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Source: The Arabidopsis Book, 2010(8)

Published By: The American Society of Plant Biologists

URL: https://doi.org/10.1199/tab.0134

First published on July 12, 2010: e0134. 10.1199/tab.0134

Glucosinolate Breakdown in Arabidopsis: Mechanism, Regulation and Biological Significance

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Glucosinolates are a group of thioglucosides in plants of the Brassicales order. Together with their hydrolytic enzymes, the myrosinases, they constitute the 'mustard oil bomb' involved in plant defense. Here we summarize recent studies in Arabidopsis that have provided molecular evidence that the glucosinolate-myrosinase system is much more than a 'two-component defense system,' and started to unravel the roles of different glucosinolate breakdown pathways in the context of plant responses to biotic and abiotic stresses.

1. INTRODUCTION

Among the secondary metabolites of *Arabidopsis thaliana* (Arabidopsis), the glucosinolates have been studied most intensively in the past decade (Rask et al., 2000; Wittstock and Halkier, 2002; Kliebenstein et al., 2005; Halkier and Gershenzon, 2006; Sønderby et al., 2010). The availability of Arabidopsis as a model plant has enabled the elucidation of most of the biosynthetic network and the key regulators for the more than 30 Arabidopsis glucosinolates, as well as the identification of diverse genes controlling glucosinolate breakdown upon tissue damage and in intact cells (see below). Glucosinolate research in Arabidopsis has thereby provided new insights into the biological roles of glucosinolates including the discovery of fundamentally new functions. This is reflected in a continuously rising number of publications on Arabidopsis glucosinolates (from twelve papers in 2000 to 61 papers published in 2009 (http://www.scopus.com)).

As anionic thioglucosides, glucosinolates are hydrophilic compounds that are unable to cross biological membranes by diffusion. They can, however, bind to receptors at the cell surface, such as chemoreceptors found in the mouth parts of Lepidopteran larvae and tarsi of butterflies and moths, thereby mediating feeding and oviposition responses in these insects (reviewed in Chew, 1988, and Louda and Mole, 1991). Biological activities beyond these neurosensory effects of intact glucosinolates arise from the products of glucosinolate breakdown (Figs. 1 and 2). Glucosinolates have long been known to be hydrolyzed by a group of endogenous β-glucosidases termed myrosinases (thioglucoside glucohydrolases (TGGs), EC 3.2.1.147). These enzymes are stored separately from glucosinolates, but get mixed with glucosinolates upon tissue damage. Hydrolysis of the thioglucosidic bond by myrosinases releases an aglucone that can either spontaneously rearrange into an isothiocyanate or be converted to alternative products such as simple nitriles, epithionitriles or organic thiocyanates depending on the presence of specifier proteins and certain structural prerequisites. Most glucosinolate hydrolysis products are volatile and lipophilic, which allows them to evaporate into the gas phase above the damaged tissue and to enter living cells, respectively. As isothiocyanates have been demonstrated to be toxic to a wide range of organisms including microorganisms, nematodes, and insects, and much less is known about the effects of the alternative products (reviewed in Wittstock et al., 2003, Burow and Wittstock, 2009, and Chew, 1988), the direct defensive function of the glucosinolate-myrosinase system has mainly been attributed to the isothiocyanates. Besides this classical pathway of glucosinolate breakdown upon tissue disruption (the 'mustard oil bomb', Matile, 1980), recent studies suggest that glucosinolates are also broken down in undamaged tissue and that this breakdown is involved in signaling and anti-pathogen defense. In this review, we summarize the current knowledge on glucosinolate breakdown in Arabidopsis, with special emphasis on its newly emerging pathways and roles.

2. GLUCOSINOLATE BREAKDOWN UPON TISSUE DAMAGE

2.1. Classical myrosinases

Classical myrosinases represent a phylogenetically distinct group within glycoside hydrolase family I (β-glycosidases) and are thought to have evolved from β-*O*-glucosidase ancestors (Xu et al., 2004). A distinguishing feature of these myrosinases is that

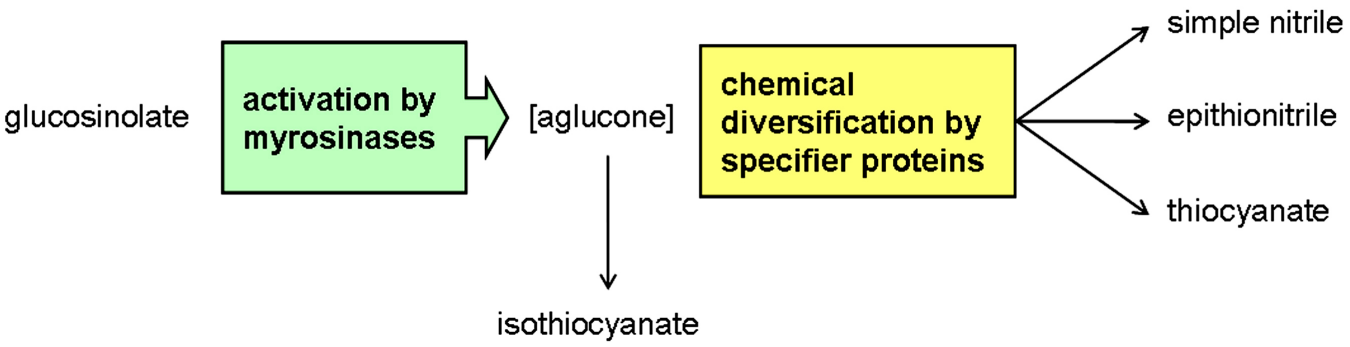

Figure 1. Glucosinolate breakdown upon tissue disruption.

Activation of glucosinolates is initiated by myrosinases (green box) which cleave the thioglycosidic bond in the glucosinolate sceleton. The resulting agluca spontaneously rearrange to form isothiocyanates unless specifier proteins (yellow box) convert them into simple nitriles, epithionitriles, or thiocyanates, depending on the biochemical properties of the specifier protein and the chemical nature of the glucosinolate side chain.

one of the catalytic glutamate residues present in the other glycoside hydrolase family I members within the peptide motif TFN**E**P (acid/base catalyst) is replaced by a glutamine residue within a TI/LN**Q**L/P motif (Burmeister et al., 1997). The function of the catalytic acid/base may be taken over by ascorbate acting as a myrosinase cofactor (Burmeister et al., 1997). Myrosinases are glycoproteins, as has been proven experimentally for a myrosinase from *Sinapis alba* seeds (http://www.pdb.org, structure ID: 1E6X) as well as for the myrosinases TGG1 and TGG2 from Arabidopsis (Ueda et al., 2006). However, heterologous expression in *Escherichia coli* yielded active TGG1 and TGG2 enzymes after reconstitution from inclusion bodies (Chung et al., 2005).

In the genome of Arabidopsis, six genes encoding classical myrosinases have been identified of which three are localized on chromosome V (*TGG1-TGG3*) and three on chromosome I (*TGG4-TGG6*) (Zhang et al., 2002a; Xu et al., 2004; Table 1). On each of these chromosomes, two of the genes (*TGG1* and *TGG2*, *TGG5* and *TGG6*) are tandem duplications separated from the third gene (*TGG3*, *TGG4*). *TGG1-TGG3* share a common gene structure composed of 12 exons and 11 introns, while *TGG4-TGG6* have 13 exons and 12 introns. Although *TGG3* and *TGG6* are expressed in specific tissues (Zhang et al., 2002b; Wang et al., 2009), both are apparently pseudogenes in several Arabidopsis accessions including Columbia-0 (Col-0) that encode nonfunctional proteins due to deletions, insertions, and premature translation termination, respectively, that result in the lack of functionally important amino acid residues (Zhang et al., 2002b; Xu et al., 2004; Wang et al., 2009).

According to Genevestigator V3 (https://www.genevestigator.com; Hruz et al., 2008), *TGG1*/*TGG2* (identical probe set) are transcribed only in the above ground organs, whereas *TGG4*/*TGG5* (identical probe set) are expressed primarily in the roots. Due to the compartmentation of the glucosinolate-myrosinase system, the actual cellular location of myrosinases in relation to the glucosinolates can be assumed to be crucial for the functionality of the system. Apparently, glucosinolates are not uniformly distributed within a single Arabidopsis leaf. Total glucosinolate levels are highest in the midvein and the outer lamina, and glucosinolate profiles differ between these sections (Shroff et al., 2008; Soenderby et al., 2010). Within the veins, glucosinolates are stored primarily in so-called S-cells in close vicinity to the phloem (Koroleva et al., 2000; Andreasson et al., 2001). For *TGG1*, the expression in scattered cells (called myrosin cells), phloem-associated cells and stomatal guard cells, respectively, of all above ground organs except the seeds has been documented by *in situ* hybridization (Xue et al., 1995), promoter-reporter gene studies (Husebye et al., 2002; Barth and Jander, 2006), immunolocalization (Ueda et al., 2006), and proteomics (Zhao et al., 2008). One study failed to detect *TGG1* expression in guard cells at the transcript level (Xue et al., 1995), and the antibody 3D7 did

Table 1. Arabidopsis thioglucosidase genes.

Name	AGI code	GH family I ID	*TGG1*	*TGG2*	*TGG3*	*TGG4*	*TGG5*	*TGG6*	*PEN2*
TGG1	At5g26000	BGLU38		**81**	**68**	**56**	**55**	**51**	**34**
TGG2	At5g25980	BGLU37	**72**		**70**	**57**	**55**	**52**	**33**
TGG3	At5g48375	BGLU39	-	-		**46**	**47**	**47**	**11**
TGG4	At1g47600	BGLU34	**49**	**49**	-		**97**	**84**	**50**
TGG5	At1g51470	BGLU35	**48**	**48**	-	**97**		**84**	**50**
TGG6	At1g51490	BGLU36	-	-	-	-	-		**38**
PEN2	At2g44490	BGLU26	**35**	**35**	-	**37**	**37**	-	

Names, AGI codes, and glucoside hydrolase (GH) family I numbers (Xu et al., 2004) are given together with the percentages of cDNA nucleotide (black numbers) and deduced amino acid (red numbers) sequence identities. *TGG1-TGG6* are referred to as classical myrosinase genes while *PEN2* encodes an atypical myrosinase. Deduced amino acid sequence identities are not given for *TGG3* and *TGG6* as these are pseudogenes.

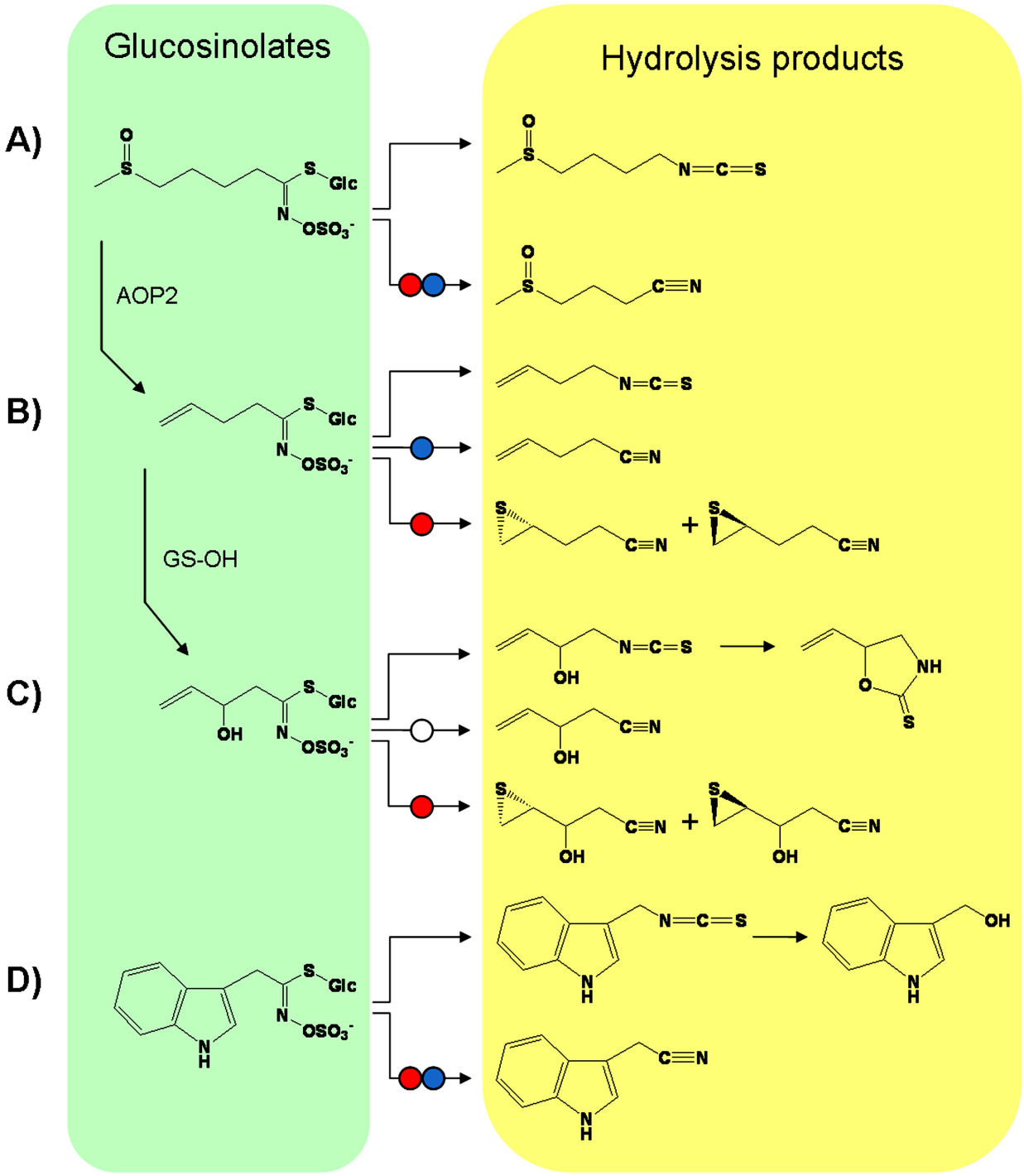

Figure 2. Chemical diversity of glucosinolate breakdown products formed upon tissue damage.

Structures of intact glucosinolates (green box) and their potential breakdown products (yellow box) in Arabidopsis are depicted for A) 4-methylsulfinylbutylglucosinolate, B) 3-butenylglucosinolate, C) 2-hydroxy-3-butenylglucosinolate, and D) indol-3-ylmethylglucosinolate. Vertical arrows and enzyme names indicate the biosynthetic link between the glucosinolates in A)-C). Arabidopsis AOP2 corresponds to GSL-ALK in Brassica species. Hydrolysis in the absence of specifier proteins (horizontal arrow, no circle) results in isothiocyanate (R-N=C=S) formation. Non-enzymatic cyclization of the isothiocyanate derived from 2-hydroxy-3-butenylglucosinolate yields goitrin (5-ethenyl-1,3-oxazolidine-2-thione, C). Indol-3-ylmethylisothiocyanate is known to further react to indole-3-carbinol, D). Red circles indicate activity of epithiospecifier protein (ESP) on alkenylglucosinolates (B, D) and on glucosinolates with other aliphatic or indolic side chains (A, D). Blue circles indicate activity of nitrile-specifier proteins (NSPs) (A, B, D). The formation of the simple nitrile from 2-hydroxy-3-butenylglucosinolate by NSPs (white circle) has not yet been demonstrated experimentally.

not detect myrosinase in guard cells (Andreasson et al., 2001), but likely reacts only with TGG2 (Ueda et al., 2006). The regulated formation of myrosin cells specifically along the veins depends on the SNARE VAM3 (At5g46860), as more and abnormally distributed myrosin cells are formed in *atvam3-4* knockout than in wild-type plants (Ueda et al., 2006). Remarkably, TGG1 is among those glycoside hydrolase family I members with the highest numbers of ESTs (Xu et al., 2004) and appears to be the most abundant protein in stomatal guard cells of rosette leaves (Zhao et al., 2008). Based on the number of available ESTs, *TGG2* is also highly expressed in above ground tissues (Xu et al., 2004). Its expression pattern based on RT-PCR and promoter activity is similar to that of *TGG1* (Xue et al., 1995; Zhang et al., 2002b; Barth and Jander, 2006; Agee et al., 2010). However, immunoblots and immunogold labeling with antibodies specific to either TGG1 or TGG2 suggest that TGG2 is much less abundant in Col-0 rosette leaves than TGG1 (Ueda et al., 2006). This is in agreement with myrosinase activity measurements on *tgg1* and *tgg2* single mutants (Barth and Jander, 2006). In contrast to *TGG1*, *TGG2* does not seem to be expressed in guard cells (Barth and Jander, 2006; Zhao et al., 2008). Highest myrosinase activity is found in young rosette leaves (about 3-week-old), while senescent rosette leaves have only low myrosinase activity (Barth and Jander, 2006; Burow et al., 2007b).

The subcellular localization of myrosinases is crucial for understanding the glucosinolate breakdown pathways in intact cells (see section 3.) and the individual roles of myrosinase isoenzymes including their interaction with other proteins. The subcellular destination of TGG1 and TGG2 is not entirely clear as prediction servers (TargetP (Emanuelsson et al., 2000) and WoLF PSORT (Horton et al., 2007)) suggest extracellular, chloroplast as well as vacuolar localization, and partially contradicting results have been obtained experimentally by using diverse methods on plants of different developmental stages. Using antibodies reacting with either TGG1 or TGG2, immunogold labeling in fact detected TGG1 and TGG2 in vacuoles in Arabidopsis leaf sections (Andreasson et al., 2001; Ueda et al., 2006), and both proteins have been identified in the rosette leaf vacuolar proteome (Carter et al., 2004). TGG2 has also been identified in the chloroplast proteome, this may, however, be due to a contamination of the chloroplast preparation (Kleffmann et al., 2004; Kley et al., 2010). Vacuolar localization is not impaired in *atvam3-4* knockout plants despite their disturbed regulation of myrosin cell formation (Ueda et al., 2006). Upon differential centrifugation, TGG1 resides in the soluble fraction, while a considerable proportion of TGG2 is detectable in aggregates precipitating at 1000x*g* (ER-body fraction; Matsushima et al., 2003) and in the microsomal pellet (100000x*g*; Ueda et al., 2006). Subcellular localization by ectopic expression of TGG1:GFP or TGG2:GFP fusions in epidermal cells of cotyledons showed, however, the presence of both proteins in the endoplasmic reticulum (ER), in ER bodies, and in transvacuolar strands (Agee et al., 2010), which are cytoplasmic strands inside the vacuole that are surrounded by tonoplast membrane (Ruthardt et al., 2005). This localization is dependent on MVP1 (At1g54030), a protein that had been annotated as myrosinase-associated protein and is involved in protein trafficking (Agee et al., 2010; see section 2.3). Interestingly, TGG2, but not TGG1, interacts with MVP1 *in vitro* as demonstrated by pull-down assays using a glutathione-*S*-transferase:MVP1 fusion and Arabidopsis leaf extracts (Agee et al., 2010). The biological importance of this interaction is currently unclear. Maybe binding of MVP1 to TGG2 regulates glucosinolate breakdown in intact tissue by a direct effect on TGG2 activity or by changing the location of TGG2 relative to the glucosinolates.

When overexpressed in the yeast *Pichia pastoris*, purified TGG1 carrying a C-terminal 6xHis-tag had a K_m of 45 μmol l^{-1} and a v_{max} of 2.2 μmol min^{-1} mg^{-1} with allylglucosinolate (sinigrin) as substrate (Andersson et al., 2009). Kinetic constants for TGG2 or using other glucosinolate substrates have not been reported. It appears, however, that TGG1 and TGG2 possess a certain substrate specificity. Crude extracts of *P. pastoris* expressing TGG1 and reconstituted inclusion bodies from TGG1-expressing *E. coli* hydrolyzed allylglucosinolate, 3-butenylglucosinolate and 4-methylsulfinylbutylglucosinolate more than twice as fast as 2(*R*)-2-hydroxy-3-butenylglucosinolate and 2-phenylethylglucosinolate (Chung et al., 2005). Similar results were obtained for TGG2, but depended on the heterologous host (Chung et al., 2005). Substrate specificity has also been observed *in planta*, as 4-methoxyindol-3-ylmethylglucosinolate is broken down less rapidly than aliphatic glucosinolates and other indolic glucosinolates in Arabidopsis leaf homogenates (Barth and Jander, 2006). Both TGG1 and TGG2 hydrolyze leaf glucosinolates upon tissue disruption as glucosinolate breakdown is basically unchanged in *tgg1* or *tgg2* single mutants (Barth and Jander, 2006). Only when both myrosinases are knocked out, myrosinase activity on allylglucosinolate as exogenous substrate is undetectable in leaf extracts, and endogenous aliphatic glucosinolates are no longer broken down in disrupted leaf tissue (Barth and Jander, 2006). However, breakdown of endogenous indolic glucosinolates still proceeds, although at low pace, indicating the presence of a breakdown pathway for these glucosinolates independent of TGG1 and TGG2 (Barth and Jander, 2006; see also section 3.2). The obvious overlap of TGG1 and TGG2 functions with respect to glucosinolate breakdown in disrupted leaves, together with the high abundance of TGG1 in guard cells as well as the aggregation tendency of TGG2 and its specific interaction with MVP1, raises the question, if these two enzymes may have other functions within or beyond plant defense (see section 3).

TGG4 and TGG5 are less well characterized than their aboveground counterparts. *TGG4* ESTs have been identified from seedlings, rosette leaves and roots, and *TGG5* ESTs from rosette leaves and roots (Xu et al., 2004). *TGG5* transcript has also been detected in the ovary (http://bbc.botany.utoronto.ca; Toufighi et al., 2005). Based on the observation that myrosinase activity is undetectable in rosette leaves when TGG1 and TGG2 are knocked-out (Barth and Jander, 2006), TGG4 and TGG5 do not seem to play a role in the breakdown of, at least, aliphatic glucosinolates in leaves. Absolute values of myrosinase activity per gram (f.w.) tissue are ten times lower in roots than in leaves of 2-week-old seedlings (Barth and Jander, 2006), despite the observation that the total glucosinolate content in roots is about half of that in leaves at the seedling stage (Petersen et al., 2002). For recombinant TGG4 and TGG5 expressed in *P. pastoris* with a C-terminal 6xHis-tag, K_m values of 245 μmol l^{-1} and 547 μmol l^{-1}, respectively, and v_{max} values of 12 μmol min^{-1} mg^{-1} and 48 μmol min^{-1} mg^{-1}, respectively, have been determined with allylglucosinolate (Andersson et al., 2009).

TGG1, TGG4, and TGG5 are activated to a different extent by up to 1.5 mM ascorbic acid *in vitro* while higher concentrations inhibit enzymatic activity (Andersson et al., 2009). Activation is

Table 2. Arabidopsis genes encoding specifier proteins.

Name	AGI code	*JAL*	*Kelch*	*ESP*	*NSP1*	*NSP2*	*NSP3*	*NSP4*	*NSP5*
ESP/ESR	At1g54040	0	5		**-/65**	**-/66**	**-/65**	**-/64**	**-/59**
NSP1	At3g16400	1	5	**-/58**		**81/88**	**91/92**	**92/96**	**-/62**
NSP2	At2g33070	1	5	**-/59**	**73/84**		**80/86**	**81/87**	**-/62**
NSP3	At3g16390	1	5	**-/58**	**88/91**	**72/82**		**91/90**	**-/61**
NSP4	At3g16410	2	5	**-/57**	**90/94**	**75/82**	**85/86**		**-/60**
NSP5	At5g48180	0	4	**-/50**	**-/53**	**-/52**	**-/51**	**-/52**	

Names (according to TAIR) and AGI codes are given together with the number of predicted Jacalin-related lectin (JAL) and Kelch domains of the encoded proteins and the precentages of cDNA nucleotide (black numbers) and deduced amino acid (red numbers) sequence identities. The first value indicates sequence identities among the JAL domains while the second value refers to sequence identities among partial sequences comprising the Kelch domains. For NSP4, the only NSP containing two JAL domains, only the second JAL domain was considered (the first JAL domain of NSP4 shares only 17-19% identity to the other JAL domains on the nucleotide level). As Col-0 does not possess ESP activity, the L*er ESP* sequence was used instead. Note that Kissen and Bones (2009) use a different nomenclature for the NSPs.

most pronounced for TGG1 (about 20-fold at 1 mM ascorbic acid). All three enzymes have been demonstrated to also possess β-*O*-glucosidase activity, but bind the chromogenic substrate 4-nitrophenyl-β-D-glucopyranoside with very low affinity (K_m 30-80 mM) (Andersson et al., 2009). Comparative studies on the substrate specificities and co-factor requirements of TGG1, TGG2, TGG4, and TGG5 would provide an opportunity to further decipher the individual roles of Arabidopsis myrosinases.

2.2. Specifier proteins

Originally discovered in *Crambe abyssinica* (Brassicaceae), specifier proteins impact the outcome of glucosinolate hydrolysis without having hydrolytic activity on glucosinolates themselves (Tookey, 1973). Most likely, they function as enzymes acting on the glucosinolate aglucone to prevent spontaneous isothiocyanate formation by catalyzing the formation of epithionitriles, simple nitriles or organic thiocyanates depending on the type of specifier protein and the chemical structure of the aglucone side chain (reviewed in Wittstock and Burow, 2007; Fig. 2). Epithionitrile formation requires the presence of a terminal double bond in the glucosinolate side chain and is catalyzed by epithiospecifier proteins (ESPs) as well as the related thiocyanate-forming protein (TFP) (Wittstock and Burow, 2007). Thiocyanate formation upon myrosinase-catalyzed hydrolysis has only been described for three glucosinolates, namely benzylglucosinolate, allylglucosinolate and 4-methylthiobutylglucosinolate (Lüthy and Benn, 1977), and happens only in the presence of TFPs (Burow et al., 2007a; Wittstock and Burow, 2007). In Arabidopsis, thiocyanate formation has not been reported. The formation of simple nitriles represents a special case, as these compounds are also produced in the absence of specifier proteins *in vitro* when the myrosinase reaction takes place at low pH values (< 5) or high ferrous ion concentrations (> 0.01 mM) (reviewed in Wittstock and Burow, 2007). Nitrile-specifier proteins (NSPs) promote simple nitrile formation at physiological pH values, but do not catalyze epithionitrile or thiocyanate formation (Burow et al., 2009; Kissen and Bones, 2009; Figs. 2 and 3). However, ESP and TFP also have nitrile-specifier activity (Burow et al., 2006b; Burow et al., 2007a).

In Arabidopsis, six specifier proteins have been identified so far (Table 2), namely one ESP (Lambrix et al., 2001) and five NSPs (Burow et al., 2009; Kissen and Bones, 2009). The corresponding genes are located on chromosomes I (*ESP*), II (*NSP2*), and V (*NSP5*), and as a cluster of three highly similar genes on chromosome III (*NSP1*, *NSP3*, *NSP4*). For the *ESP*-locus, allelic variation has been detected among different Arabidopsis accessions resulting in the presence of functional ESP in some accessions (*e.g.* Landsberg *erecta* (L*er*) and Cape Verde Islands (Cvi)) and its absence in others (*e.g.* Col-0)(Lambrix et al., 2001). Structurally, ESP and NSP5 are composed of five or four Kelch domains (Adams et al., 2000), respectively, while NSP1-NSP4 are chimeric proteins consisting of five Kelch domains and one (NSP1-NSP3) or two (NSP4) N-terminal Jacalin-related lectin (JAL) domains according to predictions by InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/; Table 2).

ESP is the best-studied member of the protein family. Its expression pattern has been investigated on the transcript, protein, and activity level using the L*er* accession (Burow et al., 2007b). QRT-PCR detected *ESP* transcript in all above-ground organs examined and traces in the roots. This correlated to a large extent with ESP amounts detected by immunoblotting and ESP activity measurements. However, there was no detectable ESP protein or ESP activity in roots and considerable discrepancies between transcript/protein levels and activities measured in cauline leaves, flowers and siliques indicating post-transcriptional regulation of ESP activity in addition to its transcriptional control (Burow et al., 2007b). Highest ESP activities (measured as the quantity of epithionitrile formed per min and mg total protein from allylglucosinolate added to crude plant extracts) were found in rosette leaves of plants before bolting and in flowers (Burow et al., 2007b). *ESP* transcript levels increase two- to fivefold upon methyljasmonate application in Arabidopsis accessions with and without ESP activity (reviewed in Burow and Wittstock, 2009). The impact of herbivory and pathogen attack on *ESP* transcript levels has only been studied in the Col-0 accession that lacks ESP activity likely due to a 10-bp deletion in a transcription factor binding site in the *ESP*-promoter sequence (Lambrix et al., 2001). Although sucking herbivores and pathogens do not seem to have a major influence on *ESP* transcript levels (De Vos et al., 2005; Schenk et al., 2000; Kempema et al., 2007), *Pieris*

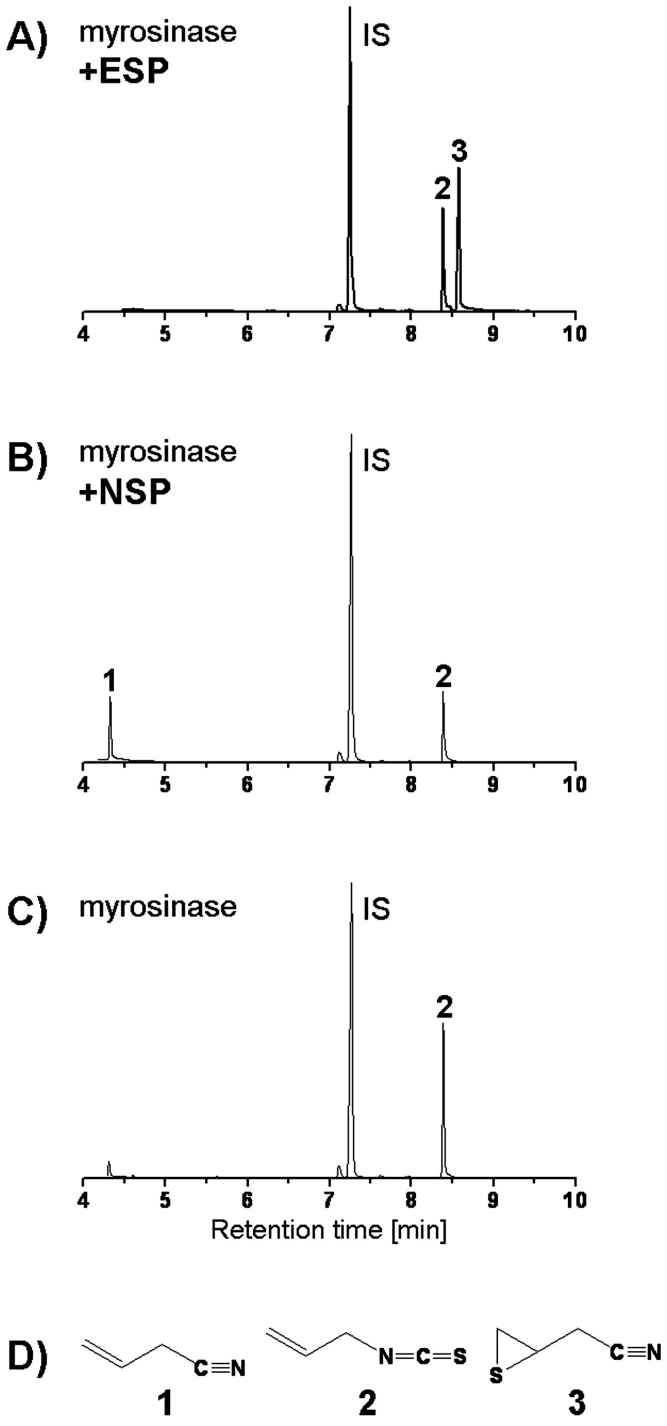

Figure 3. Effects of ESP (epithiospecifier protein) and NSP (nitrile-specifier protein) on the hydrolysis of allylglucosinolate *in vitro*.

Shown are total ion current traces of GC-MS chromatograms of enzyme assays after extraction with dichloromethane (A-C) and chemical structures of allylglucosinolate hydrolysis products (D). Allylglucosinolate (2 mM) was incubated with myrosinase in the presence of purified ESP (A), NSP1 (B), or without addition of specifier proteins (C) in 50 mM MES buffer, pH 6.0. IS, internal standard; peak numbers refer to the structure numbers in D. 1, simple nitrile; 2, isothiocyanate; 3, epithionitrile.

rapae (Lepidoptera) herbivory led to a 2.8-fold induction of *ESP* transcription (De Vos et al., 2005). Taking into account that ESP protein or activity levels have not been determined in the induction experiments, that there are indications for post-transcriptional regulation, and that most experiments have been done with the Col-0 accession, the relevance of these findings has remained unclear.

Immunolocalization confirmed the absence of ESP in Col-0 plants and in roots of L*er* plants, and showed that L*er* plants accumulate ESP in the epidermis of all above-ground organs, except the anthers (Burow et al., 2007b). In addition, ESP is found in the glucosinolate-containing S-cells of the stem of L*er* plants, but not in S-cells of their leaves. Although immunolocalization indicated a lack of ESP in the TGG1-rich stomatal guard cells in L*er* plants (Burow et al., 2007b), ESP seems to be present based on mass-spectrometric analysis of electrophoretically separated guard cell proteins of the Wassilewskija (WS) accession (Zhao et al., 2008). L*er* plants possess functional ESP, but do not accumulate alkenylglucosinolates that would give rise to epithionitrile formation upon tissue disruption. Thus, in L*er* plants, ESP can only act on agluca of alkyl-, hydroxyalkyl- and indole glucosinolates, which it converts to the corresponding simple nitriles. It is therefore interesting, that ESP localization is different in leaves of the Cvi accession that has both functional ESP and alkenylglucosinolates. In Cvi leaves, ESP is present in S-cells close to the phloem. This does, however, not result in higher ESP activity in extracts of the midrib as compared to extracts of the remaining leaf lamina further supporting some kind of posttranslational regulation (Burow et al., 2007b). In agreement with predictions by WoLF PSORT (Horton et al., 2007) and TargetP (Emanuelsson et al., 2000), it appears from the immunolocalization that ESP is a cytosolic protein. It is also detectable in the nucleus (Burow et al., 2007b) which is not unusual for small cytosolic proteins but may also indicate a specific role of ESP in regulatory processes (Miao and Zentgraf, 2007). In fact, ESP has also been termed EPITHIOSPECIFYING SENESCENCE REGULATOR (ESR/ESP) based on its interaction with the transcription factor WRKY53 involved in regulation of senescence and senescence-related phenotypes of ESP-overexpression and knockout lines (Miao and Zentgraf, 2007).

Expression of the five Arabidopsis *NSP* genes is organ-specifically and developmentally regulated. According to Genevestigator V3 (https://www.genevestigator.com; Hruz et al., 2008), *NSP2* and *NSP5* are expressed at high levels in seeds and the inflorescence. In addition, *NSP5* transcript is found in roots and senescent rosette leaves. For *NSP2*, its expression in seeds and induction by salicylic acid treatment during imbibition has been confirmed by a proteomics study (Rajjou et al., 2006). *NSP1*, *NSP3*, and/or *NSP4* transcripts (one probeset) are detected in all organs, with highest levels in seedlings and roots. *NSP1* transcript in Col-0 rosette leaves is strongly induced upon herbivore feeding, as is simple nitrile formation in leaf homogenates (Burow et al., 2009). As an *nsp1* knockout line was deficient in constitutive and herbivore-induced simple nitrile formation in rosette leaves, NSP1 seems to be the major NSP in the Col-0 rosette. Thus, despite the absence of ESP activity (see above), Col-0 is able to produce simple nitriles upon tissue disruption due to the presence of NSP(s). Both NSP1 and NSP5 have been identified mass-spectrometrically in the guard cell proteome (Zhao et al., 2008). According to WoLF PSORT (Horton et al., 2007) and TargetP (Emanuelsson et al., 2000) predictions, all Arabidopsis NSPs are cytoplasmic. This is

in agreement with the absence of NSPs in the vacuolar proteome of rosette leaves (Carter et al., 2004).

In order to measure specifier protein activity, the reaction catalyzed by specifier proteins has to be coupled to myrosinase-catalyzed glucosinolate hydrolysis, since the glucosinolate agluca, as the putative substrates, are short-lived and cannot be isolated. This is the reason why kinetic constants can not be determined for specifier proteins. Product formation by ESP and NSP1 from different glucosinolates as substrates for myrosinase has been analyzed using purified proteins expressed in *E. coli* (Burow et al., 2008; Burow et al., 2009; Fig. 3). Nitrile formation by NSP2 is evident from glucosinolate hydrolysis product profiles in homogenates of Arabidopsis expressing NSP2 under control of the CaMV35S-promoter (Kissen and Bones, 2009). From this, it seems as if at least ESP and NSP2 work on agluca of aliphatic, aromatic and indolic glucosinolates, although only low ESP activities were obtained using benzylglucosinolate as substrate for the myrosinase reaction. While the exact biochemical role of specifier proteins is currently unknown (reviewed in Wittstock and Burow, 2007), it has been shown experimentally that Arabidopsis ESP does not form a stable complex with myrosinase (Burow et al., 2006b). However, physical contact between ESP and myrosinase is required for epithionitrile formation (Burow et al., 2006b). Furthermore, ESP activity is strictly dependent on Fe^{2+} (Burow et al., 2006b; Zabala et al., 2005), but not affected by radical scavengers or exclusion of oxygen indicating a non-radical mechanism (Burow et al., 2006b). NSPs are not able to catalyze epithionitrile formation even in the presence of Fe^{2+} (Burow et al., 2009; Kissen and Bones, 2009; Fig. 3). Simple nitrile formation by NSPs is increased by Fe^{2+} (Burow et al., 2009; Kissen and Bones, 2009), but at least NSP1 activity is not dependent on Fe^{2+} (Burow et al., 2009).

The role of the N-terminal JAL domains of NSP1-NSP4 is currently unknown. It is interesting that myrosinase-binding proteins (MBPs) from *Brassica* species and corresponding proteins encoded in the Arabidopsis genome are composed entirely of JAL domains (Falk et al., 1995; Taipalensuu et al., 1997; Nagano et al., 2008), which is the reason why some NSPs had been annotated as MBP-like proteins. In *Brassica* species, MBPs have been shown to form stable complexes with myrosinases in tissue homogenates, but their function has not been resolved (Falk et al., 1995; Geshi and Brandt, 1998). The putative ancestor of the specifier protein family, At3g07720, that did not have specifier protein activity in enzyme assays with recombinant protein, but has homologues in non-glucosinolate plants and fungi, lacks a JAL domain, indicating that the presence of the JAL domain is a derived state (Burow et al., 2009). Likewise, both ESP and NSP5 as well as specifier proteins from other glucosinolate-containing plants (Matusheski et al., 2006; Burow et al., 2007a) lack a JAL domain, demonstrating that a JAL domain is not essential for catalytic activity. If the JAL domains have an impact on substrate specificity, interaction with myrosinases, regulation or localization of specifier proteins remains to be investigated.

2.3. The ESM1 and MVP1 loci

Among quantitative trait loci (QTL) that impact glucosinolate breakdown, the *EPITHIOSPECIFIER-MODIFIER1* (*ESM1*) locus (At3g14210) was found to epistatically interact with the *ESP* locus in Arabidopsis such that simple nitrile formation is suppressed and isothiocyanate production is promoted for benzylglucosinolate and alkylglucosinolates, but not for alkenylglucosinolates, when *ESM1* is functional (Zhang et al., 2006). Cloning of the *ESM1* locus revealed that it encodes a putatively ER-bound protein with high similarity to myrosinase-associated proteins (MyAPs) known to copurify with myrosinases in experiments with *Brassica* species (Zhang et al., 2006; Taipalensuu et al., 1996). Allelic variation of *ESM1* contributes to the variation of glucosinolate breakdown among different Arabidopsis accessions (Zhang et al., 2006; see below). The mechanism by which *ESM1* exerts its action on glucosinolate hydrolysis is currently unknown. A similar effect on the outcome of glucosinolate breakdown has been described for *MVP1* (*modified vacuolar phenotype 1*, At1g54030), encoding another MyAP-like protein that is closely related to ESM1. The *mvp1-1* mutant is impaired in endomembrane protein trafficking and shows a slight, but significant increase in simple nitrile formation from allylglucosinolate added to rosette leaf homogenates (Agee et al., 2010). Interestingly, MVP1 interacts with TGG2, but not with TGG1, *in vitro*.

2.4. Breakdown products, their natural variation and biological roles

As the outcome of glucosinolate breakdown depends on structural requirements regarding the glucosinolate side chain as well as on the functionality of specifier proteins, *ESM1*, and *MVP1*, there are, at least, two principle genetic sources of variability in breakdown products in different Arabidopsis accessions. First, the allelic status of various biosynthetic loci determines which structural types of glucosinolates are available as substrates for myrosinase. For example, only alkenylglucosinolates with a terminal double bond fulfill the structural requirement for epithionitrile formation. They are present in only about half of all Arabidopsis accessions (Kliebenstein et al., 2001). Secondly, the allelic status for *ESP*, *ESM1*, *MVP1* and maybe the *NSP* genes, control the conversion of the aglucone during breakdown and thereby the type of product formed. Both glucosinolate biosynthesis and breakdown are developmentally and organ-specifically regulated and influenced by environmental factors such as biotic and abiotic stresses (*e.g.* Petersen et al., 2002; Brown et al., 2003; Kruse et al., 2007; Brader et al., 2001; Wentzell and Kliebenstein, 2008; Wentzell et al., 2008; Burow et al., 2007b; Burow and Wittstock, 2009; Burow et al., 2009). This means that a single glucosinolate can give rise to up to three different types of breakdown products in Arabidopsis tissue homogenates depending on the genotype of the plant, the organ, the developmental stage, and the environmental conditions (Fig. 2). Currently, little is known about why glucosinolate breakdown varies to such a large extent, but its tight regulation suggests that specific breakdown products or certain mixtures of breakdown products serve distinct biological functions. As isothiocyanates are generally toxic, alternative breakdown pathways may also have evolved as a means to safely degrade glucosinolates in intact tissue (see section 3). Biological activities of glucosinolate breakdown products have been surveyed elsewhere (Agerbirk et al., 2009; Wittstock et al., 2003; Chew, 1988; Burow and Wittstock, 2009), and numerous studies have shown the impact of the glucosinolate-myrosinase system

on plant-insect interactions using various plant and insect species (reviewed in Hopkins et al., 2009). Here, we would like to focus on some recent studies in Arabidopsis that indicate how regulation and fine tuning of glucosinolate breakdown may enable the plant to balance its direct *vs.* indirect defense responses against generalists *vs.* specialists. Different feeding modes of herbivores (like sucking or chewing) add another layer of complexity to the defense responses as they are associated with different degrees of tissue damage.

While the defensive function of the glucosinolate-myrosinase system against chewing generalist herbivores clearly depends on glucosinolate-breakdown (Kliebenstein et al., 2002; Barth and Jander, 2006), several studies have shown that larvae of the generalist lepidopteran *Trichoplusia ni* (cabbage looper, Lepidoptera) feed more on simple nitrile-producing than on isothiocyanate-producing Arabidopsis when given a choice (Lambrix et al., 2001; Jander et al., 2001; Zhang et al., 2006). Similarly, larvae of another generalist, *Spodoptera littoralis* (egyptian cotton leafworm, Lepidoptera), develop faster on simple nitrile- than on isothiocyanate-producing lines in no-choice experiments (Burow et al., 2006a). In agreement with previous studies showing high toxicity of isothiocyanates for lepidopteran larvae (reviewed in Wittstock et al., 2003), this demonstrates that isothiocyanates are more effective as anti-herbivore defenses against generalist Lepidoptera than simple nitriles and raises the question why plants produce a large set of specifier proteins and thereby produce less toxic products.

A key to understand the biological roles of specifier proteins might be the fact that certain specialist insects (*e.g. Plutella xylostella* (diamond back moth), *Pieris rapae* (cabbage white butterfly), both Lepidoptera) have aquired adaptations to circumvent the negative effects associated with the glucosinolate-myrosinase system (Ratzka et al., 2002; Wittstock et al., 2004) and exploit isothiocyanates as well as intact glucosinolates as chemical cues to identify glucosinolate-containing plants which they use as their sole host plants (Sun et al., 2010; reviewed in Wittstock et al., 2003). Thus, if such specialist attackers are around, a plant might be better off without emission of isothiocyanates that would promote long distance attraction of ovipositing adults of these herbivores. In fact, *P. rapae* lays fewer eggs on simple nitrile-producing than on isothiocyanate-producing Arabidopsis when plants are damaged prior to the experiment (Mumm et al., 2008). This might be a consequence of the reduced amounts of isothiocyanates emitted, but in addition, indole-3-acetonitrile, a product of indol-3-ylmethylglucosinolate breakdown in the presence of ESP or NSPs (Fig. 2D), has been demonstrated to act as an oviposition deterrent for *P. rapae* (De Vos et al., 2008). Interestingly, this effect seems to be species-specific as indole-3-acetonitrile does not have a significant influence on oviposition by another specialist, *Plutella xylostella* (Sun et al., 2009). Another role of simple nitriles might be to function as signals to parasitoids of lepidopteran larvae. In wind-tunnel experiments, *P. rapae*-infested simple nitrile-producing Arabidopsis were more attractive to the specialist larval endoparasitoid *Cotesia rubecula* (Hymenoptera) than *P. rapae*-infested isothiocyanate-producing plants suggesting a role of simple nitriles in indirect defense responses (Mumm et al., 2008). In agreement with this function in tritrophic interactions, herbivory by *P. rapae* larvae results in a local induction of Arabidopsis NSP1 leading to increased simple nitrile formation in leaf homogenates as compared to uninfested plants (Burow et al., 2009).

In contrast to chewing lepidopteran larvae, aphids avoid disruption of myrosinase-containing cells by inserting their flexible stylus through the apoplast directly into the sieve elements (De Vos et al., 2007). As has been shown for the generalist *Myzus persicae* (green peach aphid, Hemiptera) feeding on Arabidopsis, intact glucosinolates are taken up by the aphids, and aliphatic glucosinolates are excreted unchanged in the aphid honeydew with no obvious negative effects on the aphids (Kim and Jander, 2007). Indole glucosinolates, however, deter aphid feeding due to the formation of antifeedant breakdown products inside the aphids (Kim et al., 2008). In plants, the breakdown pathway for indole glucosinolates is partially different from that of aliphatic glucosinolates as the corresponding isothiocyanates are unstable and form adducts with nucleophiles or decompose to indole-3-carbinols (Fig. 2D) that may also react with nucleophiles (reviewed in Agerbirk et al., 2009). As a result, the major products of indole glucosinolate breakdown in tissue homogenates in the absence of specifier proteins are ascorbigens, *i.e.* adducts of indolic isothiocyanates or indole-3-carbinols with ascorbic acid (Agerbirk et al., 2009). In *M. persicae*, a similar pathway has been identified for indol-3-ylmethylglucosinolate breakdown, namely the TGG1- and TGG2-independent formation of indole-3-carbinol followed by the reaction with cysteine and/or glutathione to yield indol-3-ylmethylcysteine and other cysteine conjugates (Kim et al., 2008). While it is currently unknown if the hydrolysis of indole glucosinolates inside the aphid happens spontaneously or is catalyzed by aphid and/or plant enzymes, it has been demonstrated that the conjugates with cysteine, but not with other amino acids, act as feeding deterrents to *M. persicae* (Kim et al., 2008). When incorporated into an artifical diet, 4-methoxyindol-3-ylmethylglucosinolate is more deterrent than 1-methoxyindol-3-ylmethylglucosinolate and their biosynthetic precursor indol-3-ylmethylglucosinolate (Kim and Jander, 2007). It is therefore interesting to note, that 4-methoxyindol-3-ylmethylglucosinolate is induced by *M. persicae* feeding on Arabidopsis (Kim and Jander, 2007).

Brevicoryne brassicae (cabbage aphid, Hemiptera) is a specialist aphid on glucosinolate-containing plants that possesses its own myrosinase and sequesters glucosinolates from its host plants for its defense against predators (Kazana et al., 2007). When infested with *B. brassicae*, Arabidopsis Ru-0 plants (accumulating allylglucosinolate as a major glucosinolate in leaves) release high amounts of allylisothiocyanate by an unknown mechanism while uninfested plants do not. There are no other large differences in volatile profiles between infested and uninfested plants. As shown by Y-tube olfactometer experiments, infested Ru-0 plants are more attractive than uninfested Ru-0 plants to *Diaeretiella rapae* (Hymenoptera: Braconidae), a specialist endoparasitoid wasp that attacks aphids on glucosinolate-containing plants (Kissen et al., 2009). When using Col-5 plants, which accumulate primarily 4-methylsulfinylbutylglucosinolate, no changes in glucosinolate-derived volatiles and parasitoid attraction upon aphid infestation are detectable, likely due to the lower volatility of isothiocyanates derived from methylsulfinylalkylglucosinolates (Fig. 2A) as compared to those derived from alkenylglucosinolates (Fig. 2B). When the latter glucosinolates are introduced to Col-5 plants by ectopic expression of the biosynthetic GLS-ALK gene from *Brassica nigra* (Fig. 2), infestation with *B. brassicae* results in the release of 3-butenylisothiocyanate, and infested plants become more attractive to *D. rapae* than uninfested plants (Kissen

et al., 2009). Isothiocyanates derived from allyl- and 3-butenylglucosinolate have previously been shown to be more attractive to *D. rapae* than the corresponding simple nitriles and epithionitriles (Pope et al., 2008). Taken together, these examples underline the link between glucosinolate biosynthesis and breakdown and its importance in ecological interactions.

3. GLUCOSINOLATE BREAKDOWN IN INTACT TISSUE

3.1. Glucosinolate turnover

Based on the observation that the total glucosinolate content per individual declines strongly during germination and seedling development, glucosinolate breakdown has long been proposed to also take place in undamaged tissue (Brown et al., 2003; Petersen et al., 2002). As the glucosinolate core structure comprises two sulfur atoms and glucosinolate biosynthetic genes are upregulated in response to sulfur fertilization, while glucosinolate content decreases during sulfur depriviation, glucosinolates have been suggested to serve as a storage form of sulfur (reviewed in Falk et al., 2007). As a breakdown pathway that would allow the mobilization of both sulfur atoms as well as the core structure nitrogen without release of toxic isothiocyanates, the involvement of myrosinase-type enzymes together with NSPs and nitrilases has been suggested (Janowitz et al., 2009; Fig. 4A). Nitrilases (EC 3.5.5.) catalyze the hydrolysis of the C-N-bond in nitriles yielding a carboxylic acid and ammonia. Arabidopsis has four genes encoding nitrilases of which three form the NIT1 group (Piotrowski, 2008). As an argument supporting this pathway, enzymes of the NIT1 group have a broad substrate specificity and accept nitriles with structural similarity to glucosinolate-derived nitriles, and the predominant isoform NIT1 (At3g44310) is most active on nitriles derived from glucosinolates (Vorwerk et al., 2001; Janowitz et al., 2009; Piotrowski, 2008). Conclusive evidence for nitrilase-dependent glucosinolate turnover *in planta* is, however, still missing.

Indications for another breakdown pathway in intact tissue come from the analysis of Arabidopsis ectopically expressing TGG4 under the control of the CaMV35S-promotor (Fig. 4A). These plants accumulate indol-3-ylmethylamine and raphanusamic acid as well as amines with side-chain structures corresponding to the structures of aliphatic glucosinolate side chains as revealed by LC-MS analysis of their DMSO extracts (Bednarek et al., 2009). Both the amines and raphanusamic acid appear to be formed as decomposition products of glutathione conjugates of glucosinolate-derived isothiocyanates (Bednarek et al., 2009). This means that ectopic expression of TGG4 leads to glucosinolate breakdown in intact tissue, likely due to an artificial distribution of myrosinase (lack of compartmentalization between myrosinase and glucosinolates), and that there is an efficient detoxification pathway for isothiocyanates in the plant. If such a pathway functions in glucosinolate turnover in intact wildtype plants has not yet been shown. At least for seedlings, the involvement of the wildtype above-ground myrosinases TGG1 and TGG2 seems unlikely, as glucosinolate turnover is largely unaffected in *tgg1 tgg2* mutant seedlings (Barth and Jander, 2006). Notably, the pathway would not mobilize the glucosinolates' nitrogen and thioglucosidic sulfur for nutrition.

3.2. Pathogen resistance and signaling

Arabidopsis is well protected against a number of non-adapted microbial pathogens by restricting the entry of these pathogens into the cells (Lipka et al., 2008). The interaction of the plant with such microbial pathogens is different from that with chewing herbivores, as it is not associated with tissue disruption, but mediated by living plant cells that protect themselves from penetration by the pathogen. As a consequence, glucosinolate breakdown has not been considered to be involved in such responses until recently when the breakdown of one specific glucosinolate, 4-methoxyindol-3-ylmethylglucosinolate, was found to be required for induced defense of Arabidopsis against the grass powdery mildew *Blumeria graminis hordei* (Bednarek et al., 2009). This discovery was, amongst others, based on the finding that one of the two induced parallel pathways conferring broad-spectrum resistance pre-invasively depends on PEN2, a peroxisomal β-glucosidase (At2g44490, BGLU26; Xu et al., 2004) that is able to hydrolyze glucosinolates, likely with a preference for indole glucosinolates (Lipka et al., 2005; Bednarek et al., 2009). As PEN2 has thioglucosidase activity despite the presence of a glutamate residue as acid/base catalyst in its active site (see section 2.1) and its position in a subclade of β-glucosidases separate from the classical myrosinases in the phylogenetic tree (Xu et al., 2004), it is considered an atypical myrosinase (Bednarek et al., 2009). Peroxisomes loaded with PEN2 accumulate at the site of fungal entry as demonstrated by fluorescence microscopy with PEN2:GFP-fusions. This suggests an active mechanism of generating high local concentrations of 4-methoxyindol-3-ylmethylglucosinolate breakdown products which might then be transferred to the apoplast by the ABC-transporter PEN3 (At1g59870) to act as antimicrobial toxins (Lipka et al., 2005; Lipka et al., 2008; Bednarek et al., 2009). The chemical nature of these compounds is still unknown. PEN2-dependent breakdown of another indole glucosinolate, indol-3-ylmethylglucosinolate, *in planta* results in the accumulation of indol-3-ylmethylamine and raphanusamic which are decomposition products of the glutathione conjugate of indol-3-ylmethyl isothiocyanate (Fig. 4B). As resistance is dependent on PEN2 and 4-methoxyindol-3-ylmethylglucosinolate, but the corresponding amine is not detectable, an alternative, PEN2-dependent processing route of 4-methoxyindol-3-ylmethylglucosinolate or another metabolic pathway of the respective glutathione conjugate is likely to function in pathogen defense (Bednarek et al., 2009; Clay et al., 2009). It is currently unclear, how PEN2 comes into contact with 4-methoxyindol-3-ylmethylglucosinolate, which is presumably stored in the vacuole. Interestingly, PEN2-dependent breakdown of 4-methoxyindol-3-ylmethylglucosinolate is also required for callose deposition in Arabidopsis seedlings as a response to the treatment with Flg22, a synthetic derivative of the microbe-associated molecular pattern (MAMP) polypeptide flagellin (Clay et al., 2009; Boller and Felix, 2009). Here, the transporter PEN3 has been suggested to be involved in triggering the movement of hypothetical complexes of indole glucosinolate breakdown products and phytochelatins to the plasma membrane where activation of callose synthase may take place (Clay et al., 2009). Thus, in this scenario, products of PEN2-dependent 4-methoxyindol-3-ylmethylglucosinolate would serve as signaling molecules rather than direct defense compounds.

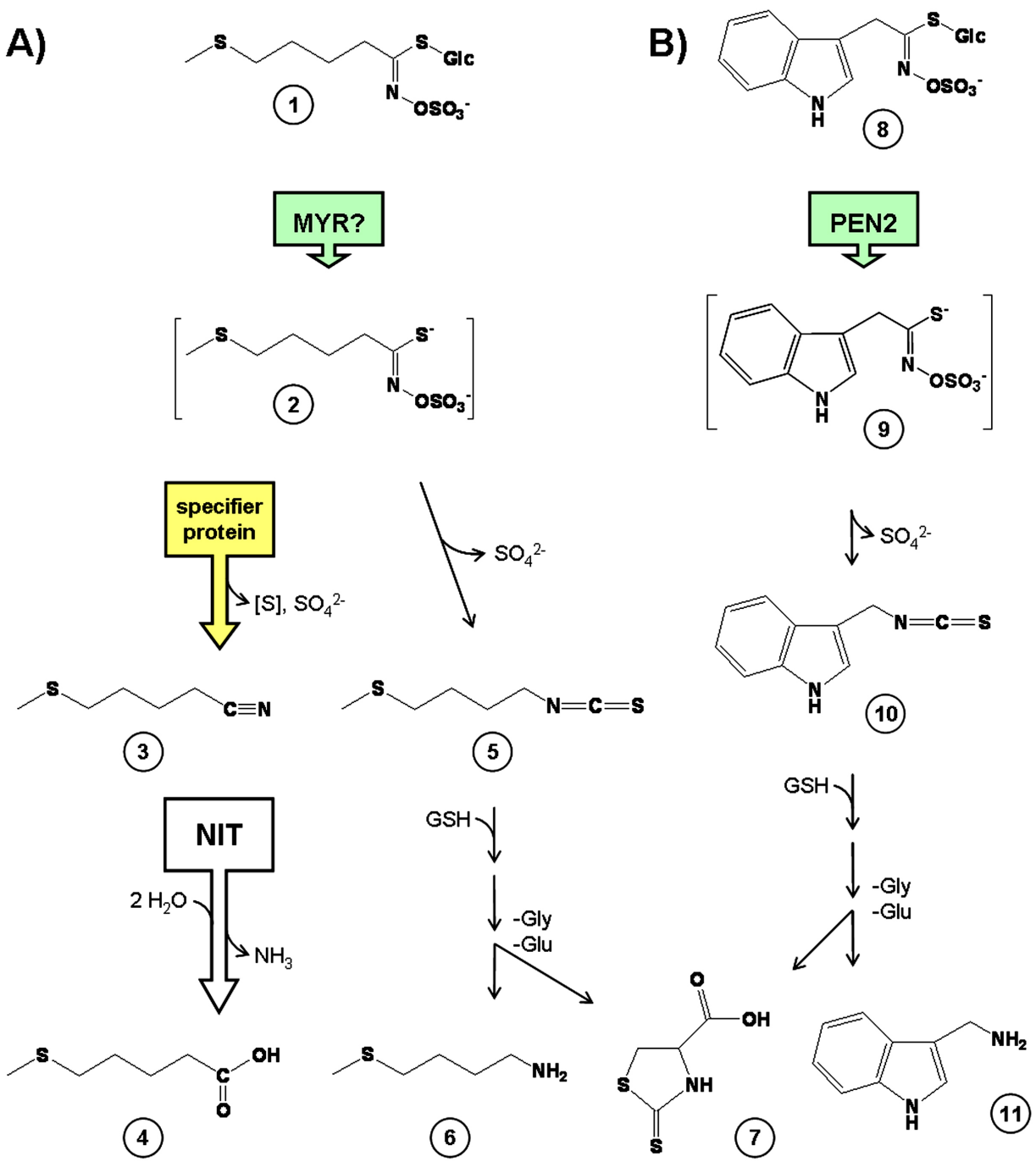

Figure 4. Glucosinolate breakdown in intact tissue.

Shown are (A) two hypothetical pathways of glucosinolate turnover (exemplified with 4-methylthiobutylglucosinolate (1)) and (B) the PEN2-dependent breakdown pathway of indole glucosinolates induced upon pathogen attack (exemplified with indol-3-ylmethylglucosinolate (8)). In A), myrosinase-type enzymes (classical myrosinases or atypical myrosinases, MYR) hydrolyze (1) to the corresponding aglucone (2). The simple nitrile (3) formed if a specifier protein is present can be further converted by nitrilases (NIT) to a carboxylic acid (4). This pathway would release both core structure sulfur atoms (as sulfate and likely as elemental sulfur [S]) as well as the nitrogen (as ammonia). In the absence of specifier protein activity, the isothiocyanate (5) would be formed instead, which might further react to a glutathione-conjugate decomposing to the amide (6) and raphanusamic acid (7). In B), hydrolysis of indol-3-ylmethylglucosinolate (8) to the corresponding aglucone (9) catalyzed by the atypical myrosinase PEN2 leads to the formation of indol-3-ylmethyl-isothiocyanate (10) which appears to be conjugated to glutathione and subsequently broken down to raphanusamic acid and indol-3-ylmethylamine (11). PEN2-dependent breakdown of 4-methoxyindol-3-ylmethylglucosinolate (not shown in the figure as the breakdown products are still unknown) is required for pathogen resistance in Arabidopsis.

PEN2 is unlikely to function in glucosinolate turnover during seedling development (see section 3.1) as it does not seem to hydrolyze aliphatic glucosinolates *in planta*. This notion is based on the observation that *cyp79B2 cyp79B3* mutant plants lacking indole glucosinolates do not show pathogen-inducible raphanusamic acid accumulation despite the fact that raphanusamic acid is a common metabolite of glucosinolate-derived isothiocyanates with both indolic and aliphatic side-chains (Fig. 4). Maybe other members of the same β-glucosidase clade are involved in glucosinolate turnover. Besides PEN2, this subclade comprises nine β-glucosidases. The subclade is a sister clade to myrosinases in a phylogenetic tree of β-glucosidases from different plant families and has been proposed to play a role in responses to biotic and abiotic stresses based on expression analysis (Xu et al., 2004). However, further experimental evidence for this is scarce. One interesting member of the PEN2-subclade that has repeatedly been associated with glucosinolate breakdown is PYK10 (At3g09260, BGLU23; Xu et al., 2004). PYK10 is a glycoprotein with about 45 % amino acid sequence identity to myrosinases. Although the natural substrates of PYK10 are unknown, the enzyme has been shown to possess β-D-glucosidase and β-D-fucosidase activity (Matsushima et al., 2004). PYK10 is highly expressed in ER bodies in Arabidopsis roots, hypocotyls and cotyledons, but is absent from rosette leaves (Matsushima et al., 2003). Methyljasmonate treatment induces ER body formation and *PYK10* transcription in rosette leaves (Matsushima et al., 2004). Similar to myrosinases in *Brassica* species that interact with jacalin-related lectins (JALs) termed myrosinase-binding proteins (MBPs) and GDSL lipase-like proteins (GLLs) termed myrosinase-associated proteins (MyAPs, which include ESM1 and MVP1 in Arabidopsis), PYK10 forms complexes with JALs and GLLs that impact its activity (Matsushima et al., 2004; Nagano et al., 2008). Interestingly, the beneficial effects of co-cultivation of Arabidopsis with the endophytic fungus *Piriformospora indica* are dependent on *PYK10* (Sherameti et al., 2008).

A role of glucosinolate breakdown in cellular signaling in response to abiotic stress has been proposed based on the observation that TGG1 is highly abundant in stomatal guard cells and that the inhibitory effect of abscisic acid (ABA) on stomatal opening is abolished in *tgg1* mutants (Zhao et al., 2008). The effect of ABA on stomatal opening results from inhibition of guard cell K^+ inward (K^+_{in}) channels. As demonstrated by whole cell patch clamp recordings, the effect of ABA on K^+_{in} channels is mimicked by administration of a commercial glucosinolate mixture to the cytosol via the patch pipette in wildtype Col-0, but not *tgg1* mutant guard cells, indicating a role of glucosinolate breakdown products (Zhao et al., 2008). Myrosinase alone does not provoke inhibition of K^+_{in} channels in wildtype guard cells when applied to the patch solution indicating the lack of glucosinolate substrates in the cytosol or insufficient enzyme activation in the absence of the appropriate triggering event (Zhao et al., 2008). The increase in ABA concentrations associated with abiotic stresses appears to require the transfer of glucosinolates from the guard cell vacuole to the cytosol and/or activation of the highly abundant TGG1 followed by glucosinolate hydrolysis. Glucosinolate breakdown products may then be involved in K^+_{in} channel inhibition (Zhao et al., 2008). If this breakdown also leads to emission of breakdown products through the stomatal pore has not been determined. This would suggest glucosinolate breakdown to function as a link between abiotic and biotic stress responses.

4. CONCLUSIONS AND PERSPECTIVES

The use of Arabidopsis as a model plant has not only allowed the identification of several new players involved in glucosinolate breakdown, but has also been indispensible for elucidating the diverse biological roles of glucosinolate breakdown products. As a result, glucosinolate breakdown seems to be much more complex than previously thought with respect to both its biochemistry and its biological roles. Besides structural requirements for the glucosinolate side-chain, the final outcome of glucosinolate breakdown in Arabidopsis depends on the interplay between hydrolytic enzymes, such as TGGs and PEN2, specifier proteins such as ESP and NSPs, modifiers such as ESM1 and MVP1 and enzymes involved in further metabolism such as nitrilases and glutathione transferases. Control and fine-tuning of glucosinolate breakdown through the regulated expression and trafficking of glucosinolates and/or components of the breakdown machinery appears to be essential for appropriate plant responses to various biotic as well as abiotic stresses. As a consequence, understanding the biological significance of the glucosinolate-myrosinase system requires the analysis of glucosinolate accumulation and breakdown with a high spatial and temporal resolution at the organ, tissue, cellular and subcellular level. Future research will likely integrate these findings at the organismic level to explore the evolutionary driving forces behind chemical diversification at the level of glucosinolate breakdown.

Acknowledgements

M.B. is a Marie Curie Intra-European Fellow. Research in U.W.'s lab is supported by the German Research Foundation (DFG).

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