The Multifunctional Enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) Converts Cysteine-Indole-3-Acetonitrile to Camalexin in the Indole-3-Acetonitrile Metabolic Network of Arabidopsis thaliana

Christoph Böttcher, Lore Westphal, Constanze Schmotz, Elke Prade, Dierk Scheel, and Erich Glawischnig

Accumulation of camalexin, the characteristic phytoalexin of Arabidopsis thaliana, is induced by a great variety of plant pathogens. It is derived from Trp, which is converted to indole-3-acetonitrile (IAN) by successive action of the cytochrome P450 enzymes CYP79B2/B3 and CYP71A13. Extracts from wild-type plants and camalexin biosynthetic mutants, treated with silver nitrate or inoculated with Phytophthora infestans, were comprehensively analyzed by ultra-performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry. This metabolomics approach was combined with precursor feeding experiments to characterize the IAN metabolic network and to identify novel biosynthetic intermediates and metabolites of camalexin. Indole-3-carbaldehyde and indole-3-carboxylic acid derivatives were shown to originate from IAN. IAN conjugates with glutathione, γ-glutamylcysteine, and cysteine [Cys(IAN)] accumulated in challenged phytoalexin deficient3 (pad3) mutants. Cys(IAN) rescued the camalexin-deficient phenotype of cyp79b2 cyp79b3 and was itself converted to dihydrocamalexic acid (DHCA), the known substrate of CYP71B15 (PAD3), by microsomes isolated from silver nitrate–treated Arabidopsis leaves. Surprisingly, yeast-expressed CYP71B15 also catalyzed thiazoline ring closure, DHCA formation, and cyanide release with Cys(IAN) as substrate. In conclusion, in the camalexin biosynthetic pathway, IAN is derivatized to the intermediate Cys(IAN), which serves as substrate of the multifunctional cytochrome P450 enzyme CYP71B15.

INTRODUCTION

Trp plays a central role in plant metabolism as a protein component, in auxin biosynthesis, and as the precursor of a great variety of secondary metabolites involved in plant defense. Prominent representatives of Trp-derived secondary metabolites in cruciferous plants include phytoanticipins, such as indole glucosinolates, and indolic phytoalexins, such as camalexin. These indole alkaloids show a great structural diversity, but most of them are substituted at the 3-position with sulfur and nitrogen-containing side chains (Pedras et al., 2000). The formation of camalexin (3-thiazol-2'-yl-indole) is characteristic of Arabidopsis thaliana (Tsui et al., 1992).

Camalexin is formed upon infection of Arabidopsis plants by a large variety of microorganisms, including bacteria, fungi, and oomycetes (Glawischnig, 2007). Also, pathogen-associated molecular patterns and toxins can trigger camalexin accumulation (Stone et al., 2000; Outob et al., 2006; Gust et al., 2007; Rauhut et al., 2009). Camalexin formation requires transcriptional activation of genes encoding biosynthetic enzymes and involves the mitogen-activated protein kinases MPK3, MPK6 (Ren et al., 2008), and MPK4 (Qiu et al., 2008). MPK4 physically interacts in the nucleus with its substrate mitogen-activated protein kinase 4 substrate 1 (MKS1) and WRKY33, a transcriptional activator of camalexin biosynthesis. Upon infection or pathogen-associated molecular pattern treatment of Arabidopsis plants, MPK4 becomes activated and phosphorylates MKS1, which results in release of the MKS1/WRKY33 complex and allows WRKY33 to activate camalexin biosynthetic genes (Qiu et al., 2008). Camalexin is also synthesized upon abiotic stress, such as treatment with heavy metal ions or UV-B irradiation; as in the biotic stress response, this response involves rapid formation of reactive oxygen species (Van Breusegem et al., 2008). Camalexin exhibits cytotoxicity (Rogers et al., 1996), particularly against eukaryotic pathogens. Some fungal strains are resistant to camalexin due to efficient degradation or export (Pedras and Khan, 2000; Stefanato et al., 2009).

In addition to indole glucosinolates and camalexin, other indolic compounds were identified in Arabidopsis, including substituted indole-3-acetonitriles (IANS) and indole-3-carbaldehydes as characteristic breakdown products of indole glucosinolate, as well as glycosylated and methylated variants of indole-3-carboxylate and its 6-hydroxy derivative (Hagemeier et al., 2001; Tan et al., 2004; Bednarek et al., 2005) (Figure 1). Metabolites of the indole-3-carboxylate class strongly...
Indole-3-carbaldehyde (I\textsubscript{3}CHO) and indole-3-carboxylic acid (I\textsubscript{3}CO\textsubscript{2}H) Published Data Are Summarized (Glawischnig, 2007; Nafisi et al., 2007). The corresponding cyp79b2 cyp79b3 CYP79B3 (Hull et al., 2000; Mikkelsen et al., 2000; Zhao et al., 2002) are unable to synthesize camalexin (Nafisi et al., 2007). In the mutant, phytoalexin deficient3 (pad3), DHCA accumulates in root exudates after 	extit{Pythium sylvaticum} infection and in leaves treated with silver nitrate (Bednarek et al., 2005; Schuhegger et al., 2006). This mutant was initially isolated in a screen for camalexin deficiency in 	extit{Pseudomonas syringae}-infected leaves (Glazebrook and Ausubel, 1994). It proved to synthesize only traces of camalexin in response to a number of biotic or abiotic treatments, normally inducing camalexin biosynthesis in wild-type plants. PAD3 encodes CYP71B15 (Zhou et al., 1999), which converts DHCA to camalexin when expressed in yeast (Schuhegger et al., 2006). Arabidopsis microsomes isolated from leaves of transgenic plants overexpressing CYP71B15 or of wild-type plants treated with silver nitrate catalyzed the same reaction, while microsomes from silver nitrate-treated pad3 leaves did not turn over DHCA. The knowledge on the biosynthesis of camalexin and related indolic compounds from Trp prior to this study is summarized in Figure 1.

Figure 1. Biosynthesis of Camalexin and Related Indolic Compounds; Published Data Are Summarized (Glawischnig, 2007; Nafisi et al., 2007). Indole-3-carbaldehyde (I\textsubscript{3}CHO) and indole-3-carboxylic acid (I\textsubscript{3}CO\textsubscript{2}H) derivatives have been identified previously (Hagemeier et al., 2001; Tan et al., 2004; Bednarek et al., 2005), but their biosynthetic origin was unclear. I\textsubscript{3}CHO was also suggested as biosynthetic intermediate in camalexin biosynthesis (Zook and Hammerschmidt, 1997). ESP, epithiospecifier protein.

accumulate in leaf and root tissue, in root exudates, and in association to the cell wall upon pathogen attack and might therefore be related to camalexin biosynthesis as precursors or side products. Despite their tentative relevance for plant defense, the biosynthetic origin of these compounds is unclear. Recently, a novel catabolic pathway of indol-3-ylmethyl glucosinolate, which recruits the P450 monooxygenase CYP81F2 (Bednarek et al., 2009; Clay et al., 2009), the atypical myrosinase PENETRATION2 (PEN2) (Lipka et al., 2005), and the ABC transporter PEN3 (Stein et al., 2006), was shown to play an essential role in plant immunity.

Camalexin originates from Trp, which is converted to indole-3-acetaldoxime (IAOx) (Figure 1). This step is shared with the biosynthesis of indole glucosinolates and catalyzed by two homologous cytochrome P450 enzymes, CYP79B2 and CYP79B3 (Hull et al., 2000; Mikkelsen et al., 2000; Zhao et al., 2002). The corresponding cyp79b2 cyp79b3 double knockout mutants (Zhao et al., 2002) are unable to synthesize camalexin (Glawischnig et al., 2004). However, metabolic intermediates downstream of IAOx, when applied to silver nitrate-treated cyp79b2 cyp79b3 leaves, have the potential to rescue the camalexin-deficient phenotype. This approach led to the identification of biosynthetic intermediates, which were converted to camalexin in vivo: (1) IAN, which can also act as auxin precursor and is known as a degradation product of indol-3-ylmethyl glucosinolate; and (2) dihydrocamalexin acid (DHCA) (Schuhegger et al., 2006; Nafisi et al., 2007). IAN is synthesized from IAOx by CYP71A13, and cyp71a13 knockout mutants have a strongly reduced capacity to synthesize camalexin (Nafisi et al., 2007). In the mutant, phytoalexin deficient3 (pad3), DHCA accumulates in root exudates after 	extit{Pythium sylvaticum} infection and in leaves treated with silver nitrate (Bednarek et al., 2005; Schuhegger et al., 2006). This mutant was initially isolated in a screen for camalexin deficiency in 	extit{Pseudomonas syringae}-infected leaves (Glazebrook and Ausubel, 1994). It proved to synthesize only traces of camalexin in response to a number of biotic or abiotic treatments, normally inducing camalexin biosynthesis in wild-type plants. PAD3 encodes CYP71B15 (Zhou et al., 1999), which converts DHCA to camalexin when expressed in yeast (Schuhegger et al., 2006). Arabidopsis microsomes isolated from leaves of transgenic plants overexpressing CYP71B15 or of wild-type plants treated with silver nitrate catalyzed the same reaction, while microsomes from silver nitrate-treated pad3 leaves did not turn over DHCA. The knowledge on the biosynthesis of camalexin and related indolic compounds from Trp prior to this study is summarized in Figure 1.

Cys was shown to be the biosynthetic precursor of the thiazole ring of camalexin (excluding the 2’ position) (Zook and Hammerschmidt, 1997). However, it was unclear whether Cys itself or rather a Cys metabolite is reacting with the indolic partner. Raphanussaminic acid, initially suggested as precursor of the thiazole ring (Bednarek et al., 2005), might be rather specifically related to glucosinolate degradation (Bednarek et al., 2009). Recently, the PAD2 gene was cloned and shown to encode glutathione synthase 1 (GSH1) (Parisy et al., 2007). This finding can be either explained by glutathione playing a regulatory role in camalexin synthesis or alternatively camalexin biosynthesis involving glutathionylation of an indolic camalexin precursor with subsequent release of Gly and Glu.

Here, an extensive mass spectrometry–based metabolomics approach was applied for the identification of a Trp metabolic network. In the biosynthesis of camalexin, IAN, a key intermediate in this network, is converted to glucosinolate degradation (Bednarek et al., 2009). Recently, the PAD2 gene was cloned and shown to encode glutathione synthase 1 (GSH1) (Parisy et al., 2007). This finding can be either explained by glutathione playing a regulatory role in camalexin synthesis or alternatively camalexin biosynthesis involving glutathionylation of an indolic camalexin precursor with subsequent release of Gly and Glu.

RESULTS

Metabolite Profiling of Camalexin Biosynthetic Mutants

To detect novel intermediates and products within the camalexin biosynthetic pathway, comparative nontargeted metabolite profiling was performed on biosynthetic mutants and corresponding wild-type lines using ultraperformance liquid chromatography–coupled electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/ESI-QTOF-MS). Among the known mutants...
impaired in camalexin accumulation, cyp79b2 cyp79b3 (Zhao et al., 2002; Glawischnig et al., 2004) and pad3 (Glazebrook and Ausubel, 1994) knockout lines defective in P450 monooxygenases catalyzing the first and the last step in camalexin biosynthesis were chosen. Both mutants were known to accumulate at most trace levels of the phytoalexin following elicitation. To probe the specificity of camalexin metabolites and intermediates dependent on the induction mode, profiling analyses were performed after abiotic and biotic stress applications, namely by treatment with silver nitrate or infection by Phytophthora infestans, respectively. Inoculation of Columbia-0 (Col-0) rosette leaves with spore suspensions of the nonadapted hemibiotrophic oomycete P. infestans results in comparably slight induction of camalexin accumulation. Therefore, experiments including biotic stress application were performed in the pen2 mutant background. Lack of PEN2 activity allows frequent initiation of invasive growth of P. infestans leading to a localized plant cell death response (Lipka et al., 2005) and about eightfold stronger accumulation of camalexin compared with Col-0 (see Supplemental Figure 1 online). The PEN2 gene encodes an atypical myrosinase capable of hydrolyzing several indol-3-ylmethylglucosinolates (Bednarek et al., 2009) and is hence not directly involved in camalexin metabolism. In addition to the analysis of leaf tissue, inoculation droplets were recollected from leaves and analyzed for secreted metabolites. Due to the low sample amount, capillary LC/ESI-QTOF-MS was used as analytical platform. To dissect camalexin-related metabolites from the entire set of metabolites upregulated after stress applications, the known biochemical relationship between mutants was used. For identification of metabolites downstream of PAD3, features (unique mass-to-charge \([m/z]\) retention time pairs) were screened that were significantly increased in intensity in treated Col-0 (pen2) samples in comparison to nontreated Col-0 (pen2) as well as in comparison to treated pad3 (pen2 pad3) and cyp79b2 cyp79b3 samples. Analogously, features that were significantly increased in intensity in treated pad3 (pen2 pad3) samples in comparison to nontreated pad3 (pen2 pad3) as well as in comparison to treated Col-0 (pen2) and cyp79b2 cyp79b3 samples were considered to be associated to metabolites upstream of PAD3. Results of data analysis applying this strategy are summarized as Venn diagrams in Figure 2 for positive ionization and in Supplemental Figure 2 online for negative ionization.

**Identification of Metabolites Downstream of PAD3**

Sixty-two features related to metabolites downstream of PAD3 were identified after abiotic stress application in positive ion mode. Among these, 38 features were putatively annotated to represent 12 metabolites (Table 1). Besides camalexin (1), two hecosides (2 and 3) and one malonyl-hecoside (4) could be identified. The aglycone elemental compositions of 2, 3, and 4 were consistent with the elemental composition of hydroxylated camalexin. 4 is the malonyl conjugate of hydroxycamalexin hecoside 3 and the most abundant of the three glycosides when assuming similar response factors. Low amounts of two camalexin dioxygenation products were characterized as 3-hydroxy-3-(thiazol-2-yl)indolin-2-one (5) and the corresponding ring-opening product 2-formamidophenyl-2’-thiazolylketone (6) (Figure 3). In addition, further minor camalexin metabolites (7 to 12) were detectable, pointing to a multitude of metabolic transformation downstream of camalexin. Whereas 7 to 10 could only be characterized by their elemental compositions, 11 and 12 were putatively annotated as oxygen-bridged camalexin dimers. A total of 88 features were mapped to putative leaf metabolites downstream of PAD3 (A, left) and 126 upstream of PAD3 (A, right). After inoculation with P. infestans, 88 (212) features were assigned to putative leaf metabolites downstream (upstream) of PAD3 (B). Nontargeted metabolite profiling of spore suspension droplets recollected from leaves revealed 65 (57) features associated to secreted metabolites downstream (upstream) PAD3 (C).
Camalexin was identified as the most abundant metabolite accompanied by significant amounts of dioxygenation products 5 and 6. In addition, the two unknown camalexin metabolites 7 and 9, which were already identified in silver nitrate–challenged Col-0 leaves, were present. To check whether *P. infestans* on its own can degrade camalexin to any of the four metabolites, spore suspensions were incubated with camalexin and analyzed by LC-MS. After incubation for 24 h, a large fraction of camalexin was metabolized and 5, 6, and 7, but not 9, were identified as major degradation products (see Supplemental Figure 3 online).

Identification of Metabolites Upstream of PAD3

A total of 126 features were detected upstream of PAD3 after abiotic stress application in positive ion mode, of which 68 were assigned to eleven putative metabolites (13 to 15 and 18 to 25) (Figure 3, Table 2). A striking feature of silver nitrate–challenged *pad3* leaves is the occurrence of four metabolites (13, 14, 18, and 19) all showing characteristic in-source fragment ions at m/z 155.060 (C10H7N2O) (see Supplemental Figure 4A online). A sum formula of C10H7N2O2S was determined for 13, and collision-induced dissociation (CID) mass spectra obtained from the protonated molecular ion indicated structural features of IAN and Cys (see Supplemental Figure 4B online). With respect to camalexin biosynthesis, 13 was hypothesized to be a Cys-IAN conjugate. To validate this hypothesis, Cys(IAN) was chemically synthesized starting from IAN and a protected L-Cys derivative. The synthesis yielded a mixture of two diastereomers that had the same chromatographic and mass spectral properties as the two isomeric compounds detected in silver nitrate–treated *pad3* leaves (see Supplemental Figure 4C online). Surprisingly, following preparative separation of the synthesized diastereomers, spontaneous epimerization was observed, which results in a slow interconversion and explains the presence of both diastereomers in the leaf extract. Based on similar fragmentation patterns and elemental compositions, 14 was putatively annotated as N-malonyl conjugate of Cys(IAN), 18 as conjugate of 3-mercaptop lactic acid and IAN [Mia(IAN)], in which the α-hydroxy acid is conjugated in the same manner to IAN as Cys in 13 and 19 as hexoside of 18 (Figure 3). Minor amounts of two metabolites (20 and 21) both displaying characteristic in-source fragment ions at m/z 171.055 (C10H7N2O+) were putatively annotated as ring-hydroxylated derivative of 18 (20) and as derived hexoside (21). In addition, a compound (15) with the same elemental composition as Cys(IAN) but a different fragmentation pattern was detected. 15 could also be identified after base-induced decomposition of Cys(IAN) at pH 12. In agreement with the literature, DHCA (22) strongly accumulated in silver nitrate–challenged *pad3* leaves (Schuhzegger et al., 2006). As observed for Cys(IAN), DHCA is further metabolized by either methylation or conjugation with amino acids. These transformations lead to accumulation of DHCA methylester (23), DHCA-Glu conjugates (24), and DHCA-Glu conjugates in silver nitrate–challenged *pad3* leaf tissue (Figure 3).

A similar set of metabolites was identified after biotic stress application. Cys(IAN), DHCA, and derived metabolites, but no DHCA methylester (23), accumulated in inoculated *pen2 pad3* leaves (Table 2). By contrast, three indole-3-carboxylic acid derivatives 26 to 28 were found to be elevated in *pen2 pad3* leaves after infection. Annotation of 29 out of 57 features revealed the presence of four metabolites upstream of PAD3 in spore suspension drops recollected from *pen2 pad3* leaves. Besides significant amounts of Cys(IAN) and DHCA, indole-3-carboxylate (29) and methyl indole-3-carboxylate (30) were found at elevated levels, which is consistent with the occurrence of indole-3-carboxylic acid derivatives 26 to 28 in leaf tissue.

### Table 1. Putative Metabolites Identified Downstream of PAD3 Accumulating after Silver Nitrate Treatment in Col-0 Leaves and Recollected Spore Suspension Droplets as Detected by LC/ESI(+)-QTOF-MS

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>AL</th>
<th>Elemental Composition</th>
<th>T&lt;sub&gt;r&lt;/sub&gt; in s UPLC</th>
<th>Quant. Ion m/z</th>
<th>No. of Annotated Differential Features&lt;sup&gt;b&lt;/sup&gt;</th>
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<td></td>
<td></td>
<td></td>
<td>(CapLC)</td>
<td></td>
<td>Leaf, AgNO&lt;sub&gt;3&lt;/sub&gt;</td>
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<tr>
<td>1</td>
<td>Camalexin</td>
<td>1a</td>
<td>C&lt;sub&gt;11&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;S</td>
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<td>12</td>
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<td>2</td>
<td>Hydroxycamalexin Hexoside #1</td>
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<td>3</td>
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<td>379.10</td>
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<td>2a</td>
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<td>320</td>
<td>465.10</td>
<td>8</td>
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<tr>
<td>5</td>
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<td>317</td>
<td>465.10</td>
<td>1</td>
</tr>
</tbody>
</table>

Total number of annotated (detected) features = 38 (62) | 43 (88) | 34 (65)

<sup>a</sup> Annotation level: 1a, compound identified using a synthesized standard; 2a, compound putatively annotated by interpretation of mass spectrometry data; 3, compound class putatively annotated.

<sup>b</sup> For feature annotation, see Supplemental Data Set 1 online. nd, not detected.
A significantly lower number of features were mapped to metabolites up- and downstream of PAD3 after analysis of negative ion metabolite profiles (see Supplemental Tables 1 and 2 online). However, annotation of these features allowed identification of the major differential metabolites and hence confirmation of analytical data obtained under positive ionization. To verify the results obtained from nontargeted analyses, annotated differential metabolites were manually quantified throughout all three independent experiments performed using quantifier ions from Tables 1 and 2 (positive ion mode) and from Supplemental Tables 1 and 2 online (negative ion mode) (see Supplemental Data Set 3 online). Relative quantification of 1 to 30 in one of the three independent experiments after abiotic and biotic stress application is shown in Figure 4.

Figure 3. Structures of Identified and Putatively Annotated Metabolites Detected Down- and Upstream of PAD3.

For annotation level of each metabolite, see Tables 1 and 2. Note that in case of compounds 2 to 4, 20, and 21, the position of the substituent on the indole ring could not be determined by mass spectrometry. Analogously, the position of the hexose moiety (Hex) in 19 is undefined. For the complete mass spectrometric characterization and additional remarks on identification, see Supplemental Data Set 2 online.

Cys(IAN) Is an Intermediate in Camalexin Biosynthesis

Based on the accumulation of Cys(IAN) and metabolites thereof in the pad3 mutant background in response to both silver nitrate application and P. infestans infection, Cys(IAN) was further investigated as candidate camalexin precursor. In vivo feeding of Cys(IAN) to the cyp79b2 cyp79b3 mutant was performed (Figure 5). External Cys(IAN) application to mutant leaves challenged with silver nitrate restored camalexin synthesis. Without silver nitrate treatment, camalexin levels were ~6% compared with treated leaves. Cys(IAN) application to pad3 leaves treated with silver nitrate yielded low (~1.4% in comparison to cyp79b2 cyp79b3), but significantly above-background levels of camalexin in pad3.

NADPH-Dependent Conversion of Cys(IAN) to DHCA and Camalexin by Arabidopsis Microsomes

In vivo complementation experiments of cyp79b2 cyp79b3 suggested that enzymes responsive to silver nitrate application are responsible for conversion of Cys(IAN) to DHCA and camalexin. To address the question of whether membrane-bound enzymes are capable of this Cys(IAN) turnover, Arabidopsis microsomes were incubated with Cys(IAN) and DHCA was identified as product (see Supplemental Figure 5 online). When leaves pretreated with silver nitrate were used for the microsome preparation and NADPH was included in the assay, an eightfold increase in Cys(IAN) turnover to DHCA and minor camalexin formation were observed. This suggested the involvement of a stress-responsive NADPH-dependent enzyme system in the formation of the thiazoline ring, for example, a cytochrome P450 enzyme.

Constitutive CYP71B15 (PAD3) Expression Is Sufficient for Camalexin Synthesis from IAN and Cys(IAN)

Leaves from 35S:CYP71B15 lines (Schuhegger et al., 2006) and Col-0 were incubated with intermediates of camalexin biosynthesis without any pretreatment (Figure 6). Camalexin amounts synthesized after application of IAN, Cys(IAN), or DHCA were significantly higher in 35S:CYP71B15 in comparison to Col-0. In particular, after Cys(IAN) application, this difference was ~20-fold. Therefore, CYP71B15 is rate limiting for conversion of Cys(IAN) to camalexin. Consequently, either high constitutive DHCA-synthesizing activity has to be postulated or CYP71B15 is a multifunctional enzyme, which catalyzes both enzymatic steps.

Cys(IAN) Is the Substrate of Multifunctional CYP71B15 (PAD3)

CYP71B15 was expressed in the yeast strain WAT11 carrying the Arabidopsis cytochrome P450 reductase 1 (ATR1) (Pompon et al., 1996; Schuhegger et al., 2006). When CYP71B15-expressing yeast microsomes were incubated with Cys(IAN) and NADPH as cosubstrate, HPLC analysis of the reaction mixture identified product peaks that comigrated with authentic DHCA and camalexin and showed their characteristic UV absorption spectra and fluorescence signals, respectively (see Supplemental Figure 6 online). DHCA identity was further confirmed by UPLC/ESI(+)-QTOF-MS. When NADPH was not included, the enzyme preparation was boiled prior to incubation, or microsomes were prepared from yeast expressing just the pYeDP60 vector, only minor product formation was observed.
Table 2. Putative Metabolites Identified Upstream of PAD3 Accumulating after Silver Nitrate Treatment in pad3 Leaves and after Inoculation with P. infestans in pen2 pad3 Leaves and Recollected Spore Suspension Droplets as Detected by LC/ESI(+)-QTOF-MS

<table>
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<th>No.</th>
<th>Name</th>
<th>ALa</th>
<th>Elemental Composition</th>
<th>Ret. Time (s)</th>
<th>Quant. Ion m/z</th>
<th>No. of Annotated Differential Featuresb</th>
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<td>406</td>
<td>155.06</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>Unknown Cys(IAN) derivative</td>
<td>3</td>
<td>C_{13}H_{13}N_{2}O_{3}S</td>
<td>300</td>
<td>155.06</td>
<td>11</td>
</tr>
<tr>
<td>18</td>
<td>Mla(IAN)</td>
<td>2a</td>
<td>C_{13}H_{13}N_{2}O_{3}S</td>
<td>300</td>
<td>155.06</td>
<td>11</td>
</tr>
<tr>
<td>19</td>
<td>Mla(IAN) Hexoxide</td>
<td>2a</td>
<td>C_{13}H_{13}N_{2}O_{3}S</td>
<td>300</td>
<td>155.06</td>
<td>11</td>
</tr>
<tr>
<td>20</td>
<td>Mla(Hydroxy)IAnon</td>
<td>2a</td>
<td>C_{13}H_{13}N_{2}O_{3}S</td>
<td>300</td>
<td>155.06</td>
<td>11</td>
</tr>
<tr>
<td>21</td>
<td>Mla(Hydroxy)IAnon Hexoxide</td>
<td>2a</td>
<td>C_{13}H_{13}N_{2}O_{3}S</td>
<td>300</td>
<td>155.06</td>
<td>11</td>
</tr>
<tr>
<td>22</td>
<td>DHCA</td>
<td>1a</td>
<td>C_{12}H_{10}N_{2}O_{2}S</td>
<td>240 (1030)</td>
<td>247.06</td>
<td>10</td>
</tr>
<tr>
<td>23</td>
<td>DHCA methylester</td>
<td>2a</td>
<td>C_{13}H_{13}N_{2}O_{3}S</td>
<td>315</td>
<td>261.07</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>DHCA-Glu conjugate</td>
<td>2a</td>
<td>C_{17}H_{17}N_{4}O_{4}S</td>
<td>265</td>
<td>376.10</td>
<td>2</td>
</tr>
<tr>
<td>25</td>
<td>DHCA-Glu conjugate</td>
<td>2a</td>
<td>C_{17}H_{17}N_{4}O_{4}S</td>
<td>265</td>
<td>376.10</td>
<td>2</td>
</tr>
<tr>
<td>26</td>
<td>I3CO_{2}Glc</td>
<td>2b</td>
<td>C_{13}H_{17}N_{2}O_{2}</td>
<td>235</td>
<td>346.09</td>
<td>18</td>
</tr>
<tr>
<td>27</td>
<td>6-GlcO-I3CO_{2}H</td>
<td>2b</td>
<td>C_{15}H_{17}N_{2}O_{3}</td>
<td>235</td>
<td>346.09</td>
<td>18</td>
</tr>
<tr>
<td>28</td>
<td>6-GlcO-I3CO_{2}Glc</td>
<td>2b</td>
<td>C_{15}H_{17}N_{2}O_{3}</td>
<td>235</td>
<td>346.09</td>
<td>18</td>
</tr>
<tr>
<td>29</td>
<td>I3CO_{2}H</td>
<td>1b</td>
<td>C_{13}H_{17}N_{2}O_{2}</td>
<td>235</td>
<td>346.09</td>
<td>18</td>
</tr>
<tr>
<td>30</td>
<td>I3CO_{2}Me</td>
<td>1a</td>
<td>C_{13}H_{17}N_{2}O_{2}</td>
<td>235</td>
<td>346.09</td>
<td>18</td>
</tr>
</tbody>
</table>

Total number of annotated (detected) features | 68 (126) | 104 (212) | 29 (57) |

aAnnotation level: 1a, compound identified using a synthesized standard; 1b, compound identified using a commercially available standard; 2a, compound putatively annotated by interpretation of mass spectrometry data; 2b, compound putatively annotated by comparison with analytical data from literature; 3, compound class putatively annotated.
bFor feature annotation, see Supplemental Data Set 1 online. I3CO_{2}H, indole-3-carboxylate; Mla, 3-mercaptolactic acid; nd, not detected.

For Cys(IAN) based on the synthesis of DHCA and camalexin an apparent K_m of 29.2 ± 1.3 μM was determined (Figure 7A), which was similar to the K_m for DHCA (Schuegger et al., 2006).

Equimolar Amounts of Cyanide Are Released during Camalexin Formation by CYP71B15

The nitrile moiety of Cys(IAN) is lost during thiazoline ring formation. This suggested that for each molecule of camalexin synthesized, one molecule of cyanide is released in the reaction catalyzed by CYP71B15. After incubation of yeast microsomes with Cys(IAN), reaction mixtures were derivatized with 2,3-naphthalene-dialdehyde (NDA) and taurine to give a fluorescent product with cyanide. The NDA/taurine derivative of cyanide was quantified by HPLC based on NDA/taurine derivates of KCN standard. Similarly to the formation of DHCA and camalexin, liberation of cyanide was dependent on NADPH and functional CYP71B15 (see Supplemental Figure 6C online). Turnover rates calculated based on the release of cyanide were similar to the rates based on the synthesis of DHCA and camalexin (Figure 7). NADPH-dependent enzymatic turnover of 50 μM Cys(IAN) yielded 0.31 nmol mg⁻¹ min⁻¹ DHCA/camalexin and 0.34 nmol mg⁻¹ min⁻¹ cyanide. This suggests that formation of camalexin by CYP71B15 is coupled to intermediary release of equimolar amounts of cyanide. NADPH-independent background formation of cyanide was considerably higher (Figure 7B), probably due to additional nonenzymatic cyanide release from Cys(IAN) during NDA/taurine derivatization (see below), which compromises exact determination of enzymatic parameters based on cyanide formation. No reproducible cyanide release was observed under conditions where Cys(IAN) was converted to DHCA and camalexin by Arabidopsis microsomes (data not shown). Possibly, Arabidopsis microsomes contain effective enzymes and corresponding accepting metabolites for cyanide detoxification.

Nonenzymatic Conversion of Cys(IAN)

A similar background turnover of Cys(IAN) to DHCA was observed in Arabidopsis and yeast microsomes without NADPH but also in yeast microsomes that were inactivated by boiling. These data suggested the existence of a nonenzymatic reaction of Cys(IAN) to DHCA. We therefore surveyed for mild oxidizing agents allowing quantitative Cys(IAN) turnover at analytical scale. Aqueous solutions of Cys(IAN) were treated with oxygen, hydrogen peroxide, or ferric chloride. However, only treatment with a large excess of ferric chloride, which is beside its oxidative properties also an effective cyanide acceptor, resulted in a smooth conversion of Cys(IAN) to DHCA and minor amounts of camalexin (product ratio ~20:1). Under similar reaction conditions, DHCA showed only a slight tendency to react to camalexin. It should be noted that Cys(IAN) slowly decomposes at pH > 10. In addition to 15, indole-3-carbaldehyde could also be identified among the reaction products.
Further Elucidation of the IAN-Dependent Metabolic Network

To identify further camalexin biosynthetic intermediates and metabolites derived from IAN, in vivo feeding experiments and nontargeted LC/MS-based analytics were combined (Malitsky et al., 2008). Because of limited commercial availability of heavy isotope-labeled IAN and the ready incorporation of, for example, 6-fluoro-IAOx into camalexin (Glawischnig et al., 2004), 6-fluoro-IAN was used as labeled tracer. IAN and 6-F-IAN were fed to nontreated and silver nitrate–challenged pad3 mutant leaves, which were analyzed by UPLC/ESI(+)-QTOF-MS. Following peak picking and alignment using the XCMS algorithm, features were searched for, which displayed an m/z shift of 17.99 ± 0.01 (theoretical mass shift for hydrogen-fluorine substitution: 17.9906 D) and a maximal retention time shift of 60 s (empirical estimation) when comparing metabolite profiles of 6-F-IAN with IAN-fed leaves (for an example, see Supplemental Figure 7 online). Based on these features, putative compound mass spectra were reconstructed and the underlying metabolites annotated by elemental composition and CID-MS/MS experiments (see Supplemental Data Set 2 online). Using this strategy, 13 pairs of nonlabeled and fluorine-labeled metabolites were identified, which accumulated after feeding of the respective precursor to nontreated and silver nitrate–challenged pad3 leaves (Figure 8; see Supplemental Table 3 online). As expected, for seven of the most abundant compounds annotated as metabolites upstream of PAD3 by analysis of biosynthetic mutants, fluorine-labeled derivatives were identified, including Cys (IAN) and derived metabolites 14, 15, and 18, as well as DHCA and derivatives 23 and 24. Furthermore, conjugates of γ-glutamylcysteine and glutathione with IAN (16 and 17) were detected by this approach and annotated on the basis of an analogous fragmentation behavior as observed for Cys(IAN) (see Supplemental Figure 7 online). Finally, indole-3-carboxylate–derived metabolites, including methyl indole-3-carboxylate (30), glucopyranosyl indole-3-carboxylate (26), N-indol-3-ylcarboxylic arylamine (32), and indole-3-carbaldehyde (31), were shown to be IAN-derived metabolites.

To clarify whether IAN or Cys(IAN) represents the branch point between the camalexin and the indole-3-carbaldehyde/
DISCUSSION

A Metabolomics Approach for the Detection of Novel Biosynthetic Intermediates and Products within the Camalexin Pathway

Nontargeted metabolite profiling has emerged as an important functional genomics tool. Comparative metabolic phenotyping of mutants and wild-type lines has a high potential for identifying functions of disrupted genes in planta (Matsuda et al., 2008). In turn, known mutations resulting in defined metabolic blocks can be used to reveal the product spectrum of a metabolic pathway downstream of this block. As recently demonstrated for flavonoid biosynthesis in Arabidopsis (Böttcher et al., 2008; Yonekura-Sakakibara et al., 2008), metabolite profiling of biosynthetic mutants represents an efficient strategy to systematically annotate the multitude of mostly unknown secondary metabolites. In this work, the same concept was applied to camalexin biosynthesis using mutants with blocks in early (cyp79b2 cyp79b3) and late (pad3) biosynthetic steps. The objectives were to elucidate the complete product spectrum downstream of PAD3 and to identify putative intermediates upstream of PAD3 allowing novel hypotheses to be deduced with respect to the central metabolic transformation between IAN and DHCA. Reverse-phase UPLC/ESI-QTOF-MS was chosen as the analytical platform because this technique was shown to facilitate separation and sensitive detection of a large fraction of the semipolar secondary metabolites, including indolic compounds, and offers superior options for structural elucidation (Clemens et al., 2006; De Vos et al., 2007). To detect metabolites as comprehensively as possible, metabolite profiles were acquired in both positive and negative ion mode. Although analysis of negative ion metabolite profiles revealed only a subset of the metabolites identified by analysis of positive ion profiles, combined analysis of data sets facilitated annotation of quasimolecular ions and allowed validation of calculated elemental compositions and relative quantification. In addition, in some cases, positive and negative ion CID mass spectra provided complementary structural information.

Eleven compounds beside camalexin were putatively annotated as metabolites downstream of PAD3 in silver nitrate–challenged Col-0 leaves. Although chemical diversification represents a widespread strategy in nature to enhance the toxicity of phytoalexins, simple camalexin derivatives, such as 6-methoxycamalexin or N-methylcamalexin, which were reported to occur in other camalexin producing species like Capsella bursa-pastoris (Jimenez et al., 1997) and Camelina sativa (Browne et al., 1991), were surprisingly lacking in Arabidopsis. Instead, major metabolic transformations of camalexin in leaf tissue include sequential hydroxylation, O-glycosylation, and malonylation, resulting in accumulation of two different hydroxycamalexin hexosides (2 and 3) and a malonyl derivative (4) as analogously reported for indole-3-carboxylate in Arabidopsis leaf and root tissue (Hagemeier et al., 2001; Bednarek et al., 2005). The respective aglycones were below the detection limit. Sequential hydroxylation and O-glycosylation were identified as camalexin detoxification reactions in the stem rot pathogen Sclerotinia sclerotiorum (Pedras and Ahiahonu, 2002) and could be functionally related to detoxification in Arabidopsis as well because of the known phytotoxicity of camalexin (Rogers et al., 1996). Based on elemental compositions, four minor compounds were putatively annotated as oxidation products of camalexin (5 to 8) and one that is devoid of sulfur as degradation product (9). Two of the oxidation products could be structurally characterized as 2,3-dihydroxylated (5) and isomeric ring-opening product (6) of camalexin. Dihydroxylation at C-2/C-3 represents...
a common catabolic pathway of indole derivatives and was reported, for instance, for the phytoalexin brassinin from *Brassica oleracea* (Monde et al., 1991). Structures and biosynthetic origin of the camalexin derivatives 10 to 12 remain to be elucidated. Based on the putative annotations, it can be assumed that 10 to 12 are products from complex oxidative processes.

In addition to the known substrate of PAD3, DHCA (22), Cys(IAN) (13) was identified to accumulate in silver nitrate–challenged *pad3* leaves. Putative annotation of nine metabolites accumulating in silver nitrate–treated *pad3* leaves revealed that DHCA and Cys(IAN) are further metabolized. Metabolization of DHCA is achieved by modification of the carboxylic acid moiety by either methylation or conjugation to the amino acids Gin and Glu. Major metabolic transformations of Cys(IAN) comprise N-malonylation and conversion to the analogous α-hydroxy acid Mia(IAN) (18), which is subsequently O-glicosylated. N-Acylation of Cys conjugates is a well-known transformation following proteolytic processing of the corresponding glutathione conjugates. N-Malonylation was recently reported as a major route of S-(fenclorim)-Cys metabolism in *Arabidopsis* (Brazier-Hicks et al., 2008). Interestingly, α-hydroxy acid 18 undergoes sequential ring hydroxylation and glycosylation similar as observed for camalexin and indole-3-carboxylate, underlining the general importance of these transformations for indole metabolism in *Arabidopsis*.

To study products and intermediates of camalexin biosynthesis after biotic stress application, the same set of profiling experiments was performed in the *pen2* mutant background inoculated with *P. infestans* spore suspensions. The experimental setup provided further the opportunity to recollect spore suspension droplets from rosette leaves and to analyze them for secreted camalexin metabolites and intermediates. The sets of metabolites identified up- and downstream of PAD3 in leaf tissue after abiotic and biotic stress application were almost identical. The only differences were the lack of DHCA methylester (23) in inoculated *pen2 pad3* leaves and the identification of indole-3-carboxylate derivatives 26 to 28, showing approximately two- to threefold stronger accumulation in inoculated *pen2 pad3* in comparison to inoculated *pen2* leaves.

Indolic compounds detected in recollected spore suspensions droplets fit in the spectrum of indolic metabolites identified in *Arabidopsis* root culture liquid after inoculation with *P. sylvaticum* (Bednarek et al., 2005). In addition to large amounts of camalexin, spore suspension droplets recollected from *pen2* leaves were found to contain four metabolites (5 to 7 and 9) whose occurrence is correlated with functional CYP79B2/B3 and PAD3. To dissect the metabolic contribution of *Arabidopsis* and *P. infestans* to the accumulation of these compounds, spore suspensions were separately incubated with camalexin. Surprisingly, the phytoalexin was readily metabolized by *P. infestans*, and the oxidation products 5 to 7 were identified as major degradation products. Hence, *P. infestans* uses similar biotransformations as described for the detoxification of camalexin in the fungus *Rhizoctonia solani* (Pedras and Khan, 2000). In contrast with 9, which seems to be associated to plant metabolism alone, the contribution of plant and oomycete to accumulation of the oxidation products 5 to 7 remains to be elucidated. Instead of camalexin, the intermediates DHCA and Cys(IAN) were identified in spore suspension droplets recollected from *pen2 pad3* leaves. In accordance with the stronger accumulation of indole-carboxylate derivatives in *pen2 pad3* leaves, droplets also displayed higher levels of indole-3-carboxylate (29) and methyl indole-3-carboxylate (30). However, increased synthesis of indole-3-carboxylate derivatives in *pen2 pad3* in response to *P. infestans* could be explained as compensation for the lack of potentially toxic camalexin.

**CYP71B15 (PAD3) Is a Multifunctional Cytochrome P450 Enzyme**

CYP71B15 (PAD3) has been previously shown to catalyze decarboxylation of DHCA to camalexin (Schuhegger et al.,
2006). Surprisingly, formation of DHCA from Cys(IAN) also is catalyzed by CYP71B15 in an NADPH-dependent manner. In accordance with the enzymatic characteristics of CYP71B15, both Cys(IAN) and DHCA rescue the camalexin-deficient phe-
notype of cyp79b2 cyp79b3 and are converted to camalexin in plants constitutively expressing CYP71B15. It is therefore likely that CYP71B15 efficiently turns over both substrates in planta.

In conclusion, CYP71B15 is a multifunctional P450 enzyme. The contribution of multifunctional P450 enzymes seems to be characteristic of camalexin biosynthesis, as CYP79B2/B3 are also thought to catalyze two consecutive oxidation reactions. Based on a detailed biochemical analysis of the founding member of the CYP79 family, CYP79A1 (P450TYR) from Sorghum bicolor, the enzyme probably catalyzes two consecutive NADPH-dependent hydroxylations of the amino nitrogen. The resulting unstable N,N-dihydroxyamino acid is dehydrated and decarboxylated nonenzymatically, yielding the oxime (Halkier et al., 1995; Sibbesen et al., 1995). By contrast, CYP71B15 modifies two different carbon positions. One intermediate, DHCA, is accumulating as major reaction product. This type of multifunctionality is rather exceptional for plant P450 enzymes. One additional example is CYP71D20 from tobacco (Nicotiana tabacum), which catalyzes the hydroxylation of 5-epi-aristolochene at both the 1- and the 3-positions, and, in addition to the final product capsidiol, the intermediate 1-deoxycapsidiol was detected in the reaction mixture (Ralston et al., 2001).

Conversion of Cys(IAN) to DHCA formally represents a multistep reaction comprising elimination of hydrogen cyanide, intramolecular cyclization, and oxidation. At present, it is unclear whether the reaction sequence is initiated by elimination of hydrogen cyanide or by oxidation. A proposed mechanism for DHCA decarboxylation and camalexin formation is shown by Schuhegger et al. (2006).

In addition to Cys(IAN), DHCA accumulates in pad3 mutants. This is in accordance with our finding that in vitro Cys(IAN) is converted also nonenzymatically to DHCA, and we suggest that this nonenzymatic conversion is responsible for the observed pad3 phenotype. To a minor extent, DHCA is decarboxylated to camalexin in FeCl3 containing solution. Thus, nonenzymatic DHCA turnover can explain the observation that traces of camalexin are synthesized in stressed pad3 leaves.

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**Figure 8.** Elucidation of the IAN-dependent network by combination of in vivo feeding experiments and LC/MS-based metabolite profiling.

(A) Metabolization of 6-fluoro-IAN after feeding to silver nitrate-challenged pad3 leaves.

(B) Relative quantification of IAN-derived metabolites after feeding of water, IAN, or 6F-IAN to nontreated or silver nitrate-treated pad3 leaves. For quantifier ions, retention times, and absolute peak area, refer to Supplemental Table 3 online.
Camalexin Biosynthesis Is Linked to Cyanide Formation

According to the enzymatic properties of CYP71B15, we propose that for each molecule of camalexin synthesized, one molecule of cyanide is released. Arabidopsis microsomes also converted Cys(IAN) to DHCA and to a minor degree to camalexin. However, we did not observe equal cyanide formation in the same reaction mixture, which suggests that cyanide is readily metabolized.

Cyanide is an efficient defense metabolite, and cyanogenic glucosides, for example, are widespread in the plant kingdom. These phytoanticipins are stored in the vacuole, and cyanide is released during tissue disruption (Zagrobelny et al., 2008). By contrast, cyanide release and camalexin synthesis are directly coupled. As camalexin biosynthesis depends on heme-containing P450 enzymes, accumulation of cyanide in situ might shut down the process, and it is therefore detoxified. Generally, plants produce cyanide as a byproduct of ethylene synthesis. Cyanide is metabolized via β-cyanoalanine, which is recycled to Asn and Asp by nitrilases of the NIT4 class (Piotrowski, 2008). Arabidopsis NIT4 transcript accumulates upon pathogen attack, for example, by Botrytis cinerea (Ferrari et al., 2007), and some coregulation with camalexin biosynthetic genes is observed (e.g., expression angler [Toufighi et al., 2005] analysis with AtGenExpress pathogen set: NIT4/CYP71A13, r = 0.777). Neither β-cyanoalanine nor γ-glutamyl-β-cyanoalanine was.

Table 3. Incorporation of IAN and Cys(IAN) into Indole-3-Carboxylic Acid Derivatives, Indole-3-Carbaldehyde, and Camalexin after Feeding to Silver Nitrate-Treated cyp79b2 cyp79b3 Leaves

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Water</th>
<th>IAN (0.25 mM)</th>
<th>Cys(IAN) (0.25 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Camalexin</td>
<td>nd</td>
<td>952 ± 290</td>
<td>2140 ± 94</td>
</tr>
<tr>
<td>26</td>
<td>13CO2Glc</td>
<td>nd</td>
<td>171 ± 45</td>
<td>11.9 ± 1.6</td>
</tr>
<tr>
<td>27</td>
<td>6-GlcO-13CO2H</td>
<td>nd</td>
<td>147 ± 14</td>
<td>8.9 ± 2.9</td>
</tr>
<tr>
<td>28</td>
<td>6-OH-13CO2Glc</td>
<td>nd</td>
<td>75.0 ± 13.3</td>
<td>nd</td>
</tr>
<tr>
<td>30</td>
<td>13CO2Me</td>
<td>nd</td>
<td>18.7 ± 4.5</td>
<td>nd</td>
</tr>
<tr>
<td>31</td>
<td>13CHO</td>
<td>nd</td>
<td>7.9 ± 4.6</td>
<td>nd</td>
</tr>
<tr>
<td>32</td>
<td>13COAsp</td>
<td>nd</td>
<td>32.9 ± 7.5</td>
<td>4.6 ± 0.6</td>
</tr>
</tbody>
</table>

Responses represent medians ± SD of three biological replicates. nd, not detected.

Camalexin Biosynthesis Is Linked to Cyanide Formation

According to the enzymatic properties of CYP71B15, we propose that for each molecule of camalexin synthesized, one molecule of cyanide is released. Arabidopsis microsomes also converted Cys(IAN) to DHCA and to a minor degree to camalexin. However, we did not observe equal cyanide formation in the same reaction mixture, which suggests that cyanide is readily metabolized.

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detected in stressed wild-type leaves by UPLC/ESI-QTOF-MS analysis (data not shown). Possibly, cyanide liberated from Cys(IAN) during camalexin biosynthesis is efficiently recycled into central metabolism via Asn and Asp, the products of NIT4-catalyzed β-cyanoalanine breakdown.

**Camalexin Biosynthesis within the IAN Metabolic Network**

The metabolomic analysis of camalexin biosynthetic mutants presented here led to a revision of the previous working model for the camalexin biosynthetic pathway via indole-3-carbaldehyde (31) (Zook and Hammerschmidt, 1997) (Figure 9). Instead of being an intermediate, 31 is a side product of camalexin biosynthesis, which is further metabolized to a number of compounds (26 to 30 and 32). Like camalexin itself, it also derives from IAN, emphasizing the emerging role of IAN as a key intermediate within the metabolic network of indolic compounds in Arabidopsis (Figure 9). IAN and indole-3-carbaldehyde derivatives are synthesized as phytoalexins or phytoanticipins in a number of Brassicaceae species (Pedras et al., 2007), demonstrating the general importance of IAN in cruciferous plants.

IAN is also a product of indol-3-ylmethylglucosinolate hydrolysis, when myrosinases act in concert with an epithospecific protein (Burow et al., 2008). It is not yet clear whether indol-3-ylmethylglucosinolate turnover feeds into the camalexin biosynthetic pathway and accounts for camalexin levels observed in cyp71a13 (Nafisi et al., 2007). Alternatively, activity of a CYP71A13 homolog, such as the closely linked CYP71A12 (Nafisi et al., 2007), could partially rescue loss of CYP71A13 function. Historically, IAN has received a lot of attention as potential intermediate in auxin biosynthesis, and recently a substantial contribution of nitrosylase-mediated IAN hydrolysis to the total pool of indole-3-acetic acid (IAA) conjugates was demonstrated for maize (Zea mays; Kriebelbaumer et al., 2007).

In Arabidopsis, IAN hydrolysis by nitrosylases in vitro is inefficient (Vorwerk et al., 2001), and an IAN-dependent auxin biosynthetic pathway appears to be relevant only under specific conditions (Piotrowski, 2008). Accordingly, we have not observed IAA/6FIAA accumulation in stressed wild-type and pad3 leaves or in stressed cyp79b2 cyp79b3 leaves incubated with IAN or 6F-IAN (data not shown).

The pad2 mutation of GSH1 results in elevated levels of Cys and reduced levels of glutathion, camalexin, and glucosinolates (Parisy et al., 2007; Schlaeppe et al., 2008), demonstrating the importance of glutathione for the biosynthesis of sulfur-containing defense compounds in Arabidopsis. To test whether the thiosulfur in glucosinolate biosynthesis derives from Cys or glutathione, Schlaeppe et al. (2008) performed incorporation experiments with 35S-labeled Cys or glutathione. As they observed ready exchange between the labeled Cys and glutathione pools, no final conclusion was possible. It can be expected that similar labeling experiments will also not be feasible for proving the origin of the thiazole ring of camalexin.

In support of a potential role of glutathione as biosynthetic precursor of camalexin, we have identified the γ-glutamylcysteine and glutathione conjugates of IAN (16 and 17) in addition to Cys(IAN) (13). Therefore, we favor the following hypothesis for Cys(IAN) biosynthesis: IAN is conjugated with glutathione by a glutathione S-transferase, yielding 17. Alternatively, 16 might be directly formed from IAN and γ-glutamylcysteine. The conjugation reaction probably requires oxidation of the methylene group adjacent to the nitrile, catalyzed, for example, by a cytochrome P450 enzyme and direct coupling of this oxygenase with glutathione S-transferase, similar to the tight link of oxygenase and glycosyltransferase in the biosynthesis of cyanogenic glucosides (Jørgensen et al., 2005). Subsequently, 17 is hydrolyzed to 16 and 13 involving peptidases and/or phytochelatine synthases (Blum et al., 2007). Alternatively, activated IAN could be directly conjugated with Cys, assuming that 16 and 17 are rather side products than biosynthetic precursors of camalexin and that the role of glutathione in this process is regulatory.

**METHODS**

**Plant Material for Metabolite Profiling**

Arabidopsis thaliana Col-0, pad3-1 (Glazebrook and Ausubel, 1994), and cyp79b2 cyp79b3 (Zhao et al., 2002) lines were grown after cold stratification for 3 d at 4°C in parallel on a soil/vermiculite mixture (3/2) in a growth cabinet with 8 h light (−150 μE m−2 s−1) at 22°C and 16 h dark at 20°C to developmental stage 3.5 (Boyes et al., 2001). Plants were sprayed with 5 mM silver nitrate or water as control and incubated for 48 h in a growth cabinet. Rosette leaves of six individual plants per genotype and treatment were harvested, immediately frozen in liquid nitrogen, pooled, and stored at −80°C until analysis. Three independent biological replicate experiments were performed on independent sets of plants grown at different times.

Arabidopsis pen2-2 (Lipka et al., 2005), pen2-1 pad3-1 (Bednarek et al., 2009), and cyp79b2 cyp79b3 mutant lines were grown after cold stratification for 3 d at 4°C in parallel on a soil/vermiculite mixture (3/2) in a phytochamber with 8 h of light (−200 μE m−2 s−1) at 22°C, 16 h dark at 20°C, and 60% relative humidity to developmental stage 3.5. Phytophthora infestans isolate 208m2 (Si-Ammour et al., 2003) was prepared for inoculations as described by Lipka et al. (2005). Rosette leaves were inoculated with 10 μL droplets of the spore suspension (concentration 5 × 106 spores mL−1) or with filtered spore suspension (pore size 0.2 μm) as control and incubated in a phytochamber with 16 h of light (−200 μE m−2 s−1) at 20°C, 8 h dark at 18°C, and 100% relative humidity. After 24 h, droplets were recollected from six individual plants per genotype and treatment, pooled, and stored at −80°C until analysis. After 48 h, rosette leaves (including droplets) of six individual plants were harvested, immediately frozen in liquid nitrogen, pooled, and stored at −80°C until analysis. Three independent biological replicate experiments were performed.

**Sample Preparation for Metabolite Profiling**

Plant material was homogenized in liquid nitrogen using a pestle and mortar, and aliquots of 5 mg were weighed into precooled 2-mL round bottom tubes (Eppendorf). After addition of 200 μL methanol/water, 80/20 (v/v) precooled at −40°C and spiked with biochanin A (5 μM; Sigma-Aldrich), phlorizin (5 μM; Fluka), anisic acid (5 μM; Sigma-Aldrich), kinetin (5 μM; Sigma-Aldrich), and N-(3-indolyacetyl)-L-valine (5 μM; Aldrich) as internal standards, the samples were immediately vortexed for 1 min, sonicated for 15 min at 20°C, and centrifuged for 10 min at 19,000g. The supernatants were transferred to new 2-mL tubes and the remaining pellets subjected to a second extraction using 200 μL methanol/water, 80/20 (v/v). The combined extracts were evaporated to dryness in a vacuum centrifuge at 30°C, thoroughly reconstituted in 200 μL methanol/
Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100 × 1.0 mm, particle size 1.8 μm; Waters) applying the following binary gradient at a flow rate of 150 μL min⁻¹: 0 to 1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 1 to 16 min, linear from 5 to 95% B; 16 to 18 min, isocratic 95% B; 18 to 20 min, isocratic 5% B. The injection volume was 3.1 μL (full loop injection). Eluted compounds were detected from m/z 100 to 1000 using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in positive and negative ion modes using the following instrument settings: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 liters min⁻¹, 190°C; capillary, −5500 V (+4000 V); end plate offset, −500 V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; in-source CID energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, argon; collision energy, 10 eV; collision RF 150/400 Vpp (timing 50/50); transfer time, 70 μs; prepulse storage, 5 μs; pulser frequency, 10 kHz; spectra rate, 3 Hz. Internal mass calibration of each analysis was performed by infusion of 20 μL 10 mM lithium formate in isopropanol/water, 1/1 [v/v], at a gradient time of 18 min using a diverter valve. For abiotic and biotic stress experiments, 144 data files [biological replicate (3) × technical replicate (4) × ionization (2) × genotype (3) × treatment (2)] were generated for each experiment.

Capillary LC/ESI-QTOF-MS–Based Metabolite Profiling of Recollected Spore Suspension Droplets

One microliter of the sample was separated on an Ultimate capillary LC system (Dionex) equipped with a modified C₁₈ column (GROMSIL ODS 4 HE, 150 × 0.3 mm, particle size 3 μm, pore size 120 Å, guard column 10 × 0.3 mm; Alttech Grom) at a flow rate of 6 μL min⁻¹ applying the following binary gradient: 0 to 5 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 5 to 45 min, linear from 5 to 95% B; 45 to 52 min, isocratic 95% B; 52 to 58 min, isocratic 5% B. Eluted compounds were detected by an API QSTAR Pulsar hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MD Sciex) equipped with an IonSpray ion source in positive ion mode as described previously (Böttcher et al., 2008). External mass calibration was performed using protonated ALJTLVS (m/z 829.3939) and a fragment ion (m/z 149.0233) originating from phthalate-type plasticizers. A total of 72 data files [biological replicate (3) × technical replicate (4) × ionization (2) × genotype (3) × treatment (2)] were generated.

Data Analysis

Nontargeted analyses of UPLC/ESI-QTOF-MS metabolite profiles acquired on an API QSTAR Pulsar: Raw data files were centroided in m/z domain, converted to mzData format using the vendor-specific Wiff to mzData Translator (www.appliedbiosystems.com), and processed with XCMS. Parameters for chromatographic peak detection using centWave were sntresh = 2, prefilter = c(3,30), ppm = 100, and peak width = c(20,60). Retention time correction was achieved in two iterations considering −200 peak groups applying the following parameter settings: minfrac = 1, bw = 60 s, mzwid = 0.05 D, span = 1, missing = extra = 0 for the first iteration and minfrac = 1, bw = 25 s, mzwid = 0.05 D, span = 0.6, missing = extra = 0 for the second iteration. After final peak grouping (minfrac = 0.75, bw = 15 s) and filling in of missing features using fillPeaks, a data matrix was exported into Microsoft Excel and processed as described above.

CID-MS/MS

MicrOTOF-Q: Precursor ions were selected in Q₁ with an isolation width of ± 4 D and fragmented in the collision cell applying collision energies in the range of 10 to 30 eV. Argon was used as collision gas. Product ions were detected using the following parameter settings: collision RF 150/400 Vpp (timing 50/50); transfer time, 70 μs; prepulse storage, 5 μs; pulser frequency, 10 kHz; spectra rate, 1.5 Hz. For CID of in-source fragment ions (pseudo-MS³), in-source CID energy was increased from 0 to 100 V.

API QSTAR Pulsar: Acquisition of MS² and pseudo-MS³ spectra was performed as described by Böttcher et al. (2008). Hyphenation of UPLC with the QSTAR was achieved via a TurbolonSpray ion source (nebulizer gas [nitrogen], 2.0 liters min⁻¹; dry gas [nitrogen], 6.5 liters min⁻¹, 400°C; curtain gas [nitrogen], 2.2 liters min⁻¹, ion spray voltage +5000 V).

Feature Annotation and Identification of Metabolites

Differential features detected in narrow retention time windows displaying a high chromatogram correlation were grouped. In combination with the raw data, individual compound mass spectra were reconstructed and features annotated as cluster ion, quasi-molecular ion, or in-source fragment ion. Annotated lists of all differential feature sets are provided in Supplemental Data Ael 1 online. Afterwards, targeted CID-MS/MS experiments of quasimolecular ions (MS²) and in-source fragment ions were performed.
(pseudo-MS²) were performed using QTOF-MS. Based on accurate mass measurements, putative elemental compositions were calculated, filtered by isotope abundance and the number of double bond equivalents and electron parity (Kind and Fiehn, 2006), and checked for consistency in relation to elemental compositions calculated for fragment ions and observed neutral losses (Konishi et al., 2007). In cases where compounds are detectable in positive and negative ion modes, elemental compositions were cross-validated by combined analysis of positive- and negative-ion mass spectral data. With respect to the metabolic mapping of the underlying features, putative molecular structures were deduced by interpretation of the fragmentation patterns. In the case of compounds 1, 5, 6, 13, 22, 29, and 30, rigorous identification by analysis of commercially available or synthesized standard compounds was achieved. Mass spectral characterization and interpretation of CID mass spectra can be found in Supplemental Data Set 2 online.

In Vivo Feeding of Camalexin Biosynthetic Precursors

For establishment of Cys(IAN) as camalexin biosynthetic intermediate and for identification and quantification of IAN metabolites, 6-week-old cyp79b2 cyp79b3 or pad3-1 mutant plants grown in 12 h light (≈100 µE m⁻² s⁻¹)/12 h dark at 21°C, were sprayed with 5 mM silver nitrate or water. After 8 h, rosette leaves were excised at the petiole and incubated in 200 µL 250 µM Cys(IAN), 250 µM IAN, 250 µM 6-fluoro-IAN (Matrix Scientific), or water for an additional 16 h. Camalexin was extracted and quantified as described (Schuhegger et al., 2006). IAN and 6F-IAN derivatives were identified and quantified by UPLC/ESI-QTOF-MS applying similar protocols for extraction, profiling, and data analysis as described above. Constitutive turnover of camalexin biosynthetic intermediates to camalexin was determined in Col-0 and 35S:CYP71B15 described above. Constitutive turnover of camalexin biosynthetic intermediates was performed as described (Schuhegger et al., 2008). IAN and 6F-IAN derivatives were identified and quantified by UPLC/ESI-QTOF-MS applying similar protocols for extraction, profiling, and data analysis as described above. Constitutive turnover of camalexin biosynthetic intermediates to camalexin was determined in Col-0 and 35S:CYP71B15 plants (Schuhegger et al., 2006). Rosette leaves were excised at the petiole and incubated in 200 µL of 250 µM IAN, 250 µM Cys(IAN), 250 µM DHCA, or water for 24 h.

Enzymatic Turnover of Cys(IAN) by Arabidopsis Microsomes and CYP71B15 (PAD3) Expressed in Yeast

Microsomes of the yeast WAT11 strain (Pompon et al., 1996) expressing CYP71B15 were prepared as described (Schuhegger et al., 2006). Arabidopsis microsomes were isolated from Col-0 rosette leaves as described previously (Schuhegger et al., 2006).

Enzyme tests with yeast and Arabidopsis microsomes were performed for 30 min at 25°C in 100 µL 50 mM KPi/2 mM DTT, pH 7.5, ≤1 mM NADPH, 200 µM substrate (variable concentrations for Km, determination), and 100 µg of microsomal protein. The reaction was stopped with 1 vol methanol and analyzed for indolic metabolites by reverse-phase HPLC (LiChroCART 250-4, RP-18, 5 µM) ([C12H10N2O2S+H]⁺: 247.0536, found: 247.0519).

We thank Alfons Gierl (Technische Universität München) for his support and encouragement and Ludger Wessjohann and Bernhard
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The Multifunctional Enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) Converts Cysteine-Indole-3-Acetonitrile to Camalexin in the Indole-3-Acetonitrile Metabolic Network of Arabidopsis thaliana

Christoph Böttcher, Lore Westphal, Constanze Schmotz, Elke Prade, Dierk Scheel and Erich Glawischnig

Plant Cell 2009;21;1830-1845; originally published online June 30, 2009;
DOI 10.1105/tpc.109.066670

This information is current as of July 1, 2017