nitrate transporter 1/peptide transporter (NRT1/PTR) family (also known as the NPF family), which is present in a wide range of plant taxa (Nour-Eldin et al., 2012). GTR1 (AtNPF2.10) and GTR2 (AtNPF2.11) were first identified and characterized in *A. thaliana* (Nour-Eldin et al., 2012), and their roles as GSL importers have been demonstrated in inter-organ transport (e.g., the seed loading process), as well as intra-organ/tissue retention and allocation of Met-derived aliphatic and indole GSLs based on symplastic or apoplastic pathways (Andersen et al., 2013; Andersen and Halkier, 2014; Madsen et al., 2014; Xu et al., 2016, 2019; Nintemann et al., 2018; Hunziker et al., 2019, 2021). Distinct from GTR1 and GTR2, the newly discovered GTR3 (AtNPF2.9) exhibits a strong preference for indole GSLs in *A. thaliana* (Jørgensen et al., 2017). It is capable of retaining indole GSLs in roots and, along with GTR1 and GTR2, participates in seed loading (Nour-Eldin et al., 2012), root exudation (Xu et al., 2016), intra-leaf distribution (Madsen et al., 2014), translocation for storage in S-cells of the stem (Xu et al., 2019), and transport of indole GSLs between roots, shoots, leaves, and flower stalks (Andersen et al., 2013; Andersen and Halkier, 2014; Jørgensen et al., 2017; Hunziker et al., 2021).

The specific ability of GTRs to alter GSL spatiotemporal distribution, especially in the seed GSL loading process, has aroused considerable interest in creating novel Brassicaceae crop cultivars that possess high GSL concentrations in vegetative parts and corresponding low concentrations in seeds (Nour-Eldin et al., 2017; Nambiar et al., 2021; He et al., 2022). Such germplasm has been generated by editing a *GTR2* homolog on chromosome A06 in *B. napus* and, most importantly, it exhibited no impairment of normal plant

growth (He et al., 2022). This result reflects the specific function of *GTR* orthologs in retaining GSLs in specific organs. In addition to the effects of GTRs, the effects of biosynthetic gene expression on GSL spatiotemporal distribution should not be neglected. It has been widely observed that the expression patterns of several biosynthetic genes make partial contributions to GSL phenotypes (Mikkelsen et al., 2000; Reintanz et al., 2001; Chen et al., 2003; Grubb et al., 2004, 2014; Redovniković et al., 2012; Kong et al., 2016). For example, *MAM1* and *MAM3* exhibited similar expression patterns during *A. thaliana* development, with distinct expression levels between tissues, although *MAM1* exhibited significantly higher expression levels that corresponded to a higher concentration of short-chain GSLs compared with long-chain GSLs (Redovniković et al., 2012). A similar phenomenon was also observed in a study of *FMO_{GS-ox6}* and *FMO_{GS-ox7}* (Li et al., 2016). Transcription factors also influence the spatiotemporal distribution of GSLs; for example, *MYB28* orthologs have been shown to maintain high levels of GSLs in leaves and low levels in seeds of *A. thaliana* and *B. napus* (Sønderby et al., 2010a; Liu et al., 2020). Therefore, observed GSL phenotypes appear at the very least to be co-determined by biosynthetic gene expression and GTRs (Tan et al., 2022), and their synergistic effects on GSL spatiotemporal distribution should be considered for engineering. It may be feasible to achieve a desired spatiotemporal GSL distribution in selected tissues and organs by molecular modification of GTRs, biosynthetic genes, and transcription factors, as well as by exploitation of unidentified GSL exporters in the future.

Selecting targets from unexploited hydrolysis genes for modification of GHPs

The well-known glucosinolate–myrosinase system, also known as the “mustard oil bomb,” plays an essential role in Brassicaceae plant defense. Myrosinases are stored in myrosin cells that are isolated from S-cells (Lüthy and Matile, 1984). When plant tissues are disrupted, myrosinases are released to interact with biologically inert GSLs and catalyze their degradation into bioactive isothiocyanates (ITCs) associated with multiple biological functions (Wittstock et al., 2016a). ITC is the major GHP type at neutral cell pH, and a hydroxy moiety at the C2 position leads to spontaneous cyclization to form oxazolidine-2-thiones (Wittstock and Halkier, 2002) (Figure 2A). The oxazolidine-2-thiones have received considerable scientific attention, as the dominant GSL (progoitrin) in classical varieties of *B. napus* exhibits a structural feature that results in an oxazolidine-2-thione product, (S)-goitrin (see section “generating multifunctional crops with defined GSL profiles and novel end uses”; Table 4). In *A. thaliana*, six genes of the β -thioglucoside glucohydrolase family (TGGs) that encode myrosinases have been identified (Figure 2A). *TGG1* and *TGG2* function in aerial plant parts, whereas *TGG4* and *TGG5* function in roots. However, *TGG3* and *TGG6* have been demonstrated to be pseudogenes and do not encode functional myrosinases (Zhang et al., 2002; Meijer et al., 2009). In addition, *PEN2* encodes an atypical myrosinase beta-glucosidase 26 that is mainly involved in hydrolysis of glucobrassicin and 4-methoxyglucobrassicin and plays an essential role in the innate immune response (Bednarek et al., 2009).

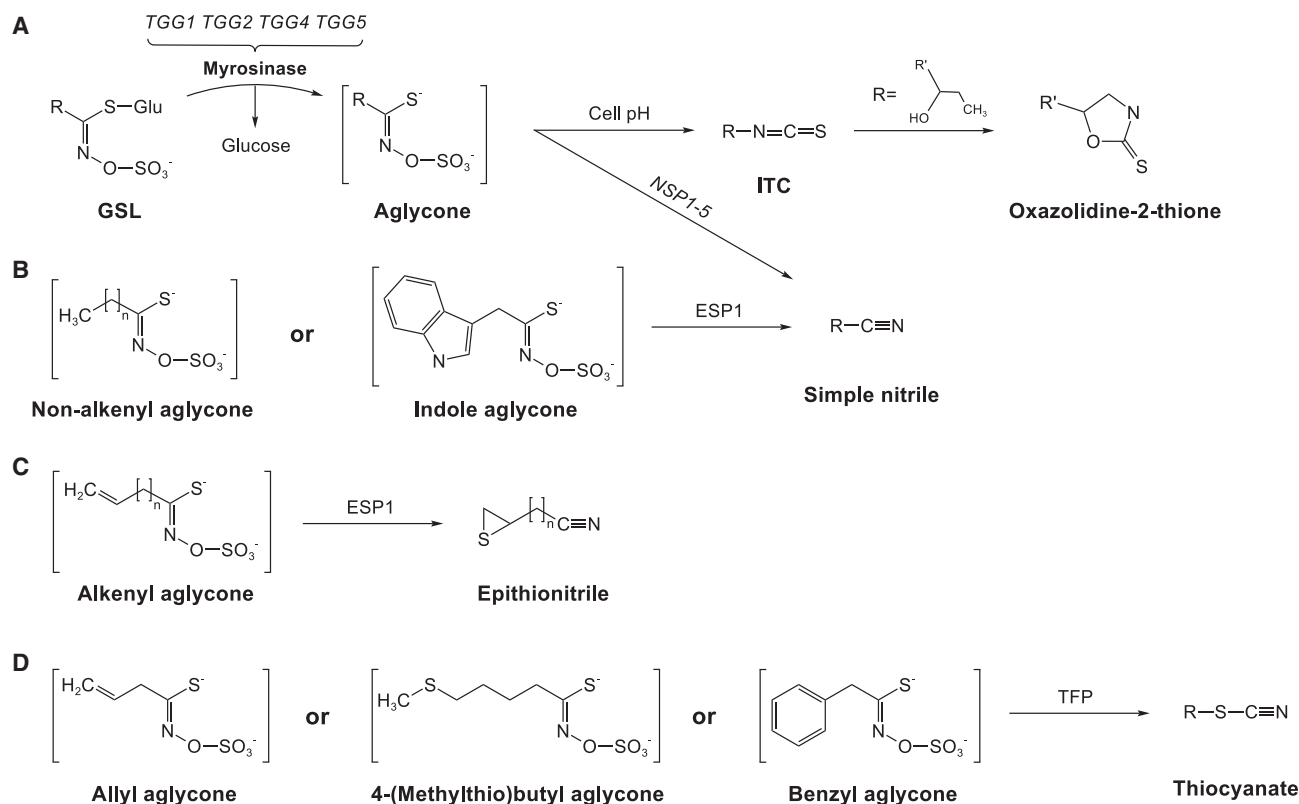


Figure 2. The production pathways of various GHPs catalyzed by myrosinases and governed by different specifier proteins.

The unstable intermediate aglycones produced by myrosinases are enclosed in square brackets. **(A)** Catalyzed by TGG-encoding myrosinases, an intermediate aglycone is produced, which can spontaneously convert into an isothiocyanate or further cyclize to form an oxazolidine-2-thione at typical cell pH. **(B)** The pathways of simple nitrile production in the presence of ESPs or NSPs. **(C)** The production of an epithionitrile from an alkenyl aglycone in the presence of ESPs.

(D) The production of a thiocyanate from an allyl, a 4-methylthiobutyl, or a benzyl aglycone in the presence of TFPs.

Information sources: Lüthy and Benn (1977); Bones and Rossiter (1996); Burow et al. (2006, 2008); Kuchernig et al. (2011); Agerbirk and Olsen (2012); Gumz et al. (2015); Wittstock et al. (2016a, 2016b).

Non-ITC GHPs can also be produced in the presence of specifier proteins at the expense of ITC formation in *A. thaliana* (Figures 2B and 2C). Nitrile-specifier proteins (NSPs) are solely responsible for the formation of simple nitriles, regardless of GSL side-chain structures (Figure 2B). Epithiospecifier proteins (ESPs) can promote formation of epithionitrile from alkenyl GSLs with double bonds at the end of side chains (Figure 2C). Apart from epithionitriles, simple nitriles can also be formed in the presence of ESPs, a process that is confined to the conversion of non-alkenyl and indole GSLs (Lambrix et al., 2001) (Figure 2B). In general, there are one ESP- and five NSP-encoding genes involved in GSL hydrolysis in *A. thaliana*. *ESP1* can be expressed in aerial tissues and organs, although its expression patterns differ among various *A. thaliana* ecotypes, some of which lack ESPs (Burow et al., 2009). *NSPs* showed distinct organ-specific expression patterns, with *NSP1* mainly functioning in seedlings and roots, *NSP2* in seeds, and *NSP3* in roots. The functions of *NSP4* and *NSP5* remain unclear (Wittstock and Burow, 2010; Wittstock et al., 2016b). Organic thiocyanates are possible GHPs formed in the presence of thiocyanate-forming proteins (TFPs), which have been found in a small subset of Brassicaceae species such as *Thlaspi arvense* (TaTFPs) and *L. sativum* (LsTFPs) (Burow et al., 2007; Kuchernig et al., 2011). To date, only three GSLs, sinigrin (allyl

GSL), glucotropaeolin (benzyl GSL), and glucoerucin (4-(methylthio)butyl GSL), have been observed that could be stably converted into relevant thiocyanates (Figure 2D). LsTFP only converts glucotropaeolin into benzyl thiocyanate, and TaTFP only converts allyl GSL into allyl thiocyanate (Burow et al., 2007; Kuchernig et al., 2011; Gumz et al., 2015). In addition to thiocyanates, simple nitriles and epithionitriles can also be produced by TFPs upon myrosinase-catalyzed GSL hydrolysis (Burow et al., 2007; Kuchernig et al., 2011; Gumz et al., 2015).

Although specifier proteins are crucial for the formation of non-ITC GHPs, external variables such as pH and the presence of metal ions (especially Fe^{2+}) can significantly affect GHP composition. Without specifier proteins, low pH ($\text{pH} < 5$) or addition of Fe^{2+} can result in the production of simple nitriles (Bones and Rossiter, 2006; Burow et al., 2006). When present, specifier protein activity can be enhanced by addition of Fe^{2+} or Fe^{3+} (ESPs), and changes in pH can also affect specifier protein activity, changing the final GHP composition (Burow et al., 2006, 2007; Kuchernig et al., 2011).

To the best of our knowledge, there have been few if any attempts to produce desired GHPs by engineering

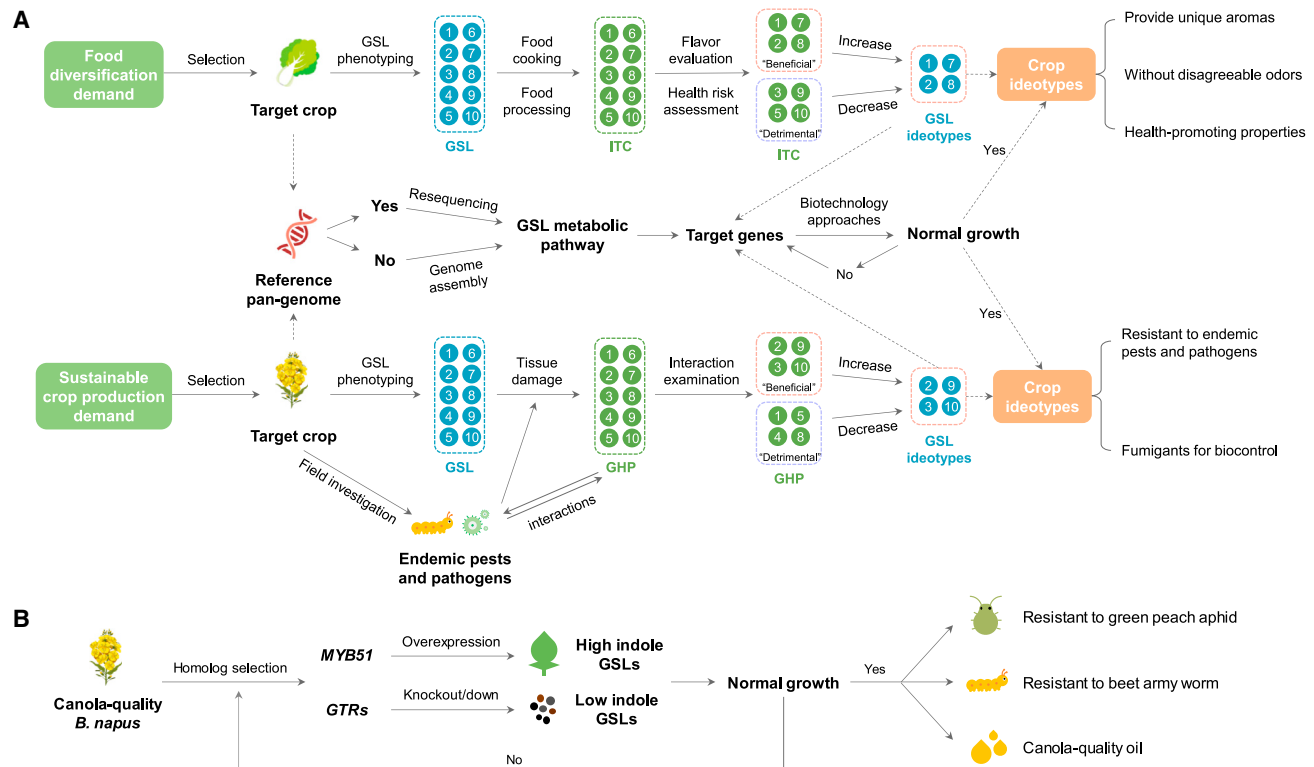


Figure 3. Roadmap for generating Brassicaceae crop ideotypes with defined GSL profiles.

(A) Two routes for developing multifunctional crop ideotypes based on expected future demand for food diversification and sustainable crop production. (B) A feasible molecular route for development of a canola-quality *B. napus* ideotype that is resistant to endemic green peach aphids.

myrosinase- and specifier protein-encoded genes in different species. Although extensive exploration and manipulation of genes involved in GSL biosynthesis and transport have created desired GSL phenotypes in crop species, these alterations manifest themselves prior to the final biological functions and desired end uses. Hence, we suggest that manipulations of genes involved in GSL hydrolysis are likely to be more profitable because they are directly associated with desired end products and biological functions. Diverse GHPs could be overaccumulated in specific tissues and organs or mass-produced *in vitro* through gene or enzyme modification or under controlled conditions such as specific pHs and $\text{Fe}^{2+}/\text{Fe}^{3+}$ concentrations. These GHPs may provide substrates for final products that meet multiple future demands, either directly or after additional processing.

GENERATING MULTIFUNCTIONAL CROPS WITH DEFINED GSL PROFILES AND NOVEL END USES

Prior to GSL engineering, it is critical to consider future demand for the development of multifunctional crops corresponding to available information on GSL and GHP functions. This may determine the selection of target crops and the roadmap for design of crop ideotypes (Figure 3A). The demand may be considered from two perspectives: food diversification and/or sustainable crop production. The former requires multifunctional crop ideotypes whose accumulated GSLs

contribute pleasing flavors to the daily diet while exhibiting health-promoting or harmless properties for humans and livestock. The latter requires multifunctional crop ideotypes whose accumulated GSLs enhance specific plant resistance to endemic pests and pathogens, thus reducing pesticide use and assisting in the development of sustainable agriculture. Regardless of any possible technical challenges in practical application, the corresponding crop ideotypes are expected to be proposed first according to specific requirements (Figure 3A).

Optional crop ideotypes based on the demand for food diversification

Food flavor is an important factor that contributes to consumer preference. Although the overall flavor of a Brassicaceae plant can derive from hundreds of odorants, it is widely believed that ITCs, the main type of GHP produced during food processing (Figure 2A), are the major distinguishing factors (Wieczorek et al., 2018; Bell et al., 2021) (Table 4). For example, considerable breeding effort has already been successful in modifying GSL profiles to reduce bitterness in Brussels sprouts (*B. oleracea* var. *gemmifera*) and ensure that they are more palatable (Fenwick et al., 1983; Van Doorn et al., 1998). More generally, there is scope for modifying GSL profiles in edible plant parts to create various crop ideotypes with distinct flavors using available biotechnological approaches. In the daily diet, pungent and spicy tastes of condiments made from sinigrin-rich species, such as wasabi, horseradish, and mustard, are

GHP	GSL ^a	Odor description	Beneficial/detrimental effects on human health ^b
Benzyl ITC	glucotropaeolin (11)	chemical, pungent, green, rotten grass, cooked, watercress-like (Masuda et al., 1996; Bell et al., 2021)	anticarcinogenic, anti-inflammatory, antidiabetic, anti-obesity, and neuroprotective activity and prevention of multiple sclerosis (Wu et al., 2021b; Sundaram et al., 2022)
3-Butenyl ITC	gluconapin (12)	green, bitter, pungent, aromatic, wasabi-like, vegetable-like, cabbage-like (Masuda et al., 1996; Bell et al., 2018, 2021)	anticarcinogenic activity (Sundaram et al., 2022) antimicrobial activity (Jang et al., 2010) prevention of postprandial hypertriglyceridemia (Washida et al., 2010)
4-Hydroxybenzyl ITC	sinalbin (23)	pungent (Bell et al., 2018)	anticarcinogenic activity (Monu et al., 2014; Jurkowska et al., 2018)
5-vinyloxazolidine-2-thiones (goitrins) ^c	progoitrin (24R) and epiprogoitrin (24S)	strongly bitter (Bell et al., 2018)	goitrogenic effect and inhibition of thyroid hormone production* (Stoewsand, 1995; Felker et al., 2016) antiviral activity (Niexing et al., 2020)
Indole-3-carbinol	glucobrassicin (43)	unpleasant (Bell et al., 2018)	cardioprotective and neuroprotective activity (Kamal et al., 2022) anticarcinogenic, antioxidant, antimicrobial, antiviral (including COVID-19) and anti-inflammatory, anti-obesity, antidiabetic, cardioprotective activity (Singh et al., 2021; Centofanti et al., 2022)
4-(Methylsulfinyl)butyl ITC (sulforaphane)	glucoraphanin (64)	no taste or flavor? (Bell et al., 2021)	anticarcinogenic activity (at least 17 types of cancer) (Kaiser et al., 2021) antimicrobial, antiangiogenic, antioxidant and cardioprotective activity (Mahn and Castillo, 2021; Mangla et al., 2021; Kamal et al., 2022) antiviral activity (including COVID-19) (Mahn and Castillo, 2021; Ordonez et al., 2022) anti-inflammatory activity (Wei et al., 2022) antidiabetic activity (Mangla et al., 2021; Wang et al., 2022a) Recovery and prevention of muscle atrophy (Vargas-Mendoza et al., 2022) Prevention of alcohol intolerance (Mahn and Castillo, 2021) Neuroprotective activity (Uddin et al., 2020)
7-(Methylsulfinyl)heptyl ITC	glucoibarin (66)	unknown	anticarcinogenic activity (DeVito et al., 2000; Rose et al., 2005a)
6-(Methylsulfinyl)hexyl ITC (hesperin)	glucohesperin (67)	unknown	anticarcinogenic activity (Trio et al., 2017) anti-inflammatory and neuroprotective activity (Jaafaru et al., 2018) antiallergic activity (Yamada-Kato et al., 2012)
8-(Methylsulfinyl)octyl ITC (hirsutin)	glucohirsutin (69)	unknown	anti-inflammatory activity (DeVito et al., 2000; Rose et al., 2005b)
5-(Methylsulfinyl)pentyl ITC (alyssin)	glucoalyssin (72)	unknown	anticarcinogenic activity (Milczarek et al., 2018; Pocasap et al., 2019) antimicrobial activity (Nowicki et al., 2021)
3-(Methylsulfinyl)propyl ITC (iberin)	glucoiberin (73)	unknown	anticarcinogenic activity (Kim and Singh, 2009; Wang et al., 2016; Pocasap et al., 2019) antioxidant activity (Ernst et al., 2013) antimicrobial activity (Nowicki et al., 2021)

Table 4. Odor descriptions and health effects of major GHPs found in important Brassicaceae species.

(Continued on next page)

GHP	GSL ^a	Odor description	Beneficial/detrimental effects on human health ^b
4-(Methylthio)but-3-enyl ITC (raphasatin)	glucoraphasatin (83)	pungent (Bell et al., 2018)	anticarcinogenic and antioxidant activity (Sundaram et al., 2022)
4-(Methylthio)butyl ITC (erucin)	glucoerucin (84)	radish-like, cabbage-like, mushroom-like (Kroener and Buettner, 2017; Wei et al., 2021)	anticarcinogenic activity (Singh et al., 2020) antioxidant activity (Cedrowski et al., 2021) anti-inflammatory, neuroprotective and cardioprotective activity (Jaafaru et al., 2018; Kamal et al., 2022) anti-obesity activity (Chae et al., 2015) antihypertensive activity (Martelli et al., 2020)
7-(Methylthio)heptyl ITC	7-(methylthio)heptyl GSL (87)	sweet, fatty, flowery, plastic, radish-like, pickle-like (Masuda et al., 1996; Kroener and Buettner, 2017)	antimicrobial activity (Shin et al., 2004) antiplatelet activity (Kumagai et al., 1994)
6-(Methylthio)hexyl ITC (lesquerellin)	glucosquerellin (88)	sweet, fatty, acidic, radish-like (Masuda et al., 1996; Kroener and Buettner, 2017)	anticarcinogenic activity (Yano et al., 2000; Korenori et al., 2013) antiallergic activity (Yamada-Kato et al., 2012) antimicrobial activity (Shin et al., 2004) antiplatelet activity (Kumagai et al., 1994)
5-(Methylthio)pentyl ITC (berteroin)	glucoberteroin (94)	radish-like, pickle-like, mushroom-like (Masuda et al., 1996; Kroener and Buettner, 2017)	Prevention of non-alcoholic fatty liver disease (Kim et al., 2021) anti-inflammatory activity (Jung et al., 2014) anticarcinogenic activity (Kim and Singh, 2009)
3-(Methylthio)propyl ITC (iberverin)	glucoiberverin (95)	strongly radish-like, pungent, sulfurous, vegetative, horseradish-like, mushroom-like, gooseberry-like (Masuda et al., 1996; Kroener and Buettner, 2017; Bell et al., 2021; Wei et al., 2021)	anticarcinogenic activity (Kim and Singh, 2009; Wang et al., 2016) antioxidant activity (Ernst et al., 2013) antimicrobial activity (Nowicki et al., 2021)
4-Pentenyl ITC	glucobrassicinapin (101)	green, acrid, pungent, peppery, sulfurous, musty, fragrant, mustard-like, horseradish-like (Masuda et al., 1996; Bell et al., 2018, 2021)	antimicrobial activity (Jang et al., 2010)
2-Phenylethyl ITC	gluconasturtiin (105)	strongly radish-like, strong watercress aroma, pungent, green, horseradish-like, gooseberry-like, tingling sensation (Masuda et al., 1996; Kroener and Buettner, 2017; Bell et al., 2018, 2021; Wei et al., 2021)	anticarcinogenic activity (Sundaram et al., 2022) antioxidant, anti-inflammatory and antimicrobial activity (Coscueta et al., 2022) cardioprotective and neuroprotective activity (Kamal et al., 2022)
Allyl ITC	sinigrin (107)	strongly pungent, spicy, bitter, sulfurous, lachrymatory, mustard-like, garlic-like, onion-like, horseradish-like (Masuda et al., 1996; Kroener and Buettner, 2017; Bell et al., 2018, 2021)	anticarcinogenic, anti-inflammatory, antimicrobial, antioxidant, and wound-healing activity (Mazumder et al., 2016) antiviral activity (including COVID-19) (Guijarro-Real et al., 2021) antidiabetic and antihyperglycemic activity (Abbas et al., 2017; Zhang and Wang, 2022) potential goitrogenic effect (Lee and Kwon, 2015)
Allyl thiocyanate	sinigrin (107)	peppery, pungent, musty, sulfurous, mustard-like, horseradish-like (Bell et al., 2018, 2021)	potential goitrogenic effect (Lee and Kwon, 2015)

Table 4. Continued

^aThe bold GSL numbers are placed in brackets after GSL names, and the order of GHPs is arranged by GSL numbers.^bThe detrimental effects of GHPs on human health are marked with an asterisk.^cGoitrins include two enantiomers: (1) (S)-goitrin, also (S)-5-vinyloxazolidine-2-thione, which is formed from the hydrolysis of progoitrin (**24R**); and (2) (R)-goitrin, also (R)-5-vinyloxazolidine-2-thione, which is formed from the hydrolysis of epiprogoitrin (**24S**).

sought after by large numbers of consumers (Table 4). Although the flavor of these three components is similar (contributed mainly by allyl ITC), wasabi tastes more moderate, fresher, and sweeter, partially owing to less gluconasturtiin (with a strongly radish-like and pungent odor) and the presence of ω -alkenyl ITCs, long-chain ω -methylthioalkyl, and ω -methylsulfinylalkyl ITCs (Uchida et al., 2012) (Table 4; Supplemental Table 1). Nevertheless, the extremely strict cultivation conditions and long growth period of wasabi make it scarce and costly for consumers (Depree et al., 1998). This leads to the prevalence of cheaper horseradish, mustard, and their by-products in the market because they are much easier to grow, manage, and process (Agneta et al., 2013). Given that the GSL profiles of wasabi and horseradish are already established (Supplemental Table 1), horseradish could be a target species for increasing the concentrations of GSLs associated with wasabi aroma through biotechnological approaches to attract interest from consumers. Other sinigrin-rich species, such as the less frequently cultivated black mustard and Ethiopian mustard (Table 1), also have the potential for genetic improvement to deliver wasabi-like flavors in their derived products.

For vegetable and oil species, the balance of flavor and health is more important (Bell and Wagstaff, 2017). To maximize the flavor of vegetables and minimize possible health risks from oil crops, there have been concerted efforts to reduce the concentrations of bitter, pungent, and health-threatening GSLs in specific organs and tissues. For example, until the 1970s, the levels (100–180 $\mu\text{mol/g}$ dry weight) of GSLs in rapeseed (Wathelet et al., 1993; Chen et al., 2018; Wang et al., 2018; Tan et al., 2022), particularly progoitrin, were recognized as having a goitrogenic effect on livestock fed protein-rich seed meal, as well as a bitter and spicy taste following extraction of oil for human consumption (Table 4). This has driven the development and extensive cultivation of canola-quality *B. napus* as a major global temperate oil crop after major modifications of seed composition that reduced levels of GSL (10–30 $\mu\text{mol/g}$ dry weight) and erucic acid (Stenfanasson and Kondra, 1975; Wathelet et al., 1993; Chen et al., 2018). Increasing evidence indicates that numerous naturally occurring GHPs detected in important Brassicaceae vegetables (sulforaphane, allyl ITC, phenethyl ITC, benzyl ITC, products of indole-3-methyl ITC such as indole-3-carbinol, etc.) have health-promoting effects (e.g., anticarcinogenic, anti-inflammatory, neuroprotective, anti-obesity, and antiviral activity) and desirable aromas, such as sweet and flowery odors (Table 4). For this reason, there is scope for developing crop ideotypes with further overaccumulation of health-promoting GSLs while maintaining or enhancing overall desirable flavor. For example, it appears that sulforaphane exhibits no significant taste or flavor (Table 4), and it has been widely proven to be one of the most potent anticarcinogenic ITCs (Garcia-Oliveira et al., 2021). This underpins the very successful commercial release of the broccoli cultivar Beneforté, with highly enriched glucoraphanin concentrations (Sivapalan et al., 2018). Similar efforts have also been made—and could be further extended—in species such as rapeseed (Liu et al., 2012), kale (Araki et al., 2013; Qian et al., 2015), mustard (Augustine and Bisht, 2015a), and several subspecies of *B. rapa* (Liu et al., 2017), as well as underutilized rocket salad (*Eruca sativa*) (Table 1). Enriching GSLs in a species can provide health-promoting effects but may also result in disagreeable odors. For instance, gluconasturtiin is

predominant in watercress edible organs (Table 1) and contributes anticarcinogenic, antimicrobial, and many other health-promoting effects, but it can also generate a high-intensity pungent odor and “tingling” taste sensation (Bell et al., 2021) (Table 4). Although the C6–C8 GSLs present in watercress contribute sweetish and flowery aromas (Tables 1 and 4; Supplemental Table 1), these may be masked because of their lower concentrations and the predominance of gluconasturtiin. Therefore, it may be feasible to enhance the overall aroma of watercress and attract more consumers by further enriching long-chain GSLs and reducing gluconasturtiin in edible organs. Alongside the intake of raw and fresh vegetables, a wider range of cooking methods may be explored to reduce undesirable odors prior to or during ingestion, or indeed following digestion (Nugrahi et al., 2015; Marcinkowska et al., 2021; Wiczeorek et al., 2021). Furthermore, our increasing understanding of aroma-active compounds in vegetable species is likely to promote development of additional aroma-associated GSLs and reduction of GSLs associated with negative odors (Engel et al., 2006; Guijarro-Real et al., 2020; Bell et al., 2021; Marcinkowska et al., 2021; Wei et al., 2021; Wiczeorek et al., 2021). It should be noted that, for a few double-high oil landraces of *B. napus*, *B. juncea*, and *B. rapa* that are still grown in India and southwest China (Kang et al., 2021), the unique aroma of the derived oil is still preferred by local consumers (Yao et al., 2020; Liang et al., 2023). This inspires us to further dissect the unique aroma compounds that could be valuable for flavor enhancement of canola oil.

Optional crop ideotypes based on the demand for sustainable crop production

In addition to affecting food flavor, GSLs can also confer resistance in Brassicaceae species. Through the glucosinolate-myrosinase system, diverse GHPs are released to play dual roles in complex plant–herbivore and plant–pathogen interactions (Figure 2A–2D). In plant–herbivore studies, insect herbivores are generally classified as generalists or specialists, depending on their feeding habits on host plants. Some generalists tend to be repelled or poisoned by GHPs, whereas several GSL specialists tend to be stimulated to oviposit or feed on Brassicaceae plant tissues (Winde and Wittstock, 2011). Over the past decade, numerous studies have demonstrated these kinds of relationships between specific GSLs/GHPs and insect herbivores (Ahuja et al., 2009; Liu et al., 2021). Likewise, similar relationships have been observed between specific GSLs/GHPs and plant pathogens (Plaszkó et al., 2021, 2022). These observations have motivated ongoing biocontrol attempts to alter concentrations of GSLs that increase resistance to specific pests and pathogens (Table 3; Supplemental Table 2). Moreover, the use of chemical pesticides may have inadvertently increased because of diminished resistance to specific pests, pathogens, and even birds in double-low oil crop production. Although there is a lack of systematic data to support this possibility, bird damage has become severe in recent years, at least in China’s canola fields, and there is a significant negative correlation between Met-derived GSLs and levels of bird damage (Zhao et al., 2016). Comprehensive field investigations will be required to verify this relationship in more regions, where yield losses will hopefully be offset by use of GSL-enriched ideotype cultivars.

The release of volatile ITCs can contribute to exogenous biocontrol in agricultural production, and the advantages of biofumigation for control of soil-borne pests and pathogens and reduction of greenhouse gas emissions have been widely demonstrated (Sarwar et al., 1998; Lazzeri et al., 2010; Peng et al., 2021; Plaszkó et al., 2021). This has been achieved through Brassicaceae crop rotation or soil incorporation of GSL-rich green tissues or seed meal (Sarwar et al., 1998). More extensive adoption of biofumigation is expected to benefit from further enrichment of specific GSLs in specific crop tissues and organs, and subsequently in their by-products. In addition to exogenous application, enhancing endogenous resistance via overaccumulation of specific GSLs could be easily achieved for oil crops because the transport of increased GSLs from vegetative organs to seeds could be disturbed by knocking out GTR proteins, thereby reducing negative effects on oil quality (Nour-Eldin et al., 2017; Nambiar et al., 2021; He et al., 2022; Tan et al., 2022). However, for vegetable species whose aerial parts are usually edible, flavor may be negatively affected if resistance is obtained by enhancing endogenous GSL concentrations (Wieczorek et al., 2018). An alternative solution is to knock out/down TGG genes to reduce the production of bitter ITCs, an approach that may be useful for vegetables usually consumed raw, although it could result in higher susceptibility of target plants to specific pests or pathogens (e.g., green peach aphid) (Borgen et al., 2012).

A roadmap for crop ideotype design to address future demand

Based on the reviewed advances and discussion of genetic mechanisms that regulate GSL biosynthesis, transport, and hydrolysis, as well as functional associations with insect herbivores, pathogens, and human and livestock health, we propose an integrated roadmap for specifying crop ideotype options that may be delivered through sophisticated metabolic engineering using combined biotechnological approaches (Figure 3A). Our expectation is that future multifunctional Brassicaceae crops and their by-products will accumulate desirable GSLs in specific organs and tissues to more precisely meet future demands for food diversification and sustainable crop production.

According to the proposed roadmap (Figure 3A), once the target crop is selected, GSL phenotyping with existing analytical strategies is required for target organs or tissues. GHP profiles are expected to be deduced based on GSL profiles that correspond to different tissue exposures, such as food processing and cooking, or tissue disruption resulting from pathogen or pest damage. For food diversification, characterization of ITCs that contribute to flavor and assessments of associated health risks for humans and livestock are necessary to ascertain which ITCs primarily contribute beneficial effects (pleasing flavors, harmless or health-promoting effects) and which contribute detrimental effects (disagreeable flavors or harmful health effects). For sustainable crop production contexts, field investigation is required to establish an inventory of endemic pathogens and pests. This may be based on reviewing available information or on contextual examination of plant–herbivore, tritrophic, or plant–pathogen interactions, which would help to ascertain which GHPs primarily contribute

beneficial effects (resistance to specific pests and pathogens) and which contribute detrimental effects (making plants susceptible or attractive to specific pests and pathogens). In addition to phenotypic evaluations, investigation of GSL metabolic pathways is also necessary. According to recent statistics, 58 Brassicaceae genomes have been sequenced, assembled, and published as of December 2022 (https://plabipd.de/plant_genomes_pa.ep), and pan-genome sequences of a few important species (e.g., *B. rapa* and *B. napus*) are also available (Song et al., 2020; Cai et al., 2021). With the published reference pan-genome sequences, detection of candidate GSL genes with existing high-throughput resequencing and bioinformatic tools is relatively straightforward. For species that lack reference genomes and pan-genome sequences, *de novo* genome assembly is now routine and is expected to be performed as a preliminary step. The complete GSL metabolic pathway could subsequently be generated via comparative genomic and pathway modelling tools. Such data frameworks, which combine information on GSL phenotypes and genotypes, will enable predictions of which GSLs can be increased and, correspondingly, which can be reduced. Finally, novel crop chemotypes can be defined and used as a template to design engineering approaches for development of target crop cultivars close to the designed ideotypes.

Biotechnological approaches for future GSL engineering

Although many ideotypes can be enumerated dependent on specific demands, the likelihood of achieving a specific GSL engineering goal will depend on the feasibility and complexity of applying available biotechnological approaches. Gene editing or transgenic approaches are available for GSL engineering, which have been demonstrated for most *Brassica* species (Li et al., 2021a; Neequaye et al., 2021; He et al., 2022; Tan et al., 2022) (Tables 2 and 3; Supplemental Table 2), and are expected to become available for a wider range of Brassicaceae species (Miao et al., 2021; Neequaye et al., 2021; Kim et al., 2022). In future GSL engineering work, selection of an appropriate combination of target genes involved in GSL biosynthesis, transport, and hydrolysis is an important preliminary step in the design of crop ideotypes. Based on this step and the proposed roadmap (Figure 3A), the most feasible route may be chosen for practical breeding. For example, green peach aphid is one of the most common generalist herbivores and usually feeds on *Brassica* species (Desneux et al., 2006). To inhibit aphid infestation in canola fields, *MYB51* and *GTRs* could be engineering targets for development of a more resistant crop ideotype (Figure 3B). Overexpression of *MYB51* homologs enhances indole GSL concentrations without affecting normal plant growth (Gigolashvili et al., 2007) and is expected to increase resistance to green peach aphids and another generalist herbivore, the beet armyworm (*Spodoptera exigua*) (Kim et al., 2008; Borgen et al., 2012). However, enrichment of indole GSLs in seeds may negatively affect the flavor of canola oil (Wieczorek et al., 2018). The knockout/knockdown of GTR homologs is expected to resolve this issue by disturbing GSL loading in the seed (Nour-Eldin et al., 2017; Nambiar et al., 2021; He et al., 2022; Tan et al., 2022; Hözl et al., 2023). Weakened performance of green peach aphids on *GTR* knockout plants has also been observed in *A. thaliana*

(Madsen et al., 2015). The final canola ideotype is expected to exhibit normal growth and enhanced resistance to green peach aphids in vegetative organs and low GSL levels in seeds, thus maintaining the quality of canola oil (Figure 3B). If normal plant growth is negatively affected after gene mutation, especially for *GTR* genes, other appropriate orthologs could be selected to evaluate plant growth effects (Figure 3B). In this route, *MYB51* could be replaced by other indole GSL biosynthesis genes such as *CYP79B2*, *CYP83A1*, *UGT74B1*, *SOT16*, *CYP81F2*, *MYB34*, *MYB122*, and even the newly characterized *WRKY33*, whose overexpression may also drive accumulation of indole GSLs and potentially increase resistance to green peach aphids, other pests, and/or pathogens (Frerigmann and Gigolashvili, 2014; Tao et al., 2022) (Table 3; Supplemental Table 2). Beyond manipulation of endogenous GSL genes, introgression of exogenous genes into target species by transformation can also promote overaccumulation of novel desirable GSLs in Brassicaceae species. For example, ectopic expression of sorghum (*Sorghum bicolor*) *CYP79A1* and *CYP71E1* caused overaccumulation of the novel GSL sinalbin in transgenic *A. thaliana* lines (Bak et al., 1999, 2000; Petersen et al., 2001), and plant morphological phenotypes and contents of other GSLs remained unaffected in *CYP79A1* transgenic lines (Kristensen et al., 2005). This approach relies on the broad substrate specificity of CYP79 enzymes in numerous species; these enzymes can catalyze the conversion of amino acids into corresponding oximes and are expected to be used in GSL engineering of more Brassicaceae species (Bak et al., 2006).

There remain several limitations and obstacles to be surmounted in the practical application of molecular engineering approaches. First, current market restrictions for genetically modified crops are strict, hindering their adoption, although such barriers may be reduced for gene editing (Pixley et al., 2022). Second, the extent to which GSL and GHP profiles can be altered by current transgenic or gene-editing approaches is still limited. This could be improved by protein engineering, which is a combination of rational design, directed evolution, and many other methods that optimize the properties of enzymes in a more controlled, targeted, and accurate manner (Kapoor et al., 2017). Proteomic engineering has been used to alter the substrate specificity of MAM synthases toward different GSLs through *in planta* and *in vivo* site-directed mutagenesis of key active residues, an effort made possible by the recent biophysical dissection of MAM synthase crystal structures in *A. thaliana* and *B. juncea* (Kumar et al., 2019; Petersen et al., 2019). A similar mutagenesis study was performed on *GTR1*, assisted by a bioinformatics protocol designed to identify the potential binding site of GSLs more efficiently than with biophysical methods (Peña-Varas et al., 2022). Therefore, as a potentially powerful method, protein engineering is expected to be applied in more GSL metabolic contexts, together with integration of further developed multi-omics techniques. Third, possible negative effects of gene modification may affect the selection of target genes and may be difficult to overcome. Fourth, genes usually exist as multiple copies within complex Brassicaceae genomes, and multiple evaluations of specific paralogs are likely to be required to determine which are functional and less likely to perturb normal plant growth. Moreover, interactions among GSL genes may differ in complex genomes. In *A. thaliana*, for example, *AtMYB28* has been shown to be a pos-

itive regulator of *AtAOP2* (Sønderby et al., 2010a), whereas three homologs encoding *BrMYB28* in *B. rapa* act as negative regulators of *BrAOP2* (Seo et al., 2016). Even in allotetraploid *B. napus*, RNAi silencing of *MAM* genes surprisingly induced biosynthesis of C3 Met-derived aliphatic GSLs (Liu et al., 2011). We expect that this problem will be progressively overcome as GSL genetic mechanisms in complex genomes are more fully elucidated in the future.

Apart from molecular approaches, traditional intra- and interspecific hybridization is still a powerful and universal approach for generating diverse crop ideotypes. Especially for field selection, GSL diversity can be increased in target crops by gene introgression, which also enables the co-selection of GSL traits and other important agronomic traits. For example, GSL diversity was enriched in resynthesized allotetraploid *B. napus* using different genome resynthesis approaches in various combinations (Kräling et al., 1990; Hill et al., 2003; Cleemput and Becker, 2012). In a resynthesized *B. napus* (AACC) germplasm pool developed by interspecific hybridization between *B. rapa* (AA) and *Brassica carinata* (BBCC), B-genome-related sinigrin is introgressed in both leaves and seeds of most accessions (Table 1; Hu et al., 2019; Qin et al., 2020). Where health-promoting GSLs exist in one species, such as glucoraphanin in broccoli, these could be further enriched to achieve higher content via recombination. Beneforté is an F₁ hybrid developed by crossing broccoli with the related wild species *B. villosa* (Traka et al., 2013). The introgressed *B. villosa* *MYB28* allele leads to a significant increase in glucoraphanin in Beneforté varieties (Sivapalan et al., 2018). Health-promoting GSLs absent in one species may also be significantly enriched to detectable levels by recombination of alleles within or between homoeologous chromosomes. In the A-genome diploid species *B. rapa*, for example, glucoraphanin is almost undetectable owing to the presence of three functional *BrAOP2* genes (Zhang et al., 2015a). Through a marker-assisted backcrossing process, two *BrAOP* genes (*BrAOP2.2* and *BrAOP2.3*) were replaced with non-functional counterparts, leading to a significant increase in glucoraphanin content (Liu et al., 2017). Following hybridization, it is crucial to determine an appropriate method of artificial selection that ensures the retention of specific alleles. This can be accelerated using a traditional marker-assisted approach or by using genomic selection and other bioinformatic analysis techniques that can detect more subtle connections between genotypes and phenotypes (Spindel and McCouch, 2016).

Overall, we hope that the collective knowledge and discussion presented in this review will stimulate development of a comprehensive toolbox and a positive outlook for sophisticated engineering of GSL profiles in a wider range of Brassicaceae species. As additional genes are waiting to be identified and their roles resolved, novel engineering targets will continue to emerge (Harun et al., 2021). This will increase the options available for modifying GSL profiles by gene-editing or transgenic approaches. There are several more potential directions for future GSL engineering that we cannot fully capture here, such as control of greenhouse growth conditions (Bell and Wagstaff, 2017) and postharvest storage, cooking, and processing protocols to obtain desired GSLs and GHPs (Wu et al., 2021a); optimization of sulfur fertilizer supply to enrich GSL accumulation (Borpatragohain et al., 2019); production of GSLs

by *in vitro* plant cell cultures (Petersen et al., 2018); and induction of GSL production by exogenous phytohormones (Wiesner et al., 2013a; Augustine and Bisht, 2015b; Wang et al., 2015) or radiation (Banerjee et al., 2016). We believe that combining such explorations will facilitate the accessibility of GSL engineering in crop breeding, ultimately enabling development of multifunctional Brassicaceae crops to meet sustainable demand in the future.

PROSPECTS

Since the initial discovery of GSLs in the early nineteenth century, our understanding of GSLs and GHPs has advanced with available chemical, genetic, and genomic analysis technologies. This has led to the thorough elucidation of variations in GSL structure, distribution, biosynthesis, hydrolysis, and transport, providing great potential for knowledge-based manipulation of GSLs and GHPs in different Brassicaceae species. However, the discussion in this review is still limited to a small number of species that have been intensively selected and domesticated. In fact, the genetic diversity harbored within the entire Brassicaceae family is far beyond our current understanding (Hu et al., 2021). Wild species are waiting to be exploited and their abundant GSL variations and other desirable traits introgressed into current major crops. They may even be used directly as novel crops through the establishment of an efficient *de novo* domestication strategy (Yu et al., 2021). At least some wild edible species, such as wall rocket (*Diplotaxis erucoides*), are expected to be developed as new crops that contain desirable GSLs (Clemente-Villalba et al., 2020; Guijarro-Real et al., 2020). Apart from exploitation of novel germplasm, advances in technology will expand the reach of GSL engineering. In particular, it is necessary to establish the feasibility of combined tissue-specific targeting of GSLs that contribute separately to human and livestock nutrition, health, and plant defense. This approach need not be applied solely to Brassicaceae species, because systems for the *de novo* production of desirable GSLs have been established in GSL-free species (e.g., potato and tobacco) (González-Romero et al., 2021; Wang et al., 2021). The underlying driver for accumulating all the relevant knowledge is the demand to produce more nutritious and healthier food for a growing human population in a sustainable manner with a reduced reliance on synthetic pesticides.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

FUNDING

This work was supported by the National Natural Science Foundation of China (32171982 and 31970564).

AUTHOR CONTRIBUTIONS

J.Z. conceptualized the manuscript. H.Q. drafted the manuscript and prepared all the tables, figures, and supplemental tables. J.Z. and G.J.K. drafted part of the manuscript and revised the manuscript, tables, figures, and supplemental tables. P.B. revised the manuscript, tables, figures, and supplemental tables. All authors approved the final manuscript for submission.

ACKNOWLEDGMENTS

We acknowledge Dr. Jinling Meng and Dr. Matthew N. Nelson for giving valuable suggestions on the manuscript. Some of the icons used in

Figure 3 were purchased and cited from the website <https://www.51yuansu.com/>. No conflict of interest is declared.

Received: May 18, 2022

Revised: February 15, 2023

Accepted: February 20, 2023

Published: February 23, 2023

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