The Biosynthetic Pathway of Indole-3-Carbaldehyde and Indole-3-Carboxylic Acid Derivatives in Arabidopsis1[W]

Christoph Böttcher2, Alexandra Chapman, Franziska Fellermeier, Manisha Choudhary, Dierk Scheel, and Erich Glawischnig*

Leibniz Institute of Plant Biochemistry, Department of Stress and Developmental Biology, 06120 Halle/Saale, Germany (C.B., D.S.); and Lehrstuhl für Genetik, Technische Universität München, 85354 Freising, Germany (A.C., F.F., M.C., E.G.)

Indolic secondary metabolites play an important role in pathogen defense in cruciferous plants. In Arabidopsis (Arabidopsis thaliana), in addition to the characteristic phytoalexin camalexin, derivatives of indole-3-carbaldehyde (ICHO) and indole-3-carboxylic acid (ICOOH) are synthesized from tryptophan via the intermediates indole-3-acetaldoxime and indole-3-acetonitrile. Based on feeding experiments combined with nontargeted metabolite profiling, their composition in nontreated and silver nitrate (AgNO3)-treated leaf tissue was comprehensively analyzed. As major derivatives, glucose conjugates of 5-hydroxyindole-3-carboxylic acid (ICOOH) are synthesized from tryptophan via the intermediates indole-3-acetaldoxime and indole-3-acetonitrile. The Biosynthetic Pathway of Indole-3-Carbaldehyde and Indole-3-Carboxylic Acid Derivatives in Arabidopsis1[W]

Based on feeding experiments combined with nontargeted metabolite profiling, their composition in nontreated and silver nitrate (AgNO3)-treated leaf tissue was comprehensively analyzed. As major derivatives, glucose conjugates of 5-hydroxyindole-3-carboxylic acid (ICOOH) are synthesized from tryptophan via the intermediates indole-3-acetaldoxime and indole-3-acetonitrile. The Biosynthetic Pathway of Indole-3-Carbaldehyde and Indole-3-Carboxylic Acid Derivatives in Arabidopsis1[W]

Based on feeding experiments combined with nontargeted metabolite profiling, their composition in nontreated and silver nitrate (AgNO3)-treated leaf tissue was comprehensively analyzed. As major derivatives, glucose conjugates of 5-hydroxyindole-3-carboxylic acid (ICOOH) are synthesized from tryptophan via the intermediates indole-3-acetaldoxime and indole-3-acetonitrile. The Biosynthetic Pathway of Indole-3-Carbaldehyde and Indole-3-Carboxylic Acid Derivatives in Arabidopsis1[W]
cruciferous plants, indole-3-carbaldehyde (ICHO) and ICOOH derivatives, such as the brassicanals, were identified as phytoalexins (for review see Pedras et al., 2011). In this work, using chemical complementation (i.e. the reconstitution of a biosynthetic pathway in a biosynthetic gene knockout mutant by feeding a downstream intermediate) in combination with nontargeted metabolite profiling, we comprehensively explore the spectrum of ICHO and ICOOH derivatives in leaves of Arabidopsis and show that, in response to AgNO₃ treatment, ICHO- and ICOOH-derived compounds accumulate in overall quantities similar to camalexin, suggesting an important role in induced chemical defense.

Aldehyde oxidases are molybdenum-containing enzymes that convert aldehydes into the corresponding carboxylic acids (Mendel and Hänisch, 2002). Arabidopsis contains four genes encoding aldehyde oxidases. ARABIDOPSIS ALDEHYDE OXIDASE3 (AAO3) catalyzes the last step in abscisic acid biosynthesis (Seo et al., 2000), and AAO4 is responsible for benzoaldehyde oxidation in siliques (Ibdah et al., 2009). AAO1 and AAO2 as homodimers and heterodimers have a rather broad substrate specificity in vitro (Akaba et al., 1999; Koiwai et al., 2000), and AAO1 was recently shown to catalyze the NADH-dependent production of superoxide anions (Zarepour et al., 2012). It was proposed that aldehyde oxidases are involved in auxin biosynthesis (Seo et al., 2000), and one substrate of the AAO1 homomer is indole-3-acetaldehyde (IAAald), which is oxidized to indole-3-acetic acid (IAA) with an apparent Km value of 39 μM (Koitai et al., 2000). However, it is not clarified to what extent IAAl is formed in vivo. While no auxin-deficient mutant phenotype of aoo1 has yet been reported, it was recently demonstrated that the major biosynthetic pathway for IAA in plants is a two-step reaction involving Trp aminotransferases and YUCCA flavin monoxygenases but not aldehyde oxidases (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Kriechbaum et al., 2012).

In this article, we present evidence that the biological role of AAO1 in Arabidopsis is in the biosynthesis of defense-related indolic compounds, and we provide a model for the biosynthetic pathway of these metabolites.

RESULTS

Identification of ICHO and ICOOH Derivatives by Metabolite Profiling of Chemically Complemented cyp79B2 cyp79B3 Mutants

We have previously shown, using 6-fluoro-IAN as a metabolic tracer, that in addition to camalexin a number of ICOOH derivatives and ICHO can be synthesized in Arabidopsis leaves from IAN (Böttcher et al., 2009). To elucidate the entire spectrum of ICHO/ICOOH derivatives accumulating in a CYP79B2/B3-dependent manner in leaf tissue, nontreated and AgNO₃-treated cyp79B2 cyp79B3 leaves were fed with IAN, ICHO, and ICOOH and subjected together with nontreated and AgNO₃-treated mock-fed wild-type and cyp79B2 cyp79B3 leaves to nontargeted liquid chromatography-mass spectrometry (LC-MS)-based metabolite profiling. In the first step, comparative analyses of mock-fed wild-type and cyp79B2 cyp79B3 metabolite profiles were performed to dissect molecular features (mass-to-charge retention time pairs) associated with metabolites whose accumulation depends on CYP79B2/B3. In the next step, molecular features with significantly increased intensity in fed cyp79B2 cyp79B3 samples in comparison with mock-fed cyp79B2 cyp79B3 samples were identified and intersected with the set of CYP79B2/B3-dependent features. Detailed analyses and Venn diagrams are shown in Supplemental Figure S1. Based on the resulting molecular feature sets, a total of 20 metabolites was detected whose accumulation depends on CYP79B2/B3 and was reconstituted in cyp79B2 cyp79B3 leaves by feeding of either ICHO or ICOOH (Table I). These metabolites were characterized by quadrupole-time-of-flight mass spectrometry (QTOFMS) and comprise ICOOH (1), its methyl, glucosyl, and malonylated glucosyl esters (2, 4, and 5), an ICOOH-Asp conjugate (3), and several Glc/malonyl Glc conjugates of 6-hydroxyindole-3-carboxylic acid (6-HO-ICOOH; 6-9). Besides ICHO (10), two glycosylated hydroxy-ICHO conjugates (11 and 12) were detected whose aglycones were identified after enzymatic hydrolysis as 5-hydroxyindole-3-carbaldehyde (5-HO-ICHO) and 4-hydroxyindole-3-carbaldehyde (4-HO-ICHO), respectively. In addition, ascorbigen (13), a glycosylated dihydroascorbigen (14), and six unknown metabolites (15–20), which so far could only be characterized by their elemental composition and fragmentation patterns, were found.

Reconstruction of the ICHO/ICOOH Metabolic Network

Compounds 1 to 20 were relatively quantified in nontreated and AgNO₃-treated mock-fed wild-type and fed cyp79B2 cyp79B3 samples (Table II). Among the nine identified ICOOH derivatives, ICOOGlc (for β-D-glucosyl indole-3-carboxylate), 6-HO-ICOOGlc (for β-D-glucosyl 6-hydroxyindole-3-carboxylate), 6-GlcO-ICOOH (for 6-hydroxyindole-3-carboxylic acid 6-O-β-D-glucoside), and 6-GlcO-ICOOGlc (for β-D-glucosyl 6-hydroxyindole-3-carboxylate 6-O-β-D-glucoside) showed the highest absolute signal intensity after feeding of cyp79B2 cyp79B3 with ICOOH. In addition, their high relative intensities, compared with AgNO₃-treated mock-fed wild-type tissue, suggest that 6-hydroxylation and O-glucosylation constitute the major metabolic reactions of ICOOH in leaf tissue. In contrast, conjugation with Asp or O-methylation, the latter occurring exclusively in AgNO₃-treated leaf tissue, represent minor metabolic transformations of ICOOH.

After chemical complementation of cyp79B2 cyp79B3 with ICHO, the compounds 5-GlcO-ICHO (for 5-hydroxyindole-3-carbaldehyde 5-O-β-D-glucoside),
Indole-3-Carboxylic Acid Biosynthesis

Table 1. Analytical data of identified ICHO/ICOOH derivatives

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>ID*</th>
<th>Elemental Composition</th>
<th>Retention Time</th>
<th>Quantiﬁer Ion</th>
<th>Observed Fragment Ions upon CIDb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Type</td>
<td>m/z</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Measured m/z</td>
<td>ppm</td>
</tr>
<tr>
<td>1</td>
<td>ICHO</td>
<td>a</td>
<td>C₉H₇NO₃</td>
<td>284</td>
<td>[M-H]⁻</td>
<td>160.0400 2.7</td>
</tr>
<tr>
<td>2</td>
<td>ICOOMe</td>
<td>b</td>
<td>C₁₀H₁₃NO₃</td>
<td>400</td>
<td>[M-H]⁻</td>
<td>174.0556 2.7</td>
</tr>
<tr>
<td>3</td>
<td>4-HO-Asp</td>
<td>d</td>
<td>C₁₀H₁₂N₂O₅</td>
<td>231</td>
<td>[M-H]⁻</td>
<td>275.0667 2.4</td>
</tr>
<tr>
<td>4</td>
<td>4-GlcO-ICHOO</td>
<td>b, c, d</td>
<td>C₁₀H₁₃NO₃</td>
<td>227</td>
<td>[M-H]⁻</td>
<td>322.0932 0.8</td>
</tr>
<tr>
<td>5</td>
<td>ICHO(MalonylGlc) c, d</td>
<td>C₁₅H₁₇NO₈</td>
<td>275</td>
<td>[M-H-CO₂]⁻</td>
<td>364.1033 1.5</td>
<td>364, 304, 244, 232, 214, 202, 160, 116, 113, 101</td>
</tr>
<tr>
<td>6</td>
<td>6-HO-ICOOGlc</td>
<td>b, c, d</td>
<td>C₁₅H₁₇NO₈</td>
<td>117</td>
<td>[M-H]⁻</td>
<td>338.0880 0.6</td>
</tr>
<tr>
<td>7</td>
<td>6-GlcO-ICOOH</td>
<td>b, c, d</td>
<td>C₁₅H₁₇NO₈</td>
<td>101</td>
<td>[M-H]⁻</td>
<td>338.0875 1.8</td>
</tr>
<tr>
<td>8</td>
<td>6-(MalonylGlcO)-ICOOH</td>
<td>c, d</td>
<td>C₁₅H₁₇NO₈</td>
<td>194</td>
<td>[M-H-CO₂]⁻</td>
<td>380.0982 1.3</td>
</tr>
<tr>
<td>10</td>
<td>ICHO</td>
<td>a</td>
<td>C₉H₇NO₃</td>
<td>288</td>
<td>[M-H]⁻</td>
<td>144.0452 2.0</td>
</tr>
<tr>
<td>11</td>
<td>5-GlcO-ICHO</td>
<td>b, c, d</td>
<td>C₁₀H₁₃NO₃</td>
<td>112</td>
<td>[M-H]⁻</td>
<td>322.0926 2.1</td>
</tr>
<tr>
<td>12</td>
<td>4-GlcO-ICHO</td>
<td>b, d</td>
<td>C₁₀H₁₃NO₃</td>
<td>155</td>
<td>[M-H]⁻</td>
<td>322.0932 2.2</td>
</tr>
<tr>
<td>13</td>
<td>Ascorbigen</td>
<td>a</td>
<td>C₉H₇NO₃</td>
<td>252</td>
<td>[M-H]⁻</td>
<td>304.0815 2.7</td>
</tr>
<tr>
<td>14</td>
<td>Dihydroascorbigl hexoside</td>
<td>d</td>
<td>C₂₃H₂₃NO₁₂</td>
<td>207</td>
<td>[M-H]⁻</td>
<td>468.1490 4.5</td>
</tr>
<tr>
<td>U15</td>
<td>Hexoside of</td>
<td>d</td>
<td>C₁₅H₁₇NO₈</td>
<td>149</td>
<td>[M-H]⁻</td>
<td>338.0869 3.6</td>
</tr>
<tr>
<td>U16</td>
<td>Hexoside of</td>
<td>d</td>
<td>C₁₅H₁₇NO₈</td>
<td>176</td>
<td>[M-H]⁻</td>
<td>338.0881 0.1</td>
</tr>
<tr>
<td>U17</td>
<td>Hexoside of</td>
<td>d</td>
<td>C₁₅H₁₇NO₈</td>
<td>213</td>
<td>[M-H]⁻</td>
<td>338.0872 2.7</td>
</tr>
<tr>
<td>U20</td>
<td>Unknown</td>
<td>–</td>
<td>C₁₀H₁₃NO₄</td>
<td>166</td>
<td>[M-H]⁻</td>
<td>231.0764 5.0</td>
</tr>
</tbody>
</table>

*Identification: a, identified using an authenticated standard; b, compound isolated by preparative HPLC from AgNO₃-treated Col-0 leaves and hydrolyzed using a β-glucosidase, with aglycone afterward identified using an authenticated standard; c, compound with identical chromatographic and mass spectral properties formed after feeding the respective aglycone to cyp79B2 cyp79B3 leaves; d, interpretation of mass spectral data. For completely interpreted CID mass spectra, see Supplemental Table S1.

ICOOGlc, 6-HO-ICOOGlc, 6-GlcO-ICOOH, and 6-GlcO-ICOOGlc appear as major derivatives, indicating that ICHO is either metabolized by 5-hydroxylation and O-glucosylation or oxidized to ICOOH and metabolized by 6-hydroxylation and O-glucosylation. Interestingly, exogenously supplied ICHO was also incorporated into ascorbigen and a glycosylated dihydroascorbiglucoside, suggesting that ICHO is reduced to a minor extent to indole-3-carbinol, which is subsequently trapped by ascorbic acid. A further minor metabolic route was 4-hydroxylation and O-glucosylation to give 4-GlcO-ICHO (for 4-hydroxyindole-3-carbaldehyde 4-O-β-D-glucoside).

It should be noted that all identified ICHO/ICOOH derivatives 1 to 20 were below the detection limit in nontreated/AgNO₃-treated mock-fed cyp79B2 cyp79B3 and could be reconstituted by feeding of cyp79B2 cyp79B3 with IAN. In contrast, camalexin was exclusively restored in cyp79B2 cyp79B3 by feeding with IAN but not ICHO or ICOOH.

Based on the chemical complementation studies, Figure 1 presents a putative biosynthetic scheme of ICHO/ICOOH derivatives.

Quantification of ICHO and ICOOH Derivative Pools

In order to quantify ICHO/ICOOH derivative pool sizes, methanolic extracts prepared from nontreated and AgNO₃-treated wild-type leaves were digested with a β-glucosidase, and the released indolic aglycones were analyzed by LC-MS (Fig. 2). In contrast to camalexin, which was near or below the detection limit, low constitutive levels of 4-HO-ICHO, 5-HO-ICHO, ICOOH, and 6-HO-ICOOH were already detectable in nontreated wild-type leaves. Following AgNO₃ treatment, pool sizes of all ICHO/ICOOH derivatives were found to be increased. Thereby, ICHO, ICOOH, and methyl indole-3-carboxylate (ICOOMe) showed the highest relative change in comparison with their constitutive accumulation levels. Notably, the total level of all ICHO/ICOOH derivatives increased.
Table II. Relative quantification of identified ICHO/ICOOH derivatives and camalexin in nontreated/AcNO$_3$-treated wild-type and cyp79B2 cyp79B3 leaves with or without chemical complementation

Compounds 1 to 20 were quantified by UPLC/ESI(−)-QTOFMS using extracted ion chromatograms of respective quantifier ions (Table I). Camalexin was quantified by UPLC/ESI(+) -QTOFMS via its protonated molecular ion. Values shown are median peak areas per fresh weight (counts mg$^{-1}$ × 10$^3$) ± SD (n = 5). w/o, Without; n.d., not detected.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Genotype/Treatment/Chemical Complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Col-0 w/o AgNO$_3$ Mock</td>
</tr>
<tr>
<td>1</td>
<td>ICOOH</td>
<td>n.d.</td>
</tr>
<tr>
<td>2</td>
<td>ICOOMe</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>N-ICO-Asp</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>ICOOGlc</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>ICOO/(MalonylGlc)</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>6-HO-ICOOGlc</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>6-Glco-ICOOH</td>
<td>1.17 ± 0.12</td>
</tr>
<tr>
<td>8</td>
<td>6-(MalonylGlcO)-ICOOH</td>
<td>n.d.</td>
</tr>
<tr>
<td>9</td>
<td>6-Glco-ICOOGlc</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>ICHO</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>5-Glco-ICHO</td>
<td>1.14 ± 0.05</td>
</tr>
<tr>
<td>12</td>
<td>4-Glco-ICHO</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>13</td>
<td>Ascorbigen</td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>14</td>
<td>Dihydroascorbigen hexoside</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td>15</td>
<td>Hexoside of C$_4$H$_7$NO$_3$</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>16</td>
<td>Hexoside of C$_4$H$_7$NO$_3$</td>
<td>n.d.</td>
</tr>
<tr>
<td>17</td>
<td>Hexoside of C$_4$H$_7$NO$_3$</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>19</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>20</td>
<td>–</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Camalexin
n.d. 30.3 ± 11.2  n.d. 16.7 ± 5.85 149.1 ± 1.00  n.d.  n.d. n.d.
was in the range of the camalexin level 24 h after AgNO₃ treatment.

**AAO1 Is Transcriptionally Coexpressed with Camalexin Biosynthetic Genes**

In addition to the in vitro substrate IAAld, heterologously expressed Arabidopsis AAO1 has been shown previously to convert ICHO into ICOOH with an apparent $K_m$ value of 4.4 μM (Koiwai et al., 2000). To evaluate whether AAO1 could be involved in the biosynthesis of pathogen-inducible derivatives of ICOOH, we performed a transcriptome coexpression analysis based on microarray experiments from the AtGenExpress pathogen compendium using the Expression Angler platform (Toufighi et al., 2005) with AAO1 as the query gene. Strikingly, significant coexpression with the camalexin biosynthetic genes CYP71A13 and CYP71B15, the transcription factor ANAC042, which is involved in the regulation of camalexin biosynthesis (Saga et al., 2012), and CYP71B6 (see below) was observed (Table III). A comprehensive coexpression analysis of AAO1 and CYP71B6 for different subsets of microarray and RNA sequencing data was performed applying the ATTED-II platform (Obayashi et al., 2014; Supplemental Table S2), confirming the coexpression of AAO1 and CYP71B6 with camalexin-related genes and with each other. This suggested that AAO1 and CYP71B6 might function in induced chemical defense.

**Enzymatic Activity of CYP71B6**

Previously, using *Escherichia coli* spheroplasts, in vitro turnover of IAN was demonstrated in the Halkier laboratory for CYP71B6 and ICHO was identified as a product (Nafisi, 2007). To prove this result using an independent heterologous expression system and to characterize CYP71B6 in more detail, it was expressed in the yeast (*Saccharomyces cerevisiae*) strain WAT11. NADPH-dependent IAN turnover to ICHO and to a lesser extent (approximately 17%) also to ICOOH was detected (Fig. 3, A and B). Similar to the turnover of Cys(IAN) by CYP71B15 (Böttcher et al., 2009), this reaction was accompanied by a stoichiometric NADPH-dependent release of cyanide, which was quantified after derivatization with 2,3-naphthalenedialdehyde and taurine (Fig. 3, A and C). When ICHO was applied as substrate, no ICOOH formation was observed (data not shown).

The kinetic properties of CYP71B6 were determined (Supplemental Fig. S2): apparent $k_{cat}$ = (92 ± 3) 10⁻³ s⁻¹; apparent $K_m$ (IAN) = 0.23 ± 0.02 μM. Strikingly, the $K_m$ value for its substrate is 1 to 2 orders of magnitude lower compared with other CYP71 enzymes (for CYP71C1, CYP71C2, CYP71C3, and CYP71C4, see Glawischnig et al., 1999; for CYP71B15, see Schuhegger et al., 2006). In conclusion, given a calculated catalytic efficiency of approximately 0.4 μM⁻¹ s⁻¹, CYP71B6 is effectively metabolizing IAN.

**Establishment of CYP71B6 and AAO1 Overexpression Lines**

AAO1 and CYP71B6 are low and moderately expressed in nontreated rosette leaves, respectively (http://bbc.botany.utoronto.ca/efp), whereas transcript levels of both genes are highly induced 18 h after AgNO₃ treatment (AAO1 more than 1,000-fold, CYP71B6...
approximately 282-fold; Supplemental Fig. S3). To investigate the effect of strong constitutive expression of these genes, CYP71B6 and AAO1 were cloned into pCAMBIA330035Su and corresponding overexpression lines were generated. Constitutive expression of the transgenes was evaluated by quantitative reverse transcription-PCR. Three CYP71B6 overexpression lines with more than 100-fold expression in relation to the wild type (approximately 194-fold [line 2], 178-fold [line 3], and 132-fold [line 4]) were identified, and lines 2 and 4 were selected for detailed analysis.

One AAO1 overexpression line with an approximately 373-fold expression level compared with the wild type was identified. To address whether the AAO1 genotype affects the in vitro turnover of ICHO to ICOOH, total protein extracts from rosette leaves of Columbia-0 (Col-0), the aao1 transfer DNA (T-DNA) insertion mutant, and 35S:AAO1 were prepared and analyzed for aldehyde oxidase activity using ICHO as substrate. ICHO oxidase activity was reduced significantly in aao1 and enhanced in 35S:AAO1 (Supplemental Fig. S4) in comparison with the wild type. This showed that AAO1 contributes significantly to ICOOH formation in vitro.

**Metabolite Profiling of Knockout and Overexpression Lines**

In order to elucidate the role of CYP71B6 and AAO1 in the biosynthesis of soluble ICHO/ICOOH derivatives, their accumulation levels in rosette leaves of the established CYP71B6 and AAO1 knockout and overexpression lines were determined relative to the wild type (Supplemental Table S3). In nontreated leaves, the accumulation levels of ICHO/ICOOH derivatives were not dependent on the CYP71B6 genotype (Fig. 4; Supplemental Table S4). In contrast, 24 h after the induction of ICHO/ICOOH biosynthesis by AgNO3 treatment, the level of ICOOGlc was consistently reduced in cyp71B6-1 and cyp71B6-2 to 82% (P = 2.0E-03) and 79% (P = 2.9E-03) of the wild-type level (Supplemental Table S5). A similar trend was observed for the other ICOOH derivatives, ICOOMe, 6-HO-ICOOGlc, 6-GlcO-ICOOH, and 6-GlcO-ICOOGlc, although without statistical significance. This suggests that CYP71B6 functions in ICOOH derivative biosynthesis in vivo (Fig. 1) at least after AgNO3 treatment and that functionally homologous enzymes exist. As observed for nontreated leaves, the accumulation levels of ICHO/ICOOH derivatives remained unchanged upon the overexpression of CYP71B6. In all lines, the level of the precursor IAN was below the detection limit.

In nontreated aao1 mutant leaves, the level of 5-GlcO-ICHO was increased significantly to 170% (P = 5.4E-08) of the wild-type level, whereas AAO1 overexpression resulted in a reduction of 5-GlcO-ICHO to 53% (P = 4.1E-04) of the wild-type level (Fig. 4). For the levels of ICOOGlc, 6-GlcO-ICOOH, and 6-GlcO-ICOOGlc, the opposite trend to that observed for 5-GlcO-ICHO became apparent in AAO1 knockout and overexpression lines compared with the wild type, albeit with a lower effect size and hence without statistical significance. However, following direct pairwise comparison of AAO1 knockout and overexpression lines, the difference in the accumulation levels of 6-GlcO-ICOOH and 6-GlcO-ICOOGlc was statistically significant (P < 2.0E-03). After AgNO3 treatment, the level of 5-GlcO-ICOH was again increased significantly in aao1 to 139% (P = 3.1E-09) of the wild-type level (Fig. 4). In contrast, AAO1 overexpression correlated with significantly decreased levels of both ICHO and 5-GlcO-ICOH, which accumulated to 64% (P = 7.7E-04) and 60% (P = 3.6E-09) of the wild-type level.

The observed chemotypes of AAO1 knockout and overexpression lines suggest that AAO1 is involved in the oxidation of ICHO to ICOOH in both nontreated and AgNO3-treated leaves (Fig. 1).

To test whether CYP71B6 and AAO1 knockout and overexpression impact the level of cell wall-associated ICOOH, cell wall preparations of nontreated and AgNO3-treated rosette leaves of wild-type and mutant
plants were hydrolyzed, and the ICOOH released was quantified (Supplemental Fig. S5). As observed for the soluble ICOOH fraction of wild-type leaves, the level of cell wall-bound ICOOH increased strongly 24 h after AgNO₃ (more than 10-fold) in comparison with nontreated leaves. Neither CYP71B6 nor AAO1 knockout or overexpression resulted in a pronounced chemotype for cell wall-bound ICOOH in AgNO₃-treated or non-treated leaves, reflecting the observations made for the soluble ICOOH fraction.

**DISCUSSION**

**Proposed Biosynthetic Pathways of ICHO-Derived Secondary Metabolites**

In a number of Arabidopsis metabolite profiling studies including different organs, derivatives of ICHO and ICOOH were detected and their concentrations were shown to increase upon pathogen infection (Hagemeier et al., 2001; Bednarek et al., 2005; Böttcher et al., 2009). Nevertheless, while the biosynthetic pathway and the biological function of the characteristic Arabidopsis phytoalexin camalexin are well understood, these metabolites have been largely neglected. This is probably due to the formation of a rather complex blend of derivatives instead of one striking major metabolite. In a previous study (Böttcher et al., 2009), we have shown that they are synthesized via CYP79B2- and CYP79B3-dependent IAOx formation and, according to incorporation experiments, via IAN. Here, we have similarly applied ICHO and ICOOH to cyp79B2 cyp79B3 leaves and subsequently used LC-MS-based metabolite profiling to explore the composition of ICHO and ICOOH derivatives. As major compounds, Glc conjugates of 5-HO-ICHO, ICOOH, and 6-HO-ICOOH were identified. The pattern of metabolites in chemically complemented cyp79B2 cyp79B3 leaves was consistent with a biosynthetic pathway via IAN, ICHO, and ICOOH for ICOOH derivatives. As observed for camalexin (Böttcher et al., 2009), hydroxylated derivatives of ICHO and ICOOH were found to accumulate, notably with a distinct substitution pattern. While 5-HO-ICHO and 6-HO-ICOOH were identified as major aglycones after β-glucosidase treatment, the levels of 6-hydroxyindole-3-carboxaldehyde (6-HO-ICHO) and 5-hydroxyindole-3-carboxylic acid (5-HO-ICOOH) were below the detection limit. This indicates that either ICHO and ICOOH are specifically hydroxylated in vivo in the 5- and 6-positions, respectively, or alternatively, intermediary 6-OH-ICHO is selectively oxidized to 6-HO-ICOOH.

Chemical complementation of cyp79B2 cyp79B3 with ICHO revealed that ICHO is incorporated to a minor extent into 4-GlcO-ICHO, ascorbigen, and dihydroascorbigen hexoside. Since ascorbigen is a well-known breakdown product of IMG (Agerbirk et al., 2009) and 4-hydroxylation of the indole ring represents a characteristic modification reaction of IMG (Pfalz et al., 2009), it can be assumed that the native accumulation of these metabolites in nontreated and AgNO₃-treated wild-type leaves is probably associated with indole glucosinolate metabolism. The artificial incorporation of exogenously supplied ICHO into these putative glucosinolate catabolites might be caused by high concentrations of ICHO in leaf tissue during the feeding experiment.

In turnip (Brassica rapa) roots, ICHO and ICOOH were identified as primary in vivo breakdown products of isotopically labeled IMG (Pedras et al., 2002). For Arabidopsis, it is currently unclear whether IMG catabolism additionally contributes, via oxidation of the primary formed indole-3-carbinol, to the accumulation of ICHO and ICOOH derivatives. However, the action of this pathway could explain the basal levels of ICHO and ICOOH derivatives in nontreated leaves.

Recently, it was speculated that camalexin could be synthesized via an alternative pathway involving GH3.5-catalyzed conjugation of ICOOH with Cys (Wang et al., 2012). We have not detected any camalexin in cyp79B2 cyp79B3 plants incubated with ICHO or ICOOH. This indicates that this reaction is not relevant in vivo and that, rather, GH3.5 overexpression results in stress reactions, including the observed induction of established biosynthetic genes.
Contribution of AAO1 and CYP71B6 to the Biosynthesis of ICOOH-Derived Secondary Metabolites in Arabidopsis

CYP71B6 and AAO1 catalyze the formation of ICHO/ICOOH and cyanide, respectively, in vitro and are transcriptionally coexpressed with known enzymes of camalexin biosynthesis. Therefore, we have analyzed the profile of IAOx-derived secondary metabolites in knockout and overexpression plants of CYP71B6 and AAO1. Most strikingly, aao1 T-DNA insertion mutants accumulate 5-GlcO-ICHO, while this derivative of the intermediate ICHO is significantly reduced in AAO1 overexpressors. This is in accordance with the frequent observation (Geu-Flores et al., 2009) that the effects on the level of a substrate (derivative) are highly significant, in this case $P < 0.001$ (Student’s $t$ test), while the effect on the product spectrum is subtle when partially redundant enzymes are expressed. Candidates for functioning redundantly to AAO1 are Arabidopsis AAO2 and CYP71B6, for which we have shown in vitro that it synthesizes ICOOH. The level of 4-GlcO-ICHO was not affected by AAO1 knockout or overexpression either in nontreated or AgNO$_3$-treated leaves, supporting the hypothesis that this ICHO derivative has a different biosynthetic origin than 5-GlcO-ICHO.

We observed reduced levels of ICOOOGlc in AgNO$_3$-induced *cyp71B6* knockout mutants in relation to wild-type plants, while for untreated plants no difference was observed. CYP71B6 is a member of a large gene family, and we speculate that other cytochrome P450 enzymes, such as CYP71A12/A13 (Klein et al., 2013), have overlapping biochemical functions and expression patterns, explaining the rather mild metabolic phenotype of *cyp71b6* knockout mutants, despite the high catalytic efficiency of CYP71B6 for its substrate IAN (approximately $0.4 \mu M^{-1} s^{-1}$) in vitro. To evaluate the function of ICHO-derived metabolites in pathogen resistance in vivo, it will be necessary to analyze multiple mutants or to engineer the whole pathway for overexpression. CYP71B6 overexpression alone did not result in a further enhancement of ICOOH derivative levels, indicating that this biosynthetic step is not rate limiting in wild-type plants.

In vitro, IAN turnover by CYP71B6 resulted in ICOOH as a product in addition to ICHO, while no direct turnover of ICHO was detected. We speculate that an $\alpha$-hydroxynitril is formed as an unstable intermediate, which decomposes to ICHO and hydrogen cyanide. Possibly, this intermediate can be oxidized a second time by CYP71B6, and subsequently, this $\alpha$, $\alpha$-dihydroxynitrile decomposes to ICOOH and hydrogen cyanide. Dual hydroxylation of the same position has been shown, for example, for CYP79 enzymes (Sibbesen et al., 1994). In the CYP71B6-catalyzed IAN turnover, stoichiometric amounts of cyanide were released in vitro. Previously, we searched for the established cyanide metabolites $\beta$-cyanoalanine and $\gamma$-glutamyl-$\beta$-cyanoalanine in extracts of AgNO$_3$-treated leaves, which were not detectable (Böttcher et al., 2009). Possibly, cyanide released by CYP71B6 and other enzymes catalyzing the same reaction is recycled efficiently into central metabolism. As both cytochrome P450s as well as AAO1 (Zarepour et al., 2012) are inhibited by cyanide, rapid removal of this by-product is necessary to prevent shutdown of the biosynthetic pathway. Recently, it was demonstrated that low amounts of cyanide (approximately 0.08 nmol g$^{-1}$ fresh weight) accumulate in response to *Botrytis cinerea* infection (García et al., 2013). We propose that, in addition to ethylene biosynthesis, IAN (this study) and Cys(IAN) (Böttcher et al., 2009) turnover might be another source of cyanide. As in response to *B. cinerea* infection camalexin alone is synthesized in concentrations...

Figure 3. CYP71B6 enzymatic activity. CYP71B6 was expressed in yeast, and enzymatic turnover of IAN (5 nmol, 200 $\mu$m) to ICHO, ICOOH, and cyanide was detected. A, Product spectrum ($n = 9$). Cyanide (CN$^-$) is a stoichiometric by-product. B and C, Representative HPLC chromatograms. B, Detection of indolic compounds at 278 nm. mAU, Milliabsorbance units. C, Detection of a cyanide derivative. Fluorescence, 418 nm (excitation)/460 nm (emission).
approximately 1,000-fold higher (Denby et al., 2004) with respect to cyanide (García et al., 2013), also here, stoichiometrically synthesized cyanide was efficiently detoxified. In mutants partially defective in cyanide detoxification, genes involved in defense are transcriptionally up-regulated (García et al., 2013). This indicates that the remaining cyanide might contribute to a positive feedback loop.

Evolution of Phytoalexin Biosynthesis in the Brassicaceae

In response to AgNO$_3$, we detected overall levels of ICHO-derived metabolites similar to camalexin, suggesting a major biological function. Recently, the phylogenetic distribution of indolic phytoalexins in cruciferous plants was studied (Bednarek et al., 2011). It was demonstrated that ICOOH derivatives are also present in the more distantly related Cardamine hirsuta in addition to camalexin-synthesizing members of the Brassicaceae. Therefore, we postulate that the formation of ICHO from IAN by enzymes such as CYP71B6 is a more ancient reaction, from which possibly the activation of IAN and subsequent conjugation with glutathione, yielding glutathione-IAN, has evolved in the common ancestor of Capsella species and Arabidopsis.

ICHO or ICOOH has been detected in species outside the Brassicaceae, including pea (Pisum sativum) seedlings, where ICHO was identified as an inhibitor of lateral bud growth (Nakajima et al., 2002), and the gymnosperm Pinus sylvestris (Sandberg et al., 1984). Here, approximately 14 pmol g$^{-1}$ fresh weight ICOOH was found in needles, which is more than 2 orders of magnitudes less than in unchallenged Arabidopsis leaves (Fig. 2). ICOOH has been attributed to the degradation of IAA. This auxin degradation pathway is not yet understood in detail. We speculate that ICHO and ICOOH or derivatives thereof can be frequently observed in plants in very small amounts as IAA degradation products. In the Brassicaceae, this route probably contributes to only a very minor extent to the biosynthesis of these compounds.

Aldehyde oxidases are widely distributed in genomes of plants, even in species for which no ICOOH derivatives are described. We hypothesize that, during the evolution of ICOOH biosynthesis in a common ancestor of Arabidopsis and Cardamine spp., an aldehyde oxidase with broad substrate specificity was recruited. Then, a pathogen-inducible expression pattern and ICHO as a preferred substrate evolved.

24 h after spraying 5 mM AgNO$_3$ (B). Metabolites for which significant differences were observed are shown. The full data set is presented in Supplemental Tables S4 and S5. Error bars indicate se (n = 16–18). Significance analysis of differences between the wild type and mutant was performed by Student’s t test (two-tailed, unequal variances; *P ≤ 10E-02, **P ≤ 10E-03, ***P ≤ 10E-04).
MATERIALS AND METHODS

Chemicals
5-HO-ICHO and 6-HO-ICHO were prepared from 5- and 6-hydroxyindole (Sigma-Aldrich) by Vilsmeier-Haack reaction using dimethylformamide and phosphoryl chloride. Ascorbigen A was prepared from indole-3-carbinol (Sigma-Aldrich) and ascorbic acid (Kiss and Neukomm, 1966). 4-HO-ICHO was purchased from Apollo Scientific (www.apolloscientific.co.uk), 4-methoxyindole-3-carbaldehyde from Chem-Impex International (www.chemimpex.com), 5-HO-ICOOH from Debye Scientific (www.debyesci.com), 6-HO-ICOOH from Sphinx Scientific Laboratory (www.sphinxscientificlab.com), and ICOOME from OlChemim (www.olchemim.cz). ICHO, 5-methoxyindole-3-carbaldehyde (5-MeO-ICHO), and ICOOOH were obtained from Sigma-Aldrich.

Plant Growth
For feeding and metabolite profiling experiments, Arabidopsis (Arabidopsis thaliana) wild-type and mutant lines were sown on a soil:vermiculite mixture (3:2), cold stratified for 3 d at 4°C, and grown in parallel in a growth cabinet with 8 h of light (approximately 150 μmol m−2 s−1)/22°C and 16 h of dark/21°C at 60% relative humidity. After 2 weeks, plants were transplanted into 5-cm pots, randomly arrayed in four-by-six grids in trays, and grown for an additional 4 weeks to developmental stage 3.5 (Boyes et al., 2001). For transformation and for RNA and protein isolation, plants were grown in a growth chamber with a 12/12-h photoperiod at 80 to 100 μmol m−2 s−1, 21°C, and 40% relative humidity.

In Vivo Feeding Experiments
Six-week-old Col-0 and cyp79B2 cyp79B3 (Zhao et al., 2002) plants were sprayed with 5 mM AgNO3, or water 1 h before the beginning of the photoperiod. After 2 h, four uniform rosette leaves per plant were excised at the petiole and immersed in PCR tubes containing 200 μL of the corresponding feeding solution (200 μM indole derivative in ethanol:water, 2:98 [v/v] or ethanol:water, 2:98 [v/v] as a control). After incubation for 24 h, rosette leaves were pooled, frozen, and homogenized in liquid nitrogen. Ground leaf material (50 ± 3 mg) was extracted twice with 200 μL of methanol:water, 80:20 (v/v) as described previously (Böttcher et al., 2009). The combined extracts were evaporated to dryness in a vacuum centrifuge at 30°C. The remaining residue was reconstituted in 100 μL of methanol:water, 30:70 (v/v), sonicated for 10 min at 20°C, and centrifuged at 16,000g for 10 min. The supernatant was analyzed by ultra-performance liquid chromatography (UPLC)/electrospray ionization (ESI)-QTOFMS in positive and negative ion mode using method 1. Five individual plants were processed for each experimental condition.

Identification of Aglycones from Glycosylated Indole Derivatives
Six-week-old, AgNO3-sprayed (5 mM, 24-h treatment) Col-0 leaves (1.5 g) were extracted twice with a total of 6 mL of methanol:water, 80:20 (v/v) as described previously (Böttcher et al., 2009). The combined extracts were evaporated to dryness in a vacuum centrifuge at 30°C. The remaining residue was redissolved in 1 mL of water:formic acid, 99.9:0.1 (v/v), sonicated for 10 min at 20°C, and centrifuged at 16,000g for 10 min. The supernatant was fractionated by preparative HPLC (YM ODS-A; 150-× 20-mm; pore size, 120 Å; particle size, 5 μm; flow rate, 10 mL min−1; gradient program: 0–1 min, isocratic, 95% A [water:formic acid, 99.9:0.1 [v/v]]), 3% B [acetonitrile:formic acid, 99.9:0.1 [v/v]); 1–31 min, linear from 5% B to 20% B; injection volume, 500 μL; fraction size, 10 μL). Fractions were evaporated to dryness in a vacuum centrifuge at 30°C, redissolved in 500 μL of water, and screened for glycosylated indole derivatives by UPLC/ESI-QTOFMS in negative ion mode using method 1. Aliquots of respective fractions were digested with β-glucosidase in sodium acetate buffer (for procedure, see below). Released aglycones were repeatedly extracted from the acidified aqueous layer with ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness in a vacuum centrifuge at 30°C, reconstituted in 50 μL of methanol:water, 30:70 (v/v), and analyzed by UPLC/ESI-QTOFMS using method 2. Aglycones were identified by comparison of retention times and mass spectral data in relation to authenticated standards.

Determination of the Total Content of Substituted ICHO/ICOOH after β-Glucosidase Treatment
Ground leaf material (100 ± 5 mg) was spiked with 1 nmol of 5-MeO-ICHO as an internal standard and extracted twice with 200 μL of methanol:water, 80:20 (v/v) as described previously (Böttcher et al., 2009). The combined extracts were evaporated to dryness in a vacuum centrifuge at 30°C. To the remaining residue, 200 μL of sodium acetate buffer (50 mM, pH 5.0, adjusted with HCl) was added, and the mixture was sonicated for 10 min at 20°C. For enzymatic deglucosylation, the mixture was incubated with 2 units of β-glucosidase (EC 3.2.1.21; Sigma-Aldrich) under continuous shaking at 37°C for 2 h. After incubation with 100 μL of HCl (1 M), the mixture was repeatedly extracted with 3 × 100 μL of ethyl acetate. The combined organic extracts were evaporated to dryness in a vacuum centrifuge at 30°C. The remaining residue was reconstituted in 100 μL of methanol:water, 30:70 (v/v), sonicated for 10 min at 20°C, and centrifuged at 16,000g for 10 min. The supernatant was analyzed by UPLC/ESI-QTOFMS using method 2.

Relative response factors (RRFs) of individual target compounds in comparison with the internal standard 5-MeO-ICHO were obtained by analysis of dilution series prepared from the pure reference compounds in methanol:water, 30:70 (v/v). For quantification, extracted ion chromatograms (mass-to-charge ratio [m/z] width ± 0.02) of corresponding protonated molecular ions were integrated: ICOOH (retention time [t] 162.06, RRF 0.28), 5-MeO-ICHO (t 176.07, RRF 0.16), 5-HO-ICOOH (retention time [t] 190 s, m/z 176.05, RRF 0.16), 6-HO-ICOOH (t 198 s, m/z 175.05, RRF 0.01), 6-HO-ICOOH (t 175.05, m/z 175.05, RRF 0.01), ICOOH (t 173.07, RRF 1.00), and camalexin (t 423 s, m/z 201.05, RRF 3.24).

Proiling of Indole Derivatives in Wild-Type and Mutant Plants
Wild-type, knockout, and overexpression plants (20 plants per genotype) were grown in parallel for 6 weeks. One hour after the beginning of the photoperiod, half of the plants was sprayed with 5 mM AgNO3, whereas the other half remained without any treatment. After 24 h, rosette leaves of eight to nine uniform plants per genotype and treatment were harvested, individually pooled for each plant, and frozen and homogenized in liquid nitrogen. Ground leaf material (100 ± 5 mg) was extracted twice with 200 μL of methanol:water, 80:20 (v/v) as described previously (Böttcher et al., 2009). The combined extracts were evaporated to dryness in a vacuum centrifuge at 30°C. The remaining residue was reconstituted in 200 μL of methanol:water, 30:70 (v/v), sonicated for 10 min at 20°C, and centrifuged at 16,000g for 10 min. The supernatant was analyzed by UPLC/ESI-QTOFMS in negative ion mode using method 1. For relative quantification of indole derivatives, extracted ion chromatograms (m/z width ± 0.02) using quantifier ions/retention times from Table I were integrated. Two independent replicate experiments were performed, resulting in a total of 16 to 18 individual plants per genotype and treatment.

Quantification of Cell Wall-Bound ICOOH
Ground leaf material (approximately 150 mg) was sequentially extracted under sonication for 15 min at 40°C with 2 × 600 μL of methanol:water, 50:50 (v/v), 2 × 600 μL of methanol, and 2 × 600 μL of acetone. The remaining residue was dried at 30°C in a vacuum centrifuge and precisely weighed into a 2-mL polypropylene tube. Alkaline hydrolysis of cell wall material (5–7 mg per sample) was performed after the addition of 500 μL of NaOH (1 M; thoroughly degassed with N2) and 5 μL of ascorbic acid (10%, w/v) for 20 h at 70°C under continuous shaking. After acidification with 60 μL of 50% HCl (37%, w/v), the mixture was extracted with 3 × 400 μL of ethyl acetate. The combined organic extracts were evaporated to dryness in a vacuum centrifuge at 30°C. The remaining residue was reconstituted in 60 μL of methanol:water, 30:70 (v/v), sonicated for 10 min at 20°C, and centrifuged at 16,000g for 10 min. The supernatant was analyzed by UPLC/photodiode array/ESI-QTOFMS in negative ion mode using method 1. ICOOH was identified by mass spectrometry and quantified by integration of the UV chromatogram at 281 nm using an external calibration.

UPLC/ESI-QTOFMS
Method 1. Chromatographic separations were performed at 40°C on an Acquity UPLC system (Waters) equipped with an HSS T3 column (100 ×
1 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, isotropic 95% A (water/formic acid, 99.9:0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9:0.1 [v/v]); 1 to 10 min, linear from 5% to 60% B; 10 to 12 min, isotropic 95% B; 12 to 14 min, isotropic 5% B. The injection volume was 2.6 μL (full loop injection). Eluted compounds were detected from m/z 100 to 1,000 using a Micromass Quattro micro QTOFMS (Bruker Daltonics) equipped with an Apollo II electrospray ion source in positive (negative) ion mode. The following instrument settings were applied: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 L min⁻¹; 190°C; capillary, −5,000 V (+4,000 V); end plate offset, −500 V; funnel 1 radio frequency (RF), 200 Volts peak-to-peak (Vpp); funnel 2 RF, 200 Vpp; in-source collision-induced dissociation (CID) energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 3 eV; collision gas, argon; collision energy, 3 eV (10 eV); collision RF, 200/400 Vpp (timing 30/50); transfer time, 70 μs; prepulse storage, 5 μs; pulse frequency, 10 kHz; mass spectra rate, 5 Hz.

Method 2. Chromatographic separations were performed at 50°C on an Aquasphere UPLC system equipped with a BEHShield RP18 column (150 × 1 mm, particle size 1.7 μm; Waters) applying the same binary gradient as described above but using a flow rate of 140 μL min⁻¹. The injection volume was 2.6 μL (full loop injection). Eluted compounds were detected from m/z 75 to 700 using a Micromass Quattro micro QTOFMS in positive ion mode. Instrument settings were as described above for positive ion mode with the exception of one parameter: collision RF, 150/200 Vpp (timing 50/50).

Mass spectra were acquired in centroid mode. Calibration of the m/z scale was performed for individual raw data files on lithium formate cluster ions obtained by automatic infusion of 20 μL of 10 mM lithium hydroxide in isopropanol/water/formic acid, 49.9:49.9:0.2 (v/v) at the end of the gradient using a diverter valve. CID mass spectra were acquired as described previously (Böttcher et al., 2009). Putative elemental compositions were determined on the basis of accurate mass and isotope pattern matching using the SmartFormula algorithm implemented in DataAnalysis 4.0 (Bruker Daltonics) and checked for consistency in relation to elemental compositions calculated for fragment ions and neutral losses from respective CID mass spectra.

Establishment of 35S:AAO1, 35S:CYP71B6, and T-DNA Insertion Lines

The AAO1 complementary DNA (cDNA) clone was kindly provided by Tomokazu Koshiba and Mitsunori Seo. AA01 and CYP71B6 coding sequences were amplified from the cDNA clone and yeast expression plasmid (see below), respectively, using the primer pairs 5'-GGCTTAAATAGTCCTTTCTC-CTTTCC-3' / 5'-GGGTAAATCACTGGCCGGTATGTA-3' and 5'-GGCTTAATGGTGAAGCAACATTGAC-3' / 5'-GGTAAATTAAACATTATTTCCTTTCTG-3', respectively, and cloned into pYEDP60u via USER technology (Nour-Eldin et al., 2006), and transformed into Saccharomyces cerevisiae WAT11 (Pompon et al., 1996). Yeast microsomes were prepared as described by Schuhegger et al. (2006). Cyanide derivatization and HPLC analysis were performed as described previously (Böttcher et al., 2009). The concentration of active P450 was determined by carbon monoxide differential spectroscopy (Omura and Sato, 1964). For analysis of the enzymatic parameters, reactions were performed in 1 mL of potassium phosphate buffer (20 mM, pH 7.5) for 20 min. The reactions were stopped by the addition of 800 μL of methanol, dried in vacuum, and resolved in 200 μL of methanol/water, 4:1 (v/v). IAN, ICHO, and ICOOH were analyzed by reverse-phase HPLC (LiChroCART 250-4, RP-18, 5 μm, Merck; flow rate, 1 mL min⁻¹; gradient: 0-2 min, isotropic, 20% A [water/formic acid, 99.7:0.3 [v/v]]; 20% B [acetonitrile]; 2-12 min, linear from 20% to 38% B; 12-13 min, linear from 38% to 100% B; 13-15 min, isotropic, 100% B) equipped with a photodiode array detector (Dionex). The product peaks were identified and quantified by comparison with authentic standards: ICOOH, tᵣ = 10.5 min, absorption maximum (λ_max) = 281 nm; ICHO, tᵣ = 11.4 min, λ_max = 297 nm. Boiled protein sample was provided as a negative control.

Yeast Expression of CYP71B6

The CYP71B6 coding sequence was amplified from total Arabidopsis Col-0 leaf cDNA using the primers 5'-GGATTTAATGTCTTTCTC-CAATTTTCCCTGAC-3'/5'-GGTTAAATTTAATCCTGGTGATGATCATT-3', cloned into pYEDP60u via USER technology (Nour-Eldin et al., 2006), and transformed into Saccharomyces cerevisiae WAT11 (Pompon et al., 1996). Yeast microsomes were prepared as described by Schuhegger et al. (2006). Cyanide derivatization and HPLC analysis were performed as described previously (Böttcher et al., 2009). The concentration of active P450 was determined by carbon monoxide differential spectroscopy (Omura and Sato, 1964). For analysis of the enzymatic parameters, reactions were performed in 1 mL of potassium phosphate buffer (20 mM, pH 7.5) for 20 min. The reactions were stopped by the addition of 800 μL of methanol, dried in vacuum, and resolved in 200 μL of methanol/water, 4:1 (v/v). IAN, ICHO, and ICOOH were analyzed by reverse-phase HPLC (MultiHigh 100 RP18-Su, Göhler Analytik; flow rate, 1 mL min⁻¹; gradient: 0-2 min, isotropic, 20% A [water/formic acid, 99.7:0.3 [v/v]]; 20% B [acetonitrile]; 2-12 min, linear from 20% to 28% B; 12-19 min, linear from 28% to 50% B; 19-22 min, linear from 50% to 100% B; 22-24 min, isotropic, 100% B) equipped with a photodiode array detector (Dionex). Quantification was based on calibration curves with authentic standards: ICOOH, tᵣ = 14.7 min; ICHO, tᵣ = 15.7 min; IAN, tᵣ = 22.0 min; λ_max = 278 nm.

Accession numbers are as follows: AAO1, A65s20960; CYP71B6, A2tg24180.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Identification of molecular features associated with ICHO/ICOOH derivatives whose accumulation depends on CYP79B2/B3 by combining chemical complementation of CYP71B6 with ICHO/ICOOH derivatives.

Supplemental Figure S2. Enzymatic parameters of CYP71B6.

Supplemental Figure S3. AAO1 and CYP71B6 transcript abundance in corresponding T-DNA insertion lines.

Supplemental Figure S4. In vitro oxidation of ICHO.

Supplemental Figure S5. Quantification of cell wall-bound ICOOH in root tissue of nontreated (gray bars) and AgNO₃-treated (24 h after treatment; black bars) wild-type (Col-0) and mutant plants.

Supplemental Table S1. CID mass spectral data of identified ICHO/ICOOH derivatives.

Supplemental Table S2. Comprehensive coexpression analysis of AA01 and CYP71B6, applying the ATTED-II platform.

Supplemental Table S3. Relative quantification of ICHO/ICOOH derivatives in nontreated leaves of wild-type and mutant/overexpressor plants nontreated and 24 h after AgNO₃ treatment.

Supplemental Table S4. Relative quantification of ICHO/ICOOH derivatives, statistical analysis.

Aldehyde Oxidase Activity in Plants

Arabidopsis leaf material (2 g) was extracted in 10 mL of protein extraction buffer 1 (50 mM Tris, 1 mM EDTA, 1 mM sodium molybdate, 10 μM FAD, and 2 mM dithiothreitol, pH 7.5), adding 0.2 g g⁻¹ fresh weight Polyclar-AT (Serva), and centrifuged at 17,400g for 15 min at 4°C. Ammonium sulfate was slowly added to the supernatant to a final concentration of 70% (4.28 g). After 1 h of stirring at 4°C and centrifuging at 17,400g for 10 min, the pellet was resuspended in 1 mL of protein extraction buffer 2 (20 mM Tris, 1 mM EDTA, 1 mM sodium molybdate, 10 μM FAD, and 2 mM dithiothreitol, pH 7.5), desalted using an NAP-10 column (GE Healthcare), and eluted in 1.5 mL of protein extraction buffer 2. Protein concentration was determined via Bio-Rad protein assay, 1.0 mg of protein was incubated with 125 μL ICHO for 30 min at 25°C in the same buffer, and the reaction was stopped by the addition of methanol (2 volumes) and analyzed by reverse-phase HPLC (LiChroCART 250-4, RP-18, 5 μm, Merck; flow rate, 1 mL min⁻¹; gradient: 0-2 min, isotropic, 20% A [water/formic acid, 99.7:0.3 [v/v]]; 20% B [acetonitrile]; 2-12 min, linear from 20% to 38% B; 12-13 min, linear from 38% to 100% B; 13-15 min, isotropic, 100% B) equipped with a photodiode array detector (Dionex). The product peaks were identified and quantified by comparison with authentic standards: ICOOH, tᵣ = 10.5 min, absorption maximum (λ_max) = 281 nm; ICHO, tᵣ = 11.4 min, λ_max = 297 nm. Boiled protein sample was provided as a negative control.

Indole-3-Carboxylic Acid Biosynthesis


Copyright © 2014 American Society of Plant Biologists. All rights reserved.


