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# Disease resistance of Arabidopsis to *Phytophthora brassicae* is established by the sequential action of indole glucosinolates and camalexin

**Klaus Schlaeppi, Eliane Abou-Mansour, Antony Buchala and Felix Mauch***
*Department of Biology, University of Fribourg, Fribourg, Switzerland*

*For correspondence (fax +41 26 300 9740; e-mail felix.mauch@unifr.ch).

## SUMMARY

**We have analysed the role of tryptophan-derived secondary metabolites in disease resistance of Arabidopsis to the oomycete pathogen *Phytophthora brassicae*. Transcript analysis revealed that genes encoding enzymes involved in tryptophan, camalexin and indole glucosinolate (iGS) biosynthesis are coordinately induced in response to *P. brassicae*. However, a deficiency in either camalexin or iGS accumulation has only a minor effect on the disease resistance of Arabidopsis mutants. In contrast, the double mutant *cyp79B2 cyp79B3*, which has a blockage in the production of indole-3-aldoxime (IAOx), the common precursor of tryptophan-derived metabolites including camalexin and iGS, is highly susceptible to *P. brassicae*. Because *cyp79B2 cyp79B3* shows no deficiencies in other tested disease resistance responses, we concluded that the lack of IAOx-derived compounds renders Arabidopsis susceptible despite wild-type-like pathogen-induced hypersensitive cell death, stress hormone signaling and callose deposition. The susceptibility of the double mutant *pen2-1 pad3-1*, which has a combined defect in camalexin synthesis and PEN2-catalysed hydrolysis of iGS compounds, demonstrates that both camalexin and products of iGS hydrolysis are important for disease resistance to *P. brassicae*. Products of iGS hydrolysis play an early defensive role, as indicated by enhanced epidermal penetration rates of Arabidopsis mutants affected in iGS synthesis or degradation. Our results show that disease resistance of Arabidopsis to *P. brassicae* is established by the sequential activity of the phytoanticipin iGS and the phytoalexin camalexin.**

**Keywords: indole glucosinolates, camalexin, glutathione, *Phytophthora brassicae*, penetration resistance.**



## INTRODUCTION

Plants have evolved a vast array of secondary metabolites, many of which contribute to defence against microbes (Dixon, 2001). Such compounds are functionally classified into constitutively produced phytoanticipins, which create a pre-formed defence barrier, and phytoalexins, which only accumulate in response to stress and form part of the inducible defences (Van Etten *et al.*, 1994). The characteristic secondary metabolites of the *Brassicaceae* (including Arabidopsis) are the sulfur-containing glucosinolates (GS) (Halkier and Gershenzon, 2006). These so-called mustard oil glucosides are recognized in daily human life as the characteristic flavor and taste of brassica vegetables, and have been reported to exert anti-tumor activity (Fahey *et al.*, 2002). GS are of crucial importance in plant defence against herbivores (Wittstock *et al.*, 2003). Of the more than 120 described GS (Fahey *et al.*, 2001), approximately 40 are detected in various ecotypes of Arabidopsis (Kliebenstein *et al.*, 2001; Reichelt *et al.*, 2002; Brown *et al.*, 2003). Based on their amino acid precursor, GS are classified into aliphatic, aromatic and indole GS. The leaves of the Arabidopsis ecotype Columbia-0 (Col-0) mainly contain the aliphatic 4-methyl-sulfinylbutyl GS and the indole GS (iGS) indol-3-ylmethyl GS (I3M) (Table S1) (Kliebenstein *et al.*, 2001). Several other aliphatic GS and the three indole GS 4-methoxy-indol-3-ylmethyl (4MO-I3M), 1-methoxy-indol-3-ylmethyl (1MO-I3M) and 4-hydroxy-indol-3-ylmethyl (4HO-I3M) are produced in lower amounts (Reichelt *et al.*, 2002).

The release of biologically active compounds from GS depends on the activity of myrosinase enzymes (β-thioglucoside glucohydrolase; EC 3.2.1.147). The 'mustard oil bomb' concept describes the glucosinolate/myrosinase system as a two-component herbivore-targeted defence mechanism in which GS and myrosinase enzymes are stored separately and come into contact upon tissue dam-

age, e.g. caused by chewing insects (Luethy and Matile, 1984). Myrosinases catalyse hydrolysis of the β-thioglucoside ester bond, and the resulting instable aglycone rearranges to form various breakdown products, typically including isothiocyanates, thiocyanates and nitriles (Bones and Rossiter, 2006). These hydrolysis products serve as feeding deterrents for non-specialized insects, but can also function as attractants for specialized insects (Wittstock *et al.*, 2003; De Vos *et al.*, 2008).

Given the wealth of information available for the role of GS in herbivore defence, their potential role in defence against microbial pathogens has received much less attention (reviewed by Halkier and Gershenzon, 2006). GS breakdown products have been reported to be toxic *in vitro* against a wide range of bacteria, fungi and oomycetes (Mari *et al.*, 1996; Brader *et al.*, 2001; Tierens *et al.*, 2001; Smith and Kirkegaard, 2002; Dhingra *et al.*, 2004; Sellam *et al.*, 2007). Several studies found a positive correlation between GS content and disease resistance (Doughty *et al.*, 1991; Tierens *et al.*, 2001; Brader *et al.*, 2006; Mishina and Zeier, 2007). Interestingly, farmers exploit the suppressive effect of GS on pathogens by using *Brassica* crop rotation as a form of bio-fumigation (Kirkegaard and Sarwar, 1998). However, apart from these studies, understanding of the contribution of GS breakdown products to disease resistance in a whole-plant context has remained elusive. Convincing novel evidence for a role for GS in disease resistance came from mutational analysis of non-host resistance of Arabidopsis. Uncharacterized hydrolysis products of the iGS 4MO-I3M were shown to play a critical role in pre-invasion resistance, which includes stages before and during penetration of the epidermal cell layer (Lipka *et al.*, 2005; Bednarek *et al.*, 2009).

A well-characterized phytoalexin of Arabidopsis is sulfur-containing camalexin (3-thiazol-2′-yl-indole; Table S1) (Tsuji *et al.*, 1992), which accumulates to high levels in response to biotic or abiotic stress (Glawischnig, 2007). Analysis of the camalexin-null mutant *pad3-1* (*phytoalexin-deficient 3-1*; Glazebrook and Ausubel, 1994), which has a defect in the conversion of dihydrocamalexic acid to camalexin (Schuhegger *et al.*, 2006), showed that accumulation of camalexin contributes to disease resistance to some pathogens, but does not have an effect on others (Kliebenstein, 2004; Kliebenstein *et al.*, 2005). The partially phytoalexin-deficient mutant *pad2-1* (*phytoalexin-deficient 2-1*; Glazebrook and Ausubel, 1994) is highly susceptible to the oomycete pathogen *Phytophthora brassicae* and other pathogens (Roetschi *et al.*, 2001; Ferrari *et al.*, 2003; Parisy *et al.*, 2007). Based on the minor effect of the camalexin null-mutation *pad3-1* on resistance to *P. brassicae*, it was concluded that camalexin deficiency is not responsible for the enhanced disease susceptibility of *pad2-1* plants in which camalexin production is only partially affected (40% of wild-type levels) (Roetschi *et al.*, 2001). The *pad2-1* mutation was mapped to the gene γ-*GLUTAMYLCYSTEINE SYNTHETASE 1*, which encodes the enzyme that catalyses the first step in the biosynthesis of glutathione (GSH) (Parisy *et al.*, 2007). Consequently, the *pad2-1* mutation reduces foliar GSH levels by 80%. Although these results highlighted the importance of GSH in disease resistance, the proximate cause of susceptibility remained ill-defined because of the multiple functions of GSH. Recently, we reported that the *pad2-1* mutant is less tolerant than wild-type to the generalist insect herbivore *Spodoptera littoralis*. The cause of this susceptibility was tracked to a deficiency in the production of GS (Schlaeppi *et al.*, 2008). Thus, the GSH deficiency of *pad2-1* negatively affects the synthesis of both camalexin and GS. This suggested the possibility that GS deficiency, alone or in combination with low camalexin levels, causes disease susceptibility to *P. brassicae*. Our aim was to test this hypothesis.

## RESULTS

### Tryptophan-derived compounds play a crucial role in disease resistance to *P. brassicae*

To assess the role of tryptophan-derived metabolites, we quantified disease resistance to *P. brassicae* in the Arabidopsis double mutant *cyp79B2 cyp79B3* (Zhao *et al.*, 2002). This mutant is defective in two cytochrome P450 enzymes that catalyse the conversion of tryptophan to indol-3-aldoxime (IAOx), and as a result does not produce either iGS or camalexin (Zhao *et al.*, 2002; Glawischnig *et al.*, 2004). Figure 1(a) shows that *cyp79B2 cyp79B3* shows a level of susceptibility equal to that of the susceptibility control *pad2-1*, whereas the wild-type Col-0 and the camalexin mutant *pad3-1* are resistant. No colonization of the host tissue was observed at 3 days post-inoculation in the incompatible interaction with Col-0 (Figure 1b). In contrast, *P. brassicae* successfully colonized *cyp79B2 cyp79B3*, spread in the intercellular space and formed asexual zoosporangia (Figure 1c) and sexual oospores (Figure 1d). As shown in Figure 1(e), which presents a quantitative comparison of disease symptom development, the deficiency of IAOx-derived compounds in *cyp79B2 cyp79B3* causes susceptibility to *P. brassicae*, whereas the lack of camalexin in *pad3-1* has only a minor symptomatic effect. The mutant *pad3-1* fails to sustain pathogen colonization and reproduction (Roetschi *et al.*, 2001). These results indicate that IAOx-derived metabolites other than camalexin play a crucial role in disease resistance to *P. brassicae*.

### The camalexin and iGS biosynthetic pathways are stimulated in response to *P. brassicae*

Quantitative real-time RTPCR was used to analyse the effect of *P. brassicae* on transcript levels of genes encoding enzymes involved in the biosynthesis of tryptophan, iGS and camalexin. Figure 2(a) shows an overview of the

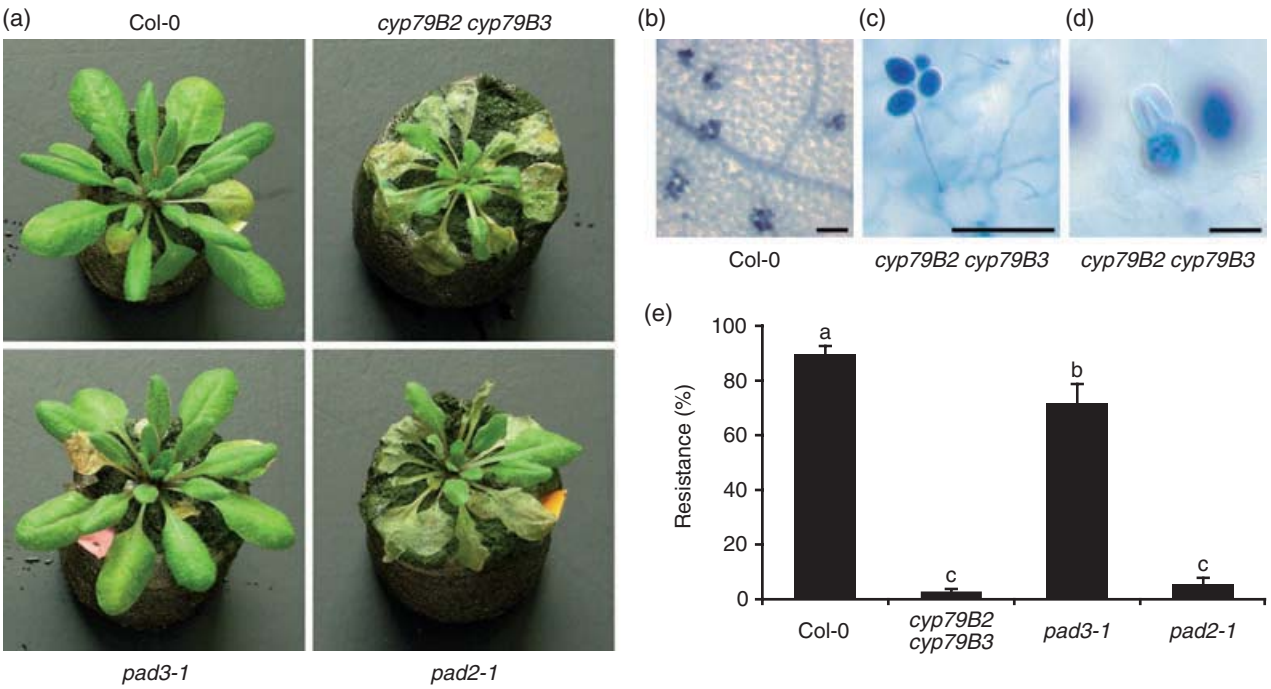


**Figure 1.** The Arabidopsis double mutant *cyp79B2 cyp79B3* is susceptible to *P. brassicae*.
(a) Four-week-old plants were spray-inoculated with zoospores of *P. brassicae* and photographed at 6 days post-inoculation. The wild-type Col-0 and the camalexin-null mutant *pad3-1* are resistant, whereas the double mutant *cyp79B2 cyp79B3* shows qualitatively similar disease symptoms to the susceptibility control *pad2-1*.
(b–d) Differential interference contrast micrographs of lactophenol/trypan blue-stained preparations at 3 days post-inoculation. In Col-0, *P. brassicae* remains confined to small areas of dead host cells stained in blue (b), but can grow and propagate in *cyp79B2 cyp79B3* by the production of zoosporangia (c) and the formation of oospores (d). Scale bars = 100 μm (b, c) and 25 μm (d).
(e) Quantitative comparison of disease resistance phenotypes. Droplets containing zoospores of *P. brassicae* were placed on leaves of 4-week-old plants, and disease resistance was scored at 5 days post-inoculation based on symptom development. Values are means ± SE for at least four independent experiments per genotype. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test).

selected genes, including the fold-change of expression observed 24 h post-inoculation (hpi) in comparison with mock-inoculated wild-type plants. With few exceptions, the transcript levels of analysed genes rapidly increased in response to *P. brassicae*. Figure S1 summarizes basal expression levels as well as transcriptional changes at 6 and 24 hpi. Surprisingly, the expression of genes encoding enzymes for aliphatic GS biosynthesis, such as *CYP79F1* (Reintanz *et al.*, 2001) and *CYP83A1* (Bak and Feyereisen, 2001), is down-regulated (Figure S1b), and the level of aliphatic GS does not change significantly in response to *P. brassicae* (Figure 2b). Although the combined level of the iGS compounds I3M and 4MO-I3M does not change much, a significant change in the iGS profile is observed upon pathogen challenge (Figure 2b). The decrease in I3M content is compensated for by a corresponding increase in the I3M derivative 4MO-I3M. Consistent with the regulation of transcripts, considerable accumulation of camalexin was measured (Figure 2c). Camalexin starts to accumulate at 12 hpi with *P. brassicae*, and reaches levels of 13 nmol/g fresh weight at 48 hpi. Hence, *P. brassicae* induces a coordinated transcriptional up-regulation of genes involved in tryptophan, camalexin and iGS biosynthesis, leading to accumulation of camalexin and qualitative differences in the iGS profile.

### The *cyp79B2 cyp79B3* double mutant shows no additional defects in classical defence responses

To exclude the possibility that *cyp79B2 cyp79B3* has additional defects that could explain its susceptibility to *P. brassicae*, the double mutant was examined for well-known classical defence reactions (Figure 3). The stress hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play essential roles in many plant–pathogen interactions (Thomma *et al.*, 2001). As shown in Figure 3(a), the *cyp79B2 cyp79B3* mutant and Col-0 showed a similar increase in transcript levels of SA-regulated *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*) (Ward *et al.*, 1991) and JA/ET-regulated *PLANT DEFENSIN 1.2* (*PDF1.2*) (Penninckx *et al.*, 1998) in response to *P. brassicae*. Hence, pathogen-induced activation of stress hormone signaling in *cyp79B2 cyp79B3* appears to be functional. The accumulation of reactive oxygen species (ROS), known as the oxidative burst, and rapid local host cell death, known as the hypersensitive response (HR), are often associated with disease resistance (Mur *et al.*, 2008). However, *cyp79B2 cyp79B3* accumulates wild-type levels of

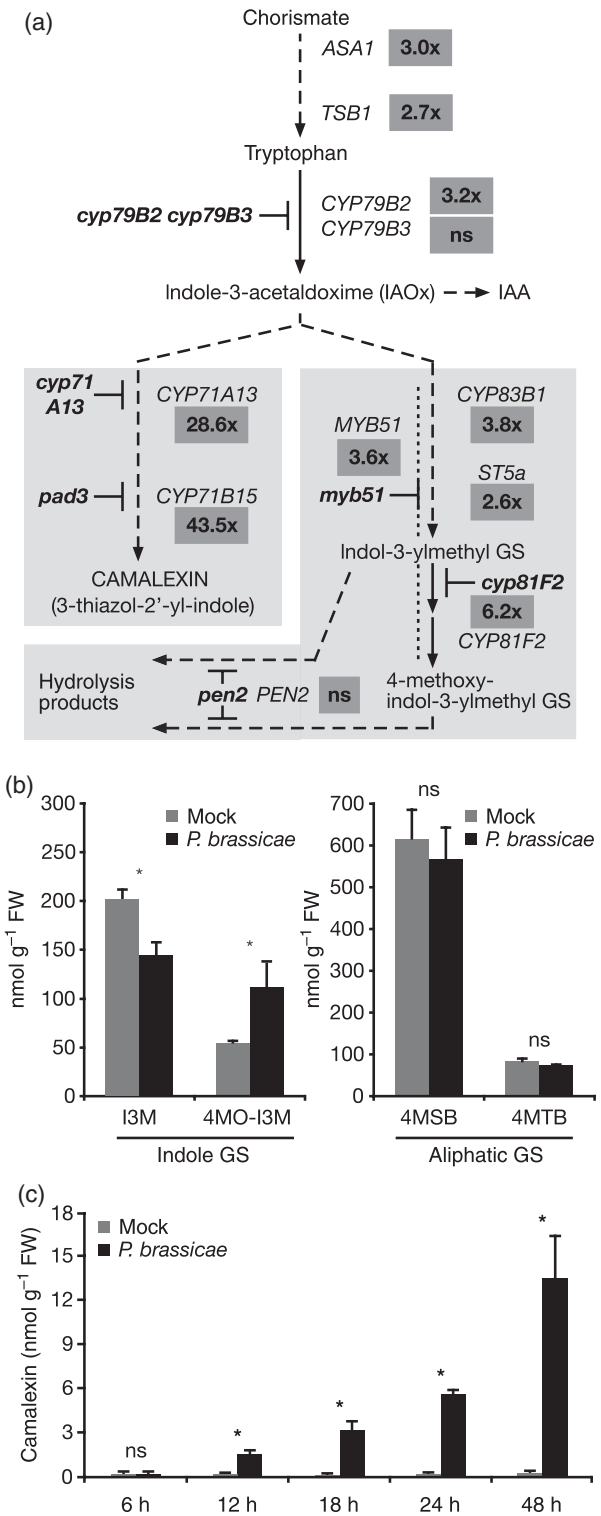


**Figure 2.** Transcriptional and product analysis of the indole pathway in response to *P. brassicae*.

(a) Schematic overview of mutants (bold letters) and selected genes (italic capitals) implicated in the biosynthesis of tryptophan, iGS and camalexin. The numbers represent the fold change in transcript levels of the respective genes in response to *P. brassicae* at 24 hpi. The transcription factor MYB51 regulates genes along the entire iGS biosynthesis pathway. Values are the means of three independent experiments. Detailed quantitative PCR results, including statistical information, are summarized in Figure S1. The abbreviations for the genes are defined in Table S1. IAA, indole-3-acetic acid.

(b) Analysis of the major indolyl and aliphatic GS upon inoculation with *P. brassicae*. Leaves of 4.5-week-old plants were droplet-inoculated with zoospores and harvested at 3 days post-inoculation. The values are means ± SE for seven independent experiments (*$P < 0.05$; ns, not significant, treatment effect; factorial ANOVA). I3M, indol-3-ylmethyl GS; 4MO-I3M, 4-methoxy-indol-3-ylmethyl GS; 4MSB, 4-methyl-sulfinylbutyl GS; 4MTB, 4-methyl-thiobutyl GS.

(c) Kinetics of camalexin accumulation of 4-week-old Col-0 plants in response to spray-inoculation with zoospores of *P. brassicae*. Values are means ± SE for triplicate samples (*$P < 0.05$, ns, not significant; Student's *t*-test). The sample size was ten leaves (two leaves each from five plants) per time point and treatment. The results were confirmed in three independent experiments.

$H_2O_2$ (Figure 3b) and shows a wild-type-like HR (Figure 3c). Furthermore, pathogen-induced callose deposition was not affected in *cyp79B2 cyp79B3* plants (Figure 3d). Finally, *cyp79B2 cyp79B3* had wild-type thiol levels (Figure S2), thus excluding the possibility that susceptibility is caused by a *pad2-1*-like deficiency in GSH. Taken together, these data suggest that the susceptibility of *cyp79B2 cyp79B3* is a direct result of the impaired production of IAOx-derived secondary metabolites.

### Combined deficiency of iGS and camalexin causes susceptibility to *P. brassicae*

The lack of camalexin in *pad3-1* does not cause susceptibility (Figure 1a,e). Therefore, the observed susceptibility of *cyp79B2 cyp79B3* to *P. brassicae* must be caused by the lack of IAOx-derived metabolites other than camalexin or by a combination of deficiencies in IAOx metabolism. IAOx also serves as a precursor for the biosynthesis of indole-3-acetic acid (IAA; Figure 2a) (Zhao *et al.*, 2002). However, the *cyp79B2 cyp79B3* double mutant has been shown to produce wild-type levels of IAA due to alternative biosynthesis pathways (Zhao *et al.*, 2002). In contrast, iGS mutants with a blockage downstream of IAOx show enhanced levels of IAA, leading to aberrant growth phenotypes (Barlier *et al.*, 2000; Mikkelsen *et al.*, 2004), that make disease resistance tests with these mutants questionable. As a compromise, we included three mutants with partial defects in iGS synthesis or degradation in our resistance assays (Figure 4). The *myb51* mutant has a defect in the expression of the transcription factor MYB51 (Gigolashvili *et al.*, 2007), and accumulates intermediate levels of iGS (Figure S3a,b). The cytochrome P450 mutant *cyp81F2* has a specific defect in the conversion of I3M to 4HO-I3M, which serves as precursor for 4MO-I3M (Pfalz *et al.*, 2009). Finally, *pen2* mutants (Lipka *et al.*, 2005) are compromised in iGS hydrolysis (Bednarek *et al.*, 2009). *PEN2* encodes an atypical myrosinase that catalyses hydrolysis of the two iGS I3M and 4MO-I3M (Figure 2a).

Figure 4 shows a comparison of disease resistance phenotypes of mutants with a lack of camalexin (*pad3-1*) and camalexin precursors (*cyp71A13*), defects in iGS synthesis (*myb51* and *cyp81F2*) or iGS hydrolysis (*pen2*), or a complete

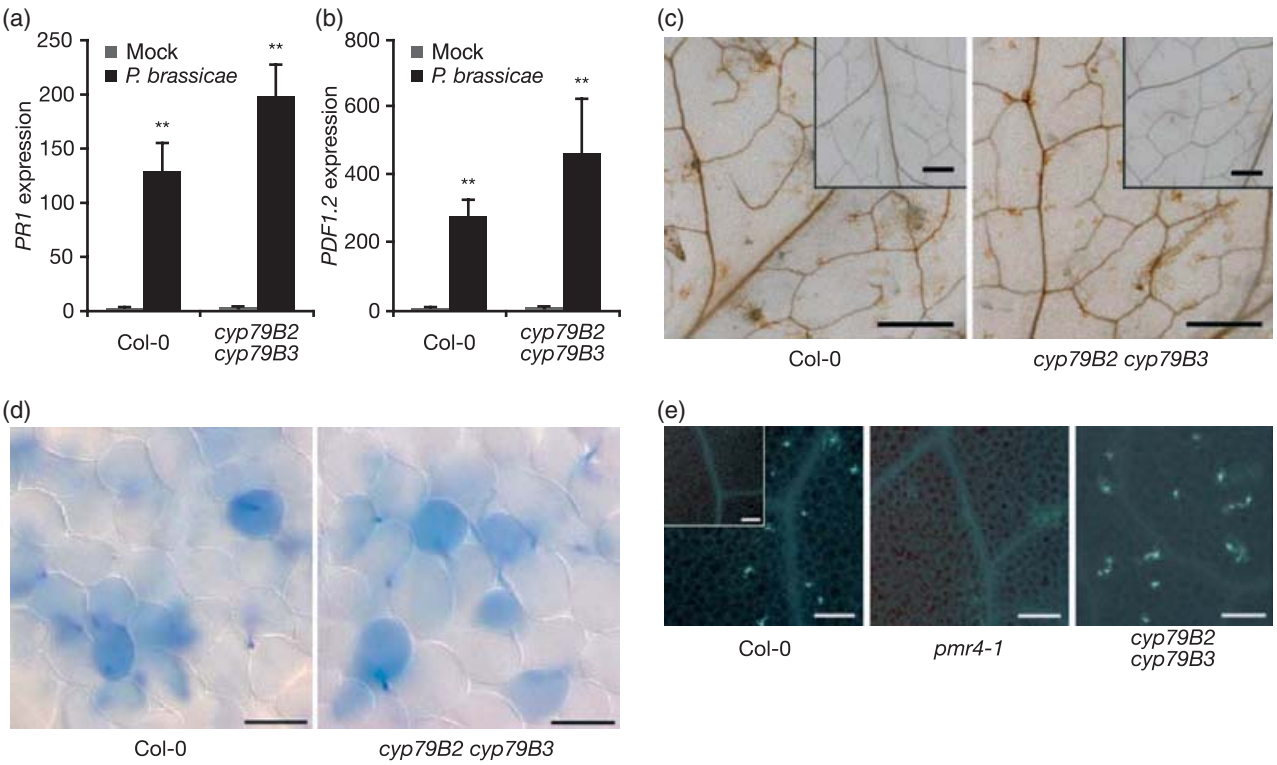


**Figure 3**. The *cyp79B2 cyp79B3* mutant has no additional defects in classical defence reactions.
(a, b) Analysis of stress hormone signaling. Transcript levels of the marker genes *PR1* for SA-controlled defence activation and *PDF1.2* for JA/ET-controlled defence activation were analysed by quantitative RT-PCR at 48 hpi. Values are means ± SE for three independent experiments (**$P < 0.001$, treatment effect; factorial ANOVA). The genotype–treatment effect was not significant.
(c) Analysis of the oxidative burst triggered by *P. brassicae*. Four-week-old plants were inoculated with zoospores of *P. brassicae*, and the *in planta* accumulation of $H_2O_2$ was analysed by DAB staining at 16 hpi. The accumulation of $H_2O_2$ at infection sites is visible as brown staining. The insets show uninoculated control leaves. Scale bars = 1 mm.
(d) Analysis of the hypersensitive response. Leaves were inoculated with zoospores of *P. brassicae*, and hypersensitive cell death was detected by lactophenol/trypan blue staining at 16 hpi. Hyphae and dead host cells are stained in blue. Scale bars = 50 μm.
(e) Analysis of callose accumulation. Inoculated leaves of Col-0, *pmr4-1* and *cyp79B2 cyp79B3* were analysed at 6 hpi for the accumulation of callose by aniline blue staining followed by fluorescence microscopy. Callose accumulation at infection sites is visible as bright spots. The inset shows staining of an uninoculated leaf. The *pmr4-1* mutant cannot accumulate callose and serves as a negative control. Scale bars = 100 μm.

lack of IAOx-derived metabolites (*cyp79B2 cyp79B3*). Mutations affecting only iGS or camalexin metabolism had no significant effect on disease resistance. However, combination of the resistant single-pathway mutants *pen2-1* and *pad3-1* resulted in susceptibility in a *pen2-1 pad3-1* double mutant. The enhanced susceptibility of *pen2-1 pad3-1* demonstrates the importance of PEN2-dependent hydrolysis of iGS for disease resistance, and supports the conclusion that the combined action of camalexin and hydrolysis products of iGS establishes disease resistance to *P. brassicae*. The fact that *pen2-1 pad3-1* is more resistant than *cyp79B2 cyp79B3* indicates that additional PEN2-independent iGS degradation pathways and/or other IAOx-derived compounds also contribute to resistance.

### The *pad2-1* mutant has defects in iGS production and hydrolysis

The iGS metabolism of *pad2-1* in response to *P. brassicae* in comparison with Col-0 was studied by analysing the expression of pathway-relevant genes and by measuring the accumulation of I3M, 4MO-I3M and PEN2-dependent production of the I3M-derived hydrolysis product indol-3-ylmethylamine (I3A; Table S1) (Bednarek *et al.*, 2009). Transcript levels of *MYB51*, *CYP83B1* and *ST5a* increase to a similar extent in *pad2-1* and Col-0 in response to *P. brassicae* (Figure S4). In addition, no significant difference in transcript levels of *PEN2* was observed between *pad2-1* and Col-0. The I3M content in mock-inoculated plants was about 20% lower in *pad2-1* compared to the wild-type, and decreased more strongly in response to *P. brassicae* within 24 hpi (Figure 5a). The decrease in I3M in pathogen-challenged wild-type plants is reflected in a 2.7-fold increase in I3A at 24 hpi, indicating rapid activation of I3M hydrolysis in response to *P. brassicae* (Figure 5c). Interestingly, I3M hydrolysis appears to occur already in unchallenged wild-type plants that accumulate measurable amounts of I3A. The enhanced decrease in the steady-state I3M content of challenged *pad2-1* is not reflected at the level of I3A. The

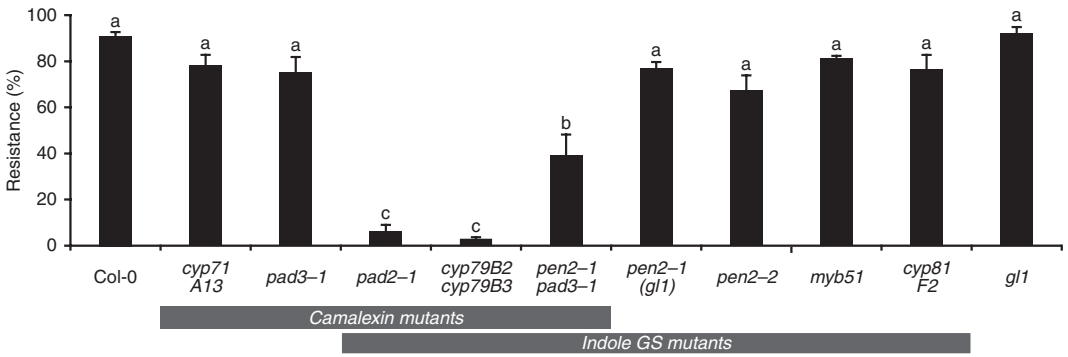


**Figure 4.** A combined deficiency in camalexin accumulation and iGS synthesis or degradation causes susceptibility of Arabidopsis to *P. brassicae*.
Droplets containing zoospores of *P. brassicae* were placed on 4-week-old plants and the infections were scored 5 days post-inoculation. Values are means ± SE for at least three independent experiments per genotype. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test). Disease resistance was determined for mutants with a deficiency in camalexin accumulation (*pad3-1* and *cyp71A13*), in iGS metabolism (*myb51*, *pen2-2* and *cyp81F2*) or in both camalexin and iGS metabolism (*cyp79B2 cyp79B3*, *pen2-1 pad3-1* and *pad2-1*). The mutant *pen2-1* is in the genetic background of *gl1* (*glabrous1*); all other mutants are in the Col-0 background.

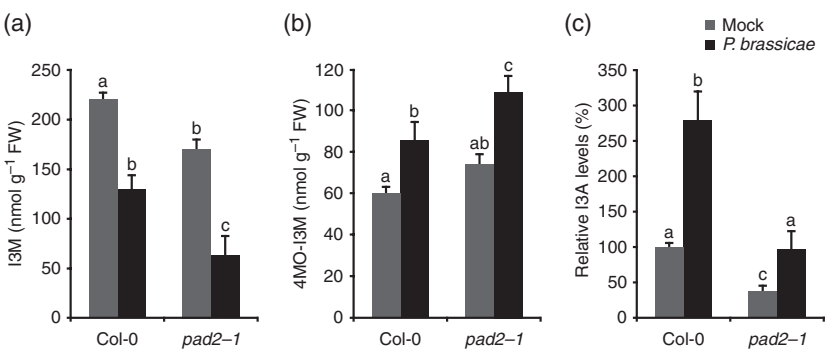


**Figure 5.** Glutathione deficiency of *pad2-1* compromises iGS synthesis and degradation.
Four-week-old plants were inoculated with *P. brassicae*. Leaf samples were harvested at 24 hpi and analysed for indolic metabolites by HPLC. Indol-3-yl-methylamine (I3A) is a *PEN2*-dependent hydrolysis product of I3M. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test).
(a, b) Values are means ± SE of four independent experiments, each with triplicate samples. The I3M levels in *pad2-1* and Col-0 differ significantly between untreated and pathogen-challenged leaves, respectively ($P < 0.05$, genotype–treatment effect; factorial ANOVA). 4MO-I3M accumulates to higher levels in *pad2-1* compared to wild-type in response to *P. brassicae* ($P < 0.05$, treatment effect; factorial ANOVA).
(c) I3A values expressed as percentages relative to mock-inoculated wild-type values. Values are means ± SE for three independent experiments, each with three samples per genotype and treatment. The pathogen-induced accumulation of I3A in the mutant *pad2-1* is significantly different from that in Col-0 ($P < 0.05$, genotype–treatment effect; factorial ANOVA).

production of I3A is strongly reduced in *pad2-1* compared to wild-type (Figure 5c). These results indicate that *pad2-1* has a reduced capacity for both I3M accumulation and degradation in response to *P. brassicae*. Together with the reported deficiency in camalexin (Roetschi *et al.*, 2001), the reduced I3M metabolism probably contributes to the susceptibility of *pad2-1*. However, the less susceptible double mutant *pen2 pad3-1* has stronger deficiencies in iGS-derived degradation products and camalexin accumulation than *pad2-1* (Figure 4). It appears, therefore, that additional deficiencies contribute to the susceptibility of *pad2-1* (Roetschi *et al.*, 2001).

A moderate increase in 4MO-I3M was measured in pathogen-challenged wild-type plants at 24 hpi, and higher levels were found in *pad2-1* (Figure 5b). As the final hydrolysis products of 4MO-I3M remain unknown, we can only speculate whether this enhancement results from redirection of the pathway caused by the reduced conversion of I3M to I3A and/or an effect of glutathione deficiency on 4MO-I3M degradation.

### GS degradation products and camalexin inhibit *in vitro* growth of *P. brassicae*

The inhibitory potential of Arabidopsis GS was tested by placing filter discs containing test solutions on *P. brassicae* germlings growing on agar plates (Figure S5a). A leaf extract containing GS was prepared from Col-0 (see Experimental procedures), and was applied in the presence or absence of either a commercial myrosinase or a protein extract from Arabidopsis containing endogenous myrosinases. When applied separately, no inhibitory effect was observed for the leaf extract, the protein extract or the

commercial myrosinase. In contrast, the GS-containing leaf extract conferred growth inhibition in the presence of the commercial myrosinase and in combination with the Arabidopsis protein extract. These findings indicate that Arabidopsis leaf extracts containing GS have a myrosinase-dependent anti-microbial activity towards *P. brassicae* at physiological concentrations. Camalexin inhibits the growth of *P. brassicae* at concentrations as low as 5 nmol/ml, with an $EC_{50}$ value of 31 nmol/ml (Figure S5b). Hence, camalexin inhibits *in vitro* growth of *P. brassicae* at concentrations that occur in pathogen-challenged leaves (Figure 2c).

**iGS play a crucial role in penetration resistance to *P. brassicae***

Microscopic analysis was performed to determine at what time point during the infection process the susceptibility of *cyp79B2 cyp79B3* is first detectable. Analysis of host penetration showed that strikingly more successful penetration events occurred in *cyp79B2 cyp79B3* compared to the wild-type (Figure 6a). To quantify this effect, we compared the number of germinated encysted zoospores at the leaf surface with the number of corresponding hyphae in the upper mesophyll. About 25% of the *P. brassicae* germlings reach the upper mesophyll layer within 6 hpi in Col-0 (Figure 6b). The rate of successful penetrations was nearly twofold enhanced in *cyp79B2 cyp79B3*, but was unaffected in *pad3-1*. A reduction of penetration resistance was also observed for other iGS mutants such as *myb51*, *cyp81F2*, *pen2-2* and *pad2-1*. The similar enhancement in penetration rates observed in *cyp79B2 cyp79B3* and *pen2-2* suggests that PEN2-mediated iGS hydrolysis plays a major role in penetration resistance to *P. brassicae*.

**iGS-mediated penetration resistance does not depend on host cell destruction**

The early protective effect of products of iGS hydrolysis prompted the question whether or not cellular destruction, i.e. in the form of an HR, is a prerequisite for iGS-mediated penetration resistance established either at the leaf surface and/or during penetration of the epidermal cell layer. To determine the timing of iGS-mediated penetration resistance in relation to HR occurrence, we monitored the kinetics of HR appearance in the epidermal cell layer by lactophenol/trypan blue staining. The HR first becomes apparent at 8 hpi during the interaction between Col-0 and *P. brassicae* (Figure 6c). At later time points, the HR is substantially more pronounced, and also occurs in the leaf mesophyll (i.e. at 16 hpi in Figure 3c). Thus, HR occurs at later infection stages than iGS-mediated penetration resistance. Cellular destruction therefore does not appear to be required for the inhibitory activity of iGS hydrolysis products.

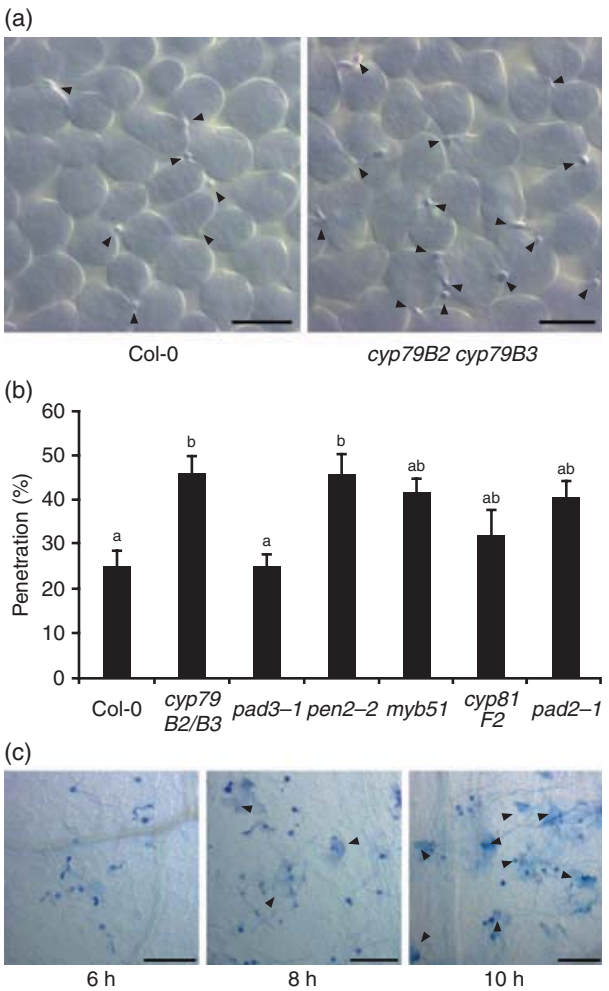


**Figure 6.** iGS play a role in penetration resistance to *P. brassicae*.
(a) Differential interference contrast micrographs with the focal plane adjusted to the upper mesophyll of lactophenol/trypan blue-stained leaves of zoospore-inoculated 3-week-old plants at 6 hpi. Hyphae of *P. brassicae* are marked with arrowheads. Scale bars = 50 μm.
(b) Quantitative analysis of penetration resistance. The number of successful penetration events was scored in the upper mesophyll and calculated as a percentage (±SE) of encysted zoospores present on the corresponding leaf surface. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test).
(c) Kinetics of hypersensitive cell death in epidermal cells. Differential interference contrast micrographs of lactophenol/trypan blue-stained leaves of zoospore-inoculated 4-week-old plants. Hypersensitive cell death (cells marked with arrowheads) is detected from 8 hpi onwards. Scale bar = 100 μm.

## DISCUSSION

Our work suggests that tissue colonization by *P. brassicae* is sequentially halted by iGS and camalexin, which act in synergy in disease resistance. iGS contribute to penetration resistance as pre-formed secondary metabolites, thereby allowing inducible defences more time to reach effective levels. Accumulation of the phytoalexin camalexin acts as an important subsequent late-acting defence barrier. Similar conclusions were drawn from an analysis of non-host resistance of Arabidopsis to the powdery mildew fungus *Erysiphe pisi* (Bednarek *et al.*, 2009). The *pad3* mutant was showed wild-type-like disease resistance, whereas *cyp79B2*

*cyp79B3* and *pen2 pad3* showed enhanced entry rates and epiphytic hyphal growth.

An intriguing new role for iGS in defence signaling was recently proposed based on the finding that iGS regulate cell wall-based accumulation of callose in response to treatment with the microbe-associated molecular pattern Flg22 (Clay *et al.*, 2009). Analysis of various iGS mutants suggested that unidentified products derived from 4MO-I3M are involved in the activation of callose deposition. Because callose deposition is a typical early defence response, this finding suggested the possibility that the susceptibility to *P. brassicae* of iGS-deficient Arabidopsis mutants might be caused by an indirect effect on callose deposition. However, we did not observe any aberrant callose phenotype in *cyp79B2 cyp79B3*, *pad2-1*, *cyp81F2* or *pen2-2* in response to *P. brassicae* (Figure 3d and data not shown). Taken together, our results show that *cyp79B2 cyp79B3* is susceptible to *P. brassicae* despite wild-type-like callose accumulation, stress hormone signaling, ROS production and hypersensitive cell death (Figure 3). Apparently, these classical defence reactions are not sufficient to protect Arabidopsis against *P. brassicae* in the absence of IAOx-derived secondary metabolites.

**Effect of *P. brassicae* on camalexin accumulation and iGS production and degradation**

Arabidopsis reacts to *P. brassicae* with a coordinated increase in the level of transcripts of genes that are involved in tryptophan, camalexin and iGS biosynthesis (Figures 2a and S1). The transcriptional activation of the tryptophan pathway and accumulation of the derived product camalexin is a well-known defence response (Tsuji *et al.*, 1992; Thomma *et al.*, 1999; Glawischnig, 2007). However, the transcriptional stimulation of the iGS pathway has mostly been studied in response to herbivorous insects (Reymond *et al.*, 2004; Mewis *et al.*, 2006), and has received less attention in the context of plant–pathogen interactions (Bednarek *et al.*, 2005).

At the metabolite level, transcriptional stimulation of the camalexin pathway results in a massive accumulation of product (Figure 2c). In contrast, *P. brassicae* affects the iGS profile but has little effect on the combined amount of the major iGS compounds (Figure 2b), despite transcriptional activation of many pathway-relevant genes (Figures 2a and S1b). It is unlikely that the transcripts are not efficiently translated or that the newly synthesized enzymes are inactive. Treatment with the stress hormone JA causes a comparable coordinated transcriptional up-regulation of the tryptophan and iGS pathways, resulting in enhanced accumulation of iGS (Brader *et al.*, 2001; Mikkelsen *et al.*, 2003; Jost *et al.*, 2005). It is therefore tempting to speculate that transcriptional activation in response to *P. brassicae* leads to an enhanced production of iGS that is compensated for by a parallel increase in iGS hydrolysis, with the result that the steady-state level of iGS compounds is not dramatically affected. Our results show a rapid initiation of hydrolysis of I3M in response to *P. brassicae* (Figure 5b). PEN2-dependent hydrolysis of iGS must occur within the first hours of the interaction, as indicated by the reduced penetration resistance of *pen2-2* detected at 6 hpi (Figure 6b). It is not currently possible to estimate the flux through the iGS pathway because the *in vivo* iGS degradation pathways and resulting products are not well known (Bednarek *et al.*, 2009). In addition, myrosinases other than PEN2 may contribute to pathogen-induced iGS hydrolysis.

**Camalexin and myrosinase-dependent GS hydrolysis inhibit the *in vitro* growth of *P. brassicae***

Camalexin and hydrolysis products of GS inhibit the *in vitro* growth of *P. brassicae* at physiological concentrations (Figure S5). Camalexin (Rogers *et al.*, 1996; Ferrari *et al.*, 2003; Sellam *et al.*, 2007) and diverse products of GS hydrolysis (Mari *et al.*, 1996; Brader *et al.*, 2001; Tierens *et al.*, 2001; Smith and Kirkegaard, 2002; Dhingra *et al.*, 2004; Sellam *et al.*, 2007) have previously been shown to inhibit the *in vitro* growth of a wide range of pathogens. The nature of the final products of GS hydrolysis depends on various reaction parameters, such as pH, temperature and the presence/absence of accessory proteins (Bones and Rossiter, 2006). Therefore, the products active in *in vitro* assays probably differ from those produced *in planta*. As a result, it is not possible to draw an accurate conclusion on the potential of *in planta*-generated products based on *in vitro* tests. However, our data do show that a GS-containing leaf extract from Arabidopsis inhibits the *in vitro* growth of *P. brassicae* at physiological levels in a myrosinase-dependent way. Similar to previous studies that indicated a protective role of GS in plant defence against pathogens (Doughty *et al.*, 1991; Tierens *et al.*, 2001; Brader *et al.*, 2006; Mishina and Zeier, 2007), we have no data on the nature of the *in planta*-generated bioactive products of GS hydrolysis.

**Contribution of iGS to disease resistance in a whole-plant context**

Genetic studies into the role of iGS in disease resistance are complicated by the fact that iGS, camalexin and IAA share a common biosynthetic pathway (Figure 2a) (Zhao *et al.*, 2002; Glawischnig *et al.*, 2004). Mutations affecting steps downstream of the common precursor IAOx lead to enhanced IAA levels and cause a severe growth-related pleiotropic phenotype. On the other hand, mutations blocking IAOx production, such as in the double mutant *cyp79B2 cyp79B3*, block the production of both iGS and camalexin, but the expected IAA deficiency is bypassed by redundant biosynthesis pathways (Zhao *et al.*, 2002). The enhanced susceptibility of *cyp79B2 cyp79B3* to *P. brassicae*, combined with the wild-type level of resistance in mutants impaired in camalexin biosynthesis alone (Figures 1 and 4),

led to the conclusion that IAOx-derived compounds other than camalexin are important in disease resistance to *P. brassicae*. The enhanced disease susceptibility phenotype of additional mutants with combined deficiencies in camalexin and iGS metabolism, such as *pad2-1* and the double mutant *pen2-1 pad3-1*, demonstrates that the combined action of iGS and camalexin is an important cornerstone of disease resistance of Arabidopsis to *P. brassicae*. The residual resistance of *pen2-1 pad3-1* in comparison with *cyp79B2 cyp79B3* suggests the involvement of additional PEN2-independent defence compounds that could either be iGS hydrolysis products released by alternative myrosinases and/or other IAOx-derived compounds. Several indolic compounds, such as indole-3-carboxylic acid (I3C) and its derivatives, which accumulate in response to pathogens (Hagemeier *et al.*, 2001; Tan *et al.*, 2004; Bednarek *et al.*, 2005), have been shown to be synthesized via IAOx (Boettcher *et al.*, 2009). The possible contribution of these compounds to disease resistance is difficult to assess in the absence of Arabidopsis mutants with a specific defect in the synthesis of I3C. Because of the lack of a suitable mutant that is completely and specifically impaired in iGS production, it remains to be determined whether complete iGS deficiency alone is sufficient to cause enhanced susceptibility to *P. brassicae*. The partially iGS-deficient mutant *myb51* remains resistant to *P. brassicae*. However, interpretation of this result is not conclusive because the reduced iGS production is compensated for by enhanced camalexin accumulation (Figure S3c).

**iGS are important for penetration resistance**

Recently, Bednarek *et al.* (2009) identified an iGS degradation pathway that plays a role in pre-invasion resistance to non-host pathogens. In comparison to wild-type plants, several mutants with defects in iGS synthesis or degradation showed decreased levels of pre-invasion resistance. These mutants include *cyp81F2*, which is defective in the accumulation of 4MO-I3M, and *pen2-1* and *pen2-2*. PEN2 was shown to mediate hydrolysis of iGS into products such as I3A and raphanusamic acid, which had not been detected previously in plant–insect interactions. However, it is not clear whether these products are toxic to microbial pathogens. Fungal entry rates were found to be similar in *cyp81F2*, *pen2* and *cyp79B2 cyp79B3* mutants, suggesting that 4MO-I3M-derived products are the major contributors to pre-invasion resistance (Bednarek *et al.*, 2009). PEN2 was also shown to be important in pre-invasion resistance of Arabidopsis to the non-host pathogen *Magnaporthe oryzae* (Maeda *et al.*, 2009). Our data reveal that PEN2-generated products contribute to host resistance of Arabidopsis to *P. brassicae*, as indicated by the enhanced susceptibility of the *pen2-1 pad3-1* double mutant (Figure 4). The double mutant *cyp79B2 cyp79B3* and *pen2-2* show a similar reduction in penetration resistance, whereas *cyp81F2* is less affected. Because *cyp81F2* still accumulates some residual 4MO-I3M (Bednarek *et al.*, 2009; Pfalz *et al.*, 2009), it is not possible to conclude from these results whether penetration resistance is entirely dependent on the products of 4MO-I3M hydrolysis or on additional iGS products from PEN2-dependent hydrolysis.

Enhanced penetration by *P. brassicae* was observed in the iGS-null mutant *cyp79B2 cyp79B3*, as well as in the partially iGS-deficient mutants *myb51* and *pad2-1*, but the camalexin-null mutant *pad3-1* shows wild-type levels of penetration (Figure 6b). Thus, iGS but not camalexin account for early penetration resistance. The partial defect in iGS metabolism in *myb51* and *pad2-1* is sufficient to compromise penetration resistance. However, mutants with decreased iGS accumulation or degradation but functional camalexin production, such as *myb51* and *pen2*, respectively, display wild-type levels of resistance (Figure 4). These data indicate that iGS degradation products provide resistance during penetration by the pathogen, and this is followed by a defensive role of camalexin, possibly together with iGS-derived products, at later stages of infection.

**The protective effect of iGS is independent of cellular destruction**

*Phytophthora brassicae* is a hemi-biotrophic pathogen that, following penetration along anticlinal walls of the epidermis, spreads in the intercellular space and causes tissue damage during later stages of pathogenesis (Roetschi *et al.*, 2001; Si-Ammour *et al.*, 2003). We provide evidence that the penetration process of *P. brassicae* is already inhibited at 6 hpi by products derived from iGS (Figure 6a,b). At this early time point, the wild-type accession Col-0 shows no detectable signs of HR (Figure 6c). iGS hydrolysis and action can apparently occur in the absence of cellular destruction. Evidence for such an alternative activation pathway comes from analysis of the atypical myrosinase PEN2. After pathogen attack, peroxisome-associated PEN2 was shown to move to sites of fungal penetration, possibly providing focused delivery of iGS hydrolysis products in living cells at the sites of cellular attack (Lipka *et al.*, 2005; Bednarek *et al.*, 2009).

**Conclusions**

Our results serve as a reminder to be cautious when interpretating disease resistance tests of mutants. Based on a minor symptomatic effect of the camalexin deficiency of *pad3-1*, we previously concluded that camalexin is of minor importance for disease resistance to *P. brassicae* (Roetschi *et al.*, 2001). Our present results reveal that camalexin is crucially important for protection, in combination with iGS. The iGS phytoanticipins have an early defensive role as inhibitors of host penetration, and the phytoalexin camalexin adds to this initial protection at later stages of the infection process.

## EXPERIMENTAL PROCEDURES

### Biological material

*Arabidopsis thaliana* was grown in Jiffy-7 peat pellets (http://www.samen-mauser.ch) in a growth chamber with a 12 h day/night photoperiod at 21°C/19°C, and a light intensity of 100 μE $m^{-2}$ $sec^{-1}$. The following mutants (all in the Col-0 background except where indicated) were used: *pad2-1* and *pad3-1* (Glazebrook and Ausubel, 1994; Schuhegger *et al.*, 2006), *cyp79B2 cyp79B3* (Zhao *et al.*, 2002), *cyp71A13-2* (Nafisi *et al.*, 2007), *hig1-1* (also known as *myb51*; Gigolashvili *et al.*, 2007), *pmr4-1* (Nishimura *et al.*, 2003), *pen2-1* (*glabrous1* background), *pen2-2* (Lipka *et al.*, 2005) and *cyp81F2-2* (Bednarek *et al.*, 2009). The *pen2-1 pad3-1* double mutant was kindly provided by S. Rosahl and L. Westphal (Leibniz Institut für Pflanzenbiochemie, Halle, Germany).

*Phytophthora brassicae* isolate D (CBS179.89) was cultivated as described previously (Roetschi *et al.*, 2001). Two methods of plant inoculation were used: (i) agar plugs containing mycelia were placed upside-down on the humidified leaf surface and empty plugs were used as control treatment (Roetschi *et al.*, 2001), or (ii) leaves were droplet-inoculated with a zoospore suspension (50 000 zoospores/ml, 10 mM dextrose). Zoospores were produced as described previously (Roetschi *et al.*, 2001). Plants were inoculated at the beginning of the dark period at 100% humidity. Plants were scored for disease resistance on a scale of 0–4, as follows: 0 = completely susceptible leaf; 1 = outgrowing lesion; 2 = confined lesion covering droplet/plug zone; 3 = a few spots observed within the droplet/plug zone; 4 = no disease symptoms. Mean disease resistance scores were transformed into percentage values for comparison of replicate inoculations.

### Biochemical analysis

The analytical methods for GS (Schlaeppi *et al.*, 2008), thiols (Parisy *et al.*, 2007) and camalexin (Chassot *et al.*, 2008) were as described previously. I3A was analysed using a Dionex P680 HPLC system (http://www.dionex.com) according to the protocol described by Bednarek *et al.* (2009). The I3A standard (unknown concentration) was kindly provided by P. Bednarek (Max-Planck Institute for Plant Breeding, Cologne, Germany).

### Quantitative RT-PCR analysis

Leaf samples were frozen in liquid nitrogen. Total RNA was extracted using the RNeasy® plant mini kit, including treatment with DNaseI (http://www.qiagen.com). The RNA samples were reverse-transcribed using an Omniscript® reverse transcription kit (http://www.qiagen.com) with 2 μg total RNA. cDNA samples were diluted 1:8, and aliquots for individual quantitative PCR runs were stored at −20°C. Quantitative RT-PCR was performed in triplicate using MESA GREEN qPCR MasterMix (http://www.eurogentec.com) in a Rotor-Gene™ 2000 apparatus (http://www.corbettlifescience.com). In a 15 μl reaction volume, 5 μl of the cDNA sample was combined with 7.5 μl of 2× SYBR Green Mix, 1.6 μl RNase- and DNase-free water, and 0.45 μl of each primer (10 μM). The cycling profile was 95°C for 15 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 25 sec and 72°C for 20 sec. The gene-specific primer pairs are listed in Table S2. The specificity of the amplicon was confirmed by melting curve analysis and agarose gel electrophoresis. Cycle threshold and efficiency values were exported from the Rotor-Gene 4.4 software for calculations (http://www.corbettlifescience.com). Expression values for the target genes were normalized to the reference index calculated from the reference genes *At4g26410*, *At3g01150*, *At1g62930* and *At2g28390* using the Excel macro 'REST' (Pfaffl *et al.*, 2002). The four reference genes (Czechowski *et al.*, 2005) were selected based on their stability of expression during *P. brassicae* infection as determined using the 'geNorm' macro (Vandesompele *et al.*, 2002). Fold induction values were calculated based on expression levels of mock-infected samples.

### Cytological analysis

Leaves inoculated with *P. brassicae* were harvested and stained with lactophenol/trypan blue to visualize pathogen structures and dead host cells (Roetschi *et al.*, 2001). *In planta* accumulation of $H_2O_2$ was revealed by 3,3′-diaminobenzidine (DAB) staining (Thordal-Christensen *et al.*, 1997). Leaves were vacuum-infiltrated with a DAB tetrahydrochloride solution (1 mg/ml) and incubated for 5 h in the dark at the infection conditions described above. Ethanol-destained leaves were analysed in 50% glycerol using a Leica DMR microscope (http://www.leica.com/) with bright-field settings. Callose depositions were stained with aniline blue (Clay *et al.*, 2009) and observed using a Leica DMR epifluorescence microscope equipped with a UV filter set (excitation 340–380 nm, emission 425 nm).

### Growth inhibition assays

GS were extracted using (70% v/v) methanol from 5-week-old Col-0 leaves for 15 min at 80°C. After centrifugation at 2000 ***g*** for 10 min, the supernatant was filtered through a glass fiber filter. After evaporation of the solvent, the residue was taken up in water and filtered through a 22 μm syringe filter. The Arabidopsis protein extract was prepared from Col-0 leaves (1 g) homogenized using a Polytron mixer (http://www.kinematica.ch) in 4 ml potassium phosphate buffer (50 mM, pH 6), centrifuged for 10 min at 12 000 ***g***, and the supernatant was desalted using PD-10 columns (http://www.6.gelifesciences.com). The resulting protein fraction was filtered through a 22 μm syringe filter. To test inhibitory effects of endogenous Arabidopsis GS to *P. brassicae*, clarified V8 tomato juice agar plates were inoculated with 5 ml of a zoospore suspension (20 000 spores/ml). After 1 h, sterile filter disks soaked in either myrosinase solution (0.3 U/ml), Arabidopsis protein extract or water were placed on the agar plates prior to application of a leaf extract containing glucosinolates (GS extract). A second application of GS extract was made after 12 h, giving to final GS concentrations ranging from 0.5× to 4× the physiological GS concentration. Growth inhibition was evidenced as inhibition zones around the filter disks 5 days post-inoculation. Growth inhibition by camalexin was tested by measuring the colony diameter of *P. brassicae* growing on clarified V8-agar plates containing various concentrations of camalexin.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Transcriptional changes in tryptophan pathway-related genes in response to *P. brassicae*.

**Figure S2.** Analysis of foliar thiol levels in uninfected Col-0, *cyp79B2 cyp79B3* and *pad2-1* plants.

**Figure S3.** Effect of mutations in the camalexin or GS pathways on the accumulation of selected tryptophan-derived secondary metabolites.

**Figure S4.** Comparison of transcript accumulation of genes involved in indole metabolism between *pad2-1* and Col-0.

**Figure S5.** Analysis of the anti-microbial activity of GS and camalexin towards *P. brassicae*.

**Table S1.** Chemical structures, common names and abbreviations for camalexin and glucosinolates.

**Table S2.** Primers used for quantitative RT-PCR analysis.

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