Biology and Biochemistry of Glucosinolates

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Annu. Rev. Plant Biol.
2006. 57:303–33

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

doi: 10.1146/annurev.arplant.57.032905.105228

First published online as a Review in Advance on January 30, 2006

1543-5008/06/0602-0303$20.00

Key Words

metabolic engineering, biosynthesis, degradation, regulation, transport, defense

Abstract

Glucosinolates are sulfur-rich, anionic natural products that upon hydrolysis by endogenous thioglucosidases called myrosinases produce several different products (e.g., isothiocyanates, thiocyanates, and nitriles). The hydrolysis products have many different biological activities, e.g., as defense compounds and attractants. For humans these compounds function as cancer-preventing agents, biopesticides, and flavor compounds. Since the completion of the *Arabidopsis* genome, glucosinolate research has made significant progress, resulting in near-complete elucidation of the core biosynthetic pathway, identification of the first regulators of the pathway, metabolic engineering of specific glucosinolate profiles to study function, as well as identification of evolutionary links to related pathways. Although much has been learned in recent years, much more awaits discovery before we fully understand how and why plants synthesize glucosinolates. This may enable us to more fully exploit the potential of these compounds in agriculture and medicine.

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INTRODUCTION

Glucosinolates: mustard oil glucosides

Glucosinolates, once known as mustard oil glucosides, have been part of human life for thousands of years because of the strong flavors and tastes they elicit in cabbage, broccoli, and other *Brassica* vegetables. In the past few decades, the importance of these nitrogen- and sulfur-containing plant secondary metabolites has increased further following discovery of their potential as cancer-prevention agents, crop-protection compounds, and biofumigants in agriculture. Moreover, the presence of glucosinolates in the model plant, *Arabidopsis thaliana*, has also helped to stimulate a vigorous research effort into these unusual amino acid–derived products. For such a widely studied group of plant compounds, glucosinolates are known from only a few angiosperm families. They have been reported almost exclusively from the order Capparales, which contains 15 families, including the Brassicaceae, Capparaceae, and Caricaceae (144). Curiously, glucosinolates are also known from the genus *Drypetes* of the family Euphorbiaceae, a genus completely unrelated to the other glucosinolate-containing families.

Chemical Structure and Hydrolysis

The approximately 120 described glucosinolates share a chemical structure consisting of a β-D-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester, plus a variable R group (**Figure 1**) derived from one of eight amino acids (49). Glucosinolates can be classified by their precursor amino acid and the types of modification to the R group. Compounds derived from Ala, Leu, Ile, Met, or Val are called aliphatic glucosinolates, those derived from Phe or Tyr are called aromatic glucosinolates, and those derived from Trp are called indole glucosinolates. The R groups of most glucosinolates are extensively modified from these precursor amino acids, with methionine undergoing an especially wide range of transformations (49). Most of the R groups are elongated by one or more methylene moieties. Both elongated and nonelongated R groups are subject to a wide variety of transformations, including hydroxylation, *O*-methylation, desaturation, glycosylation, and acylation.

Plants accumulating glucosinolates always possess a thioglucoside glucohydrolase

activity known as myrosinase, which hydrolyzes the glucose moiety on the main skeleton (140). The products are glucose and an unstable aglycone that can rearrange to form isothiocyanates, nitriles, and other products. Hydrolysis in intact plants appears to be hindered by the spatial separation of glucosinolates and myrosinase or the inactivation of myrosinase, but these components mix together upon tissue damage, leading to the rapid formation of glucosinolate hydrolysis products. Most of the biological activities of glucosinolates are attributed to the actions of their hydrolysis products (170).

Importance to Humans

Glucosinolates have long been of interest to human society because of their presence in certain Brassicaceae vegetables (cabbage, cauliflower, broccoli) and condiments (mustard, horseradish, wasabi). The distinct taste and flavors of these foods are due primarily to their isothiocyanate hydrolysis products. Indole glucosinolates and those with alkenyl R groups are especially known for causing bitterness (46).

In the past 30 years, glucosinolates have assumed major agricultural significance with the increasing importance of rapeseeds, cultivars of *Brassica napus*, *B. rapa*, and *B. juncea*, as oil crops in temperate and subtropical areas of the world. These species contain glucosinolates in all of their organs. However, plant breeders have drastically reduced the levels of seed glucosinolates to allow the protein-rich seed cake (the residue left after crushing for oil) to be sold as an animal feed supplement. One of the predominant rapeseed glucosinolates, 2-hydroxy-3-butenyl glucosinolate (**Figure 1**), forms a oxazolidine-2-thione upon hydrolysis that causes goiter and has other harmful effects on animal nutrition (63). Breeders have attempted to modify glucosinolate levels in rapeseed foliage to reduce damage from fungal and insect pests (122). In this case, the strategy is not as simple because glucosinolates and their hydrolysis products

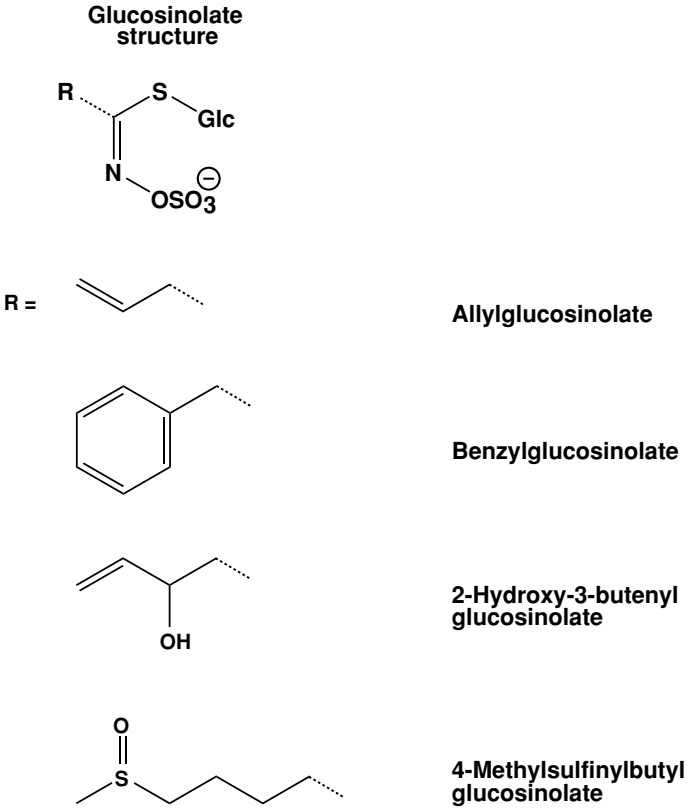

Figure 1

Chemical structure of glucosinolates. The common structure is shown, as well as examples of some specific glucosinolates cited in the text that show typical variation in the structure of the side chain.

are repellent to some insects, but often serve as attractants for others. *Brassica* cultivars are finding increased use for "biofumigation," in which harvested plant material is incorporated into agricultural soils to suppress pathogens, nematodes, and weeds (22, 164, 174). Here again glucosinolate hydrolysis products are assumed to be the active agents of the treatment.

Myrosinase: β-thioglucosidase

In the past decade, certain glucosinolates have been identified as potent cancer-prevention agents in a wide range of animal models due to the ability of certain hydrolysis products to induce phase II detoxification enzymes, such as quinone reductase, glutathione-*S*-transferase, and glucuronosyl transferases (72b, 81). Sulforaphane, the

isothiocyanate derivative of 4-methylsulfinylbutyl glucosinolate (**Figure 1**), found in broccoli, has been the focus of many of these studies (176). Sulforaphane and other isothiocyanates may prevent tumor growth by blocking the cell cycle and promoting apoptosis (81, 107, 155). Moreover, sulforaphane exhibits potential for treating *Helicobacter pylori*-caused gastritis and stomach cancer (48). These results are motivating efforts to increase the sulforaphane content of broccoli and to promote the health benefits of this vegetable.

BIOSYNTHESIS

The formation of glucosinolates can be conveniently divided into three separate phases. First, certain aliphatic and aromatic amino acids are elongated by inserting methylene groups into their side chains. Second, the amino acid moiety itself, whether elongated or not, is metabolically reconfigured to give the core structure of glucosinolates. Third, the initially formed glucosinolates are modified by various secondary transformations.

BIOSYNTHESIS: AMINO ACID CHAIN ELONGATION

The sequence of the chain-elongation pathway for amino acids participating in glucosinolate biosynthesis is based on in vivo feeding studies, the demonstration of enzyme activities in vitro, and the isolation of key intermediates. Initially, the parent amino acid is deaminated to form the corresponding 2-oxo acid (**Figure 2**). Next is a three-step cycle in which (*1*) the 2-oxo acid condenses with acetyl-CoA to form a substituted 2-malate derivative, which then (*2*) isomerizes via a 1,2-hydroxyl shift to a 3-malate derivative that (*3*) undergoes oxidation-decarboxylation to yield a 2-oxo acid with one more methylene group than the starting compound. During each round of the elongation cycle, the two carbons of acetyl-CoA are added to the 2-oxo acid and the COOH group added in the previous round is lost, for a net gain of one carbon atom. After each turn of the cycle, the extended 2-oxo acid can be transaminated to form the corresponding amino acid and enter the second phase of glucosinolate formation. Or, it can undergo additional cycles of acetyl-CoA condensation, isomerization, and oxidation-carboxylation, resulting in further elongation. Up to nine cycles are known to occur in plants (49). Similar 2-oxo acid–based chain-elongation sequences occur in leucine biosynthesis and in the TCA cycle, as well as elsewhere in plant metabolism (89).

The earliest evidence for the chain-elongation pathway came from feeding studies with radiolabeled precursors beginning in the 1960s (39, 97). More recent in vivo studies with stable isotopes (61, 62) confirmed the outline and major intermediates of the pathway. Most critical was the observation that acetate was readily incorporated into chain-elongated amino acids with the additional methylene group derived exclusively from the C-2 position (acetate methyl group). The acetate carboxyl group is lost during chain elongation or during conversion into the core glucosinolate. Additional support for the chain-elongation pathway was provided by the detection of certain intermediates in the chain elongation of methionine (32) and phenylalanine (43) and by the isolation of the chain-elongated methionine homologs themselves (69). Furthermore, the activity of the aminotransferase producing the initial 2-oxo acid from methionine (33, 60) and the activity of the condensing enzyme of the first round of methionine chain elongation (50) have been demonstrated in cell-free extracts.

The first information about the genetic basis of chain elongation came from the identification of a locus in *Arabidopsis* and *Brassica napus* that controls the chain length of methionine-derived glucosinolates (111). This locus was mapped in *Arabidopsis* using a cross between two ecotypes, Columbia and Landsberg *erecta*, whose major glucosinolates are derived from dihomomethionine and homomethionine, respectively (29). The

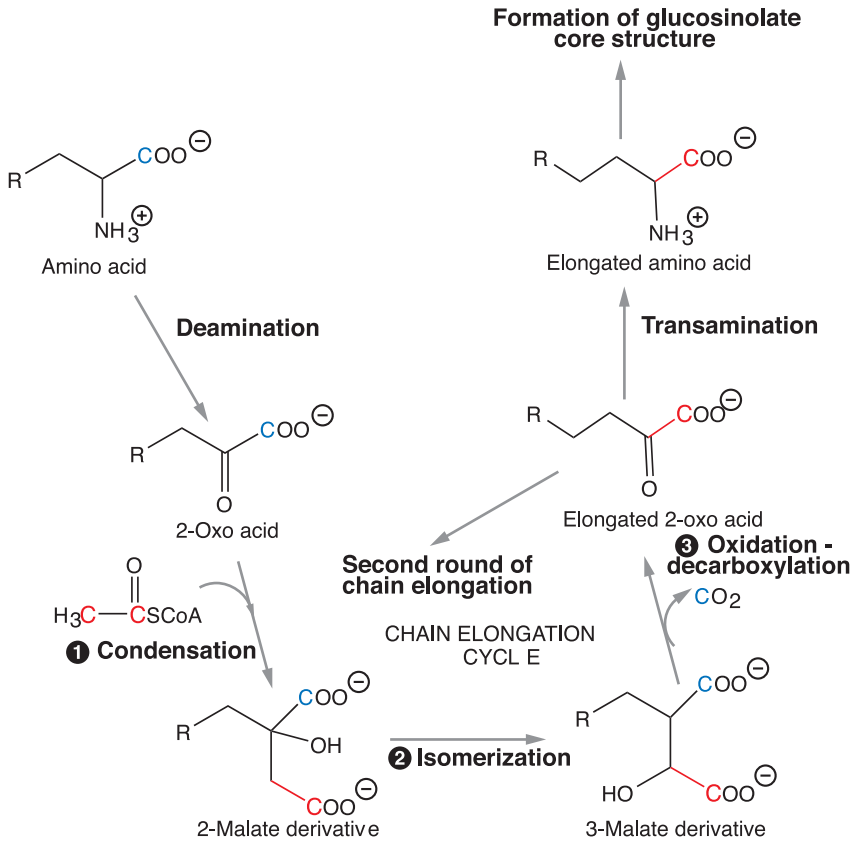

Figure 2

Amino acid chain-elongation cycle for glucosinolate biosynthesis. Illustrated is the first round of elongation. The three principal steps are: (*1*) condensation with acetyl-CoA, (*2*) isomerization, and (*3*) oxidation-decarboxylation. The carbon atoms contributed by acetyl-CoA (retained with each round) are shown in red. The carbon atom from the original COOH function (lost with each round) is shown in blue.

candidate genes were two adjacent sequences with high similarity to genes encoding isopropylmalate synthase, the enzyme catalyzing the condensing reaction of chain elongation in leucine biosynthesis. Further fine-scale mapping identified one of the two genes, *MAM1* (*Methylthioalkylmalate synthase 1*), as responsible for the chain-elongation polymorphism in Columbia and Landsberg *erecta* (91). This finding was confirmed by the isolation of missense mutants for this gene that had altered glucosinolate chain-length profiles and the heterologous expression of *MAM1* in *E. coli*, which gave an extract capable of condensing ω-methylthio-2-oxoalkanoates with acetyl-CoA to give 2-(ω-methylthioalkyl)malates. The *MAM1* gene product carried out the condensing reaction of only the first two methionine elongation cycles (152), suggesting that the second adjacent sequence (called *MAM-L* for "MAM-like") might encode the protein responsible for the remaining activities. Indeed, a *MAM-L* knockout line was recently reported to lack long-chain methionine-derived glucosinolates, but these were restored after transformation with a functional *MAM-L* gene (51). A survey of *Arabidopsis* ecotypes revealed the presence of a third *MAM*-like gene,

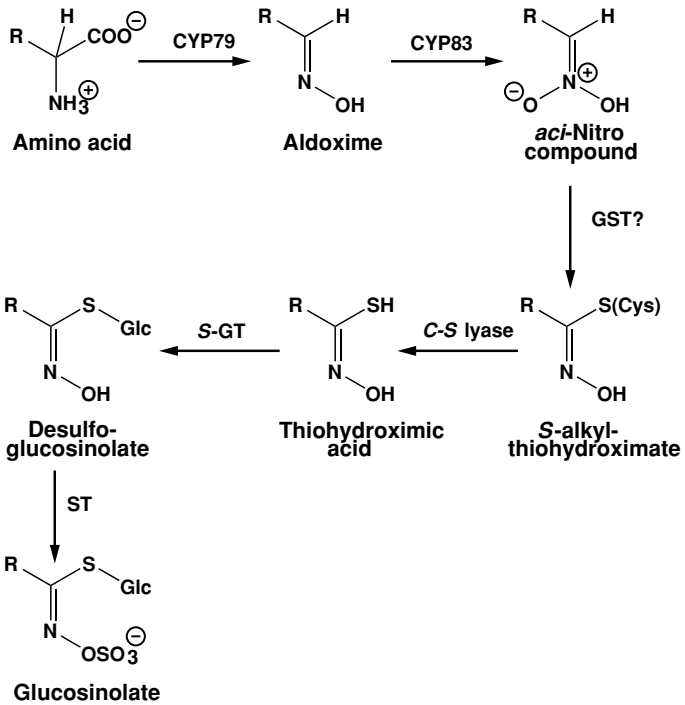

Figure 3

Biosynthesis of the glucosinolate core structure. CYP79 enzymes catalyzing the conversion of amino acids to aldoximes are the only side-chain-specific step in the pathway. The products from the CYP83s are too reactive to be isolated, but are proposed to be either *aci*-nitro compounds or their dehydrated analogs, nitrile oxides. The sulfur-donating enzyme is the only enzyme that remains to be identified, and is proposed to be a glutathione-*S*-transferase-like enzyme that uses cysteine as substrate. Abbreviations: R, variable side chain; GST, glutathione-*S*-transferase; *S*-GT, *S*-glucosyltransferase; ST, sulfotransferase.

designated *MAM2* at the same locus (90). The majority of ecotypes examined possessed functional copies of either *MAM1* or *MAM2*, but not both. A functional *MAM1* sequence was correlated with accumulation of glucosinolates having undergone two rounds of chain elongation, whereas a functional *MAM2* sequence was correlated with accumulation of glucosinolates having undergone only one round of elongation. Our knowledge of amino acid chain elongation has advanced rapidly in the past five years, yet much more information about the genes and enzymes of this segment of glucosinolate biosynthesis is necessary before we can fully understand how it is regulated and how substrates from primary metabolism are channeled to the core pathway of glucosinolate formation.

BIOSYNTHESIS: CORE STRUCTURE

The biosynthesis of the core glucosinolate structure involves intermediates common to all glucosinolates. Our knowledge on how amino acids are converted into the core glucosinolate structure has increased as research has advanced from traditional in vivo feeding studies and biochemical characterization of the enzymatic activities in plant extracts to identification and characterization of the biosynthetic genes encoding the enzymes. Here the presence of glucosinolates in the model plant *Arabidopsis* has greatly facilitated progress. The intermediates in the pathway from the amino acid to the core structure include *N*-hydroxy amino acids, aldoximes, *aci*-nitro or nitrile oxide compounds (both are too reactive to be isolated), *S*-alkyl thiohydroximates, thiohydroximic acids, and desulfoglucosinolates (**Figure 3**). The genes responsible for all these steps, except the *S*-alkylation, have been identified since 2000.

The Conversion of Amino Acids to Aldoximes

Cytochromes P450 belonging to the CYP79 family are responsible for catalyzing the conversion of amino acids to aldoximes (169). Most of the seven CYP79s in the glucosinolate pathway in *Arabidopsis* were identified using a functional genomics approach (66). This was based on the similarity of the biosynthetic pathways of glucosinolates and cyanogenic glucosides, another group of amino acid–derived natural products with aldoximes as intermediates (78). As CYP79 homologs were identified in the *Arabidopsis* genome project, they were heterologously expressed and characterized with respect to substrate specificity (66).

The function of some CYP79 genes was identified using other approaches. A screen in yeast for cDNAs conferring resistance to 5-fluoroindole (the precursor of a toxic tryptophan derivative) led to isolation of CYP79B2 (73), which together with the homolog CYP79B3 catalyze the conversion of tryptophan to indole-3-acetaldoxime (IAOx) (73, 117). A *cyp79B2/cyp79B3* double knockout is completely devoid of indole glucosinolates (178), which shows that no other source of IAOx contributes significantly to biosynthesis of indole glucosinolate. Accordingly, the plasma membrane-bound peroxidase-dependent conversion of tryptophan to IAOx (105, 106), and IAOx produced from the YUCCA pathway (177), are not involved in glucosinolate biosynthesis.

In independent genetic approaches, two mutants, *bushy* (143) and *supershoot* (150), with severe morphological alterations including several hundred axillary shoots were shown to be knockout mutants of *CYP79F1*. These mutants completely lack short-chain aliphatic glucosinolates (143). Based on this finding, it was suggested that CYP79F1 metabolizes the short-chain methionine derivatives (with one to four additional methylene groups), and that the homolog *CYP79F2* that is 88% identical at the amino acid level metabolizes the long-chain-elongated methionine derivatives (143). However, biochemical characterization of CYP79F1 and CYP79F2 showed that CYP79F1 metabolizes mono- to hexahomomethionine, resulting in both short- and long-chain aliphatic glucosinolates, whereas CYP79F2 exclusively metabolizes long-chain penta- and hexahomomethionines (34, 69). The substrate specificities of CYP79F1 and CYP79F2 explain the absence of short-chain aliphatic glucosinolates in a knockout mutant of *CYP79F1*, and why the level of short-chain aliphatic glucosinolates is not affected in a *CYP79F2* knockout mutant, whereas the level of long-chain aliphatic glucosinolates is substantially reduced (34). The results emphasize the importance of biochemical characterization of proteins because assignment of function based solely on genetic data can be misleading.

The five characterized CYP79s in *Arabidopsis* (Col-0) are responsible for aldoxime production in the biosynthesis of the major glucosinolates derived from tryptophan (CYP79B2/CYP79B3) and chain-elongated methionine derivatives (CYP79F1/CYP79F2), as well as from phenylalanine (CYP79A2) (169). However, the role of CYP79C1 and CYP79C2 is unknown. These transcripts are present at very low levels (U. Wittstock and B.A. Halkier, unpublished results), which suggests that the CYP79C homologs may be responsible for aldoxime production of low abundant glucosinolates such as those derived from, e.g., homophenylalanine, methionine (86), and tyrosine (35). It can, however, not be excluded that the in vitro activities of the recombinant CYP79s could differ from their in vivo activities, and that CYP79F1 can metabolize methionine, for example, or that CYP79A2 can convert homophenylalanine and possibly tyrosine, albeit at very low efficiency.

IAOx: indole-3-acetaldoxime

The Conversion of Aldoximes to Thiohydroximic Acids

The aldoxime-metabolizing enzyme CYP83B1 in the glucosinolate pathway of *Arabidopsis* has been identified by several approaches (11, 12, 42, 68, 149). Knockout mutants of *CYP83B1* have a characteristic high-auxin phenotype (see below). Biochemical characterization of CYP83B1 and its homolog CYP83A1 shows that aliphatic aldoximes are primarily metabolized by CYP83A1 (8, 128), whereas aromatic aldoximes derived from tryptophan, phenylalanine, and tyrosine are metabolized by both enzymes. CYP83B1 has higher affinity for these aromatic aldoximes than CYP83A1, particularly for IAOx, where there is a 50-fold difference in K_m value (8), indicating that CYP83A1 and CYP83B1 are not redundant under normal physiological conditions in the plant. Interestingly, a *cyp83A1* knockout

mutant was identified in a screen for plants having altered phenylpropanoids as it contains reduced levels of several phenylpropanoids, such as sinapoylmalate, suggesting a metabolic link between glucosinolate biosynthesis and phenylpropanoid metabolism (71). As expected, *cyp83A1* knockout mutants have reduced levels of aliphatic glucosinolates, but also increased levels of indole glucosinolates. The latter may be due to upregulation of *CYP79B2* and *CYP79B3* in the metabolically stressed plant.

The CYP83 enzymes produce an activated, oxidized form of the aldoxime, e.g., an *aci*-nitro compound or a nitrile oxide (**Figure 3**). Due to its instability, the product has not been isolated, but in vitro it reacts efficiently with nucleophilic S-donors to form *S*-alkyl thiohydroximates (11, 68). This suggests that conjugation with cysteine, the likely S-donor as evidenced by in vivo feeding studies (166), is enzymatically controlled, possibly by a glutathione-*S*-transferase-like enzyme to ensure conjugation of the proper S-donor in vivo.

In vitro, *S*-(hydroximoyl)-L-cysteine conjugates rapidly undergo internal cyclization to produce 2-substituted thiazoline-4-carboxylic acids. This suggests that the next enzyme in the pathway, the *C-S* lyase that cleaves *S*-alkyl thiohydroximate to produce the thiohydroximic acid, is tightly coupled to the *S*-donating enzyme, which in turn is tightly coupled to the CYP83 enzymes, forming a complex to carry out this sulfur chemistry without loss of reactive sulfur intermediates to the surroundings. A *C-S* lyase involved in biosynthesis of glucosinolates in *Arabidopsis* was recently identified using a bioinformatics approach (118). Metabolite profiling of the *C-S* lyase knockout mutant showed the complete absence of both aliphatic and aromatic glucosinolates. This had not previously been reported for any mutants with altered glucosinolate biosynthesis, and suggests that the *C-S* lyase constitutes a single gene family.

The Conversion of Thiohydroximic Acids to Glucosinolates

A UDP-glucose:thiohydroximic acid *S*-glucosyltransferase, UGT74B1 (At1g24100), that glucosylates phenylacetothiohydroximic acid to produce the corresponding desulfoglucosinolate was identified in *Arabidopsis* based on its homology to a patented ortholog from *Brassica napus* (65). Knockout mutants of *UGT74B1* significantly decreased, but did not abolish, glucosinolate accumulation, which suggests that additional UGTs are present in the genome. The PAPS:desulfoglucosinolate sulfotransferase, AtST5a (At1g74100), catalyzing the last step in the synthesis of the core structure was recently identified by differential RNA display of coronatine-regulated genes (136). Biochemical characterization of AtST5a and its close homologs AtST5b (At1g74090) and AtST5c (At1g18590) showed that AtST5a prefers tryptophan- and phenylalanine-derived desulfoglucosinolates, whereas AtST5b and AtST5c prefer long-chain aliphatic desulfoglucosinolates (136).

The Evolutionary Link between Glucosinolates and Cyanogenic Glucosides

Cyanogenic glucosides are widespread in the plant kingdom, being found in ferns and gymnosperms as well as angiosperms. Glucosinolates are evolutionarily younger and found only in the order Capparales and in one outgroup, the genus *Drypetes* of the Euphorbiaceae. Because both groups of natural products are derived from amino acids and have aldoximes as intermediates, it has been hypothesized that glucosinolates developed based on a predisposition for making cyanogenic glucosides. This theory is supported by the demonstration that CYP79 homologs catalyze the conversion of amino acids to aldoximes in both pathways. Consistent with an evolutionary relationship between the cyanogenic glucoside and glucosinolate

pathways, the aldoxime-metabolizing enzymes in both pathways belong to the same CYP family, as CYP71E1 metabolizes *p*-hydroxyphenylacetaldoxime in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (9), and the CYP83 enzymes, which should be assigned to the CYP71 family based on sequence homology, metabolize aldoximes in the glucosinolate pathway (11, 68, 128). In contrast to the CYP79 family of amino acid *N*-hydroxylases, the CYP71 family represents cytochromes P450 with very diverse enzymatic activities, only some of which are involved in aldoxime metabolism. A possible scenario for the evolution of glucosinolates is that a mutation in the aldoxime-metabolizing enzyme in the cyanogenic pathway resulted in the production not of the expected hydroxynitrile, but rather a toxic compound, which the plant subsequently had to get rid of (68). From this perspective, the postaldoxime enzymes of the glucosinolate pathway can be viewed as enzymes recruited from the detoxification processes to metabolize the *aci*-nitro compound or nitrile oxide. Consistent with this hypothesis, both glucosyltransferases and sulfotransferases represent detoxification mechanisms widely used in nature.

BIOSYNTHESIS: SECONDARY TRANSFORMATIONS

The initially formed parent glucosinolate is subject to a wide range of further modifications of the R group. These reactions are of biological as well as biochemical interest because they influence the direction of glucosinolate hydrolysis and the resulting activity of the hydrolysis products. The R group of glucosinolates derived from methionine and its chain-elongated homologs is especially subject to further modifications, such as the stepwise oxidation of the sulfur atom in the methylthioalkyl side chain leading successively to methylsulfinylalkyl and methylsulfonylalkyl moieties (**Figure 4**). Methylsulfinylalkyl side chains can be further modified by oxidative cleavage to afford alkenyl or hydroxyalkyl chains. Genetic loci controlling each of these conversions have been identified in *Brassica* species and in *Arabidopsis* (56, 67, 123, 132).

In *Arabidopsis*, mapping using recombinant inbred lines derived from interecotype crosses implicated a cluster of three genes in controlling oxidation of the side chain (67, 87). These genes all encode 2-oxoacid-dependent dioxygenases, members of a large family of nonmembranous, nonheme iron-containing enzymes that catalyze many hydroxylation, epoxidation, and desaturation reactions of plant metabolism (139). To date, two genes of the cluster have been functionally characterized (87). The *AOP2* gene product, which is expressed only in ecotypes accumulating alkenyl glucosinolates, converts methylsulfinylalkyl to alkenyl glucosinolates when heterologously expressed in *E. coli* (**Figure 4**). On the other hand, the *AOP3* gene product, which is expressed only in ecotypes accumulating hydroxyalkyl glucosinolates, converts a methylsulfinylalkyl to a hydroxyalkyl glucosinolate. The differences in AOP activity among ecotypes are a result of differences in the promoter regions of *AOP2* and *AOP3* and a deletion in the open reading frame of *AOP2*, which leads to a highly truncated protein (87). Inheritance studies and sequencing of BAC clones indicate that analogous 2-oxoacid-dependent dioxygenase gene clusters are present in *Brassica oleracea*, which also control glucosinolate side-chain oxidation (55, 100). Little is known about the biochemical or molecular basis of other secondary transformations, such as the esterification of free hydroxyl groups by benzoic acid, except that these reactions follow the formation of the core glucosinolate skeleton (61).

REGULATION OF BIOSYNTHESIS

In the economically important family Brassicaceae, individual species typically produce between 30–40 different glucosinolates, with

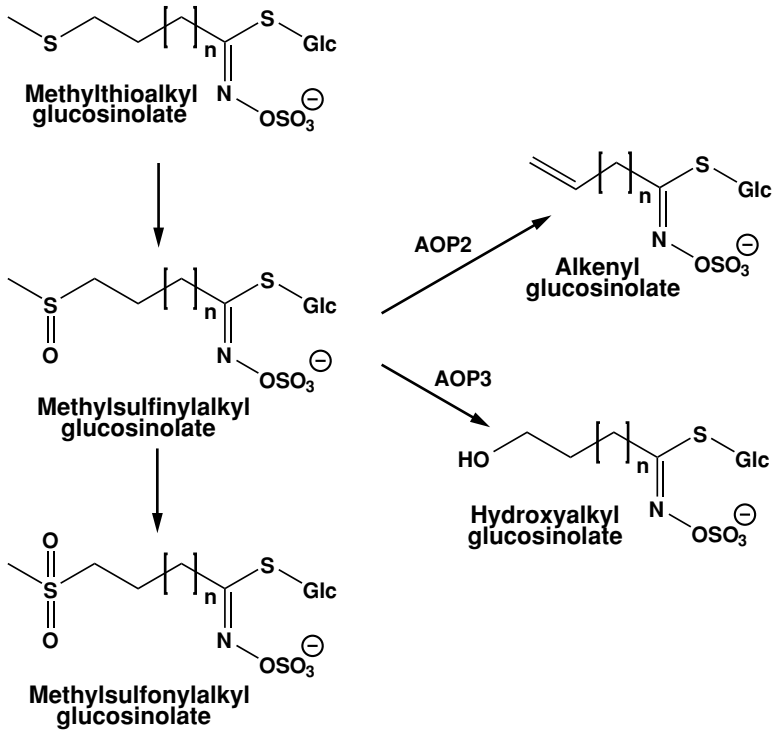

Figure 4

Some common oxidative secondary transformations of methionine-derived glucosinolates. AOP2 and AOP3 indicate the 2-oxoacid-dependent dioxygenases catalyzing these reaction types in *Arabidopsis*. For each category of glucosinolate, a different range of chain lengths is known to occur naturally (49). For methylthioalkyl and methylsulfonylalkyl, n = 1–8; for methylsulfinylalkyl, n = 1–9; for alkenyl, n = 1–5; for hydroxyalkyl, n = 0–2.

the aliphatic, methionine-derived glucosinolates contributing most to the diversity. Quantitative trait locus (QTL) analysis is a powerful method to study the qualitative and quantitative variation in glucosinolate profiles. This approach has identified four to six QTLs that control aliphatic glucosinolate concentration in seeds of *B. napus* (159, 162). In *Arabidopsis*, a QTL mapping experiment using Landsberg *erecta* (Ler) X Cape Verde Islands (Cvi-0) recombinant inbred lines has identified a number of QTLs controlling the accumulation of aliphatic, aromatic, and indole glucosinolates in leaves and seeds (85). Under these conditions, six QTLs determine total aliphatic glucosinolate accumulation, of which two are the biosynthetic loci *GS-Elong* and *GS-AOP*, six QTLs control total indole glucosinolates, and three loci regulate the less dominant aromatic glucosinolates. Five additional loci were specific to subsets of the indole glucosinolates. Except for the *GS-Elong* locus that controls both total leaf aliphatic and seed aromatic glucosinolates, no correlation was found between the QTLs for the different classes of glucosinolates, which suggests that the classes are independently regulated.

The above QTL analysis demonstrates that a large number of variable loci control glucosinolate accumulation. Apart from *GS-Elong* and *GS-AOP*, none of the loci have been cloned and characterized. However, the indole glucosinolate controlling locus *DF119L* on chromosome V maps in the vicinity of *ATR1*, the *AtMyb34* transcription factor, which has been identified as a regulator

of indole glucosinolates (31). It is therefore likely that *DF119L* is *ATR1*, although the authors argue against this proposition based on the assumption that Cvi-0 should then behave as an *ATR1 null* mutant. However, this is not a prerequisite as Cvi-0 has approximately 50% of the indole glucosinolate of L*er* (86). The dominant mutant *atr1D* was isolated in a screen for altered tryptophan regulation (*atr*) and caused elevated expression of the indole glucosinolate biosynthetic genes *CYP79B2*, *CYP79B3*, and *CYP83B1*, and the Trp biosynthetic gene *ASA1* (13). The level of indole glucosinolates is upregulated in the *atr1D* mutant, whereas an *atr1* loss-of-function mutant impairs expression of the genes and confers reduced indole glucosinolate levels. This implies that ATR1 can be manipulated to coordinately control the enzymes that synthesize indole glucosinolates.

The level and composition of glucosinolates in plants reflect both genetic and environmental factors as some glucosinolates are constitutively present and others can be induced. The induction of specific *CYP79* genes correlates with accumulation of the corresponding glucosinolates, indicating that induction is regulated at the transcriptional level (119). Several studies with different plant species have shown that methyl jasmonate and wounding induce specific indole glucosinolates (16, 17, 20, 44, 119). *CYP79B2* and *CYP79B3* appear to have different functions as *CYP79B3* is primarily induced by methyl jasmonate (20, 119), whereas *CYP79B2* is primarily induced during camalexin production (59). The pathogen response signaling molecule, salicylic acid, is a less pronounced inducer of glucosinolates. It induces specifically 4-methoxyindol-3-ylmethyl glucosinolate in several *Arabidopsis* ecotypes (84, 119). In leaves of *B. napus*, salicylic acid increases the overall level of glucosinolates, with 2-phenylethyl glucosinolate showing the highest accumulation (82). With few exceptions, the aliphatic glucosinolates in *Arabidopsis* appear to be primarily developmentally regulated (84, 119). QTL analysis of the genetic variation influencing glucosinolate profiles in *Arabidopsis* under various environmental conditions shows a high level of variation, indicating the involvement of several different signal transduction pathways (84). Cloning and characterization of the genes underlying the QTLs will generate a detailed understanding of the molecular and biochemical basis for regulating glucosinolate profiles. In a recent paper, Saito and coworkers integrated metabolomics and transcriptomics to elucidate gene-to-gene and metabolite-to-gene networks in *Arabidopsis* grown under sulfur deficiency (72). The batch-learning self-organizing mapping approach classified metabolites and transcripts according to their time-dependent pattern of changes in accumulation and expression. This allowed the re-identification of all biosynthetic genes as well as the discovery of new putative transcription factors in the glucosinolate pathway (72). Similar experiments under other abiotic or biotic stresses may identify regulators under these conditions.

Camalexin: cruciferous indole phytoalexin

DEGRADATION

Glucosinolates are degraded upon plant damage to a variety of hydrolysis products that are responsible for virtually all of the biological activities of this compound class. The process begins with myrosinase-catalyzed hydrolysis of the thioglucoside linkage, leading to the formation of glucose and an unstable aglycone (19, 140). Depending on the structure of the side chain and the presence of additional proteins and cofactors, the aglycone then rearranges to form different products, including isothiocyanates, oxazolidine-2-thiones, nitriles, epithionitriles, and thiocyanates (**Figure 5**).

Hydrolysis Products

The most common glucosinolate hydrolysis products in many species are isothiocyanates, which are formed from the aglycone by a Lossen rearrangement involving the

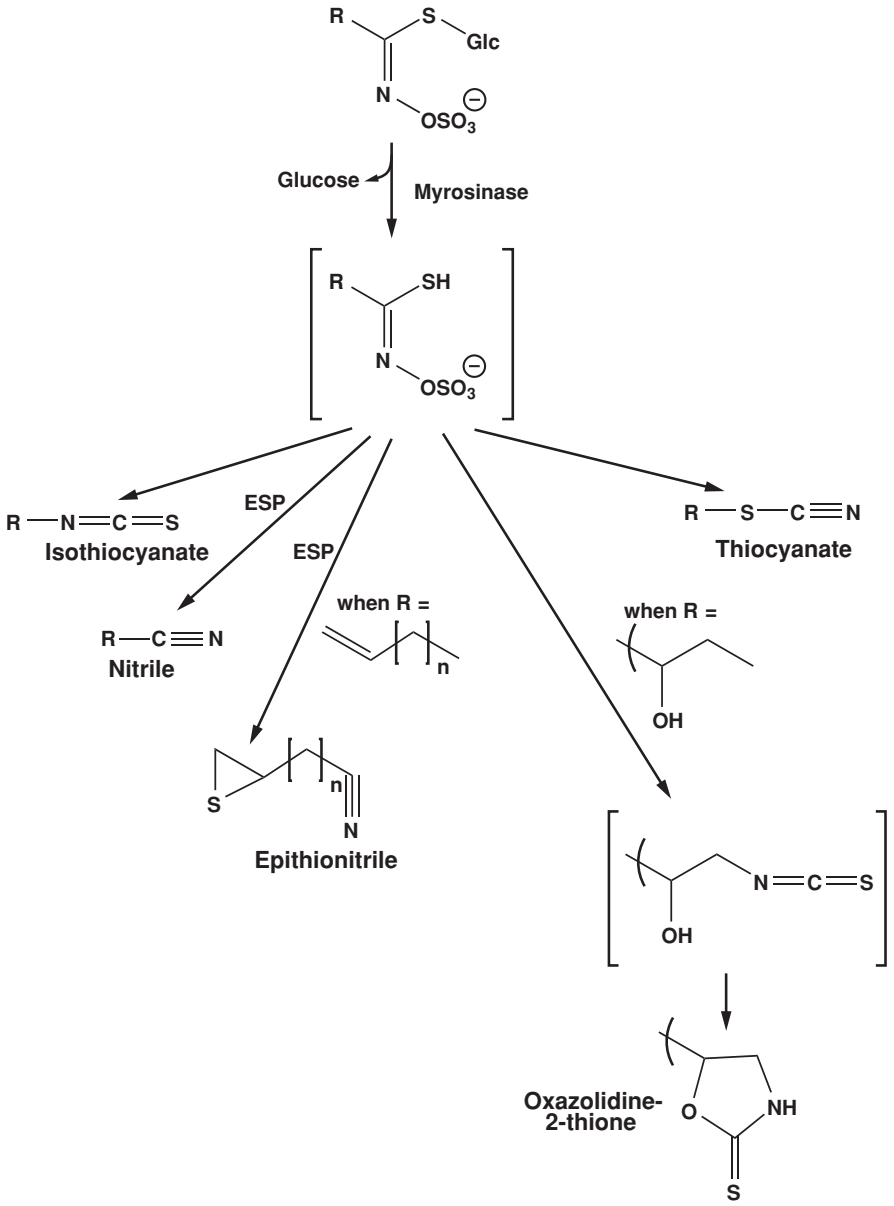

Figure 5

Outline of glucosinolate hydrolysis. Brackets indicate unstable intermediates. Abbreviations: ESP, epithiospecifier protein; R, variable side chain.

migration of the side chain from the oxime carbon to the adjacent nitrogen. When the glucosinolate side chain bears a hydroxyl group at C-2, the isothiocyanates formed are unstable and cyclize to oxazolidine-2-thiones, a class of substances known to cause goiter. In other plants, a major percentage of glucosinolate hydrolysis products are nitriles (40, 94). The formation of nitriles in vitro is favored at a pH of less than three or in the presence of

Fe^{2+} ions (54, 58). However, protein factors may be involved in nitrile formation in vivo, such as the epithiospecifier protein (ESP) (15, 52, 109, 157). When the glucosinolate side chain has a terminal double bond, ESP promotes the reaction of the sulfur atom of the thioglucoside linkage with the double bond to form a thirane ring, giving an epithionitrile (**Figure 5**). The process occurs only in the presence of myrosinase, and ESP is not known to have any catalytic abilities by itself. The recent isolation of an *Arabidopsis* gene encoding an ESP showed that this protein not only promotes the formation of epithionitriles, but also the formation of simple nitriles from a large variety of glucosinolates (94).

Other hydrolysis products include thiocyanates, which are formed from only three glucosinolates: benzyl-, allyl-, and 4-methylsulfinylbutyl-glucosinolate (**Figure 1**), all of which form stable side-chain cations. Like nitrile formation, thiocyanate production is also associated with specific protein factors (70), but these have not yet been identified. The hydrolysis of indole glucosinolates is somewhat different from that of the other glucosinolate types, because the initially formed isothiocyanates are unstable at neutral or slightly acidic pH, and are converted to further metabolites, including indole-methanols, ascorbic acid conjugates, and oligomeric mixtures (1, 27, 95).

Biochemistry and Physiology of Myrosinases

The initial catalysts in glucosinolate degradation have been the subject of many biochemical and molecular investigations (19, 140). As members of Glycoside Hydrolase Family 1 (171), myrosinases have three-dimensional structures and properties like those of certain O-glucosidases (25). The abundance of salt bridges, disulfide bridges, and H bonds apparent in the structure may promote stability in the extracellular environment in which myrosinase must function following tissue damage. Myrosinases are also heavily glycosylated with carbohydrate contributing up to 20% of their molecular masses. Glycosylation may serve to enhance protein stability as well or to protect the enzyme from being inactivated by reactive hydrolysis products. X-ray crystallography of myrosinase revealed a role for ascorbate as an essential cofactor (26). This finding helps account for the long-observed role of ascorbate in stimulating myrosinase activity at low millimolar concentrations. As the only group of β-thioglucosidases known in nature, myrosinases use only glucosinolates as substrates and have no activity toward any *O*-glycosides or other *S*-glycosides in vitro. However, among the different types of glucosinolates their substrate range is variable. Whereas most myrosinases hydrolyze multiple glucosinolate substrates, e.g., (36), some are highly specific (14, 110). In all plants investigated to date, myrosinase is encoded by a multigene family. For example, *Arabidopsis* has 4 functional myrosinase genes (171, 173) whereas *B. napus* and *Sinapis alba* each have 20 or more (140), many of which have distinct patterns of organ- and tissue-specific expression (99, 172).

The cellular and subcellular localization of myrosinase has been a question of long-standing interest in glucosinolate research because this is presumed to be part of the strategy to help keep glucosinolates and myrosinase from reacting in the intact plant but bring them together rapidly after damage, the "mustard oil bomb" sensu Lüthy & Mathile (108). For more than 100 years, it has been reported that myrosinase is localized in idioblasts with high vacuolar protein content, called myrosin cells, which are scattered in all organs of Brassicaceae species. This has been confirmed by histological, immunocytochemical and in situ hybridization methods and more recently by the use of *Arabidopsis* lines containing GUS fusion constructs with the promoters of myrosinase genes (74, 154). In these latest studies, myrosinase was found in idioblast cells of the phloem parenchyma occurring in leaves, stems, and inflorescences (6, 74, 154), and

ESP: epithiospecifier protein

Idioblast: cell that differs in form from others in same tissue

IAA: indole-3-acetic acid

also in guard cells (74, 154). When these results are taken together with the recent report that glucosinolates in *Arabidopsis* flower stalks are localized in elongated, sulfur-rich "S cells" situated just distal to the phloem (88), they suggest that myrosinase and glucosinolates are segregated in this species in separate but adjacent cells. However, the arrangement may be different in other species because, in *Brassica* spp., myrosin cells are widespread outside the vascular system (6, 154). For example, in *B. juncea* seeds and seedlings, myrosinase is located in aleurone-type cells that appear to contain glucosinolates (80). The colocalization of both of these components in one cell type means that in some cases the glucosinolate-myrosinase system might be spatially separated at the subcellular rather than the cellular level. Alternatively, myrosinases could be located in the same compartment as glucosinolates but might be inactivated by high amounts of ascorbate (19).

Extracts of myrosinase-containing plants possess a number of other proteins that coprecipitate with antibodies to myrosinase, known as myrosinase-binding proteins, myrosinase-binding protein-related proteins, or myrosinase-associated proteins (140). Many representatives of these classes are induced by wounding or insect feeding (7, 137, 138), but their relation to glucosinolate hydrolysis is still obscure. For example, certain myrosinase-binding proteins in *B. napus* seeds with lectin-like properties form high-molecular weight complexes with myrosinase that might be expected to stabilize their activities (47). However, antisense plants lacking myrosinase-binding proteins in their seeds show no changes in myrosinase activity.

METABOLIC LINKS BETWEEN GLUCOSINOLATE METABOLISM, IAA, AND OTHER INDOLE COMPOUNDS

Several lines of evidence suggest that there is a metabolic link between indole glucosinolates and the plant hormone indole-3-acetic acid (IAA). First, indole glucosinolates can be degraded into indole acetonitrile (IAN), which in turn can be hydrolyzed by nitrilases into IAA. The conversion of indole glucosinolates into IAN may require the presence of both myrosinase and an ESP (94) (see under degradation). However, an ESP is notably not present in the frequently studied *Arabidopsis* Columbia ecotype. Additional evidence for a glucosinolate-IAA link is the role of IAOx as intermediate in the biosynthesis of indole glucosinolate and as precursor of IAA, although the genes involved in conversion of IAOx to IAA remain to be identified. Several high-auxin loss-of-function *Arabidopsis* mutants gain their phenotype by blockage of postaldoxime enzymes in the glucosinolate pathway, which supposedly leads to accumulation of IAOx that is channeled into IAA. These mutants include *superroot1/afl1/rooty/hls3* (18, 30, 83, 98) (hereafter referred to as *sur1*), which is knocked out in the *C-S* lyase, and *cyp83B1/superroot2/runt1/atr4*, (11, 12, 42, 149) (hereafter referred to as *sur2*). The recently discovered *ugt74B1* mutant has a less pronounced high-auxin phenotype, probably due to redundancy (65). The gain-of-function YUCCA mutant, which has a strong high-auxin phenotype, is proposed to overproduce IAOx due to upregulation of a flavin-monooxygenase that can hydroxylate tryptamine to N-hydroxytryptamine (177). This, however, implies that the tryptamine conversion is rate limiting for IAA production, which remains to be shown.

Until recently, it was an open question whether CYP79B2 and CYP79B3, which convert amino acids to aldoximes, contribute to IAA production in wild-type *Arabidopsis* plants. Their role in IAA biosynthesis is evidenced by increased levels of free IAA in six-day-old seedlings of *CYP79B2* overexpression lines and decreased levels of free IAA in *cyp79B2cyp79B3* double mutants (178). Furthermore, decreased IAA synthesis was measured in the apical 0–2 mm of excised roots of seven-day-old seedlings of *cyp79B2cyp79B3* double mutants (103). Their role in

root-specific IAA synthesis is supported by studies with *CYP79B2* and *CYP79B3* GUS reporter constructs, which show distinct expression of each gene in the primary root meristem and at the sites of lateral root formation (103).

Another unanswered question is whether or not IAOx plays a role in IAA biosynthesis in plants that do not produce indole glucosinolates. To date, indole glucosinolates have been found in only five families of the order Capparales (64), and no *CYP79B* homologs have been identified in plant species outside these families (9). This suggests that a role of *CYP79B* homologs in IAA biosynthesis is limited to a small group of plants. On the other hand, this does not rule out that IAOx originating from other sources is an intermediate in IAA biosynthesis.

The role of IAN in IAA biosynthesis is uncertain. Attempts to rescue the high-auxin phenotype of *sur2* by crossing *sur2* to the *nit1* null mutant (NIT1 is the nitrilase with highest specificity toward IAN) were unsuccessful, which suggests that IAN is not an intermediate in the biosynthetic pathway from IAOx to IAA (11). However, Kutz et al. (93) provide evidence that, under conditions of sulfur starvation, an increase in glucobrassicin turnover and NIT3 accumulation initiate the production of extra IAA from IAN, leading to increased root growth and branching. This allows the root system to penetrate new soil areas. Interestingly, IAN was recently recognized as a phytoalexin that is induced upon fungus attack in *Brassica juncea* (134).

Based on structure similarity, it has been suggested that the indole alkaloid, brassinin, and possibly other cruciferous phytoalexins are derived from indol-3-ylmethylglucosinolate (glucobrassicin). However, camalexin (the indole alkaloid of *Arabidopsis*) is synthesized directly from IAOx by CYP79B2 and CYP79B3 (59). Furthermore, in vivo feeding studies with roots of turnip (*Brassica rapa*) using deuterated IAOx and glucobrassicin showed that IAOx, and not glucobrassicin, was incorporated into brassinin and brassinin-derived phytoalexins (133). These data suggest that the characteristic cruciferous sulfur-containing indole alkaloids are synthesized from tryptophan via IAOx by CYP79B homologs.

In *Arabidopsis*, IAOx represents a key metabolic branch point where the flow of IAOx into the biosynthetic pathways of indole glucosinolates, camalexin, and IAA must be tightly regulated (**Figure 6**). Further studies are needed to identify genes in the biosynthesis of camalexin and IAA, which will allow investigations into the organization and regulation of the different metabolons around IAOx. The emerging knowledge of IAOx synthesis and metabolism has opened an exciting area of research at the borderline between primary and secondary metabolism.

TRANSPORT IN PLANTS

Glucosinolate accumulation varies between tissues and developmental stages, both with regard to concentration and composition (23). In *Arabidopsis*, young leaves and reproductive tissues such as siliques and seeds contain the highest concentrations, whereas senescing rosette leaves contain the lowest concentrations of glucosinolates. Intermediate concentrations are found throughout the "large" organs such as the roots, leaves, and stem (23, 135). The high accumulation of glucosinolates in the seeds does not have a correspondingly high level of associated biosynthesis, which suggests the presence of an import system in this tissue (45). Several lines of evidence indicate long-distance transport of glucosinolates from parts other than the siliques to the seeds. First, glucosinolates possess the physicochemical properties required to travel in the phloem (24), fulfilling one prerequisite for long-distance transport. Secondly, examination of phloem exudates following aphid feeding has shown glucosinolate concentrations of up to 10 mM in phloem sap (114). Finally, the strongest indication of long-distance transport of glucosinolates comes from a study by Chen et al. (35). When

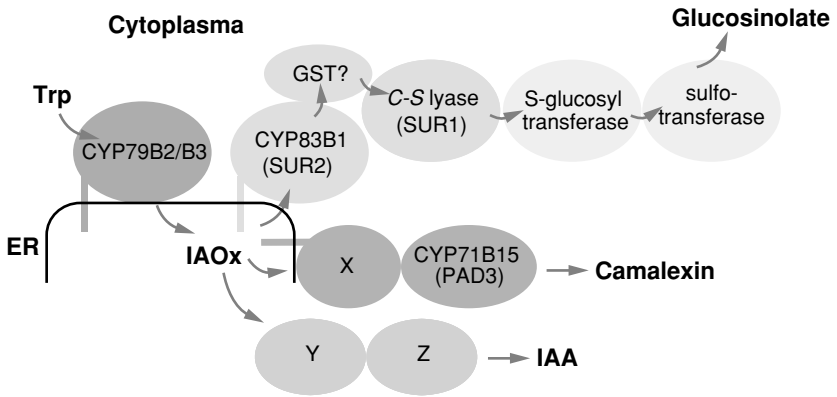

Figure 6

Visualization of a metabolic grid around indole-3-acetaldoxime (IAOx). IAOx represents a key metabolic branching point between primary and secondary metabolism as IAOx flows into the metabolons of indole glucosinolates, camalexin, and indole-3-acetic acid (IAA). The reactivity of the CYP83 product combined with the ability of the *C-S* lyase substrate to undergo internal cyclization in the absence of the *C-S* lyase suggest a very tight coupling between CYP83B1, the unidentified GST, and the *C-S* lyase. Abbreviations: GST, glutathione-*S*-transferase; SUR2, SUPERROOT2; SUR1, SUPERROOT1; ER, endoplasmic reticulum.

S-cells: sulfur-rich cells

young 35S::CYP79A1 *Arabidopsis* plants were fed [^{14}C]tyrosine to their rosette leaves, radiolabelled exogenous *p*-hydroxybenzyl glucosinolate was formed. Upon bolting, the radiolabeled glucosinolate was exported from the leaves to the seeds, possibly via transport in the phloem.

Several experiments have biochemically characterized the transport system. Excised immature *B. napus* embryos take up glucosinolates from the culture medium in an energy-dependent, carrier-mediated transport system (57). Similarly, leaf protoplasts of *B. napus* take up a variety of glucosinolates (37). Both embryo and protoplasts displayed an ability to take up glucosinolates against a concentration gradient that was dependent on a pH gradient, and was abolished by the uncoupling protonophores 2,4-dinitrophenol and carbonylcyanide *p*-chlorophenyl hydrazone (37, 57). The transport system was specific toward glucosinolates as compared to other glucosides, sugars, and amino acids. This was further supported by the facile absorption of the exogenous alkenyl glucosinolate sinigrin observed in uptake experiments using microspore-derived embryos of *B. napus* (75). The data suggest the presence of a specific H^+/glucosinolate symporter, which is unaffected by the structure of the side chain.

Several questions are unresolved regarding glucosinolate transport. First, the sulfur-rich cells (S-cells) localized in the floral stalk of *Arabidopsis* contain approximately 100 mM glucosinolate (88), and could constitute a sink for glucosinolate transport, in addition to seeds. More experiments are needed to establish whether these cells possess the biosynthetic machinery to account for their high glucosinolate content or whether they rely on import from surrounding tissues. Second, some controversy exists about the form in which glucosinolates are transported. Sulfotransferase activity has been identified in embryos of *B. napus*, which suggests that desulfoglucosinolates may be the transport form (158). Experiments with radiolabeled desulfoglucosinolates fed to silique walls and seedlings showed the glucosinolate precursor to be taken up and converted into glucosinolates (153). On the contrary, Chen et al. (35) isolated intact indole glucosinolates from phloem exudates of leaves of *B. napus*. Whether desulfoglucosinolates or

glucosinolates are the long-distance transport form of glucosinolates awaits identification and characterization of a transporter.

BIOLOGICAL FUNCTION

The activation of glucosinolates upon plant damage and the biological properties of their hydrolysis products have long suggested that the major function of these compounds in plants is to defend against herbivores and pathogens. The glucosinolate-myrosinase system has been actively investigated as a feature of plant defense for nearly 100 years, but there are still many gaps in our knowledge. Given the large literature on this subject and the regular appearance of reviews (38, 104, 170), here we emphasize only the major themes and recent contributions.

Numerous studies have demonstrated that glucosinolates exhibit outright toxicity, growth inhibition, or feeding deterrence to a wide range of potential plant enemies, including mammals, birds, insects, mollusks, aquatic invertebrates, nematodes, bacteria, and fungi (e.g., 28, 96, 131, 161). When hydrolysis products have been explicitly tested in such studies, isothiocyanates are frequently responsible for the activity of the parent glucosinolates (3, 28, 101, 156). Unfortunately, little is known about the specific mechanism by which isothiocyanates exert their toxicity aside from their general propensity to react with amino and sulfhydryl groups of proteins in vitro (79). Several recent studies have reported that plants respond to insect damage by systemically accumulating higher levels of glucosinolates, which presumably increases their resistance to subsequent attacks (2, 115, 160, 163).

As with other plant-defense compounds, the same glucosinolates that serve as general poisons and deterrents for many herbivores also attract adapted herbivores. Many insect herbivores have come to specialize on glucosinolate-containing plants and often use these compounds as cues for feeding or oviposition (53, 116, 121, 145). Attraction from a distance may be mediated by volatile hydrolysis products, whereas the intact glucosinolates can serve as contact cues for feeding or oviposition stimulation. Evidence for specialist insect attraction in such studies comes not only from behavioral experiments, but also from electrophysiological investigations in which receptor organs or cells respond directly to glucosinolates or their hydrolysis products (121, 145). Not all specialist feeders on glucosinolate-containing plants use these compounds in mediating host choice; some employ entirely different plant chemicals in this capacity (41, 112, 142).

Herbivores that specialize on glucosinolate-containing plants must have some mechanism of overcoming the toxicity of their host. In theory, they could rapidly excrete glucosinolates from their bodies, metabolize them to nontoxic derivatives, or be completely insensitive to their toxic action. The larvae of two lepidopteran species were recently shown to employ divergent metabolic strategies to circumvent the toxicity of glucosinolates. *Plutella xylostella*, the diamondback moth, cleaves the sulfate residue from the glucosinolate core structure with its own endogenous sulfatase to give a product that can no longer be hydrolyzed by myrosinase (141). *Pieris rapae*, the cabbage white butterfly, secretes a protein factor into the gut with an action similar to that of the ESP. It redirects myrosinase-catalyzed hydrolysis toward the formation of nitriles instead of isothiocyanates, and the resulting nitriles are excreted with the feces (167). Thus, both species avoid the formation of isothiocyanate hydrolysis products in their digestive tracts, which, at least for *P. rapae*, are deleterious to larval growth and survival (3). Other specialist insects that seem to feed on glucosinolate-containing plant tissue with impunity, such as the flea beetles of the genus *Phyllotreta* (129), may have similar mechanisms.

Once specialist herbivores have overcome the defensive chemistry of their hosts, they may be able to sequester plant-defensive compounds in their own tissues without harm

and exploit them in self-protection. Several examples of glucosinolate-sequestering insects were recently described, including the harlequin bug, *Murgantia histrionica* (Hemiptera) (4), the sawfly, *Athalia rosae* (Hymenoptera) (125), and the aphids, *Brevicoryne brassicae* and *Lipophis erysimi* (Homoptera) (21). As a counter example, it was shown that *Pieris* sp., which were once suspected of sequestering dietary glucosinolates, do not actually exhibit this ability (124). In *M. histrionica* and *A. rosae*, sequestration deterred predators such as birds, lizards, and ants (126, 165). The exploitation of glucosinolates by herbivores for their own defensive purposes is complicated by the fact that myrosinase is also needed to catalyze the formation of toxic hydrolysis products. In fact, the aphid *B. brassicae* does have a myrosinase activity of its own that is quite distinct from plant myrosinases (77). This enzyme is apparently stored in the aphid body separately from glucosinolates and forms isothiocyanates from the sequestered glucosinolates when the aphid is damaged or killed, serving to alarm other members of the colony (21). For other glucosinolate-sequestering insects, it is not clear if they possess an endogenous myrosinase or just rely on the myrosinase activity normally present in the guts of their enemies to make the defense effective. Some bacterial species resident in the human and rat intestine have the ability to degrade glucosinolates to isothiocyanates and other metabolites (92, 146), and this may be a widespread phenomenon in the animal kingdom. The use of plant glucosinolates by herbivores in their own defense not only extends the ecological significance of these compounds, but also underscores the intrinsic defensive value of glucosinolates to the plant that produces them.

In light of the many reports demonstrating the toxicity of glucosinolate hydrolysis products to bacteria and fungi in vitro (20, 113, 148), glucosinolates might be expected to defend plants against pathogens. In some cases, resistance to pathogens in vivo is indeed positively associated with glucosinolate content (102). But a defensive role cannot automatically be assumed because many pathogens, especially biotrophic organisms, may not cause enough cell damage to activate the glucosinolate-myrosinase system. The *Arabidopsis MAM1* mutant, which has a lower amount of 4-methylsulfinylbutyl glucosinolate than the wild-type, had increased susceptibility to *Fusarium oxysporum*, but not to other fungal and bacterial species (156). Different glucosinolate hydrolysis products seem to have varying effects on different pathogens (20, 147). Once glucosinolates are released into the soil from root exudates or the decay of plant organs, they may have important effects on the rhizosphere community. The dominant fungal species in soil near glucosinolate-containing Brassicaceae are different than the dominant fungal species found elsewhere and show increased tolerance to isothiocyanates (76). The growth of certain ectomycorrhizal species is even stimulated by the hydrolysis products of indole glucosinolates (175).

METABOLIC ENGINEERING OF GLUCOSINOLATES

There is a strong interest in being able to alter levels of specific glucosinolates in crop plants as certain glucosinolates have desirable properties in flavor, insect protection, biofumigation, and cancer prevention, whereas others have undesirable properties. To date, metabolic engineering of glucosinolate profiles has included altering the expression of one or more CYP79 enzymes. Identification of the CYP79s as the enzymes catalyzing the conversion of amino acids to aldoximes has provided important molecular tools for modulating the profile of glucosinolates (for review see Reference 120). Ectopic expression of the endogenous *CYP79* genes under the control of the strong constitutive 35S promoter has resulted in production of transgenic *Arabidopsis* lines that accumulate increased levels of benzyl (*35S::CYP79A2*) (168), indole (*35S::CYP79B2*) (31, 117), and aliphatic (*35S::CYP79F1)* (69) glucosinolates.

Overexpression of *CYP79F1* resulted in no more than a twofold greater accumulation of the homo- to tetrahomomethionine-derived glucosinolates (69, 143), which is significantly less than that for plants overexpressing *CYP79A2* or *CYP79B2*. This is likely to reflect the fact that the rate-liming step of aliphatic glucosinolate biosynthesis in these lines is the chain-elongation pathway.

Overexpression of exogenous CYP79s from the cyanogenic pathway is a means to generate novel glucosinolates. Introduction of *CYP79A1* from the biosynthetic pathway of the tyrosine-derived cyanogenic glucoside dhurrin in *Sorghum bicolor* resulted in *Arabidopsis* plants that accumulate high levels of *p*-hydroxybenzyl glucosinolate (10). Similarly, introduction of *CYP79D2* from the biosynthetic pathway of the cyanogenic glucosides linamarin and lotaustralin in *Manihot esculenta* Crantz (5) resulted in accumulation of the valine- and isoleucine-derived glucosinolates, isopropyl and 1-methylpropyl glucosinolate (119). 1-methylpropyl glucosinolate had not previously been identified in *Arabidopsis* and only trace amounts of isopropyl glucosinolate have been found in just a few ecotypes (86). The ability to alter the concentrations of not only specific endogenous glucosinolates, but also to introduce novel glucosinolates not normally present in *Arabidopsis*, reflects the fact that the postaldoxime enzymes are not specific for the nature of the side chain. The efficiency of metabolic engineering of glucosinolate profiles using CYP79 enzymes varies for the different enzymes. Several factors, such as enzyme stability, K_m value, amino acid pool size, turnover, and targeting influence the success of a metabolic engineering approach. Accordingly, metabolic engineering of glucosinolates will likely remain a largely unpredictable business, which has to be tested in vivo.

Knockout of *CYP79* genes is a means to eliminate specific glucosinolates. However, whereas knockout mutants of CYP79s metabolizing protein amino acids have mostly wild-type morphology, knockout mutants of, e.g., *CYP79F1* metabolizing chain-elongated methionine derivatives have a severe morphological phenotype with several hundred axillary shoots, reflecting an altered hormone balance (143, 150). *Arabidopsis* (Col-0) knockout mutants of *MAM1* and *MAML* are deficient in C4 and C6, C7, and C8 aliphatic glucosinolates, respectively (51, 91). One may therefore anticipate that a double knockout of these two genes will be devoid of methionine-derived, aliphatic glucosinolates.

Overexpression of regulators could be another approach to metabolic engineering. The dominant *atr1D* mutant that is upregulated in the transcripts for *CYP79B2*, *CYP79B3*, and *CYP83B1*, accumulates tenfold more total indole glucosinolates than wild type. Interestingly, although *35S::ATR1* lines produced several-fold-higher transcript levels than *atr1D*, the level of indole glucosinolates was only twofold above wild-type level (31). In comparison, *35S::CYP79B2* lines produced a fivefold increase in indole glucosinolates (117). This shows that ectopic expression of a regulator may be less optimal for metabolic engineering than a gain-of-function mutant that only overexpresses the regulator in the right cells.

Future metabolic engineering efforts with the genes from the chain-elongating pathway, the aldoxime-forming CYP79s, and the enzymes catalyzing secondary transformations provide the possibility to design crop plants enriched in desirable glucosinolates and free of undesirable glucosinolates. This will allow a detailed analysis of the biological function of the individual glucosinolates. Furthermore, with the nearly complete identification of the genes involved in the biosynthesis of the core structure, it has become a realistic possibility to engineer the glucosinolate pathway into heterologous host plants. The entire biosynthetic pathway of the evolutionarily related cyanogenic glucosides has been successfully transferred into noncyanogenic plants, resulting in plants with increased resistance to specific insects (151). The data showed that the heterologous pathway formed a metabolon in

the new host plant, and that this metabolon did not affect the morphological phenotype of the plant (130). The positive experience with engineering the cyanogenic glucoside metabolon leaves hope that the entire glucosinolate pathway may be heterologously expressed in microorganisms and heterologous plants to generate high-value products or crops.

PERSPECTIVE

The unprecedented resources supporting functional genomics research on *Arabidopsis* have led to a remarkable increase in our knowledge of the biology and biochemistry of glucosinolates, which are one of the major classes of secondary metabolites in this model plant species. The availability of genetic sequence information, large mutant collections, and tools for expression profiling, along with abundant natural glucosinolate variation among ecotypes, recombinant inbred lines, and markers for mapping have greatly facilitated the identification of genes encoding enzymes of glucosinolate biosynthesis. However, to date this success has been concentrated on the core pathway, and most of the genes and enzymes participating in the chain-elongation cycle, secondary transformations, and proteins controlling glucosinolate breakdown are still unknown. Additionally, more knowledge about the factors that regulate efflux through the pathways of biosynthesis and breakdown are necessary if we are to fully appreciate how glucosinolate accumulation is controlled.

The continued application of genetic and genomic tools along with the development of systems biology technologies to link genetic, protein, and metabolite data should ensure further progress in gene discovery. In the near future, we can expect to understand the precise biochemical and molecular mechanisms underlying how and where plants synthesize glucosinolates. The isolated genes of glucosinolate metabolism should also facilitate metabolic engineering of plants with altered profiles of glucosinolates and glucosinolate hydrolysis products. These will be invaluable for rigorously testing the physiological and ecological roles of glucosinolates in nature. Manipulation of glucosinolate metabolism will also help in exploiting the applied potential of these compounds to improve the pest resistance and health and nutrition benefits of crop plants or to generate high-value products for industry.

SUMMARY POINTS

1. Genes have now been identified in all three phases of glucosinolate biosynthesis (amino acid elongation, core structure formation, and secondary modifications); identification of the genes involved in forming the core structure is nearly complete.
2. The elongation of amino acid side chains proceeds via a three-step acid cycle involving 2-oxo acids analogous to that occurring in leucine biosynthesis. One molecule of acetyl-CoA is added and one molecule of CO_2 is lost during each turn of the cycle for a net gain of one carbon atom.
3. The cytochromes P450, CYP79B2, and CYP79B3, which convert tryptophan to indole-3-acetaldoxime, a precursor for indole glucosinolates, the indole alkaloid camalexin, and the plant hormone indole-3-acetic acid, play a role in indole-3-acetic acid biosynthesis in *Arabidopsis*.
4. The identification of genes encoding myrosinase and the epithiospecifier protein has led to new insights into what controls the mechanism and direction of glucosinolate hydrolysis and how compartmentation prevents hydrolysis in intact plants.

5. Several lines of evidence support the role of glucosinolates in plant defense, including the toxicity and deterrence of glucosinolate hydrolysis products, the existence of specific metabolic mechanisms by which adapted insects circumvent glucosinolate toxicity, and the use of glucosinolates by herbivores in their own defense.
6. Metabolic engineering of glucosinolate profiles in *Arabidopsis* has been established and provides a tool for investigating the functional role of individual glucosinolates, for example in plant-insect or plant-pathogen interactions.

FUTURE ISSUES TO BE RESOLVED

1. What are the major regulatory factors that control flux through the biosynthetic pathway? Identification of these should allow the metabolic engineering of glucosinolate profiles to advance from the empirical to the predictive stage.
2. Identification of glucosinolate transporters to address longstanding questions about transport, storage, and turnover of glucosinolates in the intact plant.
3. Are glucosinolates broken down in the intact plant through mediation of myrosinase or other factors?
4. What are the mechanisms by which glucosinolate hydrolysis products exert their toxicity on herbivores?
5. Do glucosinolates help defend plants against fungal and bacterial pathogens under natural conditions?

ACKNOWLEDGMENTS

We thank Meike Burow, Jan-Willem de Kraker, Peggy Rice, Susanne Textor, and Ute Wittstock for critical reading of the manuscript, Angela Schneider for helping manage the literature cited, and all other members of our research groups, past and present, for their contributions. The generosity of the National Danish Research Foundation, the German National Science Foundation, and the Max Planck Society in supporting glucosinolate research in our laboratories is also gratefully acknowledged.

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of Plant Biology

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