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Advancements in balancing glucosinolate production in plants to deliver effective defense and promote human health

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ABSTRACT

Glucosinolates (GSLs) are a prototypical group of bioactive compounds found in the *Brassicaceae* family that promote human health and plant defense. The GSL-myrosinase system can be induced to release multiple bioactive products when plants are subjected to mechanical damage, environmental stress, or pathogen infection. While many GSLs promote human health, some cause deleterious effects when ingested. To engineer Brassicaceae crops with lower levels of harmful GSLs without sacrificing health-promoting GSLs requires a complete understanding of the origin and advances in GSL modification. Extensive early domestication studies were conducted using classic breeding and plant nutrition. More recently, genetic modification of specific groups of GSLs or levels of GSLs in specific tissues has been partially successful. However, efforts have fallen short of delivering a reduction in potentially harmful GSLs without concomitant losses to health-promoting effects and plant defense. The latest work has been to synthetically express GSL biosynthesis pathways in non-host crops or microbial species. However, yields have been far from economically sustainable. This review discusses key advances made in GSL modification that are promising for the precise modification of GSL content and composition for optimal plant defense and human health.

1. Introduction

Glucosinolates (GSLs) are sulfur-containing secondary metabolites derived from amino acids that stand as the principal class of bioactive secondary metabolites within the *Brassicaceae* genus. The presence of GSLs was first recognized around the 17th century due to the characteristic mustard flavor of Brassicaceae crops. However, the core chemical structure of GSLs remained unknown until the 1960s. The sensory profile of GSLs is primarily bitterness, which is produced by progoitrin (PRO), gluconapin, glucobrassicin, and neoglucobrassicin [1]. Upon digestion, GSLs are converted to various degradation products (such as isothiocyanates [ITCs], nitriles, or thiocyanates) that impart distinct pungent, bitter, and sulfurous flavors to *Brassica* species [1]. Up until the 1970s, some *Brassicaceae* plants, such as oilseed rape (*Brassica napus*), were considered unsuitable for food and feed due to the detrimental effects of some GSL degradation products. The digestion of PRO, the major GSL in oilseed rape, yields goitrin, a sulfur-containing oxazolidine that inhibits the production of thyroid hormones, thereby inducing goiter, which reduces fertility and inhibits growth [2]. Consequently, researchers have dedicated decades of work to reducing GSL content in oilseed rape. In contrast, multiple GSL breakdown products provide health-promoting properties. For example, *Brassica oleracea* var. *italica* (broccoli) is rich in 4-methylsulfinylbutyl GSL (4MSB) that can be converted into sulforaphane (SF). SF belongs to the isothiocyanate (ITC) family, and it has been shown to be advantageous for human health, displaying tumor-inhibitory effects [3]. Additionally, GSLs and their degradation products provide an important native defense system for plants in Brassicales, protecting them against both biotic and abiotic stresses [4]. However, GSLs have also been reported to induce feeding and oviposition in some specialist herbivores, suggesting that some GSLs

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promote herbivory [5]. Therefore, when bioengineering GSL contents in crop species, it is crucial to precisely modify GSL pathways to balance unfavorable and favorable effects for both mammals and plants.

The first attempt to optimize the GSL content and profile in Brassicaceae involved modifying total GSL levels, both pre- and post-harvest [6,7]. Initial work relied on classical breeding techniques and fertilizer applications mainly targeted total GSLs [8,9]; however, these approaches were time-consuming, limited by genetic resources, and lacked a clear genetic basis. Cell and hairy root cultures were also applied to obtain desired GSL contents, which reduced the dependency on native plants; however, yields were very low [7]. Another approach involved targeting specific classes of GSLs for modification. GSLs are divided into three distinct groups —aliphatic, indolic, and aromatic— based on the composition of their amino acid side chains [10]. Significant progress was also made in the selective modification of GSL classes via upregulating or suppressing the expression of specific genes within their biosynthetic pathways [7]. However, there are some associated pleiotropic effects, increased susceptibility to disease, when modifying GSLs that warrant further investigation [11].

Advances in biotechnology alongside elucidation of the genetic basis of GSL biosynthesis have greatly improved precision in the modification of GSL contents. The discovery of GSL transporters has made it possible to modify GSLs in specific tissues. For example, modifications of GSL transporters in reproductive tissues selectively reduce GSL levels in seeds without influencing those in vegetative tissues. This reduces the deleterious impacts of GSL ingestion in cruciferous plants where seeds are the edible tissues [12]. However, decreases in GSL content are associated with both diminished plant defense capabilities and reduced levels of beneficial GSLs. Moreover, for plants, such as cabbage, turnip, and kale, vegetative tissues are the edible portions, requiring more precise approaches for GSL modification that target specific classes of GSLs in leaves. Here, we provide a brief overview of the early work done in GSL modification, largely through breeding and domestication. Next, we address key advances in targeted GSL modification and review promising methodologies for the future development of balanced GSL contents in Brassicales. Optimization of GSL content and composition is essential to maintain plant defense capabilities without compromising benefits to human health (Fig. 1).

2. Overview

To date, more than 130 GSL compounds have been documented (Table 1) [7]. Originating from the universally distributed cyanogenic glucosides found in plants, GSLs have independently evolved twice, once within the order Brassicales, and again in the genus *Drypetes* and *Putranjiva* [13,14]. Among these, the most agriculturally important plants belong to the *Brassicaceae* family. The widely used *Brassicaceae* model, *Arabidopsis thaliana*, has been instrumental in establishing the genetic basis of GSL regulation, metabolism, and function [15]. Compared to cyanogenic glucosides, which are primarily generated from the precursor amino acids Val, Ile, Phe, and Tyr, GSLs are derived from more diverse sources. They are generally classified into three groups: aliphatic (derived from Val, Leu, Ile, and Met), indolic (Trp), and aromatic (Phe and Tyr) GSLs [16]. The only coexistence of cyanogenic glucosides and GSLs was reported in *Carica papaya*, where they are both derived from phenylalanine [17]. The diverse structures of GSLs offer a wide array of functions, making them an ideal model for the study of plant compounds.

2.1. GSLs and plant defense

Although some volatile GSLs induce feeding and oviposition by crucifer-specialist herbivores, degradation products of GSLs are powerful compounds required for plant defense [5]. The protective effect of GSLs is mainly mediated through their bioactive properties as toxins that directly act on pathogens or herbivores. In addition, GSLs may alter plant physiology to enhance defense against plant attackers or benefit natural enemies of herbivorous insects [79]. GSLs are stored in the vacuoles of sulfur-rich S-cells, whereas the enzymes responsible for GSL hydrolysis, such as myrosinase, are located in the cytoplasm of myrosin cells [80]. Upon disruption of plant tissues, GSLs are released and make contact with myrosinase, leading to their degradation into various toxic products, including ITCs, nitriles, epithionitriles, thiocyanates, oxazolidine-2-thione, and others. This enzymatic breakdown triggers what is commonly known as the “mustard oil bomb,” which serves as a potent defense against insect herbivory, pathogen infection, and abiotic stress [79].

GSLs and their hydrolysis products activate multiple aspects of the plant's defense system, including stomatal closure, programmed cell death (PCD), callose formation, indole-3-acetonitrile (IAN)-associated defenses, emission of volatile GSLs and their hydrolyzed products, and interactions with the growth environment [81,82]. GSL-derived ITCs conjugate with glutathione to promote the generation of reactive oxygen species (ROS), which results in stomatal closure through abscisic acid [83]. Additionally, SF, a breakdown product of ITC from 4MSB, can

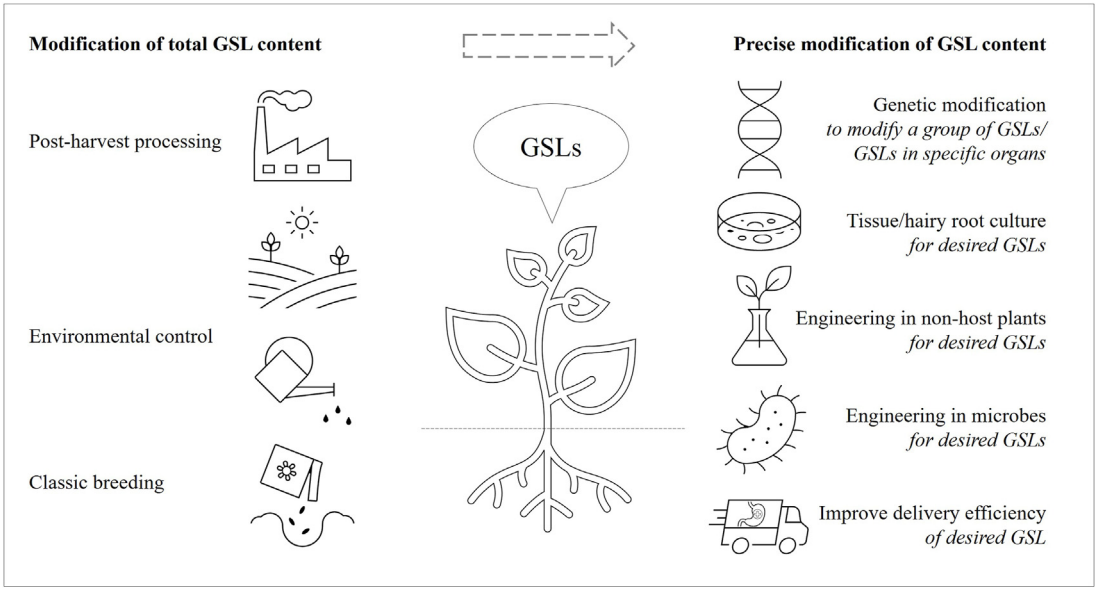

Fig. 1. Approaches to modify the glucosinolate content.

Table 1
Common GLS components and reported health benefits.

Classification	Glucosinolate	Trivial name	Abbreviation	Molecular formula	First identified/Source	Hydrolysis product	Health attributes
Aliphatic	3-Methylthiopropyl	Glucoiberverin	3MTP	$C_{11}H_{21}NO_9S_3$	*Iberis umbellata*	Iberverin	Antimicrobial [18], Anticancer (Lung) [19]
Aliphatic	4-Methylthiobutyl	Glucoerucin	4MTB	$C_{12}H_{23}NO_9S_4$	*Arabidopsis thaliana*	Erucin	Anti-inflammation [20] Antihypertension [21] Antioxidant [22] Neuroprotective [23] Anticancer (Pancreas [24], Breast [25], Ovary [26], Prostate [27], Lung [28])
Aliphatic	5-Methylthiopentyl	Glucoberteroin	5MTP	$C_{13}H_{25}NO_9S_3$	*Armoracia, Brassica, Erysimum, Hesperis, Lepidium*	6-(methyl sulfanyl) hexane nitrile	Antimicrobial [29]
Aliphatic	6-Methylthiohexyl	Glucolesquerellin	6MTH	$C_{14}H_{27}NO_9S_3$	*Alyssum maritimum, Arabis perenans*	–	–
Aliphatic	7-Methylthioheptyl	–	7MTH	$C_{15}H_{29}NO_9S_3$	*Arabidopsis thaliana*	–	–
Aliphatic	8-Methylthiooctyl	–	8MTO	$C_{16}H_{31}NO_9S_3$	*Arabidopsis thaliana*	–	–
Aliphatic	3-Methylsulfinylpropyl	Glucoiberin	3MSP	$C_{11}H_{21}NO_{10}S_3$	*Iberis umbellata*	–	Antioxidant [30]
Aliphatic	4-Methylsulfinylbutyl	Glucoraphanin	4MSB	$C_{12}H_{23}NO_{10}S_3$	*Erysimum allioni*	Sulforaphane	Anti-inflammation [31] Antioxidation [32] Neuroprotective [33] Antiobesity [34] Anticardiovascular disease [35] Anticancer [36]
Aliphatic	5-Methylsulfinylpentyl	Glucoalyssin	5MSP	$C_{13}H_{25}NO_{10}S_3$	*Aurinia leucadea (Guss.) C. Koch*	–	Antimicrobial [29]
Aliphatic	6-Methylsulfinylhexyl	Glucohesperin	6MSH	$C_{14}H_{27}NO_{10}S_3$	*Arabis perenans, L. annua*	–	–
Aliphatic	7-Methylsulfinylheptyl	Glucoibarin	7MSH	$C_{15}H_{29}NO_{10}S_3$	*Arabidopsis thaliana*	–	–
Aliphatic	8-Methylsulfinyloctyl	Glucohirsutin	8MSO	$C_{16}H_{31}NO_{10}S_3$	*Arabidopsis thaliana*	–	–
Aliphatic	Methyl	Glucocapparin	CAP	$C_8H_{15}NO_9S_2$	*Cleome spinosa*	Methyl ITC	Antimicrobial [37] Anticancer (Colon [38])
						Methyl thiocyanate	Antimicrobial [39]
Aliphatic	1-Methylethyl	Glucoputranjivin	TRA	$C_{10}H_{19}NO_9S_2$	*Lunaria annua, Sisymbrium officinale*	Isopropyl	Anti-inflammation [40] Anticancer (Gastric [41], Lung [42])
Aliphatic	2-Propenyl	Sinigrin	SIN	$C_{10}H_{17}NO_9S_2$	*Brassica juncea, Iberis umbellata, Horseradish, Wasabi condiment*	Allyl ITC	Antimicrobial [43] Anti-inflammation [44] Antihypertension [45] Antiobesity [46] Antiasthmatic [47] Anticancer (Lung [48], Bladder [49], Liver [50])
						Allyl thiocyanate	Antitubercular [51]
Aliphatic	3-Butenyl	Gluconapin	3BUT	$C_{11}H_{19}NO_9S_2$	*Arabidopsis thaliana, Alyssum maritimum, Brassica napus*	3-butenyl ITC	Antimicrobial [52] Antihypertriglyceridemia [53] Anticancer (Prostate [54], Breast, Cervical, Liver, Bone) [55]
Aliphatic	4-Pentenyl	Glucobrassicanapin	–	$C_{12}H_{21}NO_9S_2$	*Arabidopsis thaliana, Brassica insularis, Brassica napus, Brassica macrocarpa*	4-pentenyl ITC	Anticancer (Leukemia [56])
Aliphatic	2-hydroxy-3-butenyl	Progoitrin	PRO	$C_{11}H_{19}NO_{10}S_2$	*Brassica napus, Brassica oleracea, Raphanus raphanistrum, Raphanus sativus, Hirschfeldia incana*	Goitrin	Antimicrobial [57] Antithyroid [58]
Aliphatic	2-hydroxy-4-pentenyl	Gluconapoleiferin	–	$C_{12}H_{21}NO_{10}S_2$	*Armoracia, Brassica, Sisymbrium*	–	–
Aliphatic	3-Hydroxypropyl	–	3OHP	$C_{10}H_{19}NO_{10}S_2$	*Erysimum hieracifolium, Malcolmia maritima*	–	–
Aliphatic	4-Hydroxybutyl	–	4OHB	$C_{11}H_{21}NO_{10}S_2$	*Arabidopsis. thaliana*	–	–
Aliphatic	3-Methylsulfonylpropyl	Glucocheirolin	–	$C_{11}H_{21}NO_{11}S_3$	*Cheiranthus cheiri*	Thioaliphatic	Anticancer (Breast [59])
Aliphatic	4-Methylsulfinyl-3-butenyl	Glucoraphenin	–	$C_{12}H_{21}NO_{10}S_3$	*Matthiola incana*	Nitrile	

(*continued on next page*)

Table 1 (*continued*)

Classification	Glucosinolate	Trivial name	Abbreviation	Molecular formula	First identified/Source	Hydrolysis product	Health attributes
							Antimicrobial [60]
							Anticancer (Liver [61])
Aromatic	Benzyl	Glucotropaeolin	BGLS	$C_{14}H_{19}NO_9S_2$	*Tropaeolum majus, Lepidium sativum*	Benzyl ITC	Antimicrobial [62]
							Anti-inflammation [63]
							Anticancer (Bladder [64], Gastric [65]
Aromatic	2-Phenylethyl	Gluconasturtiin	2 PE	$C_{15}H_{21}NO_9S_2$	*Nasturtium officinale*	Phenethyl ITC	Antidiabetes [66]
							Antirheumatic [67]
							Anticancer (Breast [68], Cervix [69])
Aromatic	2(R)-Hydroxy-2-phenylethyl	Glucobarbarin	2R-2OH-2PE	$C_{15}H_{21}NO_{10}S_2$	*Ochrademus baccatus*	–	–
Aromatic	p-hydroxybenzyl	Sinalbin	pOHB	$C_{14}H_{19}NO_9S_2$	*Lepidium draba, Sinapis alba, Brassica oleracea*	–	–
Aromatic	4-Hydroxybenzyl	(Gluco)sinalbin	4OHB	$C_{14}H_{19}NO_9S_2$	*Sinapis alba*	4-hydroxybenzyl ITC	Antimicrobial [70]
							Anticancer (Neuroblastoma [71])
Aromatic	3-Benzoyloxypropyl	Glucomalcomiin	–	$C_{17}H_{23}NO_{11}S_2$	*Arabidopsis thaliana*	–	–
Aromatic	4-Benzoyloxybutyl	–	–	$C_{18}H_{25}NO_{10}S_2$	*Arabidopsis thaliana*	–	–
Aromatic	5-Benzoyloxypentyl	–	–	$C_{19}H_{27}NO_{10}S_2$	*Arabidopsis thaliana*	–	–
Aromatic	6-Benzoyloxyhexyl	–	–	$C_{20}H_{29}NO_{10}S_2$	*Arabidopsis thaliana*	–	–
Indolic	Indolyl-3-methyl	Glucobrassicin	I3M	$C_{16}H_{20}N_2O_9S_2$	*Arabidopsis thaliana*	Indole-3-carbinol	Anti-inflammation [72]
							Antimicrobial [73]
							Antioxidation [74]
							Anticancer [75]
Indolic	1-hydroxy-indolyl-3-methyl	1-Hydroxyglucobrassicin	1OH–I3M	$C_{16}H_{19}N_2O_{10}S_2$	*Brassica oleracea*	–	–
Indolic	4-hydroxy-indolyl-3-methyl	4-Hydroxyglucobrassicin	4OH–I3M	$C_{16}H_{20}N_2O_{10}S_2$	*Armoracia, Brassica, Isatis*	–	–
Indolic	1-Methoxy-indolyl-3-methyl	Neoglucobrassicin	1MO-I3M	$C_{17}H_{22}N_2O_{10}S_2$	*Brassica oleracea*	N-methoxyindolyl-3-carbinol	Anti-inflammation [76]
							Anticancer (Prostate [77])
Indolic	4-Methoxy-indolyl-3-methyl	4-Methoxyglucobrassicin	4MO-I3M	$C_{17}H_{22}N_2O_{10}S_2$	*Arabidopsis thaliana*	Not known	Anti-inflammation [78]

induce PCD or the hypersensitive response [84]. PCD serves to contain the pathogen at the infection site by killing the cells around it, thereby depriving the pathogen of nutrients and a suitable environment to proliferate. This localized cell death is often associated with the production of ROS and other antimicrobial compounds that help to kill the pathogen and signal other parts of the plant to strengthen its defenses. In addition, 4-methoxy-indol-3-yl-methylglucosinolate (4MO-I3M) is required for callose formation [85]. Previous studies utilizing flg22, a synthetic 22-amino acid polypeptide corresponding to the conserved region of eubacterial flagellin that strongly induces callose formation, demonstrated that both upregulation of indolic GSL biosynthesis by 4MO-I3M and GSL regulatory transcription factor *MYB51* are required for effective callose deposition [83].

Glucosinolate-producing plants have been observed to accelerate the biosynthesis of indolic and aliphatic GSLs as a defense strategy against herbivores. For example, indolic GSLs are particularly effective defenses against both chewing and sap-feeding insects [86]. Aside from their role in callose formation, indolic GSLs are linked to multiple plant defense metabolites (Fig. 2). Two major Brassicales compounds, the key phytoalexin involved in plant defense, camalexin, and the critical growth hormone indole-3-acetic acid, are both derived from IAN, a phytoanticipin that is a degradation product of I3M. As I3M and IAN are both generated through enzymatic action on a shared substrate, indole-3-acetaldoxime (IAOx), their levels are intrinsically associated. Therefore, IAN is commercially used as a fungicide and growth regulator. Additionally, IAN can be used to produce a previously unknown indole metabolite, 4-hydroxyindole-3-carbonyl nitrile (4-OH-ICN), which supplies a new indole metabolic pathway and a plant defense response distinct from the canonical camalexin pathway. 4-OH-ICN is unprecedented in plants and rare in nature, as it maintains its cyanogenic function [87]. The diversity of cyanogenic derivates enriches the repertoire of plant defense mechanisms and metabolites.

Plants of the *Brassicaceae* family emit volatile GSLs and their hydrolysis products to recruit parasitoids and predators of herbivores as an indirect plant defense [88]. This serves as an alternative defense strategy to combat certain crucifer-specialist insects, such as *Plutella xylostella*, that can detoxify defensive GSLs through desulphation. When *P. xylostella* feeds on cabbage and *Arabidopsis,* compounds such as allyl-ITC and 4MSB-ITC present in the frass of larvae are released, acting as attractants to their natural predator *Chrysoperla carnea* and endoparasitoid *Diadegma semiclausum* [88,89]. Additionally, GSLs promote defense through interaction with the growth environment. GSLs can be hydrolyzed into bioactive metabolites in the soil to defend against soil-borne pests [90]. Hydrolyzed GSLs function via three general mechanisms of action: the inactivation of the thiol bond of essential enzymes of soil-borne pests, the alkylation of the nucleophilic group of biopolymers, such as DNA, or as an uncoupling agent that fumigates pests, resulting in death [91–93]. GSL defense in plants not only persists before harvest development but also partially remains after harvest. While the defense roles of GSLs related to plant development and physiology are well studied in pre-harvest plants, research into post-harvest defense mechanisms is limited and primarily consists of phenotypic studies [94–96]. The current understanding of pre-harvest defense mechanisms of GSLs can guide future work regarding post-harvest defenses.

2.2. GSLs and health

Mammalian species utilize endogenous myrosinase and/or intestinal flora to hydrolyze GSLs, yielding bioactive compounds such as ITCs, nitriles, epithionitriles, and thiocyanates. ITCs possess antibacterial, anti-inflammatory, and anticancer activity, and can reduce the symptoms of chronic diseases (Table 1). The principal mechanism of ITCs' biological activity may be attributed to their thio group (-N═C═S). The highly electrophilic carbon atom of this group attacks thiol, amine, and hydroxyl moieties of peptides and proteins, thus impacting enzymatic activities

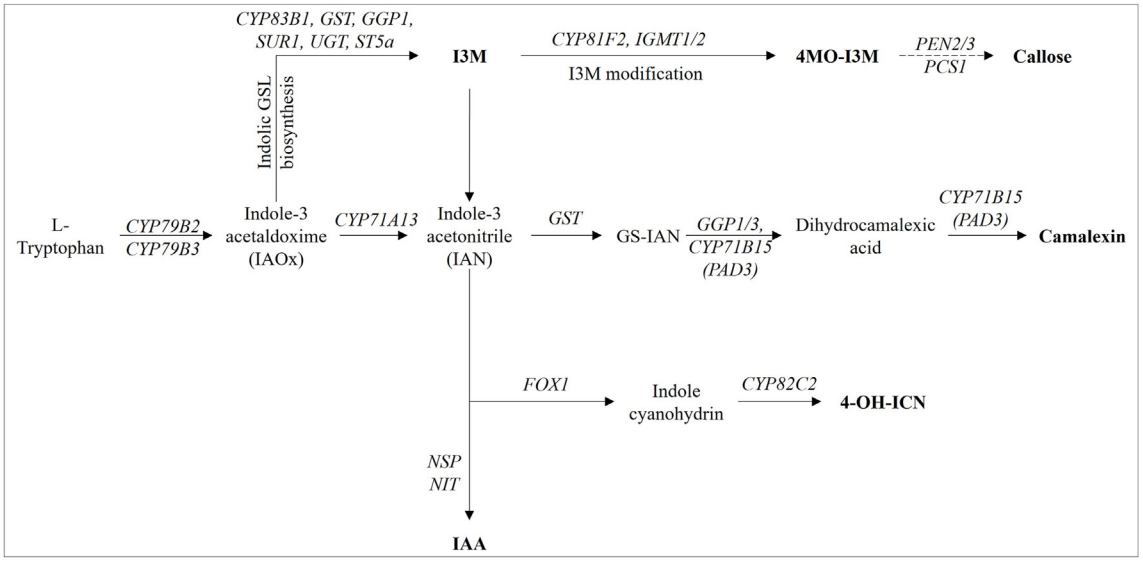

Fig. 2. Glucosinolate-related defense pathways. Abbreviations: I3M (indolyl-3-methyl glucosinolate), 4MO-I3M (4-methoxyglucobrassicin), 4-OH-ICN (4-hydroxyindole-3-carbonyl nitrile), IAA (indole-3-acetic acid), CYP83B1 (cytochrome P450 83B1), CYP81F2 (cytochrome P450 81F2), CYP79B2 (tryptophan N-monooxygenase 1), CYP79B3 (tryptophan N-monooxygenase 2), CYP71A13 (indoleacetaldoxime dehydratase), CYP71B15 (camalexin synthase), CYP82C2 (xanthotoxin 5-hydroxylase CYP82C2), GST (glutathione *S*-transferase), GGP (gamma-glutamyl peptidase), SUR1 (S-alkyl-thiohydroximate lyase SUR1), UGT (UDP-glycosyltransferase), ST5a (cytosolic sulfotransferase 16), PEN2 (beta-glucosidase 26, peroxisomal), PEN3 (ABC transporter G family member 36), FOX1 (berberine bridge enzyme-like 3), NSP (nitrile-specifier protein), NIT (nitrilase), PCS1 (glutathione gamma-glutamylcysteinyltransferase 1), IGMT (indole glucosinolate O-methyltransferase).

related to respiration, metabolism, and gene transcription. Indole-3-carbinol (I3C) and SF can inhibit various bacteria, such as *Escherichia coli* and *Helicobacter pylori*, by either hindering redox enzymes or interfering with the bioactivity of urease. Other ITCs, such as methyl ITC, allyl ITC, benzyl ITC, and phenethyl ITC, possess antibacterial properties against both gram-positive and gram-negative bacteria through multiple mechanisms, including interference with biofilm activity, perturbation of membrane integrity and redox balance, and inhibition of acetate kinase [97]. Moreover, ITCs can be synthetically modified to increase their antimicrobial properties. For example, the antimicrobial activity of a (poly)fluoroaryl substituent of SF was increased 8-fold compared to unmodified SF [98]. This may be due to the addition of the fluoroaryl group, which increases the overall electrophilicity of SF and promotes binding to target proteins. The development of ITCs with novel side chain substitutions is a promising area for future research.

Anti-inflammatory activity is another important biofunction of ITCs. Benzyl ITC, SF, and PEITC can inhibit the activity of nuclear factor-kappa B (NF-κB), the key transcription factor related to inflammation, by suppressing the activity of IκBα and IκB kinases (IKK) [99]. Besides influencing the NF-κB pathway, PEITC, SF, and benzyl-ITC also inhibit the pro-inflammatory cytokine macrophage migration inhibitory factor by directly targeting its cysteine residues [100]. Tumor necrosis factor alpha (TNF-α) is another cell signaling protein (adipokine) involved in systemic inflammation. Some synthesized indole GSLs, such as 4 ME-I3M and neo-glucobrassicin (1OH–I3M), effectively inhibit TNF-α secretion, thus preventing inflammation [78]. This anti-inflammatory property is directly related to other bioactivities of ITCs, such as anticancer activity and chronic disease mitigation. An umbrella review of 41 systematic reviews found that cruciferous vegetable intake was strongly associated with beneficial effects on gastric cancer, lung cancer, and endometrial cancer [101]. Researchers have identified multiple signaling pathways related to ITCs' anticancer capability, including Akt/NF-κB, MAPK, Wnt/β-catenin, and PTEN/PI3K/Akt [102]. PEITC and SF are proposed to prevent tumor metastasis by inhibiting matrix metalloproteinases or suppressing epithelial-to-mesenchymal transition [103]. Moreover, ITCs, such as SF and PEITC, interfere with tumor growth at the epigenetic level, regulating CpG demethylation, histone acetylation, and DNA methylation, ultimately resulting in inhibition of the long process of cancer development [104]. Many ITCs have been shown to possess anticancer capability through multiple mechanisms; however, the progress in bringing ITCs into clinical trials for cancer treatment is slow. The only ITC that has proceeded to a clinical trial is PEITC, which aims to treat lung cancer (ClinicalTrials.gov Identifier: NCT00691132).

Nevertheless, some hydrolyzed products of GSLs have side effects. For example, thiocyanate and goitrin show antithyroid effects when paired with iodine deficiency or excess intake of Brassicaceae vegetables rich in thiocyanate precursor GSLs [7]. Thiocyanate is a competitive inhibitor of iodide at the sodium/iodide symporter [105]. PRO derivatives, such as 1-cyano-2-hydroxy-3-butene (CHB), in high doses are also associated with hepatic necrosis in rats [106]. Therefore, for crops rich in GSLs that can be degraded to these harmful products, the goal of targeted breeding or genetic modification is to reduce the levels of certain GSLs. However, a challenge remains in balancing the tradeoff between toxic GSL content and native plant defense [6,7,12,107]. Further elucidation of the genetic basis of toxic GSL biogenesis may provide novel targets for future efforts.

2.3. GSL metabolism, transport, and storage

The genes, enzymes, and metabolites of the GSL biosynthesis pathway have been extensively reviewed elsewhere [15,80]. Research revealed that GSLs mainly originate from xylem and phloem parenchyma cells, as well as some non-vascular tissues such as starch sheath cells, in the case of aliphatic GSLs [108]. Parenchyma cells are distinctive, as they have thin cell walls and remain alive at maturity. They store nutrients and divide quickly, traits that are essential for plant growth and tissue repair. The abundance of GSLs in parenchyma cells suggests a key role of GSLs in post-harvest plant defense. Using CYP83A1/B1 enzymes as markers, it was demonstrated that indolic GSL biosynthesis is located solely in the phloem, while aliphatic and aromatic GSL biosynthesis occurs in both the phloem and xylem [15].

The sulfur-rich cells (S-cells) along the phloem cap are important for GSL storage; however, the sources and routes of GSL storage are unknown. The giant S-cells (length >1 mm) are thin-walled cells that maintain relatively high turgor pressure, which leads to immediate

expulsion of cell sap and contact with hydrolyzing myrosinase in neighboring idioblasts upon herbivory. S-cells are distributed along the rosette, siliques, inflorescence, and pedicle. In *Arabidopsis* flower stalks, GSL contents of >130 mM account for an impressive 84% of total sulfur content [81]. While the GSLs present in the stem are inactive, they are highly distributed in laticifer-like S-cells, which are located along the vasculature and leaf margin close to the starch sheath cells [109].

Previous research on endogenous GSL transport is limited due to the lack of effective analytical methods to study the transporters of defensive metabolites [109]. The classic approach to demonstrate metabolite transport involves functional complementation of auxotrophic *Saccharomyces cerevisiae* strains, which has successfully characterized many transporters of plant primary metabolites [7]. However, this method is not suitable for secondary metabolite GSLs, as they are not essential components for yeast growth. Moreover, transporters of defensive metabolites require substrate-induced expression and co-expression with biosynthesis genes. Recently, single-cell sampling and microscopy equipped with energy-dispersive X-rays identified two plasma membrane-localized GSL transporters, GTR1/2, and the ultimate GSL sink, S-cells [109]. It was found that accumulation of GSLs occurs through symplastic connections, either directly with adjacent biosynthesis cells or indirectly with non-adjacent cells expressing *GTR1/2* [109]. In the leaf, major GSLs are predominantly stored in the symplasm rather than in the apoplast, where they constitute only 0.12% of the total GSL content. However, the current understanding of GSL transport and storage remains incomplete; for example, GSL transport in root tissues has yet to be elucidated.

3. Advances in targeted modification of GSLs

GSL variation in different genotypes, tissues, and developmental stages of *Brassicaceae* provides diverse genetic resources to identify promising candidates for modification [4]. In broccoli, for example, the total GSL content in the seeds of different genotypes ranged from 60.46 to 218.7 μmol/g FW, with 4MSB contributing between 2.6 and 129.9 μmol/g FW and PRO contributing between 0 and 128.9 μmol/g FW [110]. Similarly, GSL profile varies in flower heads of broccoli, where the total GSL content ranged from 0.5 to 57.2 μmol/g DW, with 4MSB from 0.1 to 15.0 μmol/g DW and PRO from 0 to 4.5 μmol/g DW [111]. In the root, the total GSL content ranged from 8.5 to 73.5 μmol/g [111]. Furthermore, GSL content changes with developmental stages, as sprout tissues contain 20-fold higher GSL levels than other developmental stages [112].

3.1. Modification of total GSL content

The selection of glucosinolate content and composition is one of the most important features of *Brassicaceae* domestication. Classic breeding initiatives targeting GSL content started before 1974 and utilized phenotypic traits of numerous wild cruciferous species. The original aim was to mitigate the detrimental effects of PRO degradation products in oilseed rape, and so efforts focused largely on reducing total GSL contents. Later work proposed to increase GSL levels in broccoli, as it predominantly produces 4MSB which can be hydrolyzed to bioactive SF. Environmental control measures and post-harvest processing methods, such as fertilizer application and reduced storage temperatures, were applied to achieve higher GSL contents in broccoli. Recently, precise modification of GSL content has been accomplished through genetic modification of genes related to the biosynthesis, transport, and distribution of desired GSL components or tissues.

3.1.1. Classic breeding

Changing the total GSL content is the most cost-effective and attainable type of modification. Pioneering work applied classical breeding techniques to decrease GSL content in oilseed rape, as PRO is the predominant GSL component. Oilseed rape is one of the most economically significant crops in the Brassicales order. Besides its primary purpose in oil production, it serves as a crucial source of animal feed. Rapeseed cake, a byproduct of oilseed rape processing, boasts exceptional quality for animal feed as it is rich in high-quality amino acids (comprising 38–44%) similar to soybean proteins, with balanced amino acid compositions [113]. However, consumption of significant oilseed rape by livestock raises the issue of detrimental health effects caused by PRO degradation products. In 1974, crossing with a low GSL Polish landrace Bronowski produced a hybrid 00-variety (double low) with total GSL contents reduced from 100 to less than 20 μmol/g [6] (Table 2). This achievement marked a significant milestone in the mitigation of unfavorable effects associated with high GSL content in oilseed rape and enhanced its suitability for both agricultural and feed purposes.

Another group of breeders has prioritized plants with enhanced GSL content, especially for species rich in health-promoting GSLs such as 4MSB and I3M. A typical example is the hybrid broccoli cultivar Beneforté™, generated from a cross between *B. oleracea* var. italica and a wild variant (*Brassica villosa*) with naturally higher levels of 4MSB. Beneforté™ provides a 10-fold increase in total GSLs, primarily 4MSB, along with a remarkable 100-fold improvement in potential anticancer activity, as evidenced by *in vitro* phase II detoxification enzymatic activity in cell cultures [114]. Subsequent transcriptomic analysis revealed that the increased GSL and 4MSB content in Beneforté™ stemmed from the higher expression of *MYB28*, the central regulator of aliphatic GSL biosynthesis [115]. However, changing total GSL contents leads to a change of all detrimental and beneficial components. Resolving this dilemma requires an improved understanding of GSL regulation.

3.1.2. Environmental control

Nutrient management, such as the use of fertilizers, or stress induction, such as the modification of growth temperature, light exposure, UVB radiation and humidity, present alternative strategies for manipulating GSL contents [147,148]. Sulfur metabolism has been identified as particularly influential in this context [149] (Table 2). Sulfur is a crucial nutrient for higher plants, and cruciferous crops require high levels of sulfur to produce GSLs [150]. Each GSL component includes at least two sulfur atoms, one in the thioglucosidic bond and the other in the core structure, unless altered via side chain modification, which may include the addition or removal of sulfur. GSLs typically constitute less than 10% of the total sulfur in vegetative tissues [151]. In *Brassica juncea*, for example, the GSL content has been reported to comprise 30% of the organic sulfur fraction [8].

Application of sulfur in cruciferous sprouts, such as cabbage, broccoli, and radish, resulted in a 2–5-fold increase in the total GSL content. Moreover, these treated sprouts exhibited 22–35% higher and 34–59% lower antiproliferative activity against HepG2 human hepatocarcinoma cells and CT26 mouse colorectal cancer cells, respectively [152]. By contrast, sulfur starvation leads to reduced GSL accumulation [153]. Downregulation of GSL biosynthesis is partially due to direct or indirect control of the central sulfate limitation response regulator, *SLIM1* (*SULFUR LIMITATION1*), with this response being more pronounced in roots [154]. Dynamic responses are evident in GSL metabolism under sulphate limitation, where GSL degradation is induced while GSL biosynthesis is inhibited [155]. GSLs are, therefore, believed to provide additional storage of sulfur to buffer sulfur limitations. However, exposure of *B. juncea* and *Brassica rapa* to excessive atmospheric sulfur (H_2S and SO_2) does not affect GSL content, which would be expected if GSLs were sulfur reservoirs [116]. Additionally, seeds do not serve as a sulfur source for primary sulfur metabolism during starvation [6]. This evidence supports a role for GSLs in sulfur balance, rather than storage, in vegetative tissues.

Other nutrients influencing GSL content include nitrogen, potassium, phosphate, and calcium [156,157]. Interestingly, the form in which these nutrients are supplied impacts GSL accumulation. For example, GSL biosynthesis shows a preference for ammonium over nitrate. Research on oilseed rape unveiled the coordination of nitrogen and sulfate metabolism in leaves; ammonium upregulates nitrogen and sulfur

Table 2
Approaches for modifying total glucosinolate contents.

Treatments	Key approaches	GSL modification results	Limitations
Classic breeding	1974, 00-variety	crossed with a Polish Landrace *Bronowski* [6] 1974, first 00-variety, Canola, ↓ GLS (20 μmol/g in air-dry seeds for premium quality)	Significant improvement achieved. However, it relies on genetic resources, has an uncertain genetic background, and is time-consuming
	1998, Beneforté™	crossed with a wild variant, *Brassica villosa* [9]	
	2003, Biochemical analysis	Increased GSLs by 10 folds, induced the expression of phase II detoxification enzymes by 100 folds [114]	
	2013, Transcriptome analysis	*MYB28* is key transcription factor regulating the GSL accumulation [115]	
	2015, Other analysis	Patent filed, prevention of diabetes mellitus achieved	
Environmental control	1998, S/Sulphate	Increased S, resulted in a 0.2–50 folds increase in total GSLs; Decreased S, differentially expressed *SLIM1*, *EIL1*, and *EIL3* decreased total GSLs [7]	Results are unsustainable and less predictable
	2016, S/Gas	Excessive atmospheric sulfur (H_2S and SO_2) exposure does not affect total GSLs [116]	
	2010, K	K deficiency influenced JA biosynthesis and increased GSLs [117]	
	2015, P	P deficiency changed *PHR1* (a central regulator of phosphorus) expression, increased GSLs [118]	
	2016, N	0.5–1 fold increase in total GSLs [119]	
	2018, Ca	Calcium ameliorates the toxicity of sulfate salinity (*B. rapa*) [120]	
Post-harvest processing	1991, Low temperature	Improved total GSLs (10% increase at 0 °C and 44% at 10 °C for 4 days) in rutabaga roots [121]	Results are unsustainable and less predictable
	1995, Low temperature	Became a routine method and combined or compared with other techniques [122]	
	2001, Ultrasound	Release of GSLs from the cell in leakage, disinfect with microbes and enzymes. Used to facilitate the extraction of bioactive compounds from herbs both on small and large scales [123]	
	2019, Ultrasound	Combined with MeJA to induce the stress response [124]	
	1989, High pressure	Produced by high temperatures, such as boiling, reduced GSLs [125]	
	2007, High pressure	Enriched GSLs, SF and I3C, in the treated broccoli juice [126]	
	2008, Thermal processing	Red cabbage (*Brassica oleracea* L. *ssp.* capitata f. rubra) was treated with blanching, boiling and steaming, and steaming (1–3min) was found to be a better treatment for GSL preservation [127]	
	1997, Microwave	Reduced GSL content significantly [128]	
	2018, Microwave	Semi-continuous microwaving better preserved 4MSB compared with high-pressure processing [129]	
	2003, Pulsed electric fields	Non-thermal treatment better preserves the nutritional value of fruit juice [130]	
	2015, Pulsed electric fields	Enriched GSLs in broccoli flowers and stalks [131]	
	2012, UVB light	Induced the formation of GSLs in broccoli sprouts [132]	
	2011, Sucrose	May act as a signaling molecule that induces GSL accumulation [133]	More effective than most physical treatments but is still unsustainable and less predictable
	2016, Exogenous sucrose	Reduced post-harvest GSL loss [134]	
	1997, Essential oils	Originally used on post-harvest pathogens [135]	
	2016, Essential oils	Reduced GSL loss in broccoli sprouts [136]	
	1999, Controlled atmosphere	Useful for broccoli storage: 1–2% O_2 and 5–10 CO_2 at 1–5 °C [137]	
	2007, Controlled atmosphere	GSL levels were maintained by perforated polypropylene bag packaging [138]	
	2013, Controlled atmosphere	Superior method for preservation compared with 1MCP, with improved GSL content and extend storage at 10% O_2 and 5% CO_2 [139]	
	1997, 1- methylcyclopropene (1MCP)	Extend shelf-life by competitively inhibiting ethylene receptor [140]	Results are unsustainable and less predictable. More effective than most physical and chemical treatments post-harvest; however, more trials are required before large-scale applications.
	2010, 1MCP	Better maintained GSLs compared with 6-benzylaminopurine (6BP) [141]	
	1999, Methyl jasmonate (MeJA)	Reduced post-harvest rot caused by grey mold [142]	
	2012, MeJA	Increased the contents of indolic GSLs [143]	
	2012, 6BP	Delayed senescence and enhanced GSLs [144]	
	2013, MeJA + 1MCP	Better preserved GSLs compared with the application of MeJA or 1MCP alone [145]	
	2012, 6BP + 1MCP	Better preserved GSLs compared with using 1MCP or 6BP alone [144]	
	2003, Ethylene	Increased the levels of indolic GSLs [146]	

assimilation, and induces accumulation of nitrogen-containing components (such as amino acids) and sulfur-containing compounds (including GSLs) [158]. Further investigation revealed that *MYB28* and its redundant family member, *MYB29,* shape ammonium stress responses through iron homeostasis [159]. By contrast, potassium deficiency triggers jasmonic acid synthesis, which, in turn, induces GSL biosynthesis gene expression and accumulation [117]. Similarly, phosphate deprivation has been shown to increase GSL synthesis through the modulation of a central regulator, *PHR1*, which is responsible for the phosphate deficiency response [118].

3.1.3. Post-harvest processing

While advancements in breeding, genetic modification, agricultural practices, and environmental controls have improved food production, post-harvest losses remain a significant challenge, accounting for approximately 30% of vegetables and fruits [160] (Table 2). Once harvested, plant tissues undergo senescence, which is characterized by the loss of chlorophyll and degradation of cell structures and macromolecules. Vegetables in Brassicales are generally marketed as fresh produce. However, before consumption, Brassica vegetables are very often treated by industrial processing and/or consumer preparation. These treatments can lead to significant changes in the GSL content. Post-harvest processing and plant senescence trigger activation of the GSL-myrosinase defense system and *de novo* biosynthesis of GSLs. However, the degraded products of GSLs are unstable and lost immediately before consumption. Thus, intact GSLs are the commonly accepted food source to produce health-promoting ITCs such as SF and I3C. To harness post-harvest stress-induced *de novo* biosynthesis of GSLs, numerous studies have explored physical, chemical, and biochemical treatments to extend shelf life and reduce GSL degradation in Brassicales.

Post-harvest changes in GSL content occur via three mechanisms: leaching, enzymatic hydrolysis, and chemical degradation [161,162]. These mechanisms depend on the temperature and the presence of water or other solutions. For example, in broccoli florets, myrosinase is denatured around 70 °C; however, this threshold increases to 100 °C in immature broccoli sprouts [163]. Inherent plant properties determine the rate of a given mechanism, such that different vegetables have variable rate constants of GSL degradation [164]. The GSL type also impacts the heat-induced degradation rate. For example, indolic GSLs are less stable than aliphatic GSLs [165]. In general, chemical processes and/or preparation methods that involve minimal amounts of water and short heating times retain higher amounts of GSLs. For example, steaming, stir-frying, or microwaving tends to retain more GSLs compared to boiling [165]. The rate of formation of GSL breakdown products also depends on temperature, generating variable ratios of nitriles, epithionitriles, and isothiocyanates [166]. Another strategy to delay post-harvest senescence and modify GSL content is to maintain energy supplies by providing an exogenous supply of sucrose. Sucrose acts as an essential signaling molecule that induces GSL and anthocyanin accumulation in broccoli sprouts [134]. Equally important, a controlled atmosphere (CO_2/O_2), either through packaging or storage atmosphere control, can regulate respiration and reduce the catabolism of bioactive compounds [167].

Alternative strategies to maintain GSL content post-harvest involve preserving food quality by controlling the senescence process. One classic approach is to inhibit ethylene signaling through 1- methylcyclopropene (1MCP) application. 1MCP suppresses ethylene perception by impeding binding with ethylene receptors (ETR1/2), thereby improving the total GSL levels in broccoli florets [145]. Similarly, 6-benzylaminopurine (6BP) can reduce ethylene sensitivity and delay senescence in post-harvest foods [144]. Application of 6BP can prolong shelf life and stabilize the levels of total GSLs, chlorophyll, and peroxidases in broccoli. Moreover, significant increases in the total GSL content have been observed in post-harvest ethylene-treated broccoli heads [168]. Methyl jasmonate (MeJA) is another hormone commonly used to improve post-harvest food quality. MeJA acts as an elicitor of GSL biosynthesis, resulting in elevated levels of GSLs [168]. Additionally, melatonin (N-acetyl-5-methoxytryptamine), an abiotic and biotic antistressor, has been applied to inhibit post-harvest senescence and physiological damage in foods. Melatonin was first identified in mammals; however, in plants, it delays the browning of fresh-cut fruits, such as pear and litchi, and increases the nutritional quality of post-harvest foods such as tomato and pear [169].

Overall, recent advances in genetic modifications, environmental controls, and post-harvest processing methods have made significant progress toward achieving desired GSL profiles tailored to specific crop varieties. Classic breeding methods have successfully altered GSL profiles, albeit with challenges regarding the viability of resulting cultivars in field conditions and the intricate balance between reducing detrimental GSLs while maintaining health-promoting precursors. Environmental control measures, particularly sulfur application, have emerged as effective means of modulating GSL accumulation, highlighting the interplay between nutrient availability and GSL metabolism. Moreover, post-harvest processing strategies aim to preserve GSL content and enhance food quality, addressing the significant challenge of post-harvest losses. Moving forward, established methodologies for the modification of GSL levels should be combined with innovative biotechnological approaches and precise genetic manipulations, along with a comprehensive understanding of GSL metabolism to develop sustainable, cost-effective, and time-efficient approaches to modify GSL content in *Brassicaceae* crops.

3.2. Precise modification of GSL content

While classic breeding, fertilizer application, and post-harvest regulation have effectively yielded changes in the total GSL content, several challenges remain to be addressed. Firstly, these approaches are time-consuming and may not always be sustainable in the long term due to their reliance on external factors such as environmental conditions and resource availability. Secondly, outcomes are not always predictable, as changes in total GSL content can also affect plant defense and overall development. Finally, modifying the total GSL content influences the precursor levels of both beneficial and detrimental GSL breakdown products. To address these challenges, there is a growing need for more precise approaches to modify GSL content. This involves the application of genetic engineering techniques that allow for the specific manipulation of pathways involved in GSL biosynthesis, transport, and regulation (Table 3). Advances in biotechnology, such as genome editing technologies (CRISPR-Cas9), offer promising avenues for precise modifications of GSL content in *Brassicaceae* crops. These tools enable researchers to precisely edit the plant genome to introduce desired changes in GSL biosynthesis pathways, without affecting defense mechanisms or other aspects of plant physiology. Targeting specific genes or pathways involved in GSL metabolism allows for more predictable and controlled modifications to the GSL content.

3.2.1. Modification of a group of GSLs

Since 1994, genetic modifications have been used to manipulate GSLs, primarily through overexpression or knockdown of GSL biosynthesis genes. For instance, overexpression of the GSL biosynthesis gene *AOP2* from *B. oleracea* in *A. thaliana* induced a 2-fold increase in aliphatic GSLs [172]. *AOP2* regulates the biosynthesis of aliphatic GSLs, through the conversion of 4MSB into gluconapin, the precursor of PRO [191]. In cabbage, overexpression of *IQD1* and *MYB29* selectively increased aliphatic GSL production [192]. *IQD1* expression is induced by mechanical stimuli, prompting calcium–dependent binding to calmodulin and resulting in glucosinolate accumulation in response to biotic challenges [193]. *MYB29/29* are redundant transcription factors that negatively regulate stress signaling and aliphatic glucosinolate biosynthesis [194]. Cas9 endonuclease-based targeted mutagenesis of *MYB28/29* resulted in the complete loss of aliphatic GSL in seeds and roots,

Table 3
Approaches for the precise modification of glucosinolate content.

Modifications	Key approaches	GSL modification results	Limitations
Modify a group of GSLs	1994, ↑ biosynthesis genes	Increased methionine, improved aliphatic GSLs in *A. thaliana* mutants [170]	More accurate for changed GSLs content but less powerful for predicting the defense outcome of the resulting GSLs profile
	2006, gene introduction	Introduction of *CYP79A1* (*Sorghum bicolor*), *CYP79A2* (*A. thaliana*), *CYP79D2* (cassava *Manihot esculenta*) into *A. thaliana,* improved plant defense [171]	
	2007, overexpression	Overexpression of AOP2 [172]	
	2010, overexpression	Overproduction of phenylalanine improved benzyl GLS in *A. thaliana* mutants [173]	
	2009, 2012, gene introduction	Introduction of phenylalanine biosynthesis genes from *E. col,* increased benzyl GSLs and its ITC in *A. thaliana* mutants [174,175]	
	2017, RNAi	Knockdown of *AOP2* in *B. napus* decreased PRO (40%) and increased 4MSB (40 μmol/g) [176]	
Modify GSLs in specific organs	2012, GTR discovered	GTRs were discovered and were found to translocate GSLs to seeds for defense [177]	This modification affects the level of both beneficial and adverse GSLs
	2017, *GTR1/2*, mutation		
	2023, *UMAMIT*, mutation	Mutation of GTR paralogs in *Brassica juncea* reduced total GSL content in seeds by 60–70% and altered GSL partitioning between reproductive/biosynthetic tissues [178]	
		The loss-of-function triple mutant *umamit29/30/31* exhibits extremely low GSL content in seeds but showed no changes in the distribution of GSLs as defense compounds in plants [107]	
Modify to obtain desired GSLs	1999, Cell culture	Lower GSL contents compared with native plants (44 mmol/g DW benzyl GSLs, the highest yield of GSLs was observed in Indian cress) [179]	GSL levels are lower compared with those in the native plants. It is thus a less preferable approach for GLS production
	2008, methyl-JA, cell culture	Total GSLs increased by 2.86-fold (1.4–4 μmol/g FW) [180]	
	1999, Hairy root culture	Indian cress: 85 mmol/g DW [179]	Far from economic production due to low yield compared with that in whole plants
	2006, Hairy root culture, hormone analogue	Increased GSLs by 4.8-fold in BGLS: yielded 85.8 mmol/g FW GSLs [181]	
	2015, Hairy root culture, transgenic	Overexpression of *CYP79F1* increased GSL levels in mutants but decreased GSL contents in hairy roots [182]	
	2009, Engineering in *N. benthamiana*	The first report of *de novo* GSL synthesis in non-brassicaceous plants [183]	Still at an early stage with low yield
	2011, Engineering in *N. benthamiana*	Engineering of the indolic GSL pathway [184]	
	2012, Engineering in *S. cerevisiae*	I3M was obtained, final indolic GSL content reached 1.07 mg/L [185]	
	2018, Engineering in *E. coli*	4MSB was produced [186]	
	2018, Engineering in *E. coli*	Optimized the conversion of GSLs into ITCs at the site of cancer in vivo, achieved 95% inhibition of cancer cell growth *in vitro* and reduced tumor growth in vivo [187]	
	2019, Engineering in *E. coli*	De novo production of benzyl glucosinolate in *Escherichia coli* [188]	
	2021, Engineering in *N. benthamiana*	Engineering and optimization of the 2-phenylethylglucosinolate production in *Nicotiana benthamiana* by combining GSL biosynthesis genes from *Barbarea vulgaris* and *Arabidopsis thaliana* [189]	
	2021, Engineering in potato	Production of benzyl-GSL with improved broad-spectrum pest and disease resistance in potato through a transgenic approach [190]	

emphasizing the critical role of *MYB28/29* in specific tissues [195]. While reducing aliphatic GSL content mitigates the effects of PRO degradation byproducts, the health-promoting 4MSB level and plant defense are similarly attenuated. In another study, RNA interference silenced *AOP2* in *B. napus* and reduced PRO content while simultaneously increasing nutritional 4MSB levels [196]. However, loss of PRO content still impaired plant defense, in agreement with its role in pest and disease resistance. Thus, despite the advancements in genetic modification techniques for GSL manipulation, issues regarding the balance between reducing detrimental components and maintaining plant defense and health-promoting properties persist, requiring further research efforts and innovative modification strategies to address these challenges.

3.2.2. Modification of GSLs in specific tissues

Regulation of GSL movement from vegetative organs to reproductive organs is largely orchestrated by the GSL importers *GTR1/2/3*, which are responsible for assigning GSL distribution within and between source and sink tissues (Table 3). For example, attenuation of GSL import via knockdown of *GTR1/2* successfully decreased the total GSL content in seeds of *B. juncea* by 60–70% [178]. However, this resulted in an alteration of the overall GSL distribution in plants. To address this problem, researchers have turned their attention to newly identified GSL exporters, USUALLY MULTIPLE AMINO ACIDS MOVE IN AND OUT TRANSPORTER (UMAMIT29/30/31) [107]. The loss-of-function triple mutant *umamit29/30/31* shows very low GSL content in seeds without

altering the distribution of GSLs as defense compounds in plants. Seeds are the reproductive sink tissues and edible section of many plants in the family of Brassicaceae, such as oilseed rape. In the future, coupling the modification of GSL importers with GSL exporters may allow for complete control over seed-specific GSL production. This approach presents a promising solution to the loss of plant defense concomitant with the modification of GSL levels. However, for plants where non-seed tissues are edible, such as cabbage, turnip, and kale, the goal is to achieve targeted modifications in the leaves and roots.

3.2.3. Modification of desired GSLs

The resolution of major issues associated with GSL modification has been achieved through the combination of tissue culture and genetic engineering. The utilization of tissue culture for GSL modification in non-host organisms dates back to the 1990s and offers a practical alternative to classic methods relying on host plants and fields (Table 3). The highest GSL yield was obtained from cell cultures of Indian cress (*Tropaeolum majus*), which was reported at 44 μmol/g DW benzyl-GSL [179]. Similarly, hairy root cultures of watercress (*Nasturtium officinale*) and land cress (*Barbarea verna*) treated with the elicitors phenylalanine and cysteine increased GSL levels to 142 μmol/g DW and 236 μmol/g DW, respectively [197]. Transitioning to a transgenic approach, the introduction of GSL biosynthesis genes into non-*Brassicaceous* plants, such as tobacco (*Nicotiana benthamiana*), has enabled *de novo* biosynthesis of GSLs [198]. For example, the aliphatic GSL, 4MSB, and the indolic GSL, I3M, have successfully been synthesized in *N. benthamiana* [184,199]. More recently, this technique was applied to potato plants, resulting in the production of benzyl-GSL that conveyed improved broad-spectrum pest and disease resistance [190]. The introduction of GSL biosynthesis pathways into non-host species may be more applicable, as it is far easier to engineer high levels of the desired GSL components than to modify the blend produced by the host plants, which often has pleiotropic effects.

Recently, genetic engineering of microbial hosts has emerged as a promising avenue for large-scale production of GSLs. Initial successes include the generation of I3M in *E. coli* and *S. cerevisiae* through the integration of genes from the indolic GSL biosynthesis pathway in *A. thaliana*, which yielded a total indolic GSL content of 1.07 mg/L [185]. Similarly, aromatic GSLs, benzyl-GSL, have been synthesized in *E. coli* by introduction of the biosynthesis pathway of aromatic GSLs, with a yield of 8.3 mg/L. However, producing a detectable amount of 4MSB has been extremely challenging, and was only successful through engineering 13 GSL biosynthesis genes, six from *A. thaliana*, two from *B. rapa*, two from *B. oleracea*, and one from *Neurospora crassa*, into *E. coli* [186]. However, a key challenge lies in achieving economically sustainable yields of GSLs via genetic engineering, as current yields are significantly lower than those of native plants. Although optimization strategies have been investigated in tissue culture, the GSL yield is significantly lower than that in native plants. Nevertheless, GSL production by microbial hosts is in its early stages and holds potential as a viable solution to address this challenge.

In addition to modifying GSL content in source plants, boosting delivery efficiency offers an alternative approach to improve beneficial GSL sensitivity in humans. Nanodelivery systems offer improved solubility, stability, bioavailability, and targeting ability of hydrolysis products [200]. Recent advancements in microfluidic technology have partially addressed the issues of low reproducibility and poor controllability associated with nanodelivery and improved its potential industrial application. Encapsulating ITCs in nanoparticles, such as liposomes, micelles, and polymeric nanoparticles, protects them from harsh environmental conditions, leading to increased stability and circulation time [201,202]. Encapsulated ITCs also possess enhanced tumor accumulation and retention capability, resulting in higher anticancer activity [203]. Functional ligands can be attached to the surface of nanoparticles to further increase their targeting and anticancer ability. Moreover, multiple compounds can be simultaneously encapsulated and delivered within nanoparticles, overcoming the drawbacks of individual drugs to achieve synergistic therapeutic efficacy [204]. However, conventional preparation methods of nanodelivery systems are associated with drawbacks. Lack of controllability and poor batch-to-batch consistency are major concerns during industrial production. Fortunately, microfluidic technology allows for the preparation of nanoparticles in a more controlled manner by precisely manipulating small volumes of reaction liquids in microfluidic channels. These nanoparticles show narrow size distribution, good controllability, and low batch-to-batch variations [205]. Furthermore, microfluidic-based nanoparticle preparation can be easily scaled to an industrial level using a high aspect ratio or a parallel microfluidic device [206]. The success of the Pfizer mRNA vaccine against COVID-19 using microfluidics represents a significant milestone, demonstrating the feasibility of microfluidics for developing drug-loaded nanomedicines for market use [200]. However, the application of microfluidic-directed nanoparticles for the delivery of GSL and ITCs awaits exploration and validation.

4. Perspectives

Modification of GSL content and composition is a pivotal objective in the domestication of plants within Brassicales. Over the past seven decades, significant advances have been made in addressing key challenges surrounding GSL modification. Through classic breeding, environmental regulation, and post-harvest processing, many issues, such as efficiency, sustainability, unpredictability, and balance of the benefits and harms of GSLs and their byproducts, have been addressed. Further research into GSL-regulated post-harvest senescence and direct and indirect GSL-driven defenses may lead to the optimization of plant breeding strategies and elicitors that may be applied to improve fruit and vegetable quality. Similarly, ongoing research into GSL metabolism, transport, and storage mechanisms *in planta*, as well as GSL sensitivity and processing in mammals, is crucial for developing strategies to enhance GSL profiles in crops and control effects on human health. The work done in this area has already led to successful genetic modification of GSL biosynthesis genes, transcriptional regulators, and transporters, allowing for tissue-specific engineering of GSL levels. While considerable progress has been made in the modification of GSL content in the seeds of Brassicales crops, challenges remain for plants with non-seed tissues as the edible sections, such as cabbage, kale, turnip, and mustard. Continuation of work on GSL importers, GTRs, and their potential synergy with GSL exporters, UMAMITs, may allow for tailoring of GSL levels in non-seed tissues. Furthermore, exploring the potential of GSL degradation offers an alternative approach to alter GSL content, as it may be possible to stabilize desired GSLs and/or promote the degradation of undesired components. However, very limited research has been conducted from this perspective. In the context of health-promoting GSLs, expression of GSL biosynthesis pathways in microbes has emerged as an avenue with the potential to generate beneficial GSLs; however, optimization of microbial GSL yield is ongoing, and currently, not economically feasible. Finally, the advances made in nanodelivery may increase sensitivity or improve targeted uptake of selected GSLs in mammals and is a promising area of research in pharmaceutical applications of GSLs.

Declaration of interests

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. Given his role as Deputy Editor-in-Chief, Robert J. Henry had no involvement in the peer review of this article and has no access to information regarding its whole review process.

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Authors' contributions

B.C. and Y.Q. outlined the main sections. B.C. drafted the main sections. R.R. drafted the health sections. Y.Q. contributed text to the defense section. M.D. and R.V. offered comments on the manuscript. R.H. offered comments and edited the manuscript. H.H. offered comments on the manuscript and secured the funding.

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