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REVIEW

Improvement of glucosinolates by metabolic engineering in *Brassica* crops

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Abstract Glucosinolates (GSLs) are a class of sulfur- and nitrogen-containing, and amino acid-derived important secondary metabolites, which mainly present in plants of Brassicaceae family, including *Brassica* crops, such as broccoli, cabbage, and oilseed rape. The bioactive GSL metabolites confer benefits to plant defense, human health, and the unique flavor of some *Brassica* crops. However, certain GSL profiles have adverse effects and are known as anti-nutritional factors. This has attracted mounting attempts to increase beneficial GSLs and reduce detrimental ones in the most commonly consumed *Brassica* crops. We provide a comprehensive overview of metabolic engineering applied in *Brassica* crops to achieve this purpose, including modulation of GSL biosynthesis, ablation of GSL hydrolysis, inhibition of GSL transport processes, and redirection of metabolic flux to GSL. Moreover, advances in omics approaches, i.e., genomics, transcriptome, and metabolome, applied in the elucidation of GSL metabolism in *Brassica* crops, as well as promising and potential genome-editing technologies are also discussed.

Keywords Glucosinolate, *Brassica* crops, Metabolic engineering, Quality improvement

INTRODUCTION

Brassica is a large group of plants that consist of numerous important agricultural and horticultural crops with different edible parts which are commonly cultivated as vegetable, oilseed, and condiment, while the oil cake from rapeseed is a better feed for cattle and poultry. Three genomes (designated A, B and C) share mesohexapolyploid ancestry and pairwise combinations thereof define the *Brassica* species. The A genome (AA; n = 10) occurs in *B. rapa*, the B genome (BB; n = 8) in *B. nigra* and the C genome (CC; n = 9) in *B. oleracea*. These diploid genomes also occur in each pairwise combination to form the amphidiploid allotetraploid species *B. napus* (AACC; n = 19), *B. juncea* (AABB; n = 18) and *B. carinata* (BBCC; n = 17) (He et al. 2021; Nagaharu 1935). Nowadays, great attention has been paid to *Brassica* due to its abundance of glucosinolates (GSLs).

GSLs are a class of sulfur- and nitrogen-containing secondary metabolites, mainly distributed in the order Brassicales. The number of characterized GSLs from plants is between 88 and 137 (Blažević et al. 2020) and 17 of them have been comprehensively reported in common *Brassica* crops (Miao et al. 2017; Wu et al. 2021; Tables 1 and 2). The core structure of GSL

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Table 1 Chemical name of glucosinolates and their chemical structure

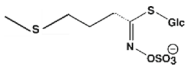
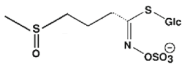
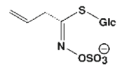
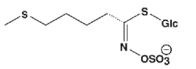
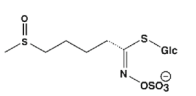
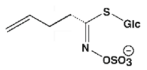
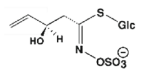
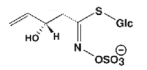
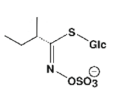
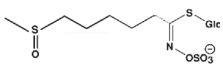
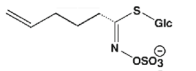
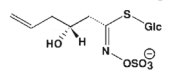
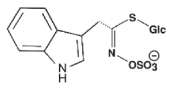
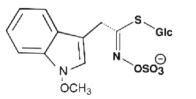
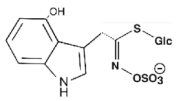
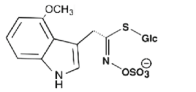
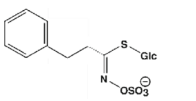
Glucosinolate (GSL)		Chemical name	Chemical structure
Aliphatic GSL	3C	3-(methylthio)propyl GSL	
		3-(methylsulfinyl)propyl GSL	
		2-propenyl GSL	
	4C	4-(methylthio)butyl GSL	
		4-(methylsulfinyl)butyl GSL	
		3-butenyl GSL	
		(R)-2-hydroxy-3-butenyl GSL	
		(S)-2-hydroxy-3-butenyl GSL	
		1-methylpropyl GSL	
	5C	5-(methylsulfinyl)pentyl GSL	
		4-pentenyl GSL	
		(S)-2-hydroxy-4-pentenyl GSL	
Indolic GSL		Indol-3-ylmethyl GSL	
		1-methoxyindol-3-ylmethyl GSL	
		4-hydroxyindol-3-ylmethyl GSL	
		4-methoxyindol-3-ylmethyl GSL	
Benzenic GSL		2-phenylethyl GSL	

Table 2 Principal GSLs identified in several common *Brassica* crops

Glucosinolate (GSL)		*Brassica oleracea*										
		Chinese kale	Broccoli	White cabbage	Savoy cabbage	Red cabbage	Kale	Collard	Tronchuda cabbage	Brussels sprouts	Cauliflower	Kohlrabi
3C aliphatic GSL	3-(methylthio)propyl GSL	−	+	+	+	+	+	−	+	−	+	−
	3-(methylsulfinyl)propyl GSL	+	+	+	+	+	+	+	+	−	+	−
	2-propenyl GSL	+	+	+	+	+	+	+	+	+	+	−
4C aliphatic GSL	4-(methylthio)butyl GSL	+	+	+	+	+	−	+	−	−	+	+
	4-(methylsulfinyl)butyl GSL	+	+	+	+	+	+	+	+	−	+	+
	3-butenyl GSL	+	+	+	+	+	+	+	+	+	+	−
	(R)-2-hydroxy-3-butenyl GSL	+	+	+	+	+	+	+	+	+	+	−
	(S)-2-hydroxy-3-butenyl GSL	−	+	−	−	+	+	−	+	+	−	−
	1-methylpropyl GSL	−	−	−	−	−	−	−	−	−	−	−
5C aliphatic GSL	5-(methylsulfinyl)pentyl GSL	+	+	−	−	+	−	−	+	−	+	+
	4-pentenyl GSL	−	+	+	+	+	−	−	+	−	+	−
	(S)-2-hydroxy-4-pentenyl GSL	+	+	+	+	+	−	−	+	−	−	−
Indolic GSL	Indol-3-ylmethyl GSL	+	+	+	+	+	+	+	+	+	+	+
	1-methoxyindol-3-ylmethyl GSL	+	+	+	+	+	+	+	+	−	+	+
	4-hydroxyindol-3-ylmethyl GSL	+	+	+	+	+	+	−	+	+	+	+
	4-methoxyindol-3-ylmethyl GSL	+	+	+	+	+	+	−	+	+	+	+
Benzenic GSL	2-phenylethyl GSL	+	+	+	+	+	+	+	+	−	+	−

Glucosinolate (GSL)		*Brassica rapa*			*Brassica napus*			*Brassica juncea*
		Turnip	Chinese cabbage	Pak Choi	Swede	Leaf rape	Oilseed rape	Mustard
3C aliphatic GSL	3-(methylthio)propyl GSL	+	−	−	+	−	−	−
	3-(methylsulfinyl)propyl GSL	+	+	+	+	−	−	−
	2-propenyl GSL	−	+	−	+	−	−	+

Table 2 continued

Glucosinolate (GSL)		*Brassica rapa*			*Brassica napus*			*Brassica juncea*
		Turnip	Chinese cabbage	Pak Choi	Swede	Leaf rape	Oilseed rape	Mustard
4C aliphatic GSL	4-(methylthio)butyl GSL	+	–	+	+	–	+	–
	4-(methylsulfinyl)butyl GSL	+	–	+	+	–	+	–
	3-butenyl GSL	+	+	+	+	+	+	+
	(R)-2-hydroxy-3-butenyl GSL	+	+	+	+	+	+	–
	(S)-2-hydroxy-3-butenyl GSL	–	–	–	–	–	–	–
	1-methylpropyl GSL	+	+	–	–	–	–	–
5C aliphatic GSL	5-(methylsulfinyl)pentyl GSL	+	+	+	+	+	+	+
	4-pentenyl GSL	+	+	+	+	+	+	–
	(S)-2-hydroxy-4-pentenyl GSL	+	–	+	+	–	+	–
Indolic GSL	Indol-3-ylmethyl GSL	+	+	+	+	+	+	+
	1-methoxyindol-3-ylmethyl GSL	+	+	+	+	–	+	+
	4-hydroxyindol-3-ylmethyl GSL	+	–	+	+	–	+	+
	4-methoxyindol-3-ylmethyl GSL	+	+	+	+	–	+	+
Benzenic GSL	2-phenylethyl GSL	+	+	+	+	–	+	+

consists of a β-D-thioglucose group, a sulfonated oxime group, and a side chain derived from amino acids (Sønderby et al. 2010). GSLs can be classified into aliphatic GSLs (derived from methionine, alanine, leucine, isoleucine, and valine), indolic GSLs (derived from tryptophan), and benzenic GSLs (derived from phenylalanine and tyrosine) according to their amino acid precursors. Generally, intact GSLs are regarded as biologically inactive, and they can be hydrolyzed by specific myrosinase into various bioactive breakdown products, such as isothiocyanates (ITCs), nitriles, epithionitriles, or thiocyanates. GSL catabolism is much more complex, as there exist classical myrosinases and atypical myrosinases, as well as myrosinase-dependent and -independent degradation pathways, which have been thoroughly reviewed recently (Blažević et al. 2020; Wu et al. 2021). Particularly, GSLs with hydroxylated side chain at carbon 3, such as (R)-2-hydroxy-3-butenyl GSL (progoitrin) and (S)-2-hydroxy-3-butenyl GSL (epiprogoitrin), cyclize to form oxazolidine-2-thiones, while indolic GSLs give unstable ITCs that react to form ascorbigens and sometime indol-3-cabinols, both of which form further indolic compounds (Blažević et al. 2020; Prieto et al. 2019). GSLs and their various hydrolysis products are important defense compounds in plants and contribute to human health and the flavor of *Brassica* vegetables.

GSL and plant defense

Well-known as 'mustard oil bomb', the GSL–myrosinase system is the core part of induced plant defense system (Kliebenstein et al. 2005), which is an 'effective weapon' for plants to resist both biotic and abiotic stress (Onkokesung et al. 2019; Salehin et al. 2019). Specifically, GSLs and their breakdown products have been shown to be toxic to many organisms that are harmful to plants, such as insects and microorganisms (Bednarek et al. 2009; Chen et al. 2020; Clay et al. 2009; de Vos et al. 2008). GSLs confer plants effective defense against generalist lepidopteran herbivores (*Spodoptera littoralis* and *Mamestra brassicae*) at least during most stages of larval development, with aliphatic GSLs having stronger effects than indolic GSLs (Jeschke et al. 2017). 4-methoxyindol-3-ylmethyl GSL could be activated by the atypical PEN2 myrosinase (a type of beta-thioglucoside glucohydrolase) for antifungal resistance (Bednarek et al. 2009). Besides, sulforaphane, the ITC product of 4-(methylsulfinyl)butyl GSL (glucoraphanin), is crucial, robust, and developmentally regulated defenses that underpin non-host resistance in the *Arabidopsis-Pseudomonas* pathosystem (Fan et al. 2011). The supplementation of allyl ITC can improve the

biofumigation process to control the root-knot nematode *Meloidogyne hapla* (Dahlin and Hallmann 2020). Moreover, the treatment of 3-butenenitrile, a nitrile, can enhance the disease tolerance of *Arabidopsis* against necrotrophic pathogens (*Pectobacterium carotovorum* ssp. *carotovorum* and *Botrytis cinerea*) (Ting et al. 2020). Compared with the extensive regulation of GSLs on plant biotic stress, they also play a role in abiotic stress (Clay et al. 2009). For instance, GSLs and ITCs can promote stomatal closure via stimulating the formation of reactive oxygen species (ROS), so as to integrate growth and stomatal regulation upon drought stress (Salehin et al. 2019; Sobahan et al. 2015).

GSL and health promotion

The most attractive biological function of GSLs and their breakdown products is their anti-cancer activity (Sundaram et al. 2021). The hydrolysis products of GSLs have been recognized to reduce the risk of various cancers, particularly ITCs that are reported to be potent anticarcinogenic compounds in lung, colorectal, breast, prostate, and other cancers both in vitro and in vivo (Gao et al. 2018; Huang et al. 2018; Lubecka et al. 2018; Núñez-Iglesias et al. 2018; Zhou et al. 2019). Generally, ITCs make contributions to cancer protection via antiproliferative (Mitsiogianni et al. 2021), pro-apoptotic (Dos Santos et al. 2020), anti-inflammatory (Rakariyatham et al. 2019), anti-migratory (Yin et al. 2019), and anti-angiogenic (Liu et al. 2018). For example, sulforaphane was reported to inhibit phase I enzymes and induce phase II enzymes in vitro and in vivo (Dinkova-Kostova and Kostov 2012). Numerous studies indicated that sulforaphane treatment could effectively induce cell cycle arrest and apoptosis in prostate, colon, and other cancer cell lines (Gamet-Payrastre 2006; Parnaud et al. 2004; Zhou et al. 2019), inhibited HDAC activity with an increase in acetylated histones in HCT116 human colorectal cancer cells (Myzak et al. 2004), and diminished the formation of mammary tumors in rats exposed to 17β-estradiol (Palliyaguru et al. 2020). Besides sulforaphane, other ITCs, including phenethyl ITC, benzyl ITC, allyl ITC, and indole-3-carbinol were also reported to inhibit cancer progression through multiple potential mechanisms, such as modulation of epigenome (Sundaram et al. 2021). Compared to ITCs, nitriles elicited a much weaker cancer-protective potential in vitro and in vivo (Basten et al. 2002; Matusheski and Jeffery 2001), while epithionitriles, such as 1-cyano-2,3-epithiopropane, had relevant cancer-preventive properties via inducing phase II enzymes (Hanschen et al. 2015). In addition to these anticarcinogenic properties, GSLs and their breakdown products, notably ITCs, also play important roles in cardiovascular protection (Wu et al. 2004), as well as protection of the central nervous system (Tanito et al. 2005) and behavior improvement of patients with autism spectrum disorder (ASD) (Singh et al. 2014).

GSL and flavor

Besides health-promoting effects, GSLs and their breakdown products attract interest from food industry, since they are responsible for some sensorial characteristics of Brassicaceae vegetables, such as taste and smell (Redovniković et al. 2008). When plant tissues are damaged, most of the volatile hydrolysis products of GSLs, especially ITCs, produce pungent and bitter taste, as well as sulfurous aroma (Rosa et al. 1996). Previous studies reported that ITCs obtained from 2-propenyl GSL, (R)-2-hydroxy-3-butenyl GSL, and 2-hydroxy-4-pentenyl GSL are responsible for bitter taste (Prieto et al. 2019), while 3-butenyl ITC and 4-pentenyl ITC for a pungent flavor (Juge et al. 2007

Adverse effects of GSL

In addition to many benefits, GSLs and their breakdown products also show some adverse effects. For instance, 1-methoxyindol-3-ylmethyl GSL and its degradation products have been shown to exert negative effects by forming characteristic DNA adducts (Gerber et al. 2011; Glatt et al. 2011). Furthermore, progoitrin has been considered as natural toxicant for its derivative has goitrogenic effects on mammals (Mithen et al. 2000). Generally, the amount of progoitrin in common *Brassica* vegetables is quite low, and this anti-nutritional effect can also be avoided by normal iodine intake (Tripathi and Mishra 2007).

To sum up, GSLs and their degradation products play different roles in terms of food, feed or in the living crop for defense. For example, Glucoraphanin and its degradation products not only reduce the risk of various human cancers (Gamet-Payrastre 2006; Palliyaguru et al. 2020; Parnaud et al. 2004; Zhou et al. 2019), but also are crucial for plant defenses against pathogen (Fan et al. 2011). Progoitrin, another kind of GSL with anticancer activity, is often enriched in rapeseed meal and could induce goiter disease in mammals (Mithen et al. 2000). And progoitrin also contributes to the special flavor of *Brassica* vegetables, combined with 2-propenyl GSL and 3-butenyl GSL. As far as food is concerned, the effects of indolic GSLs are also different. Indol-3-ylmethyl GSL degradation product indole-3-carbinol could inhibit cancer progression (Sundaram et al. 2021), whereas 1-methoxyindol-3-ylmethyl GSL is a potent

genotoxicant and can induce strong cytotoxicity for bacterial and mammalian cells at high concentration (Glatt et al. 2011). However, as defense compounds in plants, the levels of indol-3-ylmethyl GSL and 4-methoxyindol-3-ylmethyl GSL notably increase upon the activation of JA signaling as a defense response to "generalist" insect herbivores, such as green peach aphid (*Myzus persicae*), while GSLs and their degradation products are also used as oviposition and feeding cues for "specialist" insects (Chhajed et al. 2020). Therefore, elucidating the diverse functions of GSLs and their degradation products under different conditions is essential for their potential applications in food, feed and defense of crops.

Scope of this review

As GSLs exhibit potential benefits and certain negative effects, it is desirable to increase the content of beneficial GSL compositions and reduce the adverse ones. Traditional breeding is a successful approach, which created superbroccoli with increased content of glucoraphanin (Faulkner et al. 1998). The GSL contents can also be regulated by a variety of pre- and post-harvest treatments (Ilahy et al. 2020). Along with the elucidation of GSL metabolic pathway, metabolic engineering attracts great interest as a powerful tool to manipulate GSL profiles in *Brassicaceous* plants or create GSLs in none-*Brassicaceous* plants, such as microbial hosts and *Nicotiana benthamiana*. In this review, we do not cover all approaches but instead focus on metabolic engineering of GSL in *Brassica* crops and summarize advances in biotechnology application in *Brassica* plants, i.e., genome sequencing and other omics, as well as genome-editing technologies.

GLUCOSINOLATE BIOSYNTHESIS IN *BRASSICA* PLANTS AND BEYOND

Comprehensive gene inventory of GSL biosynthetic pathway has been described by several papers (Chhajed et al. 2020; Grubb and Abel 2006; Harun et al. 2020; Kittipol et al. 2019; Sønderby et al. 2010; Wang et al. 2020). The current knowledge of GSL biosynthesis is mostly based on the work in model organisms such as *Arabidopsis thaliana* and mainly has been worked out for GSLs derived from methionine and tryptophan, while the biosynthesis of other GSLs has not been so well understood until recently. Generally, GSL biosynthesis consists of three processes: chain elongation of specific precursor amino acid (methionine and phenylalanine), core structure formation, and secondary modification of amino acid side chains (Fig. 1). The chain elongation of aliphatic GSL is started by deamination of methionine by branched-chain amino acid aminotransferase (BCAT), and productions are transferred to chloroplast by bile acid transporter (BAT). Then, they are subjected to methylthioalkylmalate synthase (MAM), isopropylmalate isomerase (IPMI), and isopropylmalate dehydrogenase (IPMDH) to carry out acetyl-CoA condensation, isomerization, and oxidative decarboxylation, which can be cycled up to six times. Finally, chain-elongated derivatives of methionine are obtained via transamination process facilitated by BCAT and enter the core structure synthesis. There are five reactions required for core GSL structure formation: oxidation, oxidation with conjugation, C-S cleavage, glucosylation, and sulfation. In the whole process, cytochrome P450 monooxygenases (cytochrome P450s) of the CYP79 family and CYP83 family, *C–S* lyase (SUR), glucosyltransferase (UDP-glycosyltransferase 74 (UGT74) family), and sulfotransferase (SOT) are involved. The secondary modification of aliphatic GSL is initiated by flavin monooxygenases ($FMO_{GS\text{-}OX}$), converting methylthioalkyl to methylsulfinylalkyl GSL. The next step is oxygenation by 2-oxoglutarate-dependent dioxygenases (AOP2/GSL-ALK and AOP3/GSL-OHP) to alkenyl GSL and hydroxyalkyl GSL, respectively. Then, alkenyl GSL is converted into hydroxylated alkenyl GSL by 2-oxoacid-dependent dioxygenase (GSL-OH), and hydroxyalkyl GSL is converted into benzoylated GSLs and sinapoylated GSLs by serine carboxypeptidase-like acyltransferases (SCPL). For indolic GSL side-chain modification, cytochrome P450s of the CYP81F subfamily control the conversation of indol-3-ylmethyl GSL to 1-hydroxyindol-3-ylmethyl GSL and 4-hydroxyindol-3-ylmethyl GSL, and then indolic GSL *O*-methyltrasferases (IGMTs) are responsible for the conversion of hydroxy-GSLs to 1-methoxyindol-3-ylmethyl GSL or 4-methoxyindol-3-ylmethyl GSL.

To be noticed, the chain elongation and side-chain modification are of interest as they determine the diversity of GSL, and especially, side-chain modification is of importance due to the biological functions of GSL hydrolysis products largely depend on the structures of side chains. For instance, *B. oleracea* and *B. rapa* have the similar biosynthetic pathway of GSL but distinct GSL profiles, with 3C and 4C GSLs being the major aliphatic GSLs in *B. oleracea*, and 4C and 5C GSLs being the major ones in *B. rapa.* This characteristic is due to the different expression patterns of *MAM* family (Liu et al. 2014; Kumar et al. 2019; Petersen et al. 2019). Moreover, side-chain modification of aliphatic GSL results in various GSLs with different biological functions, e.g., many of them (such as 2-propenyl GSL, 3-butenyl GSL,

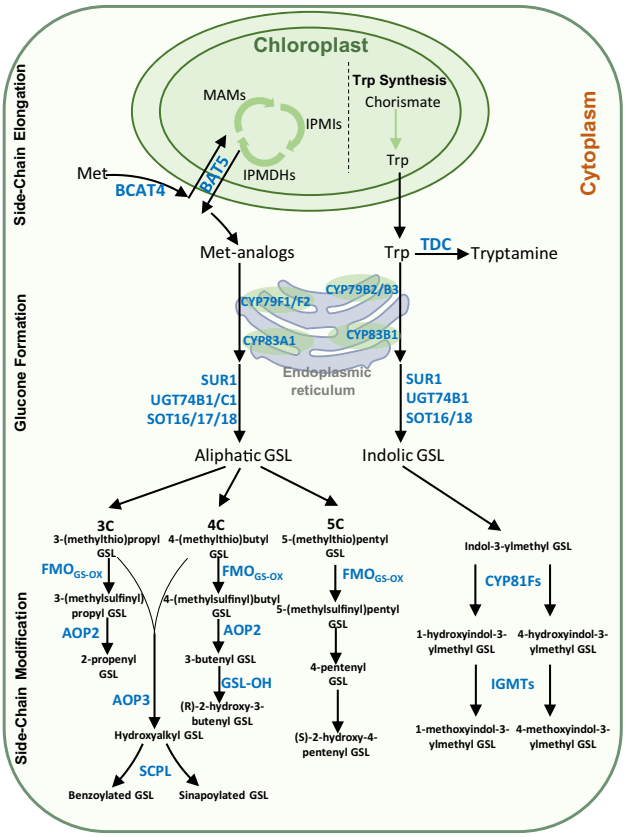


Fig. 1 Pathways of methionine and tryptophan-derived glucosinolates. Aliphatic glucosinolate biosynthesis consists of three processes: chain elongation of methionine (Met), core structure formation, and secondary modification of amino acid side chains, while indolic glucosinolate biosynthesis only consists of the last two steps by directly initiating from tryptophan (Trp). *BCAT* branched-chain amino acid aminotransferase, *BAT* bile acid transporter, *MAM* methylthioalkylmalate synthase, *IPMI* isopropylmalate isomerase, *IPMDH* isopropylmalate dehydrogenase, *CYP79F* cytochrome P450 79F, *CYP79B* tryptophan *N*-monooxygenase, *CYP83A* cytochrome P450 83A, *CYP83B* CYP83B monooxygenase, *SUR C–S* lyase, *UGT74* UDP-glycosyltransferase 74, *SOT* sulfotransferase, $FMO_{GS\text{-}OX}$ flavin monooxygenase, *AOP2* 2-oxoglutarate-dependent dioxygenase, *AOP3* 2-oxoglutarate-dependent dioxygenase, *GSL-OH* 2-oxoacid-dependent dioxygenase, *SCPL* serine carboxypeptidase-like acyltransferases, *CYP81F* CYP81F monooxygenase, *IGMT* indolic glucosinolate *O*-methyltransferase, *TDC* tryptophan decarboxylase

4-(methylsulfinyl)butyl GSL) attribute to the anticarcinogenic activity of *Brassica* vegetables (Capasso et al. 2012; Dinkova-Kostova and Kostov 2012; Soundararajan and Kim 2018; Liou et al. 2020), while some of them (such as 2-propenyl GSL, 3-butenyl GSL, 2-hydroxy-3-butenyl GSL, and 2-hydroxy-4-pentenyl GSL) contribute to the flavor of *Brassica* vegetables (Prieto et al. 2019; Zeng et al. 2021).

METABOLIC ENGINEERING OF GSL IN *BRASSICA* CROPS

To our best knowledge, various approaches are employed to engineering GSL based on enzyme and regulator genes (transcription factors) in *Brassica* crops, mainly including modulation of GSL biosynthesis, ablation of GSL hydrolysis, inhibition of GSL transport processes, and redirection of metabolic flux to GSL (Fig. 2). Successful cases are summarized in Table 3.

Engineering enzyme genes

The biosynthesis of GSL is accomplished by multi-enzymatic reactions, hence the accumulation of GSLs could be manipulated via engineering the corresponding gene expression. Zang et al. (2008b) explored metabolic engineering of aliphatic GSL in Chinese cabbage by overexpressing *Arabidopsis MAM1*, *CYP79F1*, and *CYP83A1*, respectively (Zang et al. 2008b). As expected, the accumulation of all individual aliphatic GSL was increased in *CYP83A1* transgenic line A1-1, while only 3-butenyl GSL and 4-pentenyl GSL contents were elevated in *MAM1* transgenic line M1-1. However, three *CYP79F1* transgenic (F1) lines showed inconsistent changes of GSL level. The F1-1 line exhibited increased levels of 2-hydroxy-4-pentenyl GSL, indol-3-ylmethyl GSL, and 4-methoxyindol-3-ylmethyl GSL, while the F1-2 and F1-3 lines showed reduced level of 3-butenyl GSL and 4-pentenyl GSL, and higher level of 4-hydroxyindol-3-ylmethyl GSL when compared to the wild type. Besides, Zhang et al. (2015) overexpressed *BnMAM1* or *BnCYP83A1* in *B. napus*, and obtained transgenic plants with an approximate 1.5-fold higher in aliphatic GSL level (Zhang et al. 2015). Increased amount of aliphatic GSLs was also observed in turnip (*Brassica rapa* var. *rapa*) hairy roots when overexpressing $FMO_{GS\text{-}OX}$ genes (Yang et al. 2019).

Cytochrome P450s of the CYP79 family and CYP83 family are the most crucial enzymes in the core structure formation of GSL (Bak et al. 2001; Chen et al. 2003; Hansen et al. 2001; Hull et al. 2000; Mikkelsen et al. 2000; Naur et al. 2003; Wittstock and Halkier 2000). Zang et al. (2008a) overexpressed *Arabidopsis CYP79B2*, *CYP79B3*, and *CYP83B1* in Chinese cabbage to modulate the profiles of indolic GSLs (Zang et al. 2008a). When single *CYP79B3* or *CYP83B1* was transformed, there was no change in the accumulation of indolic GSLs. Whereas, when *CYP79B2* or *CYP79B3* was expressed together with *CYP83B1*, higher levels of indol-3-ylmethyl GSL, 4-hydroxyindol-3-ylmethyl GSL, and 4-methoxyindol-3-ylmethyl GSL were obtained in transgenic plants. When all three genes were simultaneously overexpressed, no

better effects than overexpressing two genes were observed. Similar results were also found in Chinese cabbage hairy roots (Zang et al. 2009), except that only indol-3-ylmethyl GSL or 4-methoxyindol-3-ylmethyl GSL were accumulated at higher levels, and the increase of 4-methoxyindol-3-ylmethyl GSL caused a decrease of 1-methoxyindol-3-ylmethyl GSL. Moreover, both aliphatic and indolic GSL accumulations were improved when overexpressing *BnUGT74B1* in *B. napus*, and the resistance of transgenic plants to *Sclerotinia sclerotiorum* and *Botrytis cinerea* was also enhanced (Zhang et al. 2019). These cases indicate that it is possible to modulate aliphatic or indolic GSL or both by engineering one or several biosynthetic genes, although there is still some uncertainty about the outcome.

Aliphatic GSLs are the most abundant components in *Brassica* species, being about 57–97% of the total GSL content (Seo and Kim 2017). However, some of them have adverse effects on human and animal health, as well as the flavor of vegetables (Gerber et al. 2011; Glatt et al. 2011; Mithen et al. 2000). Hence, it is needed to raise the beneficial profiles and reduce the undesirable ones through fine-tuning specific GSL biosynthetic genes. Until now, the most engineered gene is *AOP2/GSL-ALK*, taking charge of the conversion of glucoraphanin to undesirable 3-butenyl GSL that is the precursor of deleterious progoitrin (Kliebenstein et al. 2001). The contents of 3-butenyl GSL and progoitrin are fairly high in some cultivable *Brassica* species including *B. rapa*, *B. napus* and *B. juncea*, while potent anticarcinogen glucoraphanin is relatively low. Thus, in *B. juncea*, high glucoraphanin level was achieved in all parts of the plant through silencing *AOP2/GSL-ALK* gene (Augustine and Bisht 2016). Meanwhile, high glucoraphanin lines also showed higher resistance to *Sclerotinia sclerotiorum*. In addition, antisense *AOP2* gene was transformed into Chinese kale, which resulted in enhanced glucoraphanin content and the ratio of 3-butenyl GSL/glucoraphanin (Qian et al. 2015). In *B. napus*, Liu et al. (2012) silenced *GSL-ALK* via RNA interference (RNAi) (Liu et al. 2012). Results showed that the concentration of glucoraphanin was enriched dramatically, while the content of progoitrin was reduced by 65% in transgenic plant seeds. Besides, the accumulation of 3-butenyl GSL and progoitrin could also be reduced by silencing the *MAM* gene family in *B. napus* canola and rapeseed, while 2-propenyl GSL was induced (Liu et al. 2011). These results indicated metabolic engineering has huge potential for improving vegetable nutrient, as well as oil and meal quality of *Brassica* crops through enriching beneficial GSL and bring down negative ones.

Besides directly engineering GSL biosynthetic genes, modulations of GSL degradation, transportation, and metabolic flux are also feasible approaches to achieve the purpose of engineering GSL. In intact plant tissue, the location of myrosinase and GSL is spatially distinct. Normally, classic myrosinase and GSL are distributed in myrosin cells/idioblasts and S-cells, separately (Hunziker et al. 2019). They come into contact upon tissue damage and GSL hydrolysis is initiated (Grubb and Abel 2006; Rask et al. 2000). Thus, Borgen et al. (2010) co-expressed barnase (ribonuclease) under the control of the seed myrosin cell-specific *Myr1.Bn1* promoter with the barnase inhibitor, barstar, under the control of the cauliflower mosaic virus 35S promoter in oilseed rape (*B. napus*), and successfully removed myrosinase-storing idioblasts in seeds without affecting plant viability (Borgen et al. 2010). The new created transgenic plants with negligible release of GSLs hydrolysis products in seeds, therefore, are suitable for seed meal production, which is an alternative to bring down GSL concentration. In addition, The GTR proteins play important roles in GSL transport process from maternal tissues to seeds

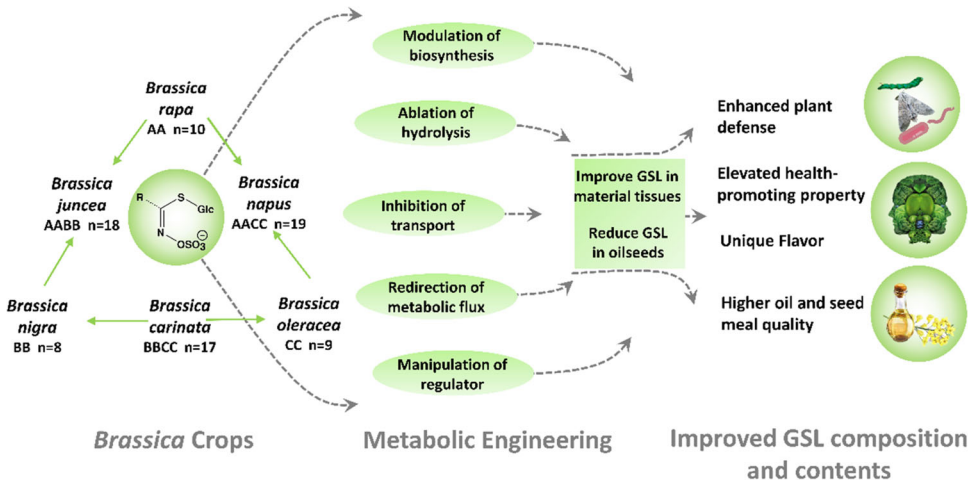


Fig. 2 Schematic diagram of metabolic engineering strategies that can be applied in *Brassica* crops to improve glucosinolate composition and contents so as to meet the specific demands

Table 3 Summarized cases of metabolic engineering of GSL in *Brassica* crops

Approach	Target species	Gene name	Source of gene	Engineering effect	References
Modulation of GSL biosynthesis	*Brassica rapa*	*CYP79B2*, *CYP79B3*, *CYP83B1*	*Arabidopsis*	Increased indolic GSL	Zang et al. (2008a, 2009)
	Brassica rapa	*MAM1*	*Arabidopsis*	Increased aliphatic GSL (3-butenyl GSL, 4-pentenyl GSL)	Zang et al. (2008b)
	Brassica rapa	*CYP83A1*	*Arabidopsis*	Increased aliphatic GSL	Zang et al. (2008b)
	Brassica napus	*MAM1* *CYP83A1*	*Brassica napus*	Increased aliphatic GSL	Zhang et al. (2015)
	B. rapa ssp. rapa	$FMO_{GS\text{-}OX}$	*B. rapa ssp. rapa*	Increased aliphatic GSL	Yang et al. (2019)
	Brassica napus	*UGT74B1*	*Brassica napus*	Increased indolic GSL and aliphatic GSL	Zhang et al. (2019)
	Brassica juncea	*AOP2*	*Brassica juncea*	Increased glucoraphanin	Augustine and Bisht (2016)
	Brassica oleracea var. alboglabra	*AOP2*	*Brassica oleracea var. alboglabra*	Increased glucoraphanin	Qian et al. (2015)
	Brassica napus	*AOP2*	*Brassica napus*	Increased glucoraphanin; decreased progoitrin	Liu et al. (2012)
	Brassica napus	*MAMs*	*Brassica napus*	Decreased 3-butenyl GSL and progoitrin; increased 2-propenyl GSL	Liu et al. (2011)
Ablation of GSL hydrolysis	*Brassica napus*	*Myr1.Bn1*	*Brassica napus*	Removed myrosinase-storing idioblasts in seeds	Borgen et al. (2010)
Inhibition of GSL transport	*Brassica rapa*	*GTR*	*Brassica rapa*	Decreased GSL in seeds	Nour-Eldin et al. (2017)
	Brassica juncea	*GTR*	*Brassica juncea*	Decreased GSL in seeds	Nour-Eldin et al. (2017)
Redirection of metabolic flux to GSL	*Brassica napus*	*TDC*	*Catharanthus roseus*	Decreased indolic GSL in seeds and the whole plants	Chavedej et al. (1994)
Modulation of GSL transcription regulation	*Brassica juncea*	*MYB28*	*Brassica juncea*	Decreased aliphatic GSL	Augustine et al. (2013), Augustine and Bisht (2019)
	Brassica oleracea var. alboglabra	*MYB28*	*Brassica oleracea var. alboglabra*	Increased aliphatic GSL	Yin et al. (2017)
	Brassica rapa	*MYB28*	*Brassica rapa*	Increased indolic GSL and aliphatic GSL	Seo et al. (2016)
	Brassica oleracea	*MYB29*	*Brassica oleracea*	Increased aliphatic GSL (glucoraphanin, 2-propenyl GSL)	Zuluaga et al. (2019)

(Nour-Eldin et al. 2012). Nour-Eldin et al. (2017) mutated GTR orthologs in *B. rapa* and *B. juncea* by using nontransgenic targeting induced local lesions in genomes (TILLING) approach, which specifically reduced seed GSL concentration by 60–70% (Nour-Eldin et al. 2017). Besides working on GSL biosynthesis and hydrolysis pathway, new methods have also been developed to introduce changes in GSL level, such as engineering metabolic flux. Chavedej et al. (1994) transformed tryptophan decarboxylase (TDC) gene into *B. napus* (canola), attempting to convert tryptophan to tryptamine rather than indolic GSLs (Chavedej et al. 1994). As expected, the indolic GSL content of mature seeds from transgenic plants was very low, even only 3% of that in control. However, the whole transgenic plants produced low level of indolic GSL, which could be harmful to plant defense in view of the importance of indolic GSL in plant resistance.

Engineering transcription factor genes

The metabolic pathway of GSL is regulated by many transcription factors, and generally, one transcription factor is in control of several biosynthetic genes. Hence, it would be more efficient to adjust GSL accumulation through modulating transcription factors. MYB28 is a vital transcription factor that directly activates aliphatic GSL biosynthetic genes (Gigolashvili et al. 2007). Several groups have reported targeted engineering of MYB28 in different *Brassica* species, including *B. juncea*, *B. oleracea*, and *B. rapa*. When *MYB28* was silenced in *B. juncea* and *B. oleracea* (Chinese kale), aliphatic GSL accumulation and related biosynthetic genes were down-regulated, while other GSL profiles and plant morphology were not affected (Augustine and Bisht 2019; Yin et al. 2017). When MYB28 was overexpressed in *B. oleracea*, only aliphatic GSL biosynthesis was boosted (Yin et al. 2017). However, when overexpressing MYB28 in *B. rapa* (Chinese cabbage), both aliphatic and indolic GSL contents were raised (Seo et al. 2016). These data indicated that the regulatory mechanism of GSL biosynthesis in *B. rapa* is different from that in other *Brassica* species and *Arabidopsis*. Moreover, MYB29 is another important transcription factor involved in aliphatic GSL biosynthesis (Gigolashvili et al. 2008), and overexpression of *BoMYB29* in *B. oleracea* could also enhance the accumulation of aliphatic GSL, such as glucoraphanin and 2-propenyl GSL (Zuluaga et al. 2019).

APPLICATION OF BIOTECHNOLOGY IN ELUCIDATION AND MANIPULATION OF GSL METABOLISM IN *BRASSICA* CROPS

Along with technologies evolve, whole-genome sequencing becomes more affordable and other omics approaches, i.e., transcriptome, and metabolome, become cheap and accurate, thus facilitate GSL metabolic pathway elucidation. To our knowledge, the genomes of all the six species of *Brassica* viz., *B. rapa* (Li et al. 2020; Wang et al. 2011), *B. oleracea* (Liu et al. 2014; Sun et al. 2019), *B. nigra* (Perumal et al. 2020), *B. juncea* (Paritosh et al. 2020), *B. napus* (Chalhoub et al. 2014) and *B. carinata* (Song et al. 2021), have been sequenced and published, which provide crucial basic references. Wei et al. (2019) carried out genome-wide resequencing in a population of diverse *B. napus* accessions with different phenotypes, and identified several loci, i.e., orthologs of *MYB28*, *MYB34*, and *AOP3*, that affecting seed GSL level (Wei et al. 2019). Similarly, Gubaev et al. (2020) applied the genotyping-by-sequencing approach in 90 advanced rapeseed accessions and found new candidate genes that potentially contribute to the control of GSL content, such as genes encoding the histone acetyltransferase HAC1 and BES1/BZR1 homolog protein 4-like (Gubaev et al. 2020).

Based on the genomic sequences, the global expression pattern of genes involved in GSL metabolism can be characterized by transcriptome sequencing (Wu et al. 2017). Besides, Kittipol et al. (2019) performed the transcriptome-based GWAS (genome-wide association studies) approach and Associative Transcriptomics (AT) among a diversity panel of 288 *B. napus* genotypes to have an insight into the underlying genetic basis controlling quantitative variation of GSLs in *B. napus* vegetative tissues, identifying that orthologues of *MYB28* were the key regulators of aliphatic GSL variation in leaves, and orthologues of *MYB29* participated in root benzenic GSL variation, as well as *MAM3* was involved in phenylalanine chain elongation for benzenic GSL biosynthesis in roots (Kittipol et al. 2019).

Furthermore, integration of transcriptome and metabolome analysis is a good way to comprehensively explore the metabolic differences between different cultivars (Park et al. 2020). With respect to evolution, comparative genome sequence confers the possibility to investigate evolutionary processes affecting genome structure and protein function that lead to the repeated evolution of GSL metabolism and diversity (Barco and Clay 2019). In addition, Yang et al. (2020) conducted transcriptome analyses combined with convergent evolution analysis of wasabi (*Wasabi japonica*), horseradish (*Armoracia rusticana*), and mustard (*B. juncea*), and uncovered gene clusters, including biosynthetic genes *CYP79A1*, *CYP83A1*, *GSTF11*, *SUR1*, and *UTG74B1*, as well as transcription factors *Dof1.1* and *IQD*, and hydrolytic gene *NIT1*, that were convergently selected. These findings were helpful in ascertaining why these three unrelated species enrich 2-propenyl GSL, and provided theoretical basis for engineering GSL metabolic pathway to fortify desirable compounds (Yang et al. 2020).

As mentioned in "Metabolic engineering of GSL in *Brassica* crops", metabolic engineering of GSL in *Brassica* plants has been conducted via several biotechnologies to specifically modulate GSL profiles, i.e., antisense RNA, RNA interference, and overexpression. However, the safety of genetically modified organisms (GMO) has been debated since its emergence. In this context, genome-editing techniques have been promoted widely by scientists, which could accurately alter DNA with targeted specificity to the target plant without introducing foreign DNA from a different species or from another cultivar of the same species, and thus gene-edited

products would have higher public acceptance than traditional GMO (Shew et al. 2018). Until now, several genome-editing techniques have been recorded, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)-associated protein 9 (CRISPR/Cas9), among which, CRISPR/Cas9 system has become more and more popular since it is time- and labor-saving as well as high efficient (Bibikova et al. 2002; Cong et al. 2013; Dreier et al. 2001; Li et al. 2011; Shalem et al. 2015). CRISPR/Cas9 has been successfully and widely applied in numerous plants, including *Brassica* crops that belong to *B. napus*, *B. oleracea*, *B. carinata,* and *B. rapa* (Dou et al. 2021; Kirchner et al. 2017; Ma et al. 2019; Sun et al. 2020; Wu et al. 2020; Xiong et al. 2019a; Zheng et al. 2020). By virtue of CRISPR/Cas9-mediated gene editing, we could not only construct specific mutant, which is helpful for gene function study but also could obtain new desirable germplasm to satisfy our demand and provide valuable resources for further breeding. For instance, seed quality was improved through alerting related genes by CRISPR/Cas9 in *B. napus* (Huang et al. 2020; Karunarathna et al. 2020; Khan et al. 2021; Okuzaki et al. 2018; Zhai et al. 2020). Besides, plant susceptibility to *Verticillium longisporum* was reduced (Pröbsting et al. 2020), resistance to *Sclerotinia sclerotiorum* was enhanced (Sun et al. 2018), and herbicide resistance was conferred (Wu et al. 2020) via mutating the corresponding genes through CRISPR/Cas9 in *B. napus.* What's more, Zaman et al. (2019) and Zhai et al. (2019) investigated the role that JAGGED (JAG) gene and INDEHISCENT homologues in pod shattering resistance, respectively, by using CRISPR/Cas9-mediated genome editing in *B. napus* (Zaman et al. 2019; Zhai et al. 2019). Sun et al. (2020) explored the function of *CRTISO* in controlling chlorophyll and carotenoid concentrations in Chinese kale (*B. oleracea* var. *alboglabra*) (Sun et al. 2020), and Xiong et al. (2019b) reported that PME37 was essential for pollen intine formation in *B. rapa* (Xiong et al. 2019b). Dou et al. (2021) created self-incompatible *B. napus* by CRISPR/Cas9 mutation of *BnS6-Smi2* which could be employed in future breeding of self-incompatible *Brassica* plants (Dou et al. 2021). Although none of them is related to GSL metabolic engineering currently, the powerful CRISPR/Cas9 will be a promising technique for the improvement of GSL profile and content in *Brassica* crops in the near future. For example, the conversion of beneficial glucoraphanin to undesirable 3-butenyl GSL could be blocked by mutating all orthologues of *AOP2* at once by CRISPR/Cas9-mediated multiple gene editing, harvesting *Brassica* crops with higher glucoraphanin and without foreign genes.

PERSPECTIVES

Metabolic engineering of GSL has been improved continuously. More and more regulators of GSL metabolism have been identified, including direct transcription factors of GSL biosynthesis and components involved in other signal transduction, and a thorough review has been given by Mitreiter and Gigolashvili (2021). However, only MYB28 and MYB29 have been engineered in *Brassica* crops, which are quite efficient in modulation of GSL accumulation. In the future, engineering other regulators are also worth consideration. For instance, MYB34 is the core transcription factor that regulates the biosynthesis of indolic GSL. Overexpression of *MYB34* in *Arabidopsis* resulted in high indolic GSL content and promoted vegetative growth. Therefore, it is potential to engineer *MYB34* in *Brassica* vegetables whose vegetative organisms are edible parts, which would be beneficial for the increase of plant resistance and yield. Heterologous expression of GSL-related gene homologues in *Brassica* crops will be a feasible strategy to introduce new beneficial GSL profiles, which would make up the shortfall of the host plants and enhance their nutritional value. What's more, identification of new genes that negatively regulate GSL metabolism in *Brassica* crops is expected, since we could knock out the negative gene via CRISPR/Cas9 technology so as to increase beneficial GSL content to meet the demand of consumers without introducing foreign genes. To be noticed, GSL metabolic pathways in *Brassica* crops have been duplicated during the evolution, resulting in multiple orthologues, which brings about extra work for engineering. However, GSL metabolism can be regulated by various internal and external signals, i.e., phytohormones, mineral nutrients, and glucose (Aarabi et al. 2016; Guo et al. 2013a, b; Miao et al. 2013, 2016; Mitreiter and Gigolashvili 2021), via some core signaling components. Especially, many of these components are very conservative and only have one orthologous gene in *Brassica* crops. And comprehensive utilization of rapid growing omics approaches, i.e., transcriptome, metabolome, proteome, and phenomics, will boost the identification of novel components and essential regulators of GSL metabolic pathways. Hence, manipulation of GSL content and composition based on engineering these conserved components will be more convenient and efficient.

Besides GSL-containing plants, several kinds of GSLs could also be synthesized in tobacco as well as microbe

Escherichia coli and *Saccharomyces cerevisiae* (Geu-Flores et al. 2009; Liu et al. 2016; Mikkelsen et al. 2010; Petersen et al. 2019; Pfalz et al. 2011; Yang et al. 2018). Although their levels are lower than that in *Brassicaceous* plants and are not suitable for large-scale production, GSL engineering in heterologous hosts has the advantage in harvesting single GSL with no need for downstream purifications. Therefore, it is still quite attractive, and lots of optimizations are needed to overcome obstacles standing in the way to commercialized production of pure GSL.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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