Research

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The role of CYP71A12 monooxygenase in pathogen-triggered tryptophan metabolism and Arabidopsis immunity

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Summary

- Effective defense of Arabidopsis against filamentous pathogens requires two mechanisms, both of which involve biosynthesis of tryptophan (Trp)-derived metabolites. Extracellular resistance involves products of PEN2-dependent metabolism of indole glucosinolates (IGs). Restriction of further fungal growth requires PAD3-dependent camalexin and other, as yet uncharacterized, indolics.
- This study focuses on the function of CYP71A12 monooxygenase in pathogen-triggered Trp metabolism, including the biosynthesis of indole-3-carboxylic acid (ICA). Moreover, to investigate the contribution of CYP71A12 and its products to Arabidopsis immunity, we analyzed infection phenotypes of multiple mutant lines combining *pen2* with *pad3*, *cyp71A12*, *cyp71A13* or *cyp82C2*.
- Metabolite profiling of *cyp71A12* lines revealed a reduction in ICA accumulation. Additionally, analysis of mutant plants showed that low amounts of ICA can form during an immune response by CYP71B6/AAO1-dependent metabolism of indole acetonitrile, but not via IG hydrolysis. Infection assays with *Plectosphaerella cucumerina* and *Colletotrichum tropicale*, two pathogens with different lifestyles, revealed *cyp71A12*-, *cyp71A13*- and *cyp82C2*-associated defects associated with Arabidopsis immunity.
- Our results indicate that CYP71A12, but not CYP71A13, is the major enzyme responsible for the accumulation of ICA in Arabidopsis in response to pathogen ingression. We also show that both enzymes are key players in the resistance of Arabidopsis against selected filamentous pathogens after they invade.

Introduction

Plants have evolved robust, innate immune systems in response to persistent colonization attempts by pathogenic microorganisms, with some of the responses involving biosynthesis and targeted secretion of antimicrobial secondary metabolites (Jones & Dangl, 2006; Piasecka *et al.*, 2015). In the model plant *Arabidopsis thaliana* (Arabidopsis), tryptophan (Trp)-derived metabolites, including the indolic phytoalexin camalexin and indolic glucosinolates (IGs), have been shown to be involved in crucial processes of the plant's innate immune system (Glawischnig, 2007; Pastorczyk & Bednarek, 2016). In addition to such metabolic compounds, indole-3-carboxylic acid (ICA) derivatives (ICAs) (Hagemeier *et al.*, 2001; Tan *et al.*, 2004; Bednarek *et al.*, 2005; Forcat *et al.*, 2010) and indole-3-carbonyl nitriles (ICNs) (Rajniak *et al.*, 2015) have been reported as end products of microbe-induced metabolic pathways.

Not only do the above listed compounds share the same precursor, but they are also involved in the first biosynthetic step of all Trp-derived specialized metabolites, the formation of indole-3-acetaldoxime (IAOx) from Trp by two redundant P450 monooxygenases, CYP97B2 and CYP79B3 (Fig. 1) (Mikkelsen *et al.*, 2000; Zhao *et al.*, 2002; Glawischnig *et al.*, 2004; Böttcher *et al.*, 2009). In Arabidopsis, this intermediate product represents the metabolic branch point for IG biosynthesis, and pathways leading to the synthesis of ICAs, ICNs and camalexin. On the IG biosynthetic pathway, IAOx is metabolized by CYP83B1 monooxygenase to the respective nitrile oxide, which is converted

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to indol-3-ylmethyl glucosinolate (I3G) in subsequent biosynthetic steps (Fig. 1) (Sonderby *et al.*, 2010). I3G can be further modified on the indole core, for instance by CYP81F2 monooxygenase that initiates formation of 4-methoxy-I3G (4MI3G) (Pfalz *et al.*, 2011). On the route to camalexin, IAOx can be metabolized to indole-3-acetonitrile (IAN) and possibly further to α-hydroxy-IAN and/or dehydro-IAN by CYP71A13 and its close homolog, CYP71A12 (Fig. 1) (Nafisi *et al.*, 2007; Klein *et al.*, 2013; Müller *et al.*, 2015). In Arabidopsis, the enzymes CYP71A13 and CYP71A12 show 89% similarity in their amino acid sequences and their corresponding genes are located in tandem on chromosome II. Metabolic phenotypes of single and double *cyp71a12* and *cyp71a13* mutant lines reveal that both monooxygenases contribute to camalexin biosynthesis when infected by *Pseudomonas syringae* and *Alternaria brassicicola* and in response to microbe-associated molecular pattern (MAMP) flg22 (bacterial flagellin epitope), silver nitrate ($AgNO_3$) or UV treatment (Nafisi *et al.*, 2007; Millet *et al.*, 2010; Müller *et al.*, 2015). The last two steps in the formation of camalexin are catalyzed by the multifunctional enzyme PHYTOALEXIN DEFICIENT 3 (PAD3) CYP71B15 monooxygenase (Schuhegger *et al.*, 2006; Böttcher *et al.*, 2009). Importantly, *pad3* mutants are completely lacking camalexin and so are frequently used when validating the function of camalexin in Arabidopsis–microbe interactions (Thomma *et al.*, 1999; Zhou *et al.*, 1999). In addition to camalexin, IAN has also been postulated as being an intermediary in ICA biosynthesis (Fig. 1) (Böttcher *et al.*, 2014). Accumulation of ICAs in single and double *cyp71a12* and *cyp71a13* mutant lines suggest that biosynthesis of these compounds by Arabidopsis in response to $AgNO_3$ toxicity depends predominantly on CYP71A12 monooxygenase, whereas specific ICAs are produced in response to UV treatment, with the contribution of CYP71A13 (Müller *et al.*, 2015). The *cyp71a12* mutant is also reported to accumulate reduced amounts of ICAs when infected with *A. brassicicola* or *Botrytis cinerea* (Rajniak *et al.*, 2015). However, the double *cyp71A12 cyp71A13* (*cyp71A12/A13*) mutant line was not tested with these two necrotrophic pathogens. The reduced accumulation of 6-*O*-glucoside of 6-hydroxy-ICA (6OGlcICA) and ICA glucose ester (ICAGlc) in leaves of *cyp71B6* and *arabidopsis aldehyde oxidase 1* (*aao1*) mutants exposed to $AgNO_3$ suggest that CYP71B6 and AAO1 enzymes contribute to the conversion of IAN via indol-3-aldehyde to ICA (Böttcher *et al.*, 2014; Müller *et al.*, 2019). Finally, experimental evidence indicates that CYP71A12 (but not CYP71A13) is also involved in ICN biosynthesis (Fig. 1). It has been proposed that α-hydroxy-IAN produced by CYP71A12 monooxygenase is metabolized by flavin-dependent oxidoreductase 1 (FOX1) to ICN, which can be further hydroxylated at the C-4 position of the indole ring by another monooxygenase, CYP82C2, to form 4-hydroxy-ICN (4OH-ICN) (Fig. 1) (Rajniak *et al.*, 2015). ICNs have been shown to be highly unstable, and in aqueous or methanolic solutions they hydrolyze to ICAs with release of HCN. Consequently, it has been hypothesized that a fraction of ICAs observed in plant extracts might be derived from the degradation of ICNs (Fig. 1) (Rajniak *et al.*, 2015).

In Arabidopsis, an effective defense against filamentous pathogens requires two autonomous, but complementary, mechanisms acting before and after invasion of its epidermal cells (Lipka *et al.*, 2008). Pathogen-inducible IG metabolism was

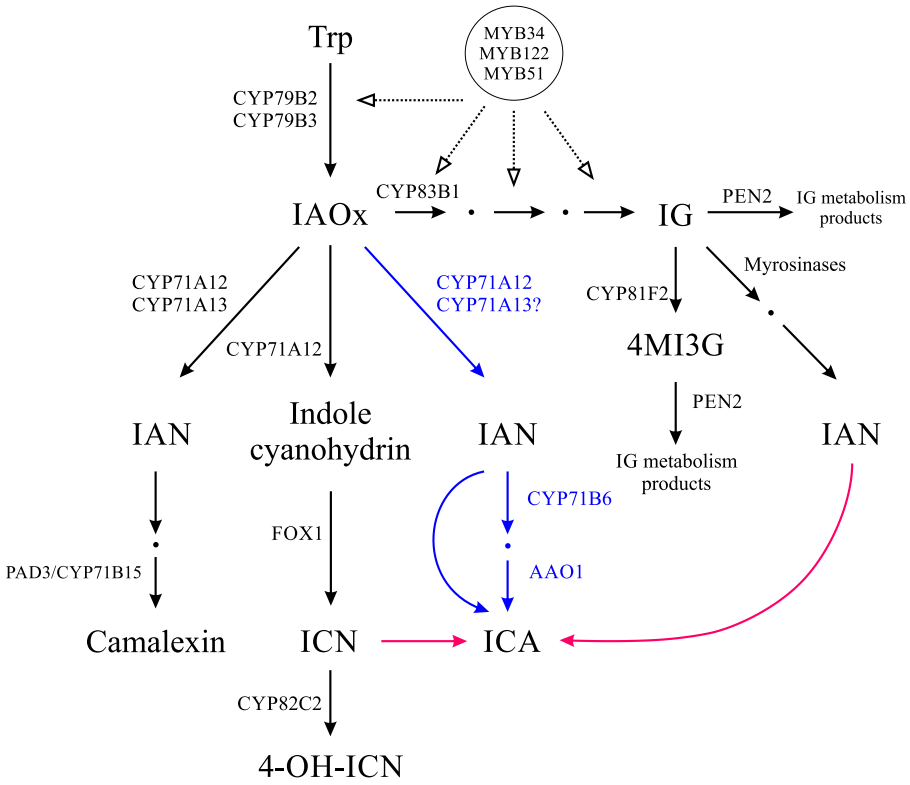

Fig. 1 Simplified scheme of biosynthesis of tryptophan derivatives and role of P450 monooxygenases in metabolism of these compounds in *Arabidopsis thaliana*. Blue color indicates validated metabolic steps involved in the biosynthesis of ICA in response to abiotic stress. Red color indicates speculative steps that can additionally contribute to ICA formation. Trp, tryptophan; IAOx, indole-3-acetaldoxime; IG, indole glucosinolates; 4MI3G, methoxy-indol-3-ylmethyl glucosinolate; IAN, indole-3-acetonitrile; ICN, indole carbonyl nitrile; 4-OH-ICN, 4-hydroxy-ICN; ICA, indole-3-carboxylic acid; AAO1, arabidopsis aldehyde oxidase 1; FOX1, flavin-dependent oxidoreductase 1; PAD3, phytoalexin-deficient 3 CYP71B15 monooxygenase; MYB, member of the myeloblastosis family of transcription factors.

originally identified as a component of extracellular resistance controlling entry of nonadapted powdery mildews into epidermal cells (Lipka *et al.*, 2005; Bednarek *et al.*, 2009). This process involves CYP81F2 monooxygenase, β-thioglucoside glucohydrolase (myrosinase) PENETRATION2 (PEN2), and glutathione *S*-transferase U13 (GSTU13), all of which are required for generating end product(s) crucial for plant immunity against nonadapted powdery mildew (Bednarek *et al.*, 2009; Piślewska-Bednarek *et al.*, 2018). As confirmed by numerous infection assays, this pathway is essential not only for controlling powdery mildew penetration, but also for extracellular resistance against numerous filamentous plant pathogens and for MAMP-triggered callose deposition (Clay *et al.*, 2009; Pastorczyk & Bednarek, 2016). Microscopic observations of *pen2* and *pad3* single and double knockout plants inoculated with conidiospores of the nonadapted powdery mildew *Erysiphe pisi* or conidiospores of the nonadapted anthracnose fungus *Colletotrichum tropicale* (formerly known as *Colletotrichum gloeosporioides*) has revealed that camalexin is not required to control pathogen entry. However, the double *pen2 pad3* mutant is not only deficient in providing resistance to powdery mildew penetration (as is *pen2*), but the double mutant additionally supports extensive growth of secondary hyphae (Bednarek *et al.*, 2009; Hiruma *et al.*, 2010, 2013). These observations suggest that successful epidermis penetration triggers immunity-contributing mechanisms at the post-invasive infection stage, which involves antibiotic activity of camalexin. Notably, the *cyp79B2 cyp79B3* (*cyp79B2/B3*) double mutant line, which lacks all Trp-derived secondary metabolites, supports even more massive growth of *C. tropicale* secondary hyphae compared with *pen2 pad3* (Hiruma *et al.*, 2013). A similarly clear difference in the susceptibility of *pen2 pad3* and *cyp79B2/B3* lines has been observed when examining the differential response of those two lines to *Phytophthora brassicae* or to adapted and nonadapted strains of the necrotrophic fungal pathogen *Plectosphaerella cucumerina* (Sanchez-Vallet *et al.*, 2010; Schlaeppi *et al.*, 2010). This striking susceptibility of the *cyp79B2/B3* double mutant indicates that Arabidopsis immunity at the post-invasive stage requires other indolic compounds in addition to camalexin. Because IG-deficient *myb34 myb51 myb122* (*myb34/51/122*) plants show the same susceptibility to *P. cucumerina* infection as the *pen2* mutant, it has been suggested that such (as yet unknown) additionally required indolic metabolites are not derived from any IGs (Frerigmann *et al.*, 2016). ICAs, the biosynthesis of which is known to be induced by pathogen infection in Arabidopsis and related Brassicaceae species, are the main candidates for potential involvement in the restriction of post-invasive pathogen growth (Bednarek *et al.*, 2011). However, the role of ICAs in plant immunity has not yet been experimentally verified. In addition, it has been recently reported that the *cyp82C2* mutation increases Arabidopsis susceptibility to *P. syringae*, *A. brassicicola* and *B. cinerea*, indicating a possible role of 4OH-ICN in providing resistance against bacterial and fungal pathogens (Rajniak *et al.*, 2015).

In this study we investigated the function of several enzymes in the pathogen-triggered biosynthesis of ICAs. In addition, we used combinations of *pen2*, *pad3*, *cyp71A12*, *cyp71A13* and *cyp82C2* mutants to decipher the function of various IAN-derived compounds in Arabidopsis immunity against *P. cucumerina* and *C. tropicale*. Overall, our study revealed a major contribution of CYP71A12 to the pathogen-triggered ICA biosynthesis and important functions of this monooxygenase in the restriction of post-invasive pathogen growth in Arabidopsis.

Materials and Methods

Plant material

Arabidopsis thaliana seeds *of cyp71A12* (GABI_414A10), *cyp71A13* (SALK_105136), *aao1* (SALK_069221), *cyp71B6* (GABI_305A04), *fox1* (GABI_813E08) and *cyp82C2* (GABI_183B09) T-DNA lines were obtained from Nottingham Arabidopsis Stock Center. The isolation of homozygous mutant lines was performed according to standard procedures. For oligonucleotide sequences of the primers LP, RP and BP, see Supporting Information Table S1. The *pen 2-2*, *pen2 pad3* and *cyp79B2 cyp79B3* mutant lines were reported by Bednarek *et al.* (2009). The TALEN-generated *cyp71A12 cyp71A13* was described by Müller *et al.* (2015). The *myb34/51/122* triple mutant line was reported by Frerigmann *et al.* (2016).

Generation and selection of multiple mutant lines

The *pen2 cyp71A12* and *pen2 cyp82C2* double mutant lines, *pen2 pad3 cyp82C2* and *pen2 cyp71A12 cyp71A13* triple mutant lines as well as *myb34/51/122 cyp71A12A/A13* quintuple mutant line were generated by standard genetic crosses followed by identification of the mutant alleles. For genotyping of *pen2*, *cyp82C2*, *cyp71A12* T-DNA lines, oligonucleotide sequences of primers are listed in Table S1. Single nucleotide deletion in *PAD3/CYP71B15* (Zhou *et al.*, 1999; Nafisi *et al.*, 2007) was confirmed by digestion of 600 bp PCR product obtained with LP 5′-GTCGCGATCTCTTCGAAAGA-3′ and RP 5′-GAGAGCATCTCCATCGTTCTC-3′ with *Hind*III restriction enzyme (WT digestion pattern 400 bp + 200 bp, *pad3* mutation – no digestion of the PCR product). The TALEN mutation in the *cyp71A12 cyp71A13* double mutant line was confirmed according to Müller *et al.* (2015). Mutations in the *myb34/51/122* triple mutant line were confirmed according to Frerigmann *et al.* (2016).

HPLC analysis of specialized metabolites

Four-week-old plants of selected mutant lines were inoculated with *P. cucumerina* spore suspension (isolate BMM (Brigitte Mauch-Mani), 1.25×10^8 ml^{-1}). Leaf samples for quantitative analysis of soluble Trp derivatives were collected 48 h post-inoculation and extracted in dimethyl sulfoxide as described by Bednarek *et al.* (2009). For the analysis of cell wall-bound ICA, 6-wk-old plants were inoculated as described above with *P. cucumerina* spore suspension. Leaf samples (~2 g) were collected 48 h post-inoculation. Purification of cell walls and extraction of the cell wall-bound fraction of ICA was performed according to Tan *et al.* (2004) with some modifications. Extracts

of soluble and cell wall-bound compounds were subjected to HPLC analysis on an Agilent 1100 HPLC system equipped with diode array and fluorescence detectors. Samples were analyzed on an Atlantis T3 C18 column (150 × 3 2.1 mm, 3 mm; Waters, Millford, MA, USA) according to the same protocols as reported earlier (Bednarek *et al.*, 2009).

RNA extraction and expression analysis by qRT-PCR

For gene expression analysis of *CYP71A12/A13* in respective mutant lines inoculated with *Pc*BMM, total RNA extraction and cDNA synthesis were performed using a Qiagen RNeasy Plant Mini Kit and Omniscript RT Kit, respectively, according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) analysis was performed using iTag Universal SYBR Green Supermix in a Bio-Rad CFX Connect Real-Time System. Relative quantification of expression levels of target genes was performed using the comparative Ct method and normalized to *ACTIN2*. For gene expression of *CYP71A12*, *CYP82C2* and *PAD3/CYP71A15* in *C. tropicale*–Arabidopsis interactions, total RNA was extracted using PureLink (TRIzol plus RNA Purification, Life Technologies) and treated with DNase (RQ1 RNase-free DNase; Promega) to remove DNA contamination. qRT-PCR analysis was performed using Takara TB Green Premix Ex Taq and a Thermal Cycler Dice Real Time System TP800 (Takara, Shiga, Japan). Arabidopsis UBIQUITIN-CONJUGATING ENZYME 2 (At5g25760) was used as an internal control for normalizing the level of cDNA. For amplification of all analyzed genes, a standard qRT-PCR protocol was applied with 1 min elongation time. Oligonucleotide sequences used for qRT-PCR analysis are listed in Table S1.

Disease resistance analysis

Plectosphaerella cucumerina Three-week-old Arabidopsis plants grown as previously described (Sanchez-Vallet *et al.*, 2010) were inoculated with a spore suspension (4×10^6 spores ml^{-1}) of adapted (BMM) and nonadapted (2127) strains of *P. cucumerina*. Disease progression in the inoculated plants was estimated by relative quantification of fungal DNA by means of qRT-PCR as previously described by Sánchez-Rodríguez *et al.* (2009). The qRT-PCR results are mean values ± SD from two technical replicates.

Colletotrichum tropicale To investigate the entry trail of *C. tropicale* (formerly *Colletotrichum gloeosporioides*) (Kosaka & Takano, 2018) and the development of primary biotrophic hyphae, 2 µl drops of a *C. tropicale* conidial suspension (*c.* 5×10^5 conidia ml^{-1}) were drop inoculated with 0.05% (w/v) glucose onto the cotyledon of each 12-d-old-plant, and the inoculated plants were subjected to microscopic analysis at 14 h post-inoculation as described by Hiruma *et al.* (2010). To investigate the expansion of necrotrophic hyphae at the post-entry and post-invasive defence stages, 5 µl drops of *C. tropicale* conidial suspension (*c.* 2.5×10^5 conidia ml^{-1}) were inoculated with 0.1% (w/v) glucose onto each of the leaves of 4-wk-old plants. After incubation, the inoculated plants were subjected to lesion development analysis and Trypan Blue staining.

Statistical analysis

The statistical significance of observed differences was validated with two-way or one-way analyses of variance (ANOVAs) followed by post hoc Tukey's honest significance test. These tests were performed using XLSTAT software (Addinsoft).

Results

CYP71A12, but not CYP71A13, is required for pathogen-triggered accumulation of ICAs

Metabolite profiling of *cyp71A12* knockout plants indicates the crucial contribution of CYP71A12 monooxygenase to the $AgNO_3$-, UV- and pathogen-triggered accumulation of ICAs (Müller *et al.*, 2015; Rajniak *et al.*, 2015). Analysis of the double *cyp71A12/A13* mutant suggested that CYP71A13 provides an additional minor contribution to the UV-induced accumulation of these compounds (Müller *et al.*, 2015). To assess the role of both monooxygenases in the pathogen-triggered biosynthesis of ICA derivatives, we inoculated leaves of *cyp71A12*, *cyp7A13* and *cyp71A12/A13* mutant lines together with wild-type (WT) plants (Col-0 ecotype) with a spore suspension of the host-adapted pathogenic strain of the necrotrophic fungus *Plectosphaerella cucumerina BMM* (*PcBMM*), which is able to colonize Col-0 plants (Ton & Mauch-Mani, 2004). We used HPLC coupled with UV-detection (HPLC/UV) to analyze the accumulation of 6OGlcICA and ICAGlc in leaves of control and *PcBMM*-inoculated plants (Fig. 2a). Our analysis revealed that the pathogen triggered significantly lower accumulation of both ICA derivatives in the *cyp71A12* single mutant line than in WT plants. However, despite this deficiency observed in the infected leaves, WT-like concentrations of both ICAs accumulated in the leaves of control *cyp71A12* plants (Fig. 2a), suggesting that CYP71A12 monooxygenase is required for mediating pathogen-triggered, but not for the constitutive biosynthesis of 6OGlcICA and ICAGlc. In contrast to the *cyp71A12* genotype, concentrations of both analyzed ICAs in extracts obtained from leaves of the *cyp71A13* genotype inoculated with *PcBMM* were significantly higher than the respective concentrations in inoculated WT plants. Moreover, the double knockout line *cyp71A12/A13* retained the capacity to synthesize *cyp71A12*-like amounts of 6OGlcICA and ICAGlc, indicating that the CYP71A13 monooxygenase does not significantly contribute to the pathogen-triggered formation of ICA (Fig. 2a). This in turn suggests that either CYP71A12 acts in a partially redundant manner with a monooxygenase(s) other than CYP71A13 or that ICA might be produced in an alternative metabolic pathway.

Because ICA has also been reported to occur in the fraction comprising cell-wall-bound metabolites, we analyzed the amounts of ICA in the hydrolysates obtained from cell walls isolated from leaves of naïve and *PcBMM*-inoculated Col-0, *cyp71A12* and *cyp71A12/A13* plants. Our HPLC/UV analysis revealed that the *cyp71A12* mutation reduced the pathogen-triggered, but not constitutive, accumulation of cell-wall-bound

ICA, whereas an additional mutation in *CYP71A13* did not affect our measured ICA concentrations (Fig. S1). Overall, our experiments reveal that CYP71A12, but not CYP71A13, contributes to the biosynthesis of the soluble and cell-wall-bound fractions of ICAs in Arabidopsis.

IG hydrolysis does not significantly contribute to pathogen-triggered formation of ICAs

Conversion of IAOx by the enzymes CYP71A12 and CYP71A13 is not the only known source of IAN in Arabidopsis. This compound can also form during myrosinase-mediated hydrolysis of IGs (Fig. 1) (Müller *et al.*, 2015). To address this proposed link between IG metabolism and ICA biosynthesis, we generated a quintuple *cyp71A12/A13 myb34/51/122* mutant to analyze the accumulation of 6OGlcICA and ICAGlc in leaves of this line. The transcription factors MYB34, MYB51 and MYB122 control IG biosynthesis, and consequently triple *myb34/51/122* mutant plants are depleted of these metabolites (Frerigmann & Gigolashvili, 2014). As already reported, we had observed a lower constitutive accumulation of 6OGlcICA and ICAGlc in leaves of the *myb34/51/122* control than in Col-0 (WT) plants (Fig. 2b), possibly in response to the impact of MYB34, MYB51, and MYB122 transcription factors on the constitutive expression of monooxygenases *CYP79B2* and *CYP79B3* (Frerigmann *et al.*, 2016). However, we did not observe any significant difference in the accumulation of either ICAs between *PcBMM*-inoculated leaves of Col-0 and *myb34/51/122* mutant, suggesting that after inoculating Arabidopsis with a pathogen, expression of *CYP79B2* and *CYP79B3* is controlled by other transcription factors. Similarly, we did not observe any significant reduction in 6OGlcICA and ICAGlc concentrations in the *cyp71A12/A13 myb34/51/122* mutant relative to the *cyp71A12/A13* double mutant (Fig. 2b). Overall, our results indicate that IG metabolism does not significantly contribute to ICA accumulation, at least under our experimental conditions.

CYP71B6 and AAO1, but not FOX1, have a minor contribution to ICA-derivative accumulation in response to *P. cucumerina* infection

Based on the reduced accumulation of some ICAs in the respective knockout lines, CYP71B6 monooxygenase and AAO1 have been proposed as enzymes that convert IAN to indole-3-aldehyde

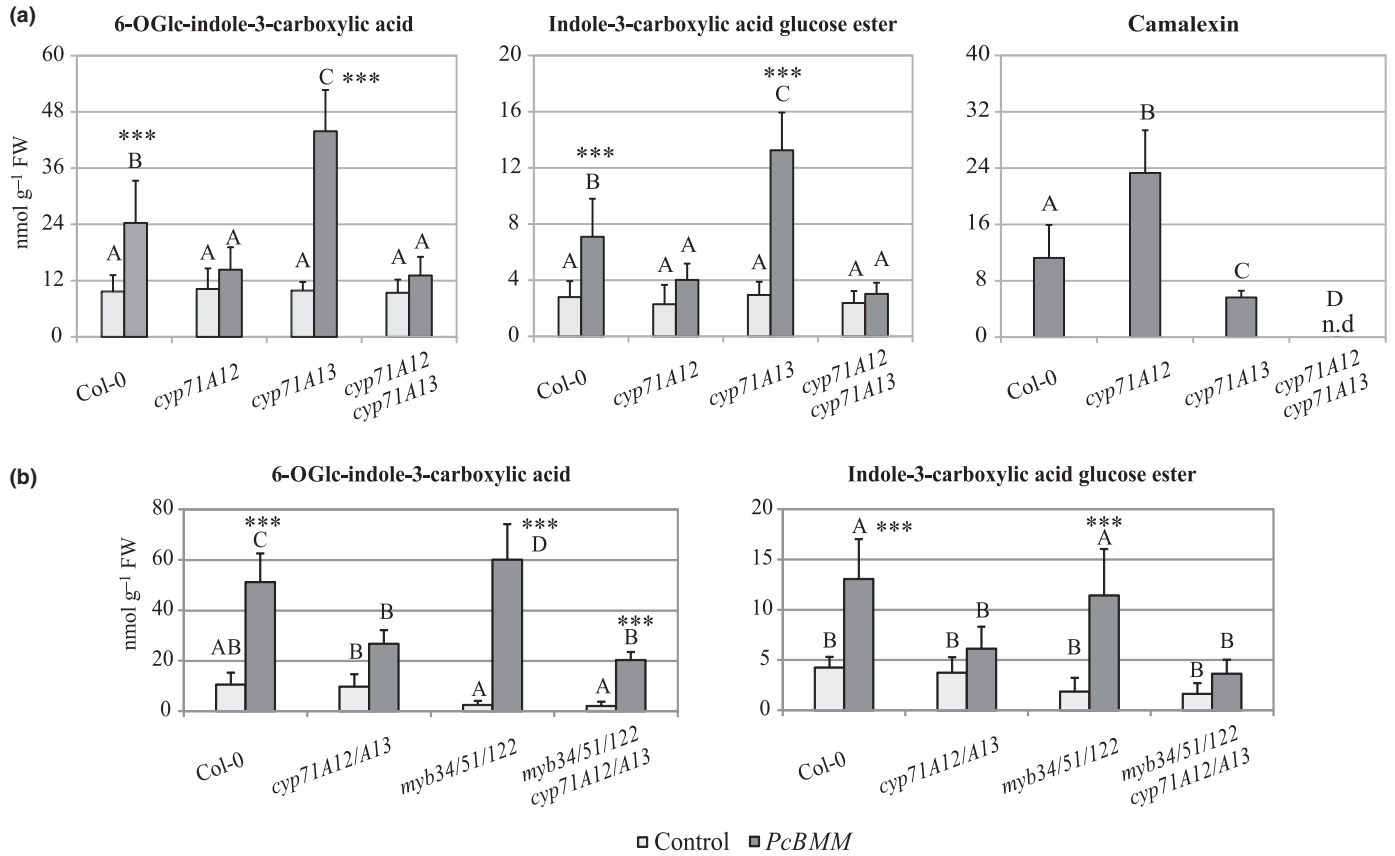

Fig. 2 CYP71A12, but not indole glucosinolates, contributes to the biosynthesis of indole-3-carboxylic acid derivatives in *Arabidopsis thaliana*. (a) Accumulation of selected Trp derivatives in *cyp71a12* and *cyp71a13* mutant lines. (b) Accumulation of selected Trp derivatives in indole glucosinolate-deficient mutant lines. Accumulation of respective metabolites in leaves of Col-0 and indicated mutant plants 48 h post-inoculation with spores of *Plectosphaerella cucumerina BMM*. Results are means ± SD from three independent experiments with tree biological replicates in each ($n = 9$). Significantly different statistical groups indicated by two-way ANOVA followed by post-hoc Tukey's honest significance test are shown with different capital letters. For camalexin accumulation, a one-way ANOVA followed by Tukey post-hoc test was used. Values marked with asterisks are significantly different from respective controls (***, $P < 0.0001$). FW, fresh weight.

and later to ICA as Arabidopsis responds to $AgNO_3$ toxicity (Böttcher *et al.*, 2014). To determine if these enzymes also contribute to the pathogen-triggered biosynthesis of ICA, we analyzed the accumulation of 6OGlcICA and ICAGlc in *cyp71B6* and *aao1* mutant plants. Our results revealed a slight, but significant, reduction in the accumulation of 6OGlcICA in *P. cucumerina*-inoculated leaves, indicating that CYP71B6 and AAO1 contribute to ICA biosynthesis during infection (Fig. S2).

It has been proposed that ICA can be produced in Arabidopsis, or form artificially during sample preparation, via degradation of ICN, whose formation is dependent on CYP71A12 and FOX1 enzyme activity (Rajniak *et al.*, 2015). To test if such degradation contributes to the pools of analyzed ICA derivatives, we examined concentrations of 6OGlcICA and ICAGlc in samples prepared from leaves of the *fox1* mutant line. Our HPLC analysis revealed that concentrations of both compounds in analyzed leaf extracts are not compromised by a defect in the *FOX1* gene (Fig. S3), clearly demonstrating that pathogen-triggered accumulation of ICAs involves CYP71A12 moonooxygenase, but is independent of FOX1 enzyme activity.

Redirection of IAOx between CYP71A12 and CYP71A13

Our metabolite profiling of *cyp71A12* and *cyp71A13* single mutant lines revealed that 6OGlcICA and ICAGlc, the biosynthesis of which are dependent on the presence of the enzyme CYP71A12, over-accumulate in the *cyp71A13* line during its immune response to *PcBMM* fungi. Conversely, we found that under the same conditions, the accumulation of camalexin, whose biosynthesis in leaves is mainly mediated by CYP71A13, is significantly higher in the *cyp71A12* single knockout mutant than in WT plants (Fig. 2). These differences in camalexin and ICA accumulation suggest that a T-DNA insertion in one of the two *CYP71A* genes leads to an elevated expression of the other homolog during the immune response in Arabidopsis. To test this hypothesis, we performed qRT-PCR analysis of *CYP71A12* and *CYP71A13* expression in *cyp71A12* and *cyp71A13* mutant lines after inoculating Arabidopsis with *PcBMM* fungi. We found that the *cyp71A12* mutant line overexpresses *CYP71A13* relative to WT plants, whereas disruption of *CYP71A13* results in significantly higher expression of *CYP71A12* (Fig. 3). Overall, our results reveal that the dysfunction of one of the analyzed monooxygenases leads to the overexpression of its counterpart enzyme. Consequently, the remaining biosynthetic intermediate (IAOx) can be redirected into the other branch of the biosynthetic pathway, resulting in the over-accumulation of its resulting end product. By contrast, our HPLC analysis revealed that IG accumulation is not affected in the *cyp71A12* and *cyp71A13* single and double mutants (Fig. S4), suggesting that the IAOx pool used for camalexin and ICA biosynthesis cannot be fed into the IG branch of Trp metabolism.

CYP71A12 contributes to immunity to *P. cucumerina* infection

Metabolic analysis indicated that *cyp71A12* plants are clearly defective in the biosynthesis of 6OGlcICA and ICAGlc triggered by *P. cucumerina*, suggesting that this genotype could be used to test the immune function of ICAs. Earlier infection assays with *B. cinerea* and *A. brassicicola* did not reveal any enhanced susceptibility of the *cyp71A12* mutant to these two necrotrophic fungi (Rajniak *et al.*, 2015), suggesting that CYP71A12 and ICAs are unnecessary for conferring Arabidopsis immunity toward fungal pathogens. Our initial infection assays with *PcBMM* also did not reveal enhanced susceptibility of *cyp71A12* towards this pathogen (Fig. S5). However, we assumed that as it was done with *pad3*, penetration resistance has to be diminished first to observe effects of the *cyp71A12* mutation on pathogen development (Bednarek *et al.*, 2009; Hiruma *et al.*, 2010; Sanchez-Vallet *et al.*, 2010; Schlaeppi *et al.*, 2010). To this end, we generated a set of double and triple mutants by combining *cyp71A12* and *cyp71A13* knockouts with the *pen2* line, which is impaired in its ability to control pathogen entry. The *cyp82C2* knockout line was included in this experiment to differentiate the potential immune function of ICA derivatives and 4OH-ICN, the biosynthesis of which was reported to be dependent on CYP71A12 and CYP82C2. We then inoculated the generated mutant lines with a spore suspension of adapted (*PcBMM*) and nonadapted (*Pc2127*) strains of *P. cucumerina*. Opposite to *PcBMM*, the *Pc2127* strain is not able to colonize Col-0 plants (Sanchez-Vallet *et al.*, 2010). Our determination of fungal biomass with qRT-PCR of the fungal β-*tubulin* gene did not reveal any higher susceptibility of *pen2*

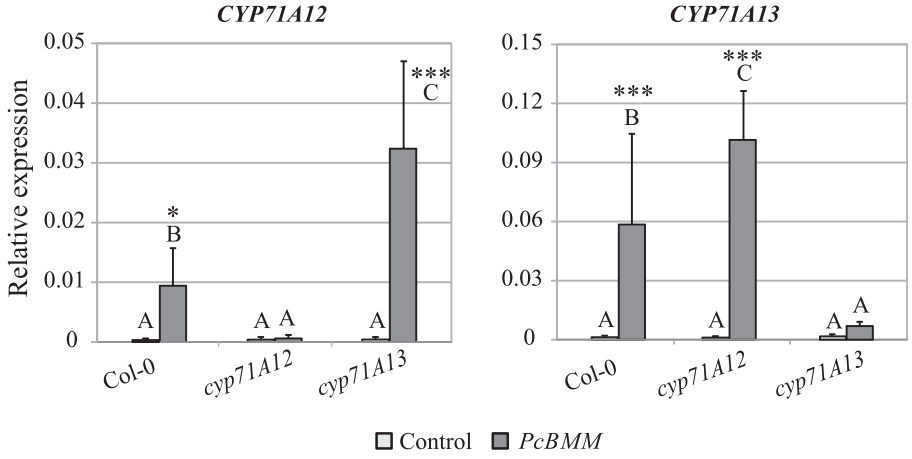

Fig. 3 Mutations in *CYP71A12* and *CYP71A13* induce expression of the counterpart gene in *Arabidopsis thaliana*. Relative expression of *CYP71A12* and *CYP71A13* in leaves of Col-0 and respective mutant lines 24 h after inoculation with *Plectosphaerella cucumerina BMM* spores. Data are means ± SD from three independent experiments each with four biological replicates ($n = 12$). Significantly different statistical groups indicated by two-way ANOVA followed by post-hoc Tukey's honest significance test are shown by different capital letters. Values marked with asterisks are significantly different from respective controls (*, $P < 0.05$; ***, $P < 0.0001$).

cyp71A12 or *pen2 cyp82C2* mutants to *PcBMM* and *Pc2127* fungal strains as compared to the single *pen2* mutant line (Fig. 4). However, our analysis showed that the *pen2 cyp71A12/13* triple mutant was significantly more susceptible to *Pc2127* compared with the *pen2 pad3* line. These results indicate that the products of CYP71A12 activity in Arabidopsis are significant in resisting nonadapted *P. cucumerina* strains, but only in the simultaneous absence of PEN2-dependent products and camalexin. Despite the enhanced susceptibility of the *pen2 cyp71A12/A13* mutant, this genotype was still less susceptible to infection by the *PcBMM* and *Pc2127* fungal lines compared with the double *cyp79B2/B3* line, indicating that apart from CYP71A12 and CYP71A13 monooxygenases other IAOx-converting enzymes are probably of significance in the reaction of Arabidopsis to *P. cucumerina*.

To gain more insight into the nature of the CYP71A12-dependent metabolites responsible for the infection phenotypes we observed, we generated and tested the triple *pen2 pad3 cyp82C2* line in our pathogen assays. Similar to the *pen2 cyp71A12/A13* line, the *pen2 pad3 cyp82C2* line was also more susceptible to *P. cucumerina* infection compared with the *pen2 pad3* line (Fig. 5), suggesting that at least part of the susceptibility we observed in the *pen2 cyp71A12/A13* lines might result from a 4OH-ICN deficiency. By contrast, we found opposite differences in the susceptibility of *pen2 cyp71A12/A13* and *pen2 pad3 cyp82C2* lines toward both tested *P. cucumerina* isolates (Fig. 5), thus indicating a differential contribution of CYP82C2- and CYP71A12-downstream products to the interactions with *PcBMM* and *Pc2127* strains.

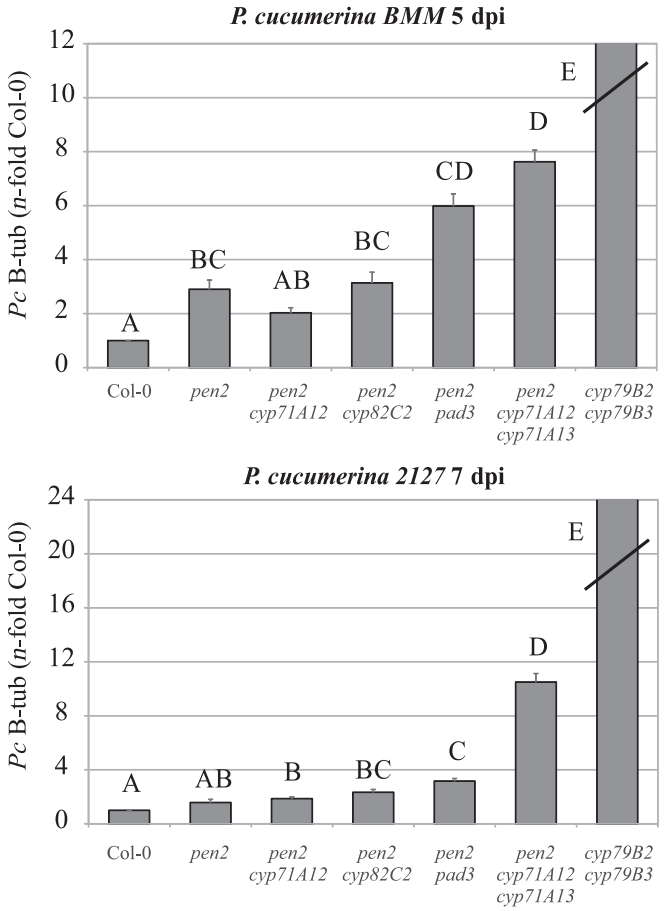

Fig. 4 CYP71A12 contributes to *Arabidopsis thaliana* resistance against *Plectosphaerella cucumerina*. Real-time PCR quantification of *P. cucumerina* DNA (*Pc* β-*tubulin*) in leaves of inoculated plants at 5 d post-inoculation (dpi) (strain *BMM*) and 7 dpi (strain *2127*). Values are means ± SD from two independent experiments with three biological replicates in each ($n = 6$) and are presented as the average of the *n*-fold of fungal DNA levels, relative to the wild-type (Col-0) plants. Capital letters indicate significantly different statistical groups of genotypes (one-way ANOVA, followed by Tukey's post-hoc test). An additional three experiments were performed with similar results.

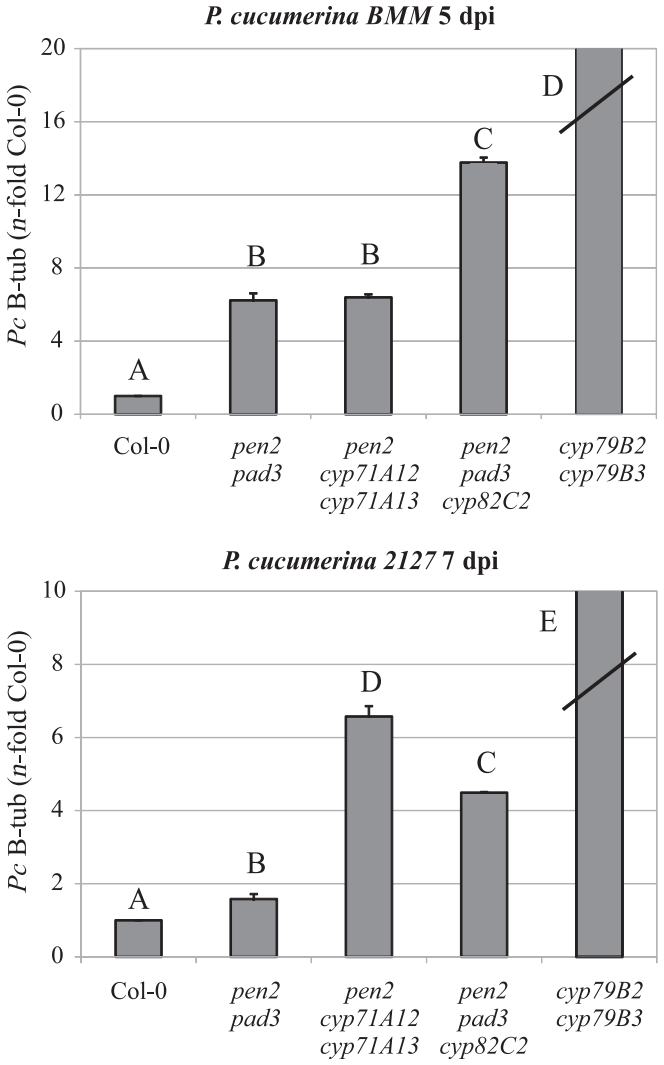

Fig. 5 Function of CYP82C2 in *Arabidopsis thaliana* resistance against *Plectosphaerella cucumerina* depends on camalexin accumulation. Real-time PCR quantification of *P. cucumerina* DNA (*Pc* β-tubulin) in leaves of inoculated plants at 5 d post-inoculation (dpi) (*P. cucumerina BMM*) and 7 dpi (*P. cucumerina 2127*). Values are means ± SD from one experiment with three biological replicates in each ($n = 3$) and are presented as the average of the *n*-fold fungal DNA levels, relative to the wild-type (Col-0) plants. Capital letters indicate significantly different statistical groups of genotypes (one-way ANOVA, followed by Tukey's post-hoc test). An additional two experiments with three biological replicates in each were performed and gave similar results.

Downstream products of CYP71A12 activity are unnecessary for defensive mechanisms acting at the pre-invasion stage of *C. tropicale* infection

We have shown that the enzymes CYP71A12 and CYP71A13 jointly contribute to the immunity of Arabidopsis against *P. cucumerina*. However, the lifestyle and infection strategy of this pathogen makes it difficult to distinguish defects in the pre- and post-invasive stages of *P. cucumerina* growth. To gain the information needed, we performed infection assays in Arabidopsis with the nonadapted hemibiotrophic ascomycete *C. tropicale* (Kosaka & Takano, 2018). First, we determined if CYP71A12 and CYP71A13 contribute to the biosynthesis of ICAs and camalexin when Arabidopsis is inoculated with *C. tropicale*, in a manner similar to its reaction to the *PcBMM*. To do this, we performed HPLC analysis of samples from *C. tropiclae*-inoculated leaves of *pen2*, *pen2 pad3 pen2*, *cyp71A12*, and *pen2 cyp71A12/A13* plants. Our analysis revealed that *cyp71A12* and *cyp71A13* gene mutations impact the accumulation of ICAs and camalexin during the plant's reaction to the hemibiotrophic pathogen in manner similar to its reaction against the necrotrophic *PcBMM* fungal strain (Fig. S6). We then tested the possible influence of CYP71A12 in restricting pathogen penetration by drop-inoculating a conidiospore suspension of *C. tropicale* onto leaves of *cyp71A12 cyp71A13*, *cyp82C2* and *pad3* mutant lines together with WT plants and two *pen2* mutant alleles. Our macroscopic examination revealed necrotic lesions only on the leaf surfaces of the *pen2* mutant alleles, which are impaired in restricting pathogen growth at the pre-invasive stage of infection; no lesions occurred on the *cyp71A12/A13*, *cyp82C2* or *pad3* lines (Fig. 6a). When we microscopically examined fungal entry rates of *C. tropicale* into epidermal cells, we confirmed that only the *pen2* Arabidopsis line showed fungal penetration (Fig. 6b). Overall, our results indicate that end products of the CYP71A12- and CYP71A13-dependent branch of Trp metabolism are not involved in the control of pathogen entry in Arabidopsis.

Products of CYP71A12 activity, but not 4OH-ICN, are required to terminate post-invasive growth of *C. tropicale*

Infection phenotypes of *cyp79B2/B3* and *pen2 pad3* double knockout lines indicate that the termination of post-invasive *C. tropicale* growth in Arabidopsis requires other Trp-derived compounds in addition to camalexin (Hiruma *et al.*, 2013). To determine if these Trp-derived metabolites include products formed through the CYP71A12-dependent branch of Trp metabolism, we examined the expansion of *C. tropicale* hyphae from the initial site of infection to mesophyll cells in Arabidopsis mutant lines we had already used in the assays with *P. cucumerina* (Fig. 7). Our microscopic analysis of plant–pathogen contact sites revealed significantly more frequent expansion of fungal hyphae into leaves of the double *pen2 cyp71A12* line than into leaves of the *pen2* mutant line. This confirms the important contribution of CYP71A12-dependent compounds at the post-invasive stage of *C. tropicale* infection. By contrast, necrotrophic hyphae expansion was rarely observed in the *pen2 cyp82C2* double mutant line,

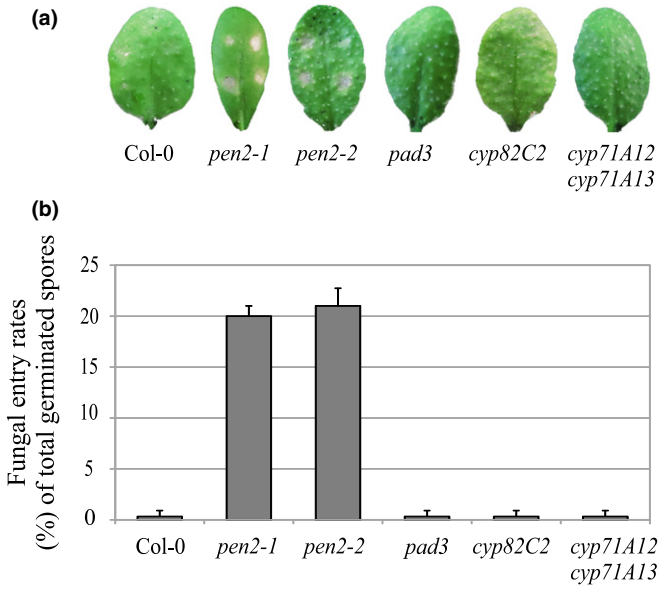

Fig. 6 CYP71A12, CYP71A13 and CYP82C2 are dispensable for the penetration resistance of *Arabidopsis thaliana* to *Colletotrichum tropicale*. (a) Leaves of indicated genotypes 4 d after inoculation with conidia of *C. tropicale*. *Colletotrichum tropicale* (2.5×10^5 conidia ml^{-1}) was inoculated onto 4-wk-old plant. The photograph was taken 4 d after the inoculation. Necrotic lesions indicate defects in extracellular resistance. (b) Entry rates of germinated *C. tropicale* conidiospores observed on the indicated genotypes. At 14 h post-inoculation, the entry rate of fungal hyphae was determined by using light microscopy. The results are means ± SD from three independent experiments, where at least 100 germinating conidia were counted in each experiment ($n \geq 300$).

which indicates that weakened resistance against secondary penetration in the *pen2 cyp71A12* genotype does not depend on 4OH-ICN deficiency (Fig. 7). In addition, the *pen2 cyp71A12/A13* triple knockout experienced more intense hyphae proliferation than the *pen2 pad3*, *pen2 cyp71A12* and *pen2 pad3 cyp82C2* mutant lines. Collectively, our results suggest that compounds formed via the CYP71A12-dependent branch of Trp metabolism act in concert with camalexin to control further expansion of *C. tropicale* hyphae from penetrated epidermal cells. However, secondary hyphae penetration into mesophyll cells of the *pen2 cyp71A12/A13* triple knockout was significantly lower than in the double *cyp79B2/B3* mutant line (Fig. 7). This hindrance of secondary hyphae spread in the *cyp71A12* mutant could be promoted by ICAs remaining in the *cyp71A12* mutant. Alternatively, resistance against *C. tropicale* might require additional Trp-derived compounds whose formation depends on CYP79B2/3 enzyme activity and not on CYP71A12/13 enzyme activity.

Expression of genes encoding monooxygenases involved in IAOx metabolism is strongly induced in response to pathogen penetration

Infection phenotypes of *pen2 pad3* and *pen2 cyp71A12 cyp71A13* mutant lines suggest that accumulation of camalexin and ICAs is required to restrict the post-invasive growth of *C. tropicale* successfully. To examine the transcriptional control

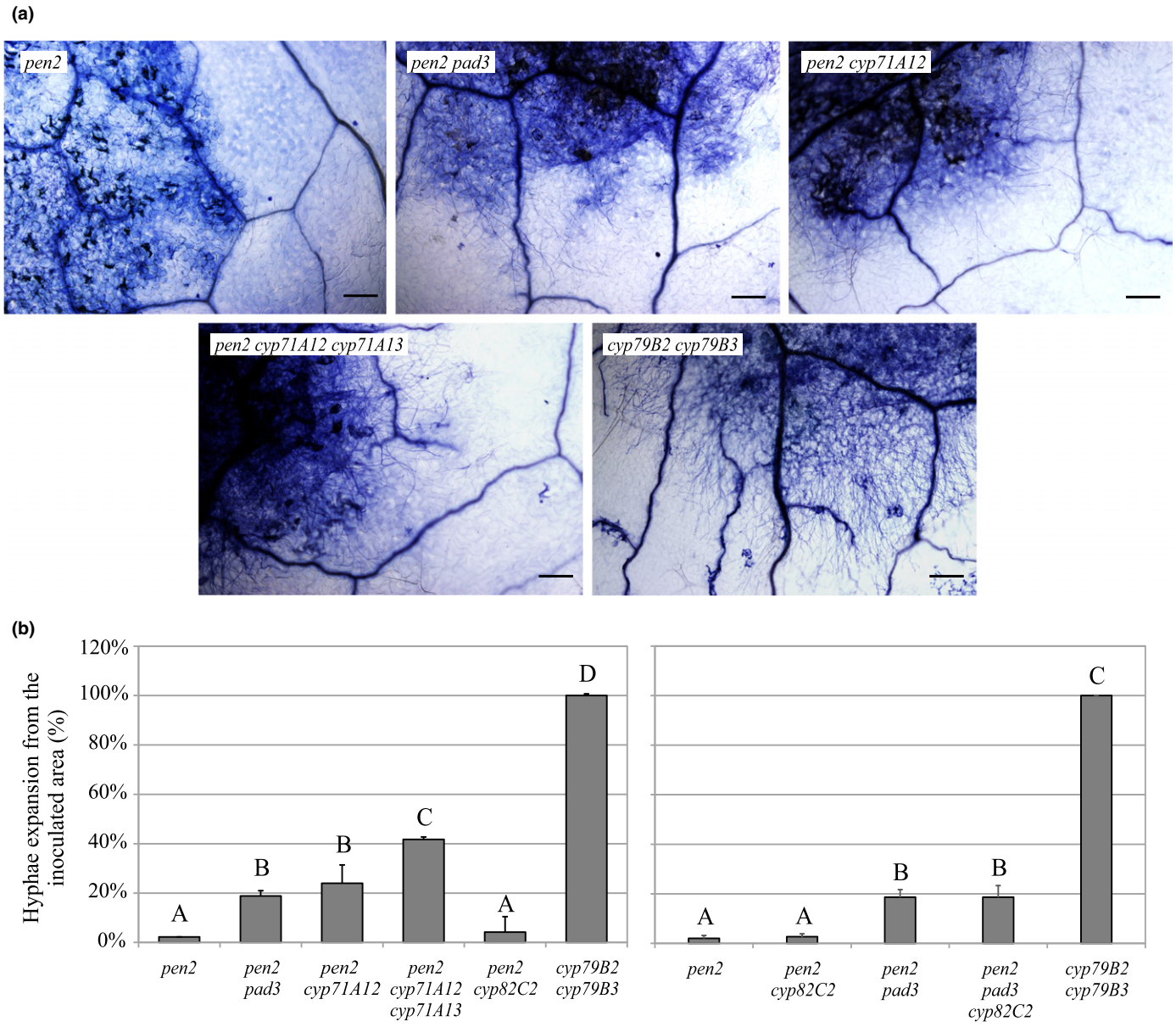

Fig. 7 CYP71A12 and CYP71A13, but not CYP82C2, contribute independently to the post-invasive resistance of *Arabidopsis thaliana* towards *Colletotrichum tropicale*. (a) Micrographs of hyphal extension of *C. tropicale* from inoculated areas to outside areas in each mutant line. Bars, 200 μm. (b) Determination of the expansion rate of invasive necrotrophic hyphae of *C. tropicale* from the inoculated area to outside areas. The results are means ± SD from three independent experiments, where in each at least 50 inoculated sites were investigated per genotype ($n \geq 150$) for the extension of invasive hyphae to outside areas. Capital letters indicate significantly different statistical groups of genotypes (one-way ANOVA, followed by Tukey's post-hoc test).

of genes involved in the biosynthesis of these compounds, we performed a qRT-PCR analysis of *CYP71A12 PAD3/CYP71A15* and *CYP82C2* expression in leaves of the *pen2* mutant and WT plants, both inoculated with conidiospores of *C. tropicale*. Our analyses revealed that expression of the tested genes was only slightly induced in WT plants (Fig. 8), whereas all three genes were transcribed with much higher efficiency in the *pen2* mutant plants, suggesting that successful penetration into epidermal cells is necessary to trigger efficient biosynthesis of the IAOx-derived metabolites required to terminate post-invasive pathogen growth.

Discussion

Biosynthetic pathway leading to ICA

In this study, we investigated biosynthetic pathways leading to the pathogen-triggered biosynthesis of ICA derivatives and their function in the immunity of the model plant Arabidopsis (Fig. S7). Our results indicated that CYP71A12 monooxygenase is essential in the pathogen-triggered accumulation of these compounds (Fig. 2). CYP71A12 has been reported to convert IAOx to IAN, which is then thought to be converted to ICA via indole-

3-carbaldehyde (Böttcher *et al.*, 2014; Müller *et al.*, 2015). Candidate enzymes contributing to the relevant reactions, CYP71B6 and AAO1, have been identified based on an analysis of gene co-expression and on the observed partial deficiency of the pertinent mutants involved in the $AgNO_3$-induced ICA biosynthetic pathway (Böttcher *et al.*, 2014; Müller *et al.*, 2019). Similar to Arabidopsis's response to $AgNO_3$ toxicity, our analysis revealed that *cyp71B6* and *aao1* mutant plants are only minimally limited in accumulating ICA derivatives in response to *P. cucumerina* (Fig. S2), suggesting that either other enzymes (redundant to CYP71B6 and AAO1) provide a major contribution in ICA biosynthesis or there exists an alternative biosynthetic route for ICA accumulation. CYP71A12 together with CYP71A13 have been reported to convert IAN to α-hydroxy-IAN *in vitro*, albeit at different efficiencies, and to further convert α-hydroxy-IAN to dehydro-IAN (Klein *et al.*, 2013). It is possible that ICA can be derived from any of the alternative products of CYP71A12 activity without any contribution of CYP71B6 and/or AAO1 enzymes. Rajniak *et al.* (2015) suggested that ICA can be formed during cyanogenic hydrolysis of ICN, which is produced from α-hydroxy-IAN by FOX1 activity (Fig. 1). However, our HPLC analysis of extracts from *fox1* mutant leaves did not reveal any defects in constitutive or *PcBMM*-triggered ICA accumulation, excluding a significant contribution of this alternative pathway to the observed accumulation of ICAs (Fig. S3).

Functional differentiation of CYP71A12 and CYP71A13

Despite a clear contribution of CYP71A12 to the pathogen-triggered biosynthesis of ICAs, we did not detect any defects in the constitutive accumulation of 6OGlcICA and ICAGlc in *cyp71A12* mutants (Fig. 2a), suggesting the existence of an enzyme(s) that is redundant with CYP71A12. An immediate candidate for such an enzyme is CYP71A13. This monooxygenase is highly homologous with CYP71A12 and its close proximity on the chromosome of the relevant genes suggests that the two enzymes were formed in a gene duplication event. Moreover, both enzymes have similar *in vitro* enzymatic activities and are partially redundant (with some degree of tissue specificity) on the biosynthetic pathway leading to camalexin (Fig. 2a) (Nafisi *et al.*, 2007; Millet *et al.*, 2010; Klein *et al.*, 2013; Müller *et al.*, 2015). In addition, experimental results have suggested that both these monooxygenases contribute to the UV-induced accumulation of ICAs, but that CYP71A13's involvement is less pronounced (Müller *et al.*, 2015). Our analysis of the accumulation of 6OGlcICA and ICAGlc in leaves of *cyp71A13* and *cyp71A12/A13* mutants did not reveal any significant contribution of CYP71A13 to the pathogen-triggered biosynthesis of these metabolites (Fig. 2a). A similar observation has been made regarding ICN biosynthesis, but only single *cyp71A12* and *cyp71A13* mutants have been thoroughly investigated (Rajniak *et al.*, 2015). Overall, it appears that despite the high homology and compensatory involvement of both monooxygenases in camalexin production, CYP71A13 is the enzyme that is most probably specialized in the biosynthesis of camalexin, whereas CYP71A12 additionally contributes to the formation of a different set of IAOx-derived products. These differences in produced metabolites can result from the unique *in vivo* enzymatic activities of CYP71A12 and CYP71A13, their different cellular or subcellular locations, or their different abilities to form complexes with downstream enzymes involved in their distinctive biosynthetic pathways. Regardless, the lack of a major contribution of CYP71A13 to ICA biosynthesis suggests that another CYP71A12 homolog, for instance CYP71A18, might play a role in synthesizing ICA.

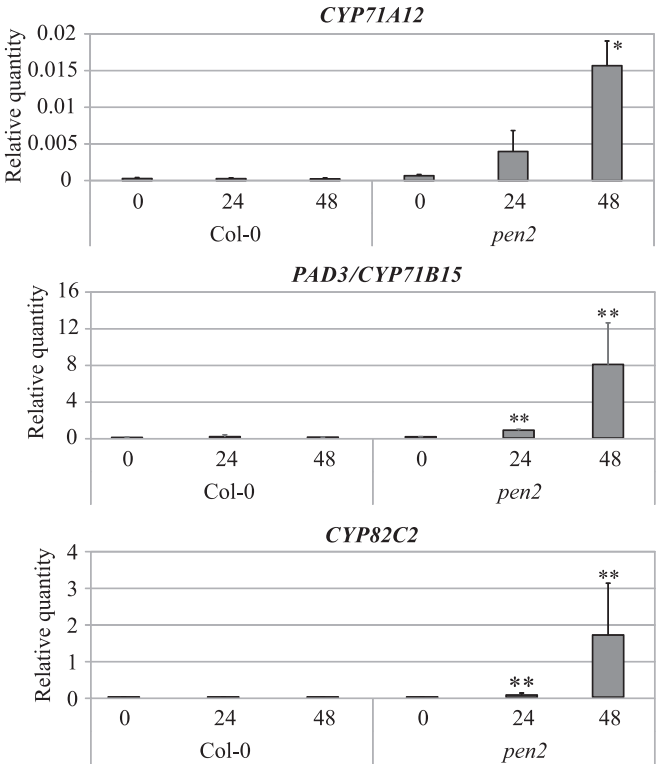

Fig. 8 Expression of genes linked to restriction of post-invasive fungal growth in *Arabidopsis thaliana* is induced with successful pathogen penetration. Relative expression of *CYP71A12*, *PAD3/CYP71B15* and *CYP82C2* in Col-0 and *pen2* lines 24 and 48 h after inoculation with *Colletotrichum tropicale* conidiospores. Results are means ± SD from three independent experiments with three biological replicates in each ($n = 9$). Significantly different statistical groups of genotypes indicated by ANOVA (Tukey's test) are indicated by capital letters. Values marked with asterisks are significantly different from respective controls (Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$).

Biosynthetic link between IGs and ICAs

Myrosinase-mediated hydrolysis of glucosinolates leads to the formation of unstable aglycones that can decompose to a variety of products, including nitriles whose formation is supported by specifier proteins (Wittstock *et al.*, 2016). Consequently, hydrolysis of I3G may lead to the formation of IAN, which is thought to contribute to ICA biosynthesis (de Vos *et al.*, 2008; Müller *et al.*, 2015). Alternatively, in the absence of specifier protein I3G, aglycone degrades to unstable indol-3-ylmethtyl isothiocyanate, which in turn decomposes to indole-3-carbinol (Wittstock *et al.*, 2016). Indole-3-carbinol can be further oxidized to

indole-3-aldehyde and then to ICA (Bednarek *et al.*, 2005; Müller *et al.*, 2015). In line with the proposed contribution of IG hydrolysis to ICA biosynthesis, we found that the IG-deficient *myb34/51/122* mutant constitutively accumulates very low concentrations of ICA derivatives (Fig. 2b). However, it is difficult to determine if low accumulation of ICAs results directly from the IG deficiency or from a very low constitutive expression of *CYP79B2* and *CYP79B3* genes that were observed in *myb34/51/122* plants (Frerigmann & Gigolashvili, 2014). In contrast to naïve plants, the expression of both *CYP79B* genes is not controlled by MYB34, MYB51 or MYB122 transcription factors in pathogen-challenged plants (Frerigmann *et al.*, 2016). However, we observed a lack of significant difference in the accumulation of analyzed ICAs between leaves of *cyp71A12/A13* and *cyp71A12/A13 myb34/51/122* plants inoculated with *P. cucumerina*, indicating that the input of IG hydrolysis into ICA biosynthesis is negligible, at least under our experimental conditions.

Function of CYP71A12 and CYP82C2 in the restriction of post-invasive fungal growth

Our pathogen assays indicate that similarly to *PAD3/CYP71B15*, the effect of the mutation in *CYP71A12* on plant immunity becomes obvious in the *pen2* background (Figs 6, 7) (Bednarek *et al.*, 2009; Sanchez-Vallet *et al.*, 2010; Schlaeppi *et al.*, 2010). This mutation effect agrees with the observed impact of the *pen2* mutation on the expression of *CYP71A12* and *PAD3/CYP71B15* in *C. tropicale*-inoculated leaves (Fig. 8) and explains the proposed functioning of camalexin and ICAs in post-invasive immunity. Notably, our results specified in detail the differential contribution of CYP71A12-dependent products in Arabidopsis's defense against the specific pathogens we tested. During Arabidopsis's interaction with *P. cucumerina*, the functioning of ICAs and ICNs could be revealed only in the absence of camalexin (Fig. 4), whereas functioning of ICAs in resistance against secondary hyphal penetration by *C. tropicale* was independent of camalexin (Fig. 7). In addition, our analysis of *pen2 cyp82C2*, *pen2 pad3 cyp82C2* and *pen2 cyp71A12/A13* mutants suggests that during Arabidopsis's interaction with *P. cucumerina*, 4OH-ICN is the principal CYP71A12 downstream product contributing to the observed infection phenotypes (Fig. 5), whereas 4OH-ICN seems to be unnecessary for restricting post-invasive growth of *C. tropicale* (Fig. 7). Interestingly, unlike *cyp82C2* plants, *cyp71A12* mutants that are also 4OH-ICN-deficient did not exhibit enhanced susceptibility against *B. cinerea* and *A. brassicicola* (Rajniak *et al.*, 2015), suggesting that 4OH-ICN deficiency is not the only explanation for the infection phenotype observed in *cyp82C2* mutants. Interestingly, in addition to the function in Trp metabolism, CYP82C2 monooxygenase has also been reported as JASMONIC ACID-HYPERSENSITIVE1 (JAH1) (Liu *et al.*, 2010). In line with this designation, *cyp82C2* mutant plants show reduced expression of the plant defensin *PDF1.2* gene in response to methyl jasmonate treatment, suggesting that this mutant is defective in JA-dependent immune responses (Liu *et al.*, 2010). As indicated by the infection phenotype of the JA-insensitive *jar1* mutant, JA-signaling is important for resistance against *P. cucumerina*, but not against *Colletotrichum higginsianum* (Liu *et al.*, 2007; Pétriacq *et al.*, 2016). These findings correlate with the enhanced susceptibility we found in the *pen2 pad3 cyp82C2* line to *P. cucumerina*, but not to *C. tropicale* (Figs 5, 7), suggesting that a defect in JA-signaling could have contributed to the phenotype we observed. This could be further addressed by JA-pathway analysis and ICN measurements in the generated mutant lines infected with *P. cucumerina* and *C. tropicale*. However, direct quantitative analysis of ICNs in plant extracts is quite challenging, if possible at all, due to the high instability of these compounds (Rajniak *et al.*, 2015).

ICA metabolic network in Arabidopsis immunity

Our analysis of CYP71A12-deficient mutants proves that this monooxygenase has an important function in Arabidopsis immunity, particularly in the restriction of post-invasive fungal growth. As discussed above, infection phenotypes observed in *cyp82C2* lines during this and other studies indicate that biosynthesis of 4OH-ICN can only partially, if at all, explain susceptibility to infection observed in *cyp71A12* mutants. It is therefore very likely that ICAs contribute to Arabidopsis immunity against selected filamentous pathogens after they invade. However, the molecular mechanism underlying this function remains obscure, especially because ICA does not inhibit *in vitro* growth of *P. cucumerina* hyphae excluding direct antifungal activity of this compound (Gamir *et al.*, 2014). Recently published results have indicated that exogenously applied ICA induces ABA-dependent callose deposition when *P. cucumerina* infects (Gamir *et al.*, 2018). It has therefore been postulated that ICAs might function as regulatory or signaling molecules in Arabidopsis's immune response. However, even if true, the importance of the various alternative substitutions observed within the many identified ICA derivatives remains obscure (Böttcher *et al.*, 2014). For instance, IG functioning in extracellular resistance and aphid deterrence requires substitution at the C-4 position of the indole ring (Bednarek *et al.*, 2009; Pfalz *et al.*, 2009), suggesting that particular substitution(s) of ICA could also be of functional significance. Moreover, it is difficult to predict whether the role of ICAs is linked to the soluble fraction or cell-wall-bound fraction of these compounds (Hagemeier *et al.*, 2001; Tan *et al.*, 2004; Forcat *et al.*, 2010). In this context, it is also of interest where the ICA biosynthesis is taking place in relation to fungal colonization. Such questions can be only answered when the enzymes involved in ICA metabolism and extracellular transport are identified.

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Author contributions

MP, AK, EG, AM, YT and PB conceived the project, designed experiments and wrote the article. MP, AK, MP-B, G L, HF and KK performed the experiments and analyzed the data with the support and supervision of EG, AM, YT and PB. MP and AK contributed equally to this work

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 CYPO71A12 is involved in the biosynthesis of the cell-wall-bound fraction of indole-3-carboxylic acid.

Fig. S2 CYP71B6 and AAO1 contribute to *Plectosphaerella cucumerina*-triggered accumulation of indole-3-carboxylic acid derivatives.

Fig. S3 FOX1 is dispensable for the accumulation of indole-3-carboxylic acid derivatives in Arabidopsis leaves.

Fig. S4 Constitutive and pathogen-inducible biosynthesis of indole glucosinolate is independent of CYP71A12 and CYP71A13.

Fig. S5 The *cyp71A12* mutant is not affected in its immunity against *Plectosphaerella cucumerina BMM*.

Fig. S6 CYP71A12 and CYP71A13 contribute differentially to the biosynthesis of indole-3-carboxylic acid derivatives and camalexin.

Fig. S7 Biosynthesis of indole-3-carboxylic acid within the network of pathogen-triggered tryptophan metabolism.

Table S1 Sequences of oligonucleotides used for PCRs.

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