Published in "Biology of Plant-Microbe Interactions vol. 7: 1-7, 2010"
which should be cited to refer to this work.

Indole glucosinolates are an important first layer defense of Arabidopsis against *Phytophthora brassicae*

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Summary

We are interested in the role of indole glucosinolates (iGS) and the phytoalexin camalexin in diseases resistance of Arabidopsis to the oomycete pathogen *Phytophthora brassicae*. Transcript profiling revealed that many genes involved in iGS or camalexin biosynthesis are up-regulated upon inoculation. Mutants with defects in either iGS metabolism or camalexin accumulation remain resistant to *P. brassicae*. Interestingly, the combined deficiency in iGS and camalexin in the double mutants *cyp79B2 cyp79B3* and *pen2-1 pad3-1* results in susceptibility. Hence, Arabidopsis defence against *P. brassicae* relies on the combined action of iGS and camalexin. The constitutively produced iGS appear to play an early role in penetration resistance whereas the inducible camalexin is important at later stages of the infection.

Introduction

The Arabidopsis mutant *pad2-1* accumulates reduced levels of camalexin and is susceptible to *Phytophthora brassicae*. Camalexin deficiency is, however, not the cause of susceptibility as evidenced by the disease resistance phenotype of other camalexin-deficient mutants (Roetschi et al., 2001). What does then explain the susceptibility of *pad2-1* to *P. brassicae*? Recently, we reported that *pad2-1* is less tolerant to the herbivorous insect *Spodoptera littoralis*. The reduced tolerance is linked to a deficiency of *pad2-*

1 in the insect-induced accumulation of glucosinolates (GS; Schlaeppi et al., 2008). This suggested the possibility that GS deficiency is causing, alone or in combination with low camalexin levels, disease susceptibility to *P. brassicae*.

GS (ß-thioglucoside-N-hydroxysulfates) are characteristic secondary metabolites of the *Brassicaceae* including Arabidopsis. GS are constitutively produced and become biologically active as aglycones after hydrolysis by myrosinase enzymes. The hydrolysis products serve as feeding deterrents for non-specialized insects but can also function as attractants for specialized insects (Halkier and Gershenzon, 2006). Several studies indicate a possible defensive role of GS in disease resistance (Doughty et al., 1991; Brader et al., 2006). Genetic dissection of non-host resistance identified the atypical iGS-specific myrosinase PEN2 and the cytochrome P450 enzyme CYP81F2 involved in the formation of 4-Methoxy-indole-3-ylmethyl GS (4MO-I3M). Specific PEN2-dependent hydrolysis products of 4MO-I3M GS were shown to be critical to pre-invasion resistance of Arabidopsis to a number of non-host pathogens (Bednarek et al., 2009).

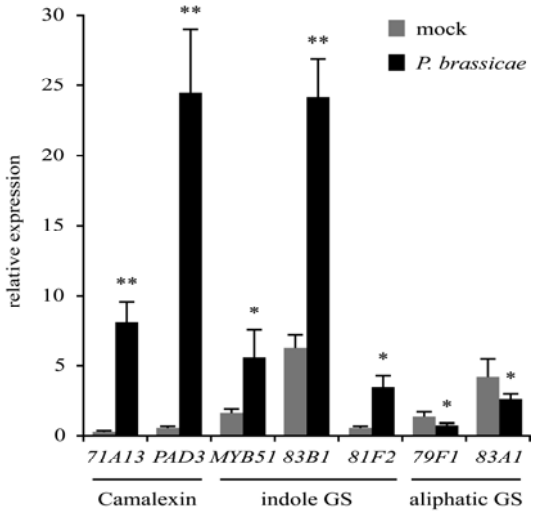

Fig. 1. Transcript levels of genes involved in camalexin-, iGS- and aGS-biosynthesis in response to *P. brassicae.* Quantitative RT-PCR analysis of mock- and *P. brassicae*-inoculated 4-week-old Col-0 leaf samples collected at 24 hpi. Expression levels are normalized relative to the expression level of the reference gene *PTB* (At3g01150). The values represent the mean (±SE) of 3 independent experiments (** $P < 0.001$, * $P < 0.05$; Student's *t*-test).

Results and Discussion

Camalexin and iGS pathways are transcriptionally up-regulated in response to P. brassicae

Transcriptome analysis of the Arabidopsis - *P. brassicae* interaction indicated a stimulation of the tryptophan-derived biosynthesis of camalexin and iGS (Mauch et al., 2009). Quantitative PCR confirmed the up-regulation of a selection of biosynthetic genes and the transcription factor MYB51/HIG1 that regulates the iGS pathway (Fig.1). In contrast, genes encoding enzymes involved in the biosynthesis of aliphatic GS (aGS) are down-regulated. Overall, the transcriptional changes of Arabidopsis in response to *P. brassicae* indicate a possible implication of camalexin and iGS but not aGS in disease resistance to *P. brassicae*.

The combined action of iGS and camalexin is important in disease resistance of Arabidopsis to P. brassicae

The two cytochrome P450 monooxygenases CYP79B2 and CYP79B3 catalyze the conversion of tryptophan to indole-3-acetaldoxime (IAOx), which acts as a precursor of camalexin-, iGS- and auxin-biosynthesis. The double mutant *cyp79B2 cyp79B3* is deficient in camalexin (Glawischnig et al., 2004) and iGS biosynthesis, but contains normal auxin levels due to redundancy in auxin biosynthesis (Zhao et al., 2002). Figure 2B shows the susceptibility phenotype of *cyp79B2 cyp79B3* to *P. brassicae* compared to the resistant wildtype (Fig. 2A). Several mutants with reduced levels of iGS (*hig1-1*; Gigolashvili et al., 2007), with defective iGS-specific myrosinase (*pen2-1*; Lipka et al., 2005) or with an altered iGS profile (*cyp81F2*; Bednarek et al., 2009) remain resistant to *P. brassicae* (Fig. 2C). Similarly, the camalexin-deficient mutants *pad3-1* (Glazebrook and Ausubel, 1994) and *cyp71A13* (Nafisi et al., 2007) remain resistant to *P. brassicae* (Fig. 2D). Thus, mutations affecting either iGS or camalexin metabolism have only a minor effect on disease resistance. In contrast, *pad2-1* and the double mutant, *pen2-1 pad3-1*, with a deficiency in camalexin accumulation and iGS hydrolysis show compromised disease resistance. Two scenarios might explain the differential susceptibility of the double mutants *cyp79B2 cyp79B3* and *pen2-1 pad3-1*: (1) IAOx- derived secondary metabolites others than iGS and camalexin may contribute to disease resistance or (2) additional enzymes other than PEN2 are capable for the hydrolysis of iGS. Overall, our data indicate that the combined lack of tryptophan derived secondary metabolites, including camalexin and iGS, is causing susceptibility to *P. brassicae*.

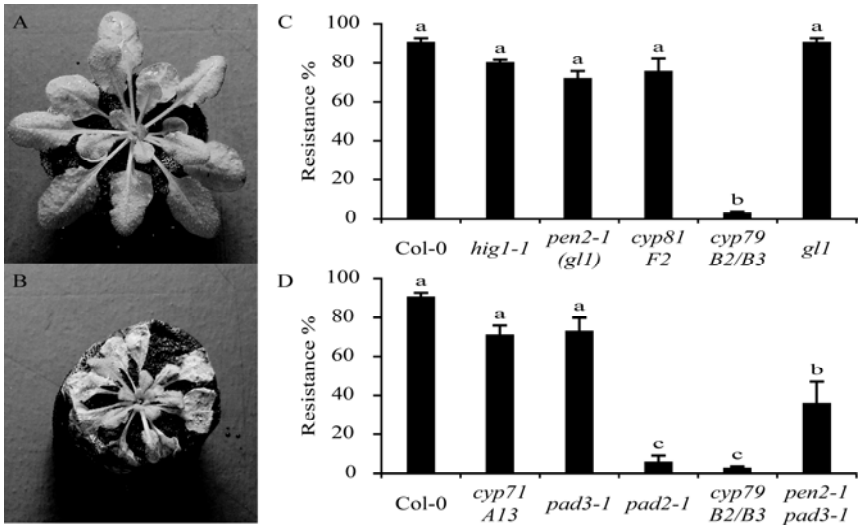

Fig. 2. Disease resistance phenotypes of camalexin and iGS mutants.

Four-week-old plants were infected with zoospores of *P. brassicae* and the disease resistance was scored 5 dpi. Disease resistance phenotypes of Col-0 (A) and *cyp79B2 cyp79B3* (B). Quantitative analysis of disease resistance (C-D) of the mutants *hig1-1*, *pen2-1* (*gl1* background) and *cyp81F2*, which have defects in iGS metabolism and the camalexin-deficient mutants *cyp71A13* and *pad3-1*. The double mutant *pen2-1 pad3-1* is affected in both camalexin and iGS metabolisms. Values are the mean (±SE) of at least 3 independent experiments per genotype. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test).

iGS and camalexin contribute sequentially to disease resistance

Microscopic characterization of the infection process revealed a 2-fold enhancement in the number of spores successfully penetrating the epidermal cell layer of *cyp79B2 cyp79B3* in comparison to the wildtype (Fig. 3A). This effect is detectable already at 6 hpi. Reduced penetration resistance was also observed in *hig1-1* and *pad2-1*, but not in the camalexin-deficient mutant *pad3-1*. Hence, iGS play a specific early role in penetration resistance. Because *pad2-1* also allows increased penetration, the susceptibility of *pad2-1* might at least partially be explained by the combined deficiency of both classes of secondary metabolites. Figure 3B shows that in wild-type plants, camalexin begins to accumulate at 12 hpi and reaches important levels at 48 hpi. As evidenced by *in-vitro* assays (Fig. 3C), these camalexin levels (48h) are in a range relevant for growth inhibition of *P. brassicae*. In summary, we conclude that, contrary to previous reports (Roetschi et al., 2001), camalexin contributes to disease resistance to *P. brassicae*.

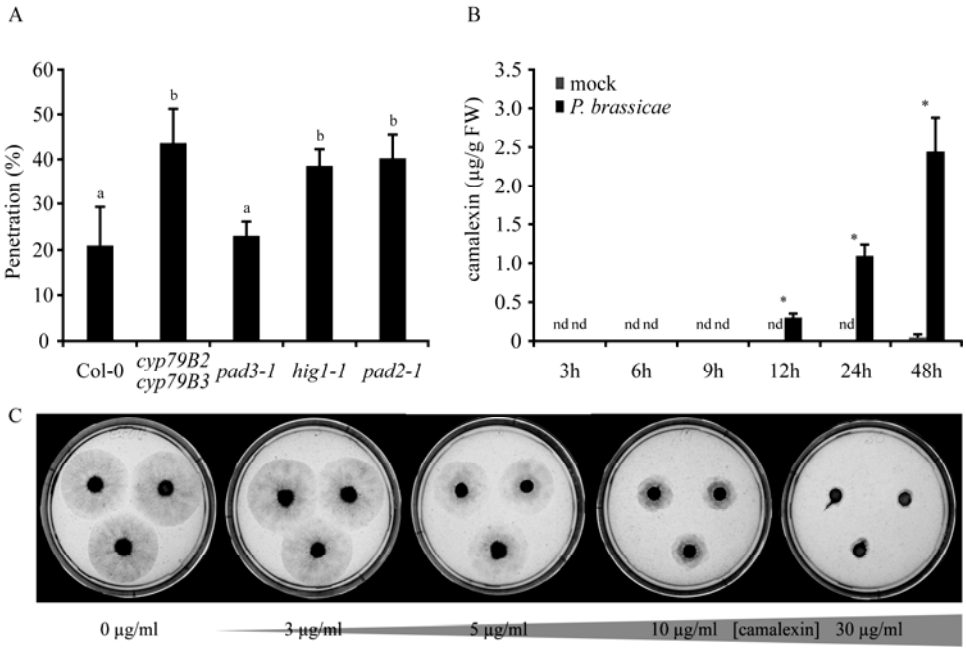

Fig. 3. iGS function in penetration resistance in the absence of camalexin.

(A) Increased pathogen entry is observed in *cyp79B2 cyp79B2*. Four-week-old plants were infected with *P. brassicae* and leafs sampled at 6hpi. The number of penetration events was scored in the upper mesophyll and calculated as percentage (±SE) of spores present at the leaf surface. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test). (B) Camalexin accumulation of 4-week-old wild-type plants in response to *P. brassicae* infection. Values (±SE) are from 3 independent replicates (nd = not detected (detection limit of 0.2 ng standard), * $P < 0.05$; Student's *t*-test). (C) Antimicrobial activity of camalexin to *P. brassicae* was tested on V8-agar plates containing different concentrations of camalexin. The colonies were photographed at 4 dpi. An EC50 value of 6.3 µg/ml camalexin was calculated from 6 replicate recordings of radial growth.

Conclusions

The disease resistance phenotypes of different mutants shows that the combined action of both iGS and camalexin is required for full resistance of Arabidopsis to *P. brassicae*. The iGS phytoanticipins function at initial stages of defence in penetration resistance whereas the phytoalexin camalexin acts as second late-acting defence mechanism.

Acknowledgements

This work was supported by grant 31003A-116531 to F.M. from the Swiss National Science Foundation. We acknowledge the authors cited in the text for providing seeds of mutants. We thank L. Westphal and S. Rosahl (IPB Leibnitz, Halle, Germany) for seeds of the *pad3-1 pen2-1* double mutant. We thankfully acknowledge H-J. Schoonbeek for the donation of synthetic camalexin.

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