

Review

Glucosinolate Biosynthesis and the Glucosinolate–Myrosinase System in Plant Defense

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Abstract: Insect pests represent a major global challenge to important agricultural crops. Insecticides are often applied to combat such pests, but their use has caused additional challenges such as environmental contamination and human health issues. Over millions of years, plants have evolved natural defense mechanisms to overcome insect pests and pathogens. One such mechanism is the production of natural repellents or specialized metabolites like glucosinolates. There are three types of glucosinolates produced in the order Brassicales: aliphatic, indole, and benzenic glucosinolates. Upon insect herbivory, a “mustard oil bomb” consisting of glucosinolates and their hydrolyzing enzymes (myrosinases) is triggered to release toxic degradation products that act as insect deterrents. This review aims to provide a comprehensive summary of glucosinolate biosynthesis, the “mustard oil bomb”, and how these metabolites function in plant defense against pathogens and insects. Understanding these defense mechanisms will not only allow us to harness the benefits of this group of natural metabolites for enhancing pest control in Brassicales crops but also to transfer the “mustard oil bomb” to non-glucosinolate producing crops to boost their defense and thereby reduce the use of chemical pesticides.

Keywords: glucosinolates; myrosinase; degradation product; mustard oil bomb; plant defense

1. Introduction

Glucosinolates, once referred to as mustard oil glucosides, have long been part of human life and agriculture due to their influence on the distinctive flavor and aroma of brassicaceous vegetables, involvement in plant defense, and auxin homeostasis [1–3]. Glucosinolates occur in members of the Brassicaceae family including cabbage, broccoli, and mustard [4,5]. Additionally, they occur in sixteen other plant families for a total of 4700 species [6]. In the past few decades, the importance of these sulfur-containing specialized metabolites has attracted attention because of their health promoting activities (e.g., anti-carcinogenesis) [7–9], potential functions in non-host resistance to bacteria and fungi [10,11] and insect defense [3,12–14]. Glucosinolates form a network with other metabolic pathways which play important roles in plant growth, development, and interaction with the environment [15,16]. Some glucosinolates can also act as antioxidants against oxidative stresses [17]. The presence of 36 different glucosinolates in the reference plant, *Arabidopsis thaliana* [18,19], has inspired

extensive research and progress in these amino acid-derived natural compounds, leading to elucidation of their biosynthetic pathways, identification of regulatory factors and mechanisms, as well as revelation of crosstalk with other pathways [3,20,21]. Glucosinolates coexist with myrosinases, which hydrolyze glucosinolates into different bioactive degradation products [22,23] (Figure 1). The specialized glucosinolate–myrosinase system is often referred to as the “mustard oil bomb” for deterring herbivore attack. Here, we describe recent advances made in the biosynthesis of glucosinolates, the “mustard oil bomb”, and how they function in plant defense against pathogens and insects. Understanding this unique chemical defense system will not only allow scientists to harness this group of natural metabolites for enhancing pest control in the order Brassicales, but also transfer these natural defense compounds to non-glucosinolate producing plants and crops [24] (in a tissue-specific manner) for enhancing disease resistance and thereby reducing the use of chemical pesticides.

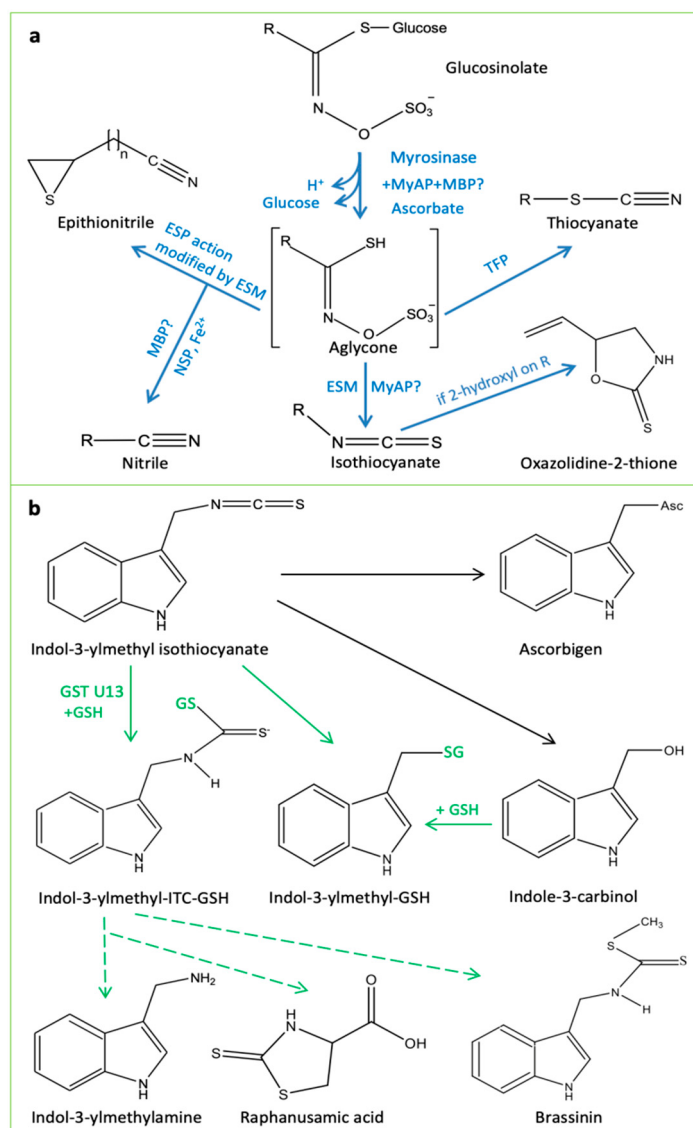


Figure 1. Glucosinolate structure and degradation products from the “mustard oil bomb”. Factors affecting formation of different biological active metabolites include potential myrosinase-interacting proteins, iron, glutathione, and glutathione S-transferase. (a) Sulfate is liberated in all reactions leading from the aglycone. Elemental sulfur (possibly S₈) is an additional product in the reaction forming a simple nitrile. The structures marked epithionitrile and oxazolidine-2-thione represent examples of

these product types, depending on specific R-groups in the precursor glucosinolates. (b) The pathways in green represent putative indole ITC-derived products which lack biochemical evidence for activity (adapted from [25,26]). Black line represents a spontaneous reaction. ESM, epithiospecifier modifier; ESP, epithiospecifier protein; GSH, glutathione; GSTU13, glutathione S-transferase U13; MBP, myrosinase binding protein; MyAP, myrosinase associated protein; NSP, nitrile specifier protein; TFP, thiocyanate-forming proteins.

2. Diversity of Glucosinolate Structure and Biosynthesis

The basic core structure of all the glucosinolates consists of a β -thioglucose residue linked via a sulfur atom to a (Z)-N-hydroximosulfate ester, plus a variable side chain (R group) derived from an amino acid [2,27,28] (Figure 1). Different precursor amino acids, variation in side-chain length caused by chain elongation, and extensive side-chain modifications lead to the chemical diversity of approximately 137 suggested glucosinolates with 88 confirmed structures [28]. This gap between the suggested and confirmed glucosinolates is attributed to efficiency and accuracy of current structural identification tools such as liquid chromatography, mass spectrometry, and nuclear magnetic resonance [28]. Glucosinolates can be classified according to their precursor amino acids, i.e., those derived from methionine, alanine, leucine, isoleucine, valine, or glutamate are aliphatic glucosinolates; those made from tryptophan are indole glucosinolates; and those synthesized from phenylalanine and tyrosine are benzenic glucosinolates. Aliphatic glucosinolates represent the most diverse group in *Arabidopsis* and many other species of the order Brassicales [28,29]. Glucosinolate biosynthesis involves many genes, enzymes, and transcription factors [30]. This section focuses on the biosynthetic pathways of different types of glucosinolates.

2.1. Biosynthesis of Aliphatic Glucosinolates

Figure 2 summarizes the current knowledge of glucosinolate biosynthesis, which is mostly gained from studies in *A. thaliana*. To initiate methionine-derived aliphatic glucosinolate biosynthesis, branched-chain amino acid aminotransferase 4 (BCAT4) catalyzes deamination of methionine to a 2-oxo acid, which is transported to chloroplasts via bile acid transporter 5 (BAT5). There 2-oxo acid undergoes condensation with acetyl-CoA by methylthioalkylmalate synthase 1 (MAM1), MAM2, and MAM3 [30–32]. The crystal structures of MAMs allowed identification of key active site residues responsible for controlling the MAM specificity for different 2-oxo substrates, thus accounting for side-chain length diversity of the aliphatic glucosinolates [33]. After condensation, the 2-malate derivative isomerizes through isopropylmalate isomerase large subunit 1 (AtLeuC1), isopropylmalate isomerase small subunit 1 (AtLeuD1), and AtLeuD2, followed by oxidative decarboxylation catalyzed mostly by isopropylmalate dehydrogenase 1 (IPMDH1) [30,34,35]. After transamination of the chain-elongated 2-oxo acids by BCAT3, the chain-elongated methionines enter the core biosynthetic pathway and are further metabolized by CYP79F1 and CYP79F2 to produce aldoximes. CYP83A1 converts the aldoximes to unidentified intermediates (previously assumed as *aci*-nitro compounds) [36], which are conjugated with glutathione (GSH) by glutathione S-transferase F11 (GSTF11) and GSTU20, and then cleaved by gamma-glutamyl peptidase 1 (GGP1) and C-S lyase (Super root 1 (SUR1)) to produce thiohydroximates. It should be noted that although the *aci*-nitro compounds are still used in recent literature (e.g., [37]), the chemical structure of the CYP83 products is yet to be elucidated.

The thiohydroximates are then S-glucosylated by UDP-glucosyltransferase 74C1 (UGT74C1) to form desulfoglucosinolates [30,38]. Sulfotransferase 17 (SOT17) and SOT18 catalyze the sulfation of desulfoglucosinolates to form intact glucosinolates [30,37,39]. Aliphatic glucosinolates can undergo side-chain modifications, including sulfur oxygenation by flavin-monooxygenases (FMOs) and form hydroxylated alkenylglucosinolates from sulfinylglucosinolates through alkenylhydroxalkyl-producing 2 (AOP2), AOP3 and 2-oxoglutarate-dependent dioxygenase (GS-OH) [30,37] (Figure 2). AOP2 is responsible for 3-butenylglucosinolate and 2-propenylglucosinolate production in *Brassica oleracea*, while the GS-OH-related enzyme plays a role in 4-(methylsulfonyl)-3-butenylglucosinolate biosynthesis in radish [40–42]. In general, GS-OH family enzymes play diverse roles in the biosynthesis of different

plant metabolites including flavonoids, nucleic acids, and alkaloids [43]. AOP3 is responsible for the formation of hydroxyalkylglucosinolates [44], and it acts with MAM2 to produce short-chain aliphatic glucosinolates [45]. Transcriptomic co-expression analysis has revealed that almost all the genes involved in methionine chain elongation and core structure pathways are coordinately regulated and coexpressed [46,47].

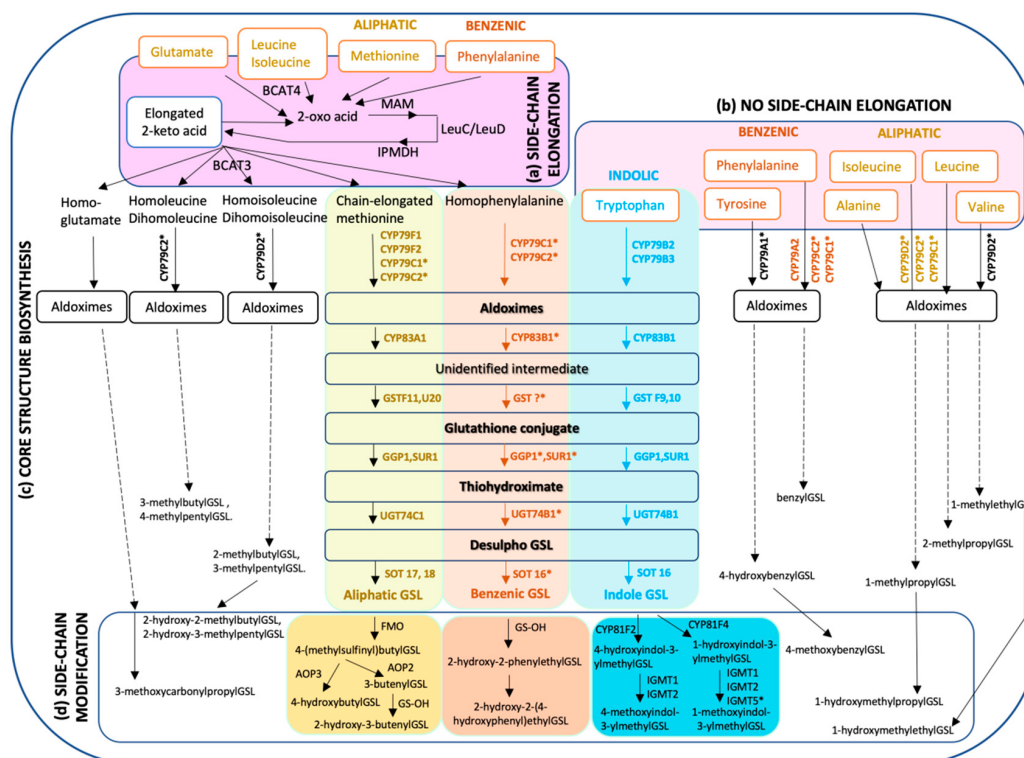


Figure 2. Biosynthetic pathways of aliphatic, indole, and benzenic glucosinolates. Aliphatic glucosinolate biosynthesis includes methionine, leucine, and isoleucine chain elongation, core structure synthesis, and side-chain modifications. It also includes isoleucine, valine, alanine, and glutamate core structure synthesis and side-chain modification. Benzenic biosynthesis includes phenylalanine chain elongation, no side-chain elongation of phenylalanine and tyrosine, core structure synthesis, and side-chain modification. Indole glucosinolate biosynthesis includes core structure synthesis and side-chain modifications of indole glucosinolates. **(a) Chain elongation:** BCAT, branched-chain amino acid aminotransferase; MAM, methylthioalkylmalate synthase; AtLeuC, isopropylmalate isomerase large subunit; AtLeuD, isopropylmalate isomerase small subunit; IPMDH, isopropylmalate dehydrogenase. **(b) No chain elongation:** CYP79A1, cytochrome P450 79A1; CYP79A2, cytochrome P450 79A2; CYP79C1, cytochrome P450 79C1; CYP79C2, cytochrome P450 79C2; CYP79D2, cytochrome P450 79D2. **(c) Core structure synthesis:** CYP79B2, cytochrome P450 79B2; CYP79B3, cytochrome P450 79B3; CYP79F1, cytochrome P450 79F1; CYP79F2, cytochrome P450 79F2; CYP83A1, cytochrome P450 81A1; CYP83B1, cytochrome P450 83B1; GSTF9, glutathione S-transferase F9; GSTF10, glutathione S-transferase F10; GSTF11, glutathione S-transferase F11; GSTU20, glutathione S-transferase TAU 20; GGP1, gamma-glutamyl peptidase 1; SUR1, super root 1; UGT74B1, UDP-glucosyl transferase 74B1; UGT74C1, UDP-glucosyl transferase 74C1; SOT16, sulfotransferase 5a; SOT17, sulfotransferase 5c; SOT18, sulfotransferase 5b. **(d) Side-chain modifications:** FMO, flavin-monooxygenase glucosinolate S-oxygenase; AOP2, alkenyl hydroxalkyl-producing 2; AOP3, alkenyl hydroxalkyl-producing 3; GS-OH, 2-oxoglutarate-dependent dioxygenase; CYP81F1, cytochrome P450 81F1; CYP81F2, cytochrome P450 81F2; CYP81F3, cytochrome P450 81F3; CYP81F4, cytochrome P450 81F4; IGMT1, indole GSL O-methyltransferase 1; IGMT2, indole GSL O-methyltransferase 2; IGMT5, indole GSL O-methyltransferase 5. * Indicating those genes and/or enzymes were characterized in heterologous systems, not in native species.

2.2. Biosynthesis of Indole Glucosinolates

Indole glucosinolate biosynthesis starts with conversion of tryptophan to indole-3-acetaldoxime by CYP79B2 and CYP79B3. CYP83B1 then catalyzes the aldoxime to produce an uncharacterized intermediate, which undergoes sulfur incorporation and thiohydroximate formation through the activities of GSTF9, GSTF10, GGP1, and SUR1. In a similar manner to aliphatic glucosinolate biosynthesis, UGT74B1 is required for thiohydroximate glucosylation, and SOT16 is responsible for the sulfation step to produce intact indole glucosinolates. In terms of modifications, CYP81Fs catalyze hydroxylation of indole glucosinolates, e.g., CYP81F2 is responsible for the production of 4-hydroxyindole glucosinolate [30,36,48–50] (Figure 2). In addition, CYP86A7 and CYP71B26 may be responsible for the hydroxylation of indole glucosinolates, especially at 1-position [51]. The hydroxyindole glucosinolates can be further metabolized to methoxyindole derivatives through indole glucosinolate methyltransferases 1 and 2 (IGMT1 and IGMT2) [50]. In addition, methylation of 4-hydroxyindol-3-ylmethyl glucosinolate (4MI3G) is controlled by cytoplasmic protein phosphatase 2A regulatory subunit B'γ (PP2A-B'γ), which physically interacts with IGMTs and regulates the IGMT activities in catalyzing the O-methylation at the 4-position [52]. Recently, it was reported that methylation of 1-hydroxyindol-3-ylmethylglucosinolate can take place via indole glucosinolate O-methyl transferase 5 (IGMT5) [53]. Furthermore, the PP2A-B'γ may affect catabolism of indole glucosinolates through direct regulation of the phosphorylation of myrosinase TGG1 involved in glucosinolate hydrolysis [54]. Posttranslational modification analysis of glucosinolate metabolic enzymes is an interesting research direction.

2.3. Knowledge Gaps in the Biosynthesis of Glucosinolates

Although the biosynthetic pathways of methionine-derived aliphatic glucosinolate and tryptophan-derived indole glucosinolate have been well-studied in *A. thaliana* over the past few decades [3,27,28,30,33–35,48], knowledge gaps exist between the aldoxime and thiohydroximate steps in terms of the unidentified intermediates and the reaction mechanisms. In addition, the enzymatic and genetic details of the biosynthesis of aliphatic glucosinolates from other precursors are poorly known, apart from limited results obtained in heterologous systems (Figure 2). For example, when a *Manihot esculenta* cyanogenic glucoside producing CYP79D2 was expressed in *A. thaliana*, isoleucine and valine-derived isopropyl and methylpropylglucosinolates were produced [55]. A recent study overexpressed CYP79C1 and CYP79C2 in *Nicotiana benthamiana*. When leucine was provided as substrate, the transgenic plants produced 2-methylpropylglucosinolate, whereas benzylglucosinolate was produced when phenylalanine was used [56]. Information on the biosynthesis of alanine and glutamate-derived glucosinolates is scarce. Similarly, research on benzenic glucosinolate biosynthesis is also lacking. As described above, the CYP79Cs may be involved in the production of both aliphatic and benzenic glucosinolates [56]. Another study showed that overexpression of a *Sorghum bicolor* CYP79A1 in *A. thaliana* led to the production of tyrosine-derived *p*-hydroxybenzylglucosinolate [57]. A recent study showed evidence that CYP79Fs use homophenylalanine as a precursor in benzenic glucosinolate biosynthesis [58]. The results clearly indicate that the substrate specificity of CYP79s may be broad and not limited to one group of amino acid precursors [59,60]. Interestingly, aliphatic MAM enzymes were found to produce homophenylalanine [61], suggesting potentially broad substrate specificity and versatility of enzymes in glucosinolate biosynthesis [62]. Although these results from the heterologous systems present exciting opportunities for synthetic biology applications of glucosinolate biosynthesis, their relevance and significance in native plant species is yet to be investigated. In addition, the transcription factors that regulate benzenic glucosinolate biosynthesis remain elusive [27].

3. Regulation of Glucosinolate Biosynthesis

Glucosinolate biosynthesis is regulated by many different factors. For example, environmental regulation has been long well-known [2,11,14,36,63–65]. In addition, great progress has been

made in transcriptional regulation, e.g., the identification of subgroup 12 R2R3-MYB transcription factors and components acting upstream, including MYB28 and MYB29 [46], basic nuclear-localized calmodulin-binding protein, IQ-domain1 (IQD1) [66], and ethylene-insensitive3-like transcriptional factor (SLIM1) [67]. In *Brassica* species, this subgroup 12 consists of 55 MYBs that have been reported to play roles in glucosinolate biosynthesis [68]. Here, we describe different regulators and molecular mechanisms.

3.1. Transcriptional Regulators Controlling Glucosinolate Biosynthesis

MYB28, MYB76, and MYB29 transcription factors (also known as high aliphatic glucosinolate 1 (*HAG1*), *HAG2*, and *HAG3*, respectively) were found to regulate aliphatic glucosinolate biosynthesis in three different ways. First, Hirai et al. used gene co-expression analysis and found that MYB28 and MYB29 are co-regulated with known genes in glucosinolate biosynthesis [46]. Second, a quantitative trait loci (QTL) analysis identified that MYB28 is located within a genomic region that determines aliphatic glucosinolate levels [69]. Third, MYB28, MYB29, and MYB76 were identified in a screen for their transactivation potential toward biosynthetic genes of aliphatic glucosinolates [70,71]. Overexpression of MYB28, MYB29, and MYB76 resulted in increased accumulation of aliphatic glucosinolates (but not indole glucosinolates) in leaves and suspension cells, increased expression of aliphatic glucosinolate biosynthetic genes, and repression of the indole glucosinolate pathway [46,69,71]. Knockout mutants of MYB28 showed significantly decreased levels of short- and long-chain aliphatic glucosinolates, whereas only short-chain glucosinolates were reduced in *myb29* mutant [69,71], suggesting that MYB29 regulates the production of short-chain aliphatic glucosinolates, while MYB28 controls both short- and long-chain products. The double mutant *myb28myb29* had almost completely abolished production of aliphatic glucosinolates, suggesting that MYB28 and MYB29 are the master regulators, and MYB76 only plays an accessory role [48,69–71].

The first identified positive regulator of indole glucosinolate biosynthesis is altered tryptophan regulation 1 (*ATR1/MYB34*) [72]. A dominant overexpression allele, *atr1D*, confers constitutively activated expression of genes encoding anthranilate synthase alpha subunit 1 (*ASA1*) and tryptophan synthase beta subunit 1 (*TSB1*) in tryptophan synthesis and in the core pathway (*CYP79B2*, *CYP79B3* and *CYP83B1*), while the expression of *CYP79F1* is not altered. Consequently, there was a ten-fold increase of indole glucosinolates in the *atr1D* compared to wild type [72]. By contrast, a loss of function mutant *atr1-1* showed reduced expression of *CYP79B2*, *CYP79B3*, and *CYP83B1*, and decreased levels of indole glucosinolates [72]. Phylogenetic analysis shows that two other MYB factors, MYB51 and MYB122, are closely related to MYB34, and that they all belong to the subgroup 12 R2R3-MYB transcription factor family [73–75]. Subsequent studies have demonstrated that MYB51 and MYB122 are also positive regulators controlling indole glucosinolate biosynthesis. For example, MYB51 overexpression lines showed elevated accumulation of indol-3-ylmethylglucosinolate and a *myb51* knockout mutant had decreased levels of indol-3-ylmethylglucosinolate [76]. Likewise, overexpression of MYB122 led to increased indol-3-ylmethylglucosinolate and indole-3-acetic acid (IAA). However, this result can only be observed in the presence of a functional MYB51, suggesting that MYB51 plays a dominant role in shoots [76]. In roots, MYB34 is a major player involved in controlling the biosynthesis of indole glucosinolates [77].

MYC2, -3, and -4 basic helix loop helix (bHLH) transcription factors are involved in plant defense response through jasmonate (JA) signaling. It was found that total glucosinolate level in the *myc2/3/4* triple mutant was less than 1% of that in *A. thaliana* wild type [78]. Another study showed that bHLH05 plays a role in indole glucosinolate biosynthesis through interaction with MYB51, and bHLH04 and bHLH06 also affect glucosinolate metabolism [75]. Another group of AP2 transcription factors involved in ethylene signaling can also affect glucosinolate biosynthesis. For example, ethylene response factor 6 (ERF6), together with its upstream kinases mitogen-activated protein kinase 3 (MPK3) and MPK6, controls the expression of MYB51, MYB122, *CYP81F2*, *IGMT1*, and *IGMT2* involved in indole glucosinolate biosynthesis and has a positive effect on plant immunity [79]. Other ERFs, including

ERF96, ERF102, and ERF107, enhance plant defense against a fungal pathogen *Verticillium longisporum* via activation of indole glucosinolate biosynthesis [80]. Furthermore, SLIM1 functions as a central transcriptional regulator controlling sulfate uptake. It represses glucosinolate biosynthesis and activates glucosinolate degradation under sulfur deficiency [67]. Moreover, IQD1 modulates expression of the genes involved in aliphatic and indole glucosinolate biosynthesis [66]. Gain- and loss-of-function *iqd1* alleles correlate with significant but mild increases and decreases in glucosinolate levels, respectively. Overexpression of *IQD1* induces transcription of the genes encoding key enzymes in indole glucosinolate biosynthesis, while genes related to aliphatic glucosinolate biosynthesis and glucosinolate degradation were downregulated [66]. The molecular mechanism underlying IQD1 transcriptional activity is still unknown. Another known transcription factor positively controlling glucosinolate biosynthesis is DNA-binding with one finger (Dof) 1.1, which can be activated by mechanical wounding and herbivore attack [81]. It was shown to regulate the transcription of *CYP83B1* in indole glucosinolate biosynthesis. Overexpression of *AtDof1* caused moderately increased levels of aliphatic and indole glucosinolates [81].

3.2. Signaling Networks in the Control of Glucosinolate Biosynthesis

Glucosinolate metabolism is responsive to many different environmental or endogenous stimuli such as sugar, pathogen challenge, herbivore damage, wounding, mineral nutrient, JA, salicylic acid (SA), ethylene, and other phytohormones [2,36,49,63–65]. Sugar signaling regulates glucosinolate biosynthesis. For example, treatment of *B. oleracea* (var. *alboglabra*) with a mixture of glucose and gibberellic acid (GA) enhanced glucosinolate production [82]. External glucose treatment of *A. thaliana* seedlings enhanced expression of *MYB34*, *MYB51*, and *MYB122* genes, and thereby increased the levels of indole glucosinolates [83]. Pathogen and bacterial flagellin peptide flg22 can activate *MYB51* and *WRKY* transcription factors and consequently increase indole glucosinolates, which are important in plant immunity [10,11,84–86]. It is well-known that cellular reactive oxygen species (ROS) and redox changes are early immune responses to pathogen infection [17,87]. Recent evidence suggests redox may play a role in glucosinolate biosynthesis. For example, GST and catalase can function as antioxidants [88]. Under mild stress conditions, *A. thaliana* mutants in glutathione synthesis had increased biosynthesis of short-chain aliphatic glucosinolates, but not the long-chain aliphatic glucosinolates [89], while mutation of GST and catalase led to glucosinolate deficiency [90]. These results are consistent with the redox regulation of IPMDH1 involved in the methionine chain-elongation process of aliphatic glucosinolate biosynthesis [35]. Redox regulation of other glucosinolate enzymes has not been reported and should be examined in the future.

Sulfur and nitrogen availability affect the type and amount of glucosinolates [91–93]. Sulfur deficiency induces sulfur deficiency induced genes (*SDI1* and *SDI2*). *SDI1* was shown to bind *MYB28* and inhibit aliphatic glucosinolate production [94]. By contrast, an increase in sulfur supply enhanced the production of both aliphatic and indole glucosinolates [95]. Use of hydrogen sulfide and sulfur dioxide can maintain glucosinolate biosynthesis, but not to the levels of sulfate [96]. Under sulfate deficiency, synchronized repression of many genes in glucosinolate biosynthesis was observed using an integrated transcriptomics and metabolomics approach [65,97]. Nitrogen is an essential element of amino acids, the precursors for glucosinolate biosynthesis. Ammonium as a nitrogen source could enhance glucosinolate biosynthesis [98]. Raphanusamic acid, a breakdown product from all glucosinolate structures, can be used to indicate increased accumulation of glucosinolates under different nutrient conditions [92]. For example, potassium deficiency led to accumulation of glucosinolates [99,100], while manganese deficiency decreased glucosinolate biosynthesis [101]. Selenium enhanced indole glucosinolate production and decreased aliphatic glucosinolates in radish [102]. However, selenium supplementation in broccoli decreased glucosinolate production due to downregulation of the genes for biosynthesis of methionine and phenylalanine [103,104]. In general, molecular mechanisms underlying the nutrient effects on glucosinolate biosynthesis are lacking.

Many external elicitors affect glucosinolate biosynthesis. For example, salt potentiates glucosinolate production in *A. thaliana* and enhances defense against pathogen infection [105]. External elicitors, including methyl jasmonate (MeJA), SA, glucose, and wounding, enhance glucosinolate production [106,107]. MeJA treatment resulted in increased levels of aliphatic and indole glucosinolates [64,108,109]. Interestingly, the expression of *MYB29* and not *MYB28*, was induced by MeJA, suggestive of its role in MeJA signaling [46]. In addition, *MYB76* was dramatically induced in response to wounding [70]. In a *myc2* mutant, MeJA-induced expression of *MYB51* was enhanced, but the MeJA-mediated *MYB34* expression was decreased, indicating that *MYC2* positively regulates MeJA-mediated *MYB34* expression and negatively regulates *MYB51* expression [110]. In addition to its association with MeJA signaling, *MYB51* plays an important role in mediating indole glucosinolate biosynthesis and defense against pathogens and herbivores. For example, *MYB51* was induced by pathogens or pathogen elicitors [111], and overexpression of *MYB51* resulted in an increased resistance to generalist herbivore [76].

Recently, a lamin-like little nuclei 1 (*LINC1*) was found to regulate JA signaling. Mutation of *LINC1* decreased expression of JA transcriptional repressor jasmonate zim-domain (*JAZ*) genes and enhanced glucosinolate production upon pathogen infection [112]. This result is consistent with the observed overexpression of glucosinolate biosynthetic genes in *A. thaliana jaz* quintuple mutant [113,114], indicating the importance of JA signaling in glucosinolate biosynthesis and plant defense [76,111,115]. With this knowledge, treatment of *Cardamine hirsuta* roots with JA stimulated glucosinolate production in the shoots and enhanced herbivore defense [116]. Similarly, JA treatment of *B. oleracea* (var. *italica*) elevated its resistance to cabbage looper (*Trichoplusia ni*) due to enhanced production of indole glucosinolates [117]. Similar results of enhanced glucosinolate production and insect defense were observed in other species [118–121]. Recently, a chloroplast retrograde signaling pathway was shown to increase glucosinolate biosynthesis and boost plant immunity through regulating JA and SA signaling [122]. Another phytohormone brassinosteroid (BR) also plays a regulatory role in glucosinolates biosynthesis as revealed from an *A. thaliana* BR-overproduction line [123,124] and a *brassinosteroid insensitive 1 (bri1)* mutant. Enhancing BR signaling abolished glucosinolate biosynthesis [123,124]. By contrast, blocking BR signaling in the *bri1* mutant showed enhanced expression of *MAM1*, *MAM3*, *BCAT4*, and *AOP2* genes and increased total glucosinolate content [125], indicating BR is a negative regulator of glucosinolate production. Absciscic acid (ABA) is also a negative regulator because an ABA-deficient mutant *aba1-1* contained more 4MI3G and exhibited aphid resistance [126]. Interestingly, under drought conditions, *A. thaliana* increased aliphatic glucosinolate levels through a transcriptional cascade mediated by the auxin-sensitive Aux/IAA repressors, thereby enhancing drought tolerance. Loss of the Aux/IAA repressors led to reduced glucosinolates and decreased drought tolerance [127]. These results highlight a novel drought response pathway through auxin signaling and glucosinolate biosynthesis.

3.3. Crosstalk of Glucosinolate Biosynthesis with Other Metabolic Pathways

Crosstalk between indole glucosinolate biosynthesis and those of indole-3-acetic acid (IAA) and camalexin has been well-studied [2,27,128,129]. IAA and camalexin are connected to indole glucosinolates through indole-3-acetaldoxime (IAOx) and indole-3-acetonitrile (IAN). When indole glucosinolate biosynthesis was blocked, increased IAA production from IAOx and IAN was observed [129]. This is a classic example of metabolic flux and pathway channeling. Many years ago, the connection between glucosinolate biosynthesis and phenylpropanoid pathway was hinted at by a study of *A. thaliana* reduced epidermal fluorescence2 (*ref2*, also known as *cyp83a1*) mutant [130]. The phenylpropanoid–glucosinolate interaction was thought to be mediated via inhibition of caffeic acid *O*-methyl transferase and caffeoyl-CoA *O*-methyl transferase by aliphatic aldoximes, thereby decreasing phenylpropanoid production [130]. Later, results from a *ref5 (cyp83b1)* mutant demonstrated that IAOx accumulation can limit the production of phenylpropanoids [131]. Recently, it was found in the *ref2* and *ref5* mutants that Kelch domain F-boxes (*KFBs*) degraded the rate-limiting enzyme

for phenylpropanoid biosynthesis, phenylalanine ammonia lyase. Disruption of the *KFBs* restored phenylpropanoid biosynthesis [21]. The crosstalk between phenylpropanoid and glucosinolate biosynthesis may be attributed to IAOx and mediator subunit 5 (MED5) [132]. In addition, flavone 3'-O-methyltransferase 1 (OMT1) responsible for methylation of 3'-hydroxy group in flavonoids [133] displayed activity for methylation of hydroxyl-indole glucosinolates [51]. In a proteomic study of *cyp79b2/b3* mutant, OMT1 was found to be connected to FMO1, suggesting its role in sulfinylglucosinolate formation [51,134]. Furthermore, the *cyp79b2/b3* mutant showed changes in the expression levels of 6,7-dimethyl-8-ribityllumazine synthase and 2-methyl-6-phytyl-1,4-hydroquinone methyltransferase, which are involved in riboflavin and tocopherol biosynthesis, respectively [134]. Another enzyme that affects glucosinolate biosynthesis is pectin acetyl esterase 9 (PAE9). Its mutation led to decreased production of glucosinolates and camalexin. PAE9 was shown to be required for constitutive upregulation of defense-related compounds, and its function involves redox reactions [135]. The regulatory mechanisms of these newly discovered cross talks are intriguing and remain to be unraveled in future studies.

4. Glucosinolate–Myrosinase System: The “Mustard Oil Bomb”

As discussed in previous sections, glucosinolates play important roles in plant response to diverse environmental factors and defense against pathogens and insects. Although intact glucosinolates may confer resistance to certain insects [14], the major roles of glucosinolate degradation products have long been recognized [136]. The enzymes catalyzing glucosinolate hydrolysis are myrosinases (β -thioglucosidases) [137,138]. The glucosinolate–myrosinase system represents a “two-component” defense system, often referred to as the “mustard oil bomb” [139]. Upon tissue damage (e.g., by insect chewing), the contact of glucosinolates with myrosinases activates the rapid generation of an unstable aglycone—a thiohydroximate-O-sulfate intermediate which undergoes elimination of the sulfate group, leading to the formation of biologically active chemicals, including nitriles, epithionitriles, thiocyanates, oxazolidine-2-thiones, and/or isothiocyanates (Figure 1) to defend against pathogens and insect herbivores [63,140]. Since the details of “mustard oil bomb” have previously been intensively reviewed [140,141], here, we will just briefly describe the system and focus on its functions in plant defense.

Now it is known that the “mustard oil bomb” contains several components, i.e., glucosinolates, myrosinases, and myrosinase-interacting proteins which include myrosinase-binding proteins (MBPs), myrosinase-associated proteins (MyAPs), and specifier proteins [140,142]. The glucosinolate degradation products from the “mustard oil bomb” have diverse biological functions [2,22,138,140], and their production depends on several factors, such as plant species, glucosinolate profiles (especially side-chain structures), reaction conditions, and hydrolyzing enzymes (e.g., pH, ferrous ions, GSH, MBP, MyAP, nitrile specifier protein (NSP), thiocyanate-forming protein (TFP), epithiospecifier modifier (ESM), and epithiospecifier (ESP)) [138,140,143] (Figure 1).

4.1. Myrosinases and Myrosinase-Interacting Proteins

Myrosinases are distributed in different types of cells like myrosin cells, guard cells, phloem associated cells, and aleurone-type cells [140,144]. The roles of a bHLH transcription factor FAMA and its interacting protein, SCREAM, in myrosin cell development as well as in guard cell differentiation support the notion that myrosinases originated in stomatal guard cells and were then co-opted into myrosin cells near the veins [144]. There are two types of myrosinases: typical (classical) and atypical. Typical myrosinases are glycosylated, use ascorbate as a cofactor, have catalytic residues QE, and take glucosinolates as the only substrates. By contrast, atypical myrosinases do not require ascorbate, have EE catalytic residues, and take glucosinolates as well as O-glucosides as substrates. In *A. thaliana*, β -thioglucoside glucohydrolase (TGG) 1–6 are typical myrosinases. Penetration 2 (PEN2), PYK10, and other β -glucosidases (BGLUs) are atypical myrosinases [138,140,141,145]. It should be noted that bacteria and aphids seem to have atypical myrosinases [138]. *TGG1* and *TGG2* are expressed

in shoots [146], while *TGG4* and *TGG5* are expressed in roots [147]. *TGG3* and *TGG6* (previously reported to be pseudogenes) were found to be expressed in specific flower tissues [148,149]. However, knowledge of their biological functions is lacking. The TGGs seem to exhibit a low degree of substrate specificity. *PEN2* and *PYK10* catalyze the hydrolysis of indole glucosinolates in plant defense [10,11,150]. Although *PEN2*-catalyzed indole glucosinolate degradation plays an important role in pathogen defense [10,11], it is not required for the protection of age-related leaf senescence [151]. Another myrosinase, *BGLU30*, is responsible for decreases of glucosinolates upon exposure to the dark [152,153]. Both *BGLU28* and *BGLU30* participate in glucosinolate degradation under plant sulfur deficiency [154]. Oxazolidinethionase was recently suggested to be involved in glucosinolate hydrolysis by converting 1,3-oxazolidine-2-thione to 1,3-oxazolidin-2-ones [155]. However, its interaction with myrosinases has not been tested. Several myrosinase-interacting proteins have been shown to affect the formation of different glucosinolate degradation products [140]. For example, a MyAP-like ESM was found to favor isothiocyanate production and protect *A. thaliana* from herbivory [156]. However, there has been little evidence for complex formation between myrosinases and their interacting proteins in the reference plant. Systematic studies to characterize the protein interactions, signaling pathways that regulate myrosinase activities, and in vivo glucosinolate turnover/degradation products are needed for understanding the specific functions of the “mustard oil bomb” in different biological contexts.

4.2. “Mustard Oil Bomb” in Bacterial and Fungal Pathogen Defense

The role of “mustard oil bomb” in insect defense has been studied for more than 100 years, while its role in plant pathogen defense was only realized about a decade ago [10,11,157]. Physical barriers like the cuticle on plant surfaces represent an important plant defense system against pathogens. *A. thaliana* myrosinase *tgg* mutants showed disrupted and irregular cuticles. This phenotype correlates with decreased levels of fatty acids and their phytol esters, glucosinolates, and indole compounds in the *tgg1 tgg2* mutant [158]. However, the direct link between myrosinases and cuticle development is not known. Recently, *A. thaliana* polyunsaturated fatty acid mutants were found to display a cuticle permeability defect and strong resistance to a necrotrophic fungus, *Botrytis cinerea*. Large amounts of 7-methylsulfonylheptylglucosinolate (7MSOP) were found on the plant surface [159]. It is not clear if 7MSOP may affect cuticle composition and permeability and whether 7MSOP degradation plays a role in fungal resistance. Stomatal pores on the leaf surface represent the major entry points of bacterial invasion, and both myrosinases and glucosinolates are present in stomatal guard cells [140]. Overexpression of *TGG1* promotes stomatal closure and delays stomatal opening as an immune response against *Pseudomonas syringae* [160]. Unlike insect damage-triggered activation by myrosinase, glucosinolates are actively turned over in plant cells and recruited for broad-spectrum antifungal defense [10,161]. Upon fungal attack of *A. thaliana*, *CYP81F2* catalyzes the accumulation of 4MI3G, which is hydrolyzed by the atypical *PEN2* myrosinase. The hydrolysis products are then transported to the cell periphery at the fungal entry sites by a *PEN3* plasma membrane-resident ABC transporter for antifungal defense [10]. Although this study discussed the potential implication of indole-3-acetonitrile, it did not characterize the bioactive degradation product. Recently, glucosinolate-derived isothiocyanates were found to impact mitochondrial function in fungal cells [161]. Therefore, it is important to profile the glucosinolate degradation products in plant pathogen defense. Another study showed that the *PEN2*- and *PEN3*-dependent 4MI3G metabolism is also required for the microbe-associated molecule pattern (MAMP)-triggered defense response, highlighting the link of glucosinolate hydrolytic products as signaling molecules in plant innate immune response to both adapted and non-adapted microbial pathogens [11]. *PEN2* mutation led to accumulation of indole glucosinolates [162,163] and, consequently, this mutation prevented plant cells from programmed cell death triggered by flg22 due to the failure to form glucosinolate hydrolysis products [163]. It is intriguing how pathogens and MAMP specifically activate *PEN2*, but not TGGs in this study. Clearly, *TGG1* plays a role in stomatal immunity [160], highlighting the existence of cell-type specific regulations.

The action of glucosinolate degradation in plant defense is affected/regulated by many factors. GSH is not only involved in glucosinolate biosynthesis (Figure 1) but also affects glucosinolate breakdown product formation. Recently, GST U13 was identified as an indispensable component of the PEN2–indole glucosinolate immune pathway. It transfers glutathione to form complexes with unstable breakdown products to yield different protective metabolites against pathogens [25] (Figure 1). In addition, zinc was shown to increase sulfur assimilation, amino acid biosynthesis and, consequently, glucosinolate production [164], and enhance plant immune response [165,166]. Furthermore, iron deficiency induced the expression of a bHLH transcription factor IAA-LEUCINE RESISTANT3 (ILR3), which triggers the expression of genes involved in long-chain glucosinolate biosynthesis. The increase in the glucosinolate levels correlated with enhanced pathogen defense [167]. Moreover, soil type and biofumigation have been shown to be important in “mustard oil bomb”-mediated pathogen defense. For example, sandy soil enhanced the toxic effect of allyl isothiocyanate on a *Meloidogyne hapla* nematode [168]. Crop rotation with *Brassica* species and mixtures of rapeseed and mustard have been used in agriculture practice to control plant pathogens, utilizing the “mustard oil bomb” [169,170]. Despite many studies focusing on indole glucosinolates and their degradation products, aliphatic glucosinolates can also be important in plant defense against specific pathogens. The fungus *B. cinerea* with a broad plant host range showed variable sensitivity to different glucosinolates and their hydrolysis products [171]. Interestingly, a Brassicaceae-specific fungus *Alternaria brassicicola* has developed adaptation to indole glucosinolates and can cope with their hydrolysis products, but it was strongly deterred by aliphatic isothiocyanates derived from aliphatic glucosinolate breakdown [171]. Therefore, glucosinolate engineering for plant pathogen defense needs to consider the pathogen species and the bioactive metabolites concerned.

5. Role of the “Mustard Oil Bomb” in Plant Insect Defense

Insect herbivores cause more than 20% yield loss to agricultural crops worldwide despite the increased use of pesticides [172]. Crop damage is mostly caused by insects chewing on leaves, fruits, or shoots/roots. In addition, phloem-sucking insects can open entry points for infection by bacteria, fungus, and viruses. For example, citrus greening disease is caused by *Candidatus Liberibacter* transferred by phloem-feeding psyllids. This disease has almost devastated the Florida citrus industry. The “mustard oil bomb” has specifically evolved in Brassicales to defend against insect herbivores (Figure 3). The pungent glucosinolate degradation products repel insect herbivores. For example, isothiocyanate derived from 2-propenylglucosinolate inhibits the gut cathepsin protease of a corn earworm (*Helioverpa armigera*), causing decreased growth and death of the insect [173]. Although insect feeding habits span the entire range from monophagous to highly polyphagous, many studies contrast selected “specialist” and “generalist” insects for simplicity. However, although many glucosinolate-adapted insects are restricted to glucosinolate-containing plants, they are rather polyphagous within that group of ca. 4700 species. These highly glucosinolate-specialized insects combine metabolic handling of the “mustard oil bomb” with behavioral adaptations such as use of glucosinolates and hydrolysis products as cues for oviposition and feeding. This is the case for the discussed *Pieris* and *Plutella* species. In this section, we will briefly describe examples of the adaptations of highly glucosinolate-adapted insects to the “mustard oil bomb”, followed by a general discussion of glucosinolate effects on insects with a generalist type of host plant range, not restricted to glucosinolate-containing plants.

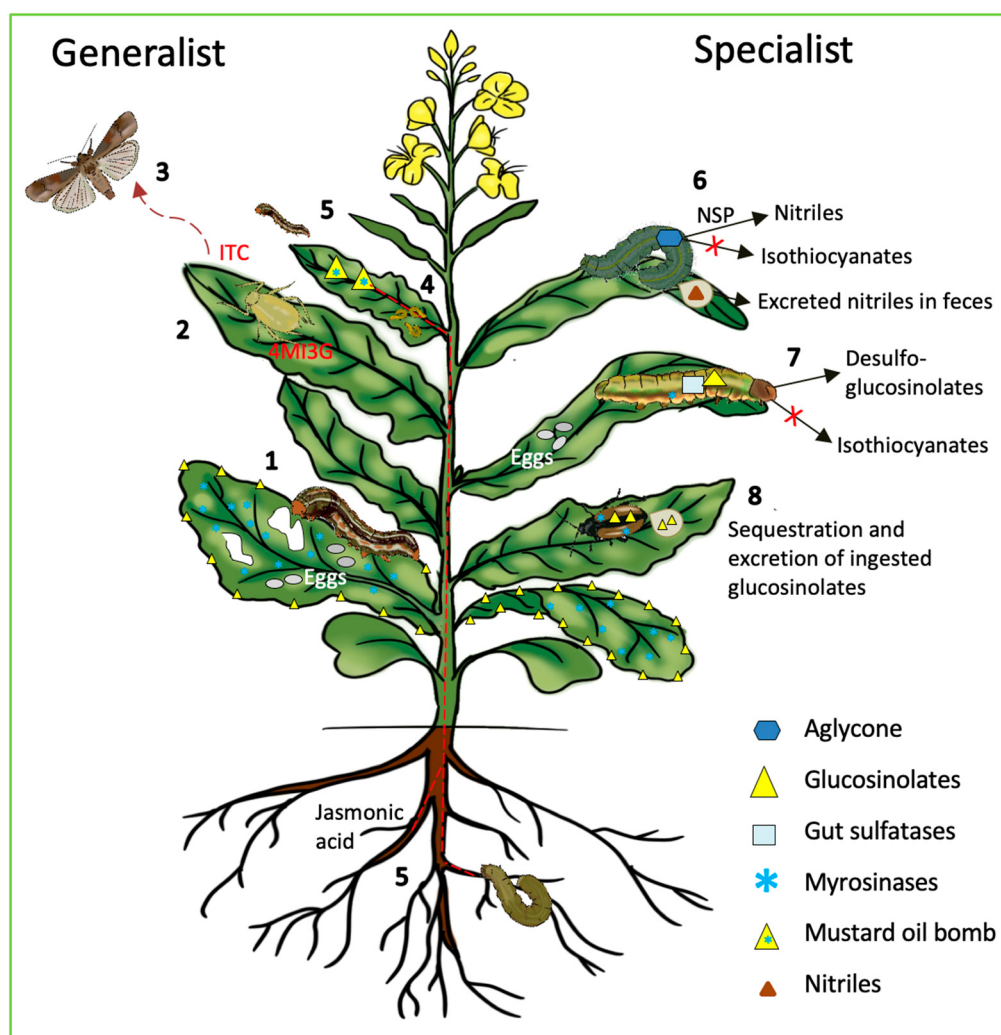


Figure 3. Role of the “mustard oil bomb” in plant interactions with generalist and specialist insects. **Generalist insects:** (1) Insects adjust their oviposition and feeding away from glucosinolates and myrosinases to avoid triggering the “mustard oil bomb”; (2) Aphid feeding leads to increased synthesis of 4-methoxyindol-3-ylmethylglucosinolate (4MI3G); (3) Volatile isothiocyanate (ITC) repels insects and provides protection to plants; (4) Increase in glucosinolates affects larval growth and development; (5) Systemic defense/priming by jasmonic acid treatment of the roots or root herbivory repels insects from attacking leaves. **Specialist insects:** (6) Conversion of unstable aglycone from the “mustard oil bomb” to nitriles (less toxic than ITC) in feces in the presence of nitrile specifier protein (NSP); (7) Conversion of glucosinolates to desulfoglucosinolates (by insect sulfatase) that cannot be hydrolyzed by myrosinases; (8) Sequestration and excretion of ingested glucosinolates before being broken down to form toxic products.

5.1. Disarming the “Mustard Oil Bomb”

During thousands of years of evolution, specialist insects have developed different strategies to disarm the “mustard oil bomb” in the Brassicales plants (Figure 3). One strategy is to convert glucosinolates to desulfoglucosinolates that cannot be hydrolyzed by myrosinases. Specialist insects like the diamondback moth (*Plutella xylostella*) have sulfatases in their gut, which hydrolyze the sulfate ester bond of glucosinolates and prevent formation of toxic hydrolysis products (e.g., isothiocyanates) [139,174]. Over 80% of glucosinolates were converted to the desulfo form after ingestion, thereby enhancing insect reproductive capability [175]. Another strategy used by specialist insects is the production of nitriles of lower toxicity than isothiocyanates. The unstable aglycone

intermediates are directed to produce nitriles instead of isothiocyanates by NSP [176] (Figure 1a). A third strategy used by a group of insects is to rapidly absorb glucosinolates in their digestive tracts and excrete out before being hydrolyzed by myrosinases [136]. For example, the horseradish flea beetle (*Phyllotreta armoraciae*) can quickly eliminate ingested glucosinolates via sequestration and excretion [177]. With these adaptive strategies, specialist insects may seek plants/tissues with high glucosinolate contents, which provide them with a competitive edge. It should be noted that plants can still mount a defense against the specialists. For example, the diamondback moth is very destructive to most cruciferous plants, but not to plants like wintercress (*Barbarea vulgaris*). In addition to glucosinolates, the *Barbarea* plants produce triterpenoid saponins, which are toxic to the insects [178,179]. Another way to overcome insect detoxification mechanisms is plant-mediated RNAi. Silencing of a GST important for glucosinolate detoxification in a phloem-feeding whitefly (*Bemisia tabaci*) was successful in eliminating the pest [180].

5.2. “Mustard Oil Bomb” in Tug of War between Plants and Insects

Plant–insect interactions have been a constant tug-of-war. When plants encounter insect feeding (e.g., the green peach aphid (*Myzus persicae*)), they increase the synthesis of 4MI3G from indol-3-ylmethylglucosinolate in the leaves [14]. This response provides protection to the plants even in the absence of myrosinases by affecting fecundity of the aphid [14]. On the other hand, although generalists do not have the same adaptive strategies as the specialists, they adjust their oviposition and feeding behavior to avoid detonating the “mustard oil bomb” (Figure 3). In *A. thaliana*, glucosinolates are allocated mostly in outer lamia to create a barrier for insects that usually approach from leaf edges. The larvae of corn earworm (*Helicoverpa zea*) prefer feeding from the inner lamia of leaves compared to the outer side [181].

In general, glucosinolate-producing plants were found to boost indole glucosinolate biosynthesis as a defense response to insect herbivores. Aliphatic and benzenic glucosinolates often show minor increases or even decreases [157,182]. This may be attributed to activation of the JA signaling cascade by the herbivory and/or wounding, which induces expression of indole glucosinolate biosynthetic genes and eventually increased indol-3-ylmethylglucosinolate and 4MI3G levels. However, a recent study with two generalist caterpillars, the African cotton leafworm (*Spodoptera littoralis*) and the cabbage moth (*Mamestra brassicae*) showed that *A. thaliana* aliphatic glucosinolates have a greater negative effect on larval growth and development than indole glucosinolates [175]. Unfortunately, this study did not determine changes of aliphatic and indole glucosinolates in the plants after the caterpillar attack. Therefore, the results may be considered controversial. It is possible that indole glucosinolates have an inhibitory effect at high doses when they are highly induced. On the other hand, engineering aliphatic glucosinolates may be more effective for insect defense.

Interestingly, plants seem to have developed “memory”, also called a prime response from previous incidences of herbivore attacks, which prepares plants to respond strongly and more rapidly following the next attack [116,183–185]. In addition, shoot glucosinolate levels increased upon attack by underground insect herbivory, providing systemic resistance [186,187]. Root JA treatment had a similar effect on enhancing shoot defense against aboveground herbivore challenge [116]. Chewing insects like caterpillars induce JA and glucosinolate biosynthesis in plants [188,189] through activating expression of genes related to JA and ethylene pathways, but not the SA pathway [190,191]. The molecular mechanisms underlying the priming and systemic effects might involve long-distance signaling molecules, but they are not currently known. However, such priming mechanisms may not be universal. Despite high glucosinolate levels in Chinese cabbage (*B. pekinensis*), the green peach aphid (*M. persicae*) grew much better on pre-infested plants [192]. These pre-infested cabbages had a high amino acid to sugar ratio in the phloem sap and high amino acid concentration in plant leaves [192]. Thus, nutrition acquisition seemed to play a larger role in aphid colonization than avoiding plant defense. This explanation is supported by the preference of *M. persicae* to grow on young leaves with

more amino acids, carbohydrates, and glucosinolates rather than old leaves with low glucosinolate contents [193,194].

Compared to chewing insects, which may induce changes in both aliphatic and indole glucosinolates (mostly), phloem-feeding aphids caused specific increase of indole glucosinolates in aphid feeding tissues [14]. On the other hand, the green peach aphid showed impaired growth when fed on an artificial diet containing indole glucosinolates [14]. In addition, phloem-feeding insects have more involvement of SA compared to JA [191,195]. Because of the phloem-feeding guild, these insects may be able to minimize contact with myrosinase by avoiding myrosin cells and, hence, their vulnerability to the “mustard oil bomb” [139,196]. Considering drought stress and many other environmental factors affect plant glucosinolate biosynthesis and levels [197,198], environment, pest, and plant constitute a pest disease triangle. Therefore, studying plant insect interactions under different environmental conditions is essential, and the results can have close-to-real-life applications.

In addition to glucosinolate increases following insect attack, myrosinase levels also showed increases after insect herbivore feeding [199]. Although it remains unknown whether the myrosinase level is a limiting component in the plant “mustard oil bomb”, the decrease in myrosinases of the *A. thaliana* *tgg1 tgg2* mutant significantly increased generalist growth. As for specialists, the mutant did not have affected feeding nor reduced insect performance due to low feeding stimulants from glucosinolate degradation [146]. Clearly, myrosinase activity is important in plant insect defense. Attack of *B. rapa* by the specialist herbivore *P. xylostella* increased myrosinase activity, but generalist cabbage moth (*M. brassicae*) larval feeding caused a significant decrease of myrosinase activity in *B. napus* [138]. Suppression of myrosinase activity may be an effective way for insects to avoid detonating the “mustard oil bomb”. Myrosinase activity may be regulated by glycosylation, phosphorylation, and redox environment [54,200]. Investigating myrosinase posttranslational modifications, activity regulation, and protein complex formation with myrosinase-interacting proteins during plant defense against insects is an exciting future research direction and will generate novel insights.

6. Conclusions

This review summarizes the current knowledge of glucosinolate biosynthesis and regulation as an important natural plant defense system. It highlights how specialized metabolites can be modulated by different internal and external factors, including signaling molecules, nutrients, metabolic regulation/crosstalk, pathogens, as well as insect herbivores. Moreover, it describes the plant “mustard oil bomb” and how it functions in plant defense against pathogens, generalist and specialist insects, and how insects avoid and/or disarm the “mustard oil bomb”. In spite of current progress, there still exist many knowledge gaps regarding molecular networks regulating the “mustard oil bomb”, functions of different myrosinase-interacting proteins, mobile signals underlying the priming mechanism, myrosinases, and other mechanisms evolved in insects to deal with this particular plant defense system. Future systemic studies harnessing the power of multi-omics will help to connect the missing dots not only in the plant cells but also in the pest disease triangle that includes plants, pests, and environmental factors. As the frontier of biological research moves into the single-cell level, studying plant defense at a single-cell resolution will have paramount significance, especially considering the cell type-specific organization of the “mustard oil bomb”. Although this defense system is largely limited to plants in the order Brassicales, its scientific value and potential agricultural/ecological impacts should be recognized. In addition, improved understanding of the “mustard oil bomb” in the framework of the plant disease triangle will allow us to harness the benefits of this group of natural specialized metabolites through synthetic biology for human health and crop disease/pest control. For example, this knowledge may facilitate production of the cancer-preventive glucosinolates in different organisms (e.g., yeast and vegetables), and the transfer of these natural defense compounds to non-glucosinolate-producing crops (in a cell and/or tissue-specific manner) to enhance disease resistance without compromising crop yield. In addition, it may help reduce the use of chemical pesticides, and thereby have a positive impact on the environment and human health.

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