Biphenyl 4-Hydroxylases Involved in Aucuparin Biosynthesis in Rowan and Apple Are Cytochrome P450 736A Proteins

Debabrata Sircar, Mariam M. Gaid, Cornelia Chizzali, Dennis Reckwell, David Kaufholdt, Till Beuerle, Giovanni A.L. Brogini, Henryk Flachowsky, Benye Liu, Robert Hänsch, and Ludger Beerhues*

Institute of Pharmaceutical Biology (D.S., M.M.G., D.R., T.B., B.L., L.B.) and Institute of Plant Biology (D.K., R.H.), Technische Universität Braunschweig, 38106 Braunschweig, Germany; Plant Pathology, Institute of Integrative Biology, Eidgenössische Technische Hochschule Zürich, 8092 Zurich, Switzerland (C.C., G.A.L.B.); and Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Horticultural and Fruit Crops, 01326 Dresden, Germany (H.F.)

ORCID IDs: 0000-0002-2788-119X (D.S.); 0000-0002-7290-0628 (M.M.G.); 0000-0001-9337-4527 (C.C.); 0000-0001-5449-0170 (D.R.); 0000-0001-5037-6653 (T.B.); 0000-0001-8527-565X (B.L.); 0000-0002-2147-2731 (L.B.).

Upon pathogen attack, fruit trees such as apple (Malus spp.) and pear (Pyrus spp.) accumulate biphenyl and dibenzofuran phytoalexins, with aucuparin as a major biphenyl compound. 4-Hydroxylation of the biphenyl scaffold, formed by biphenyl synthase (BIS), is catalyzed by a cytochrome P450 (CYP). The biphenyl 4-hydroxylase (B4H) coding sequence of rowan (Sorbus aucuparia) was isolated and functionally expressed in yeast (Saccharomyces cerevisiae). SaB4H was named CYP736A107. No catalytic function of CYP736 was known previously. SaB4H exhibited absolute specificity for 3-hydroxy-5-methoxybiphenyl. In rowan cell cultures treated with elicitor from the scab fungus, transient increases in the SaB4H transcript level was observed with phenylalanine ammonia lyase transcript levels preceding phytoalexin accumulation. Transient expression of a carboxyl-terminal reporter gene construct directed SaB4H to the endoplasmic reticulum. A construct lacking the amino-terminal leader and transmembrane domain caused cytoplasmic localization. Functional B4H coding sequences were also isolated from two apple (Malus × domestica) cultivars. The MdB4Hs were named CYP736A163. When stems of cv Golden Delicious were infected with the fire blight bacterium, highest MdB4H transcript levels were observed in the transition zone. In a phylogenetic tree, the three B4Hs were closest to coniferaldehyde 5-hydroxylases involved in lignin biosynthesis, suggesting a common ancestor. Coniferaldehyde and related compounds were not converted by SaB4H.

Rosaceous species include a number of valuable fruit trees, which are widespread in the temperate regions of the world. Economically important fruits are the pome fruits, such as apple (Malus spp.) and pear (Pyrus spp.), the stone fruits, including cherry, peach, plum, apricot, and almond (all Prunus spp.), and the berry fruits, such as strawberry (Fragaria spp.) and blackberry and raspberry (both Rubus spp.). All these fruits are widely consumed for their tastes, nutritional values, and contents of health-promoting metabolites. In addition, the Rosaceae include a number of widespread ornamental species. A large variety of rose (Rosa spp.) cultivars were bred, and hawthorn (Crataegus spp.) and rowan (Sorbus spp.) are common as ornamental trees.

Over the centuries, cultivars with favorable properties were selected; however, vegetative propagation by grafting led to genetic uniformity and decreased resistance to pests and diseases (Norelli et al., 2003; Gessler and Patocchi, 2007). The defense potential of plants consists of multiple strategies, one of which is the accumulation of phytoalexins (Dixon, 2001). Plants have elaborated an amazing array of more than 200,000 natural products, many of which confer selective advantage against herbivore and pathogen attacks (Dixon, 2001; Hartmann, 2007). Two closely related classes of phytoalexins are biphenyls and dibenzofurans, which are formed by apple, pear, and related fruit trees. This group of pome-bearing species has long been known as Rosaceae subfamily Maloideae but recently was reclassified as the subtribe Malinae of the subfamily Amygdaloideae (temporarily...
Pyrinae of the Spiraeoideae; Campbell et al., 2007; Potter et al., 2007).

Two decades ago, Kokubun and Harborne (1995) identified biphenyls and dibenzofurans as the phytoalexins of the Malinae. So far, 10 biphenyls and 17 dibenzofurans have been detected, which are formed de novo in response to biotic and abiotic stress (Chizzali and Beerhues, 2012). The occurrence of pathogen-induced biphenyl and dibenzofuran compounds is confined to the Malinae (Kokubun and Harborne, 1995). However, some taxa other than the Malinae contain biphenyls and dibenzofurans as preformed constituents, which contribute to the constitutive defense barrier (Hüttner et al., 2010). The majority of the biphenyl and dibenzofuran phytoalexins were found in response to fungal attack, either natural infection or artificial inoculation (Chizzali and Beerhues, 2012). A single article reports the accumulation of biphenyls and dibenzofurans after bacterial challenge (Chizzali et al., 2012b). The phytoalexins inhibit spore germination, germ tube development, and mycelial growth as well as bacterial propagation, although the underlying mechanisms of antimicrobial action are still unknown (Chizzali and Beerhues, 2012).

The most common biphenyl phytoalexin is aucuparin, which was detected in eight Malinae species after pathogen attack (Chizzali and Beerhues, 2012). The scaffold of this compound is formed by biphenyl synthase (BIS), a type III polyketide synthase, which catalyzes the condensation of the unusual starter substrate benzoyl-CoA with three molecules of malonyl-CoA to yield 3,5-dihydroxybiphenyl (Fig. 1; Liu et al., 2004, 2007). The benzoyl moiety of the starter substrate stems from L-Phe, whose carbon skeleton is channeled into the phytoalexin biosynthetic pathway by the action of phenylalanine ammonia lyase (PAL; Hanson and Havir, 1981). Little is known about the conversion of the PAL product, trans-cinnamic acid, to benzoyl-CoA in the Malinae. Benzaldehyde dehydrogenase was detected in chitosan-treated rowan (Sorbus aucuparia) cell cultures, which serve as an in vitro system for studying biphenyl and dibenzofuran metabolism in the Malinae (Gaïd et al., 2009; Hüttner et al., 2010). The first complementary DNA (cDNA) encoding BIS was isolated from these cell cultures after elicitor treatment (Liu et al., 2007). The elicitors that efficiently induce phytoalexin formation in rowan cell cultures include preparations from the fungus Venturia inaequalis, the causal agent of scab, and the bacterium Erwinia amylovora, the causal agent of fire blight (Hüttner et al., 2010). These infections are among the most devastating diseases encountered in apple orchards (MacHardy, 1996; Vanneste, 2000). Recently, the downstream enzymes that metabolize the BIS product, 3,5-dihydroxybiphenyl, have been studied in cell-free extracts and microsomes from rowan cell cultures after the addition of an aqueous elicitor preparation from V. inaequalis (Khalil et al., 2013). Interestingly, the sequence of the biosynthetic reactions leading from 3,5-dihydroxybiphenyl to aucuparin was O-methylation → 4-hydroxylation → O-methylation (Fig. 1). The alkylation steps were catalyzed by two distinct O-methyltransferases, and the hydroxylation reaction was catalyzed by a cytochrome P450 (CYP), which was named biphenyl 4-hydroxylase (B4H; Khalil et al., 2013).

Here, we report the isolation of the SaB4H coding sequence using a subtracted cDNA library of rowan and the recently published genome sequence of apple (Malus × domestica ‘Golden Delicious’). In addition, the MdB4H coding sequences of cv Golden Delicious and cv Holsteiner Cox were cloned. The expression of SaB4H and MdB4H was analyzed in rowan cell cultures after treatment with elicitor from the scab fungus and in apple stems after infection with the fire blight bacterium, respectively. Besides functional and phylogenetic analyses, the subcellular localization of SaB4H reporter fusions was studied in Nicotiana benthamiana.

**RESULTS**

**cDNA Cloning of SaB4H Starting with a Subtracted Rowan cDNA Library**

Pools of mRNA were isolated from chitosan-treated and nontreated rowan cell cultures and used to construct
a suppression subtraction hybridization (SSH) cDNA library, which consisted of differentially expressed transcripts. A total of 2,000 cDNA clones were sequenced and bioinformatically processed (Supplemental Fig. S1), leading to the detection of 15 CYP contigs. The individual sequences were used to search the apple genome sequence, which is available via the Rosaceae Genome Database (GDR). Apple sequences sharing a high degree of similarity with the rowan contigs were filtered against CYP sequences in the data bank related to the modification of products of the shikimate pathway. These in silico analyses, details of which are listed in Supplemental Table S1, resulted in the selection of six apple unigenes that shared high sequence identity (87.5%–97%) with the rowan contigs 62, 120, and 162. Gene-specific primers were derived and led to the amplification of a transcript from *V. inaequalis*-treated rowan cell cultures. After 5’ and 3’ extensions, the resulting full-length cDNA was reamplified using a proofreading polymerase. The cDNA consisted of a 1,500-bp coding sequence (cds), a 188-bp 5’ untranslated region (UTR), a 260-bp 3’ UTR, and a 32-bp poly(A) tail. The cds encoded a 499-amino acid protein with a predicted molecular mass of 56.9 kD and a pI of 6.82. The protein was named CYP736A107, based on the standardized CYP nomenclature system (Nelson, 2009). Its structural analysis revealed the presence of motifs that are characteristic of plant CYPs (Fig. 2). The highly conserved heme-binding domain FxxGxRxCxG (Chapple, 1998) was present as FGSGRRRCAG. The N-terminal membrane anchor comprising 19 amino acids was followed by the Pro-rich region PPGPRGP [consensus (P/I)PGPx(G/P)xP; Schalk et al., 1999]. In addition, the KETLR(K) helix and the PERFE motif were present (Bak et al., 2011). The amino acid sequence of CYP736A107 shared 39.6% and 40.1% identities with functionally established flavonoid 3’-hydroxylases from *Epimedium sagittatum* (ADE80941) and *Brassica napus* (ABC58722), respectively.

**cDNA Cloning of Class II CPR from *V. inaequalis*-Treated Rowan Cell Cultures**

NADPH-cytochrome P450 reductase (CPR) is the electron-transfer partner for CYPs (Jensen and Møller, 2010). The apple unigene MDP0000167955 shared high sequence similarity with CPR genes in the data bank and the only CPR transcript fragment present in the SSH cDNA library. Using gene-specific primers and a proofreading polymerase, an SaCPR transcript was cloned from *V. inaequalis*-treated rowan cell cultures. The SaCPR cDNA contained a 2,139-bp cds encoding a 712-amino acid polypeptide with a predicted molecular mass of 78.8 kD. The protein shared 81% sequence identity each with *Gossypium hirsutum* CPR (ACN54324) and poplar (*Populus trichocarpa*) CPR3 (AAK15261) as well as 75.5% and 65.5% identities with Arabidopsis (*Arabidopsis thaliana*) CPR2 (AAK17169) and CPR1 (CA46814), respectively. The conserved binding domains for cofactors and substrates (FMN, FAD, NADPH, and CYP) and the membrane anchor near the N terminus were present (Supplemental Fig. S2). The extended N-terminal sequence upstream of the membrane anchor identified the rowan enzyme as a class II CPR (Ro et al., 2002).

**Functional Characterization of CYP736A107**

The CYP736A107 cds and the CPR cds were cloned in the same expression vector and heterologously expressed in yeast (*Saccharomyces cerevisiae*), from...
which microsomes containing the two proteins were isolated. Based on the carbon monoxide difference spectrum (Supplemental Fig. S3), the CYP expression level was 286 nmol g⁻¹ microsomal protein. Microsomes were incubated with 3-hydroxy-5-methoxybiphenyl, which was recently found as a B4H substrate using microsomes from elicitor-treated rowan cell cultures (Khalil et al., 2013). In the presence of NADPH, 3-hydroxy-5-methoxybiphenyl was 4-hydroxylated to yield noraurauparin, as demonstrated by gas chromatography-mass spectrometry (GC-MS) in comparison with an authentic reference compound (Fig. 3). In addition, identical retention time values and UV spectra were observed in HPLC-diode array detection (DAD) analysis (Supplemental Fig. S4). No product formation was found in assays that contained heat-denatured protein or microsomes from yeast cells harboring the empty vector. Nor was CYP736A107 activity detected when the rowan CPR cDNA was omitted from the vector, indicating the lack of interaction with yeast CPR. Substrates structurally related to 3-hydroxy-5-methoxybiphenyl were converted neither under standard assay conditions nor at varying pH values (5.5–10.5), except for 3,5-dihydroxybiphenyl. However, the relative activity was less than 2%, leading to the formation of only trace amounts of 3,4,5-trihydroxybiphenyl (Table I). Notably, 2-hydroxy-4-methoxydibenzofuran, which has the same substitution pattern as 3-hydroxy-5-methoxybiphenyl, was not converted. Furthermore, stilbenes and one-ring compounds were not substrates. Nor did the enzyme accept cinnamic acid derivatives, such as ferulic acid and coniferaldehyde. Thus, CYP736A107 was identified as SaB4H.

Enzyme activity was strictly dependent on NADPH, whose replacement with NADH reduced the activity to approximately 15%. In the presence of both NADPH and NADH, the activity was increased by approximately 15%. The pH and temperature optima were 7.5 and 30°C, respectively. B4H activity was linear with time up to 45 min and with increasing CYP concentrations in the standard assay up to 15 pmol, as determined by carbon monoxide difference spectroscopy. The $K_m$ values for 3-hydroxy-5-methoxybiphenyl and NADPH were $2.4 \pm 0.07$ and $72.3 \pm 7.5 \mu M$, respectively (Table II). Electron transfer from NADPH was exclusively due to rowan CPR, because yeast CPR alone failed to accomplish detectable SaB4H activity. The turnover number ($k_{cat}$) and the catalytic efficiency ($k_{cat}/K_m$) with 3-hydroxy-5-methoxybiphenyl were $0.342 \text{ min}^{-1}$ and $2.375 \text{ M}^{-1} \text{s}^{-1}$, respectively (Table II). When SaCPR was incubated with cytochrome $c$ as an artificial electron acceptor (Urban et al., 1994), the $K_m$ value for NADPH was $51 \pm 5.4 \mu M$. No appreciable loss of SaB4H activity was observed upon storage of microsomal preparations in Tris-EDTA-glycerol buffer at −80°C for 6 months. At 4°C, the enzyme activity decreased to about 20% to 30% within 24 h.

Expression of SaPAL, SaBIS1, and SaB4H in V. inaequalis-Treated Rowan Cell Cultures

Transcript levels in rowan cell cultures after the addition of an aqueous extract from the scab-causing fungus were analyzed by reverse transcription

**Figure 3.** GC-MS analysis of SaB4H assays. The enzymic product (noraurauparin) was analyzed as a trimethylsilyl derivative resulting from N-methyl-N-(trimethylsilyl) trifluoroacetamide treatment. The control incubation contained heat-denatured microsomes.
(RT)-PCR (Fig. 4). The gene-specific primer pairs used led to the amplification of 407-, 882-, 379-, and 300-bp fragments of SaPAL, SaBIS1, SaB4H, and SaActin transcripts, respectively. Actin mRNA served as a control for equal template amounts. SaB4H transcripts were detectable as early as 0.5 h after the onset of elicitation. The mRNA level peaked at 4 h and decreased slowly thereafter. Similar changes were found for the SaBIS1 transcript level, which, however, increased more slowly before reaching the maximum at 4 h. A significantly faster increase was observed for the SaPAL mRNA level, which already peaked after 1 to 2 h and returned rapidly to the basal SaPAL expression level that was present at the onset of elicitation.

<table>
<thead>
<tr>
<th>Substrate Formula</th>
<th>Activity %</th>
<th>Substrate Formula</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxy-5-methoxybiphenyl</td>
<td>100</td>
<td>Resorcinol OH</td>
<td>0</td>
</tr>
<tr>
<td>3,5-Dihydroxy-biphenyl</td>
<td>&lt;2</td>
<td>Orcinol monomethyl ether OH</td>
<td>0</td>
</tr>
<tr>
<td>3,5-Dimethoxy-biphenyl</td>
<td>0</td>
<td>Orcinol OH</td>
<td>0</td>
</tr>
<tr>
<td>2',3,5-Trihydroxy-biphenyl</td>
<td>0</td>
<td>3,5-Dihydroxy-benzoic acid OH</td>
<td>0</td>
</tr>
<tr>
<td>2,5-Dihydroxy-biphenyl</td>
<td>0</td>
<td>Benzoic acid OH</td>
<td>0</td>
</tr>
<tr>
<td>2-Hydroxy-4-methoxydibenzoferan</td>
<td>0</td>
<td>Ferulic acid OH</td>
<td>0</td>
</tr>
<tr>
<td>2,4-Dihydroxy-dibenzoferan</td>
<td>0</td>
<td>Coniferaldehyde OH</td>
<td>0</td>
</tr>
<tr>
<td>Pinosylvin</td>
<td>0</td>
<td>4-Coumaric acid OH</td>
<td>0</td>
</tr>
<tr>
<td>Resorcinol monomethyl ether</td>
<td>0</td>
<td>Cinnamic acid OH</td>
<td>0</td>
</tr>
</tbody>
</table>
Subcellular Localization of SaB4H Reporter Fusions in *N. benthamiana*

In silico analysis of SaB4H using protein target prediction software revealed the presence of a 19-amino acid N-terminal leader and membrane anchor (Fig. 2). To study the effect of this domain on subcellular localization, a modified yellow fluorescent protein called Venus (Kaufholdt et al., 2013) was fused to the 3' end of the SaB4H cds (35S::SaB4H-Venus). In addition, a construct containing the 27 N-terminal amino acids including the membrane anchor sequence (35S::SIG-Venus) and a construct lacking the membrane anchor and starting with amino acid 30 (35S::TRUNC-Venus) were generated. All three constructs were placed under the control of the 35S promoter from *Cauliflower mosaic virus* and were transiently expressed in *N. benthamiana* leaves using *Agrobacterium tumefaciens* infiltration. The resulting fluorescence distribution patterns were studied using confocal laser scanning microscopy. Both the SaB4H-Venus and the SIG-Venus fusions showed perinuclear and reticulate distribution, indicating endoplasmic reticulum (ER) association (Fig. 5, A and C, respectively). A construct harboring an ER retention signal (HDEL-DsRed) was expressed as a positive control, leading to a similar fluorescence distribution pattern to the expression of SaB4H-Venus (Fig. 5B, 1 and 2). Coexpression of the ER marker HDEL-DsRed and the SaB4H-Venus constructs resulted in colocalization of the proteins, as indicated by a whitish color in the case of a perfect match (Fig. 5B, 3). The occasional occurrence of fluorescent spots may be due to either ER-derived vesicles containing protein constructs or aggregates of Venus-DsRED fusions, which are caused by the high level of expression (Fig. 5B, 3). Finally, a construct that lacked the membrane anchor sequence (TRUNC-Venus) failed to target the protein to the ER; therefore, fluorescence was present in the cytoplasm (Fig. 5D).

Detection of SaB4H Activity in Microsomes from *N. benthamiana*

Microsomes from *N. benthamiana* leaves transiently expressing the 35S::SaB4H-Venus construct were incubated with 3-hydroxy-5-methoxybiphenyl and NADPH. Subsequent HPLC analysis demonstrated the formation of norauccuparin (Supplemental Fig. S5). The identity of this enzymatic product was confirmed by cochromatography with a sample of authentic reference compound and UV spectroscopy. No norauccuparin formation was observed in control assays containing the microsomal preparation from leaves of *N. benthamiana* after infiltration with *A. tumefaciens* that contained the empty vector.

Expression of *MdB4H* in Fire Blight-Infected Stems of Apple ‘Golden Delicious’

The cv Golden Delicious was selected because its genome sequence is available (Velasco et al., 2010).

<table>
<thead>
<tr>
<th>Table II. Steady-state kinetic parameters of B4Hs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data are means ± SD of three replicates.</td>
</tr>
<tr>
<td>Enzyme</td>
</tr>
<tr>
<td>SaB4H</td>
</tr>
<tr>
<td>Md(GD)B4H</td>
</tr>
<tr>
<td>Md(HC)B4H</td>
</tr>
</tbody>
</table>

Figure 4. Changes in the SaPAL, SaBIS1, and SaB4H expression levels in *V. inaequalis*-treated rowan cell cultures. A, The expression patterns were studied by RT-PCR. B to D, The transcript levels of SaPAL (B), SaBIS1 (C), and SaB4H (D) were normalized on the basis of the actin transcript amounts. SD values are indicated (n = 3).
Real-time PCR was used to quantify Md(GD)B4H mRNA amounts in cv Golden Delicious stems after inoculation with *E. amylovora*. As observed previously with cv Holsteiner Cox, which is used as a model system (Chizzali et al., 2012a), infection with the fire blight pathogen caused the typical shepherd’s crook symptom at the shoot tip (Vanneste, 2000) and resulted in the formation of a transition zone that was framed by the necrotic and the healthy stem segments (Fig. 6A). Md(GD)B4H expression in *E. amylovora*-infected stems of cv Golden Delicious was measured in relation to that observed in mock-inoculated control stems 6 d post inoculation, based on the previous observations with cv Holsteiner Cox (Chizzali et al., 2012a). A single PCR product was amplified using gene-specific primers for MdB4H, and no primer dimers were observed. The Md(GD)B4H transcript level in the transition zone was approximately 1,350 times that in the respective stem segment of mock-inoculated control shoots (Fig. 6B). The Md(GD)B4H mRNA amount in the healthy stem segment, which later turns into the transition zone, was approximately 1,150 times higher than in the respective stem zone after mock inoculation. Isolation of mRNA from the necrotic stem segment above the transition zone was not feasible.

**Phytoalexin Detection in Fire Blight-Infected Apple ‘Golden Delicious’**

*E. amylovora*-inoculated stems of cv Golden Delicious were dissected in necrotic, transition, and healthy zones (Fig. 6A), which were extracted with methanol (80%). Phytoalexins were identified by GC-MS using a library containing the previously structure-elucidated biphenyls and dibenzofurans from elicitor-treated rowan cell cultures (Hüttner et al., 2010; Chizzali et al., 2012b). Three biphenyls, noraucuparin, aucuparin, and 2'-hydroxyaucuparin, were detected in the transition zone (Supplemental Fig. S6). The flanking necrotic and healthy zones as well as segments from mock-inoculated shoots lacked detectable amounts of phytoalexins.

**MdB4H Transcript Cloning from Fire Blight-Infected Apple**

The transition zone of cv Golden Delicious served as an mRNA source to amplify the Md(GD)B4H coding sequence. Md(GD)B4H turned out to be identical to the apple unigenes MDP0000152900 and MDP0000149697 (GDR). The transition zone of fire blight-infected stems...
RNA isolation from the necrotic tissue was not feasible.


Phylogenetic Reconstruction of B4H and Related Amino Acid Sequences

A neighbor-joining tree was constructed using CYPs that are functionally characterized and contribute to various pathways of plant secondary metabolism (Fig. 7). The accession numbers and the CYP nomenclature of these proteins are indicated in Supplemental Table S2. The majority of the included CYPs catalyzed hydroxylation reactions, besides three functionally established CYPs that accomplish C-C and C-O coupling steps. The CYP-cam gene of Pseudomonas putida was used to root the tree. The B4H sequences from rowan and apple ‘Golden Delicious’ and ‘Holsteiner Cox’ grouped together. They were closest to a cluster that consisted of CA5H, also referred to as ferulate 5-hydroxylases, from various angiosperm species. B4H and CA5H share around 40% amino acid sequence identity (Fig. 2). The clade that consisted of the B4H and CA5H enzymes grouped together with a clade that comprised flavonoid 3’-hydroxylases and flavonoid 3’,5’-hydroxylases.

DISCUSSION

Aucuparin is the most abundant biphenyl phytoalexin formed in species of the economically valuable Malinae. Its substitution pattern consists of a para-hydroxy group that is framed by two methoxy functions. While the 3- and 5-hydroxy groups originate from the BIS-catalyzed biosynthesis of the scaffold, the para-hydroxy group is introduced by B4H. SaB4H and MdB4H were named CYP736A107 and CYP736A163, respectively, based on the standardized CYP nomenclature system (Nelson, 2009). So far, no catalytic functions have been demonstrated for CYP736 proteins, which are absent in several taxa, such as Arabidopsis and monocots, despite their early emergence (Nelson and Werck-Reichhart, 2011). However, recent studies have functionally related CYP736 proteins to plant defense and cross talk with microorganisms, either pathogens or symbionts. Three CYP736A25 variants are differentially regulated at the transcriptional and posttranscriptional levels in grape (Vitis vinifera) lines, which are either susceptible or tolerant to Xyliella fastidiosa (Cheng et al., 2010). In soybean (Glycine max), CYP736A34 is highly coexpressed with genes involved in root and Rhizobium spp.-induced nodule development (Guttikonda et al., 2010). However, these plant species do not form biphenyl and dibenzofuran phytoalexins.

Plant genome sequencing projects have revealed a vast number of CYPs, leading to the annotation of more than 5,000 sequences in the databases (Nelson and Werck-Reichhart, 2011). CYPs are among the largest plant gene superfamilies and constitute around 1% of the protein-coding genes. The angiosperm sequence diversity is encapsulated by 59 CYP families (Nelson et al., 2008). Arabidopsis, rice (Oryza sativa), poplar, and grape contain 245, 332, 310, and 315 CYPs, respectively (Nelson et al., 2008). Papaya (Carica papaya), with 142 CYPs, is nearly minimalist in the CYP collection of plants, whereas the human genome contains only 57 CYP genes. However, the biochemical functions of the majority of CYP proteins are still unknown (Schuler and Werck-Reichhart, 2003; Mizutani, 2012). In Arabidopsis, only 60 of the 245 CYPs are functionally clarified (Mizutani and Ohta, 2010). Even within the same subfamily, each CYP may be responsible for different roles. In this study, the SaB4H gene was identified by combining information from the recently published apple genome sequence (Velasco et al., 2010) and a newly established rowan SSH cDNA library.
Figure 7. Neighbor-joining tree illustrating the evolutionary relationships between B4H and CYPs involved in secondary metabolism. Numbers at the branch points are bootstrap values from 1,000 replicates and Poisson correction. The scale bar = 0.2 amino acid substitutions per site. The accession numbers of the amino acid sequences included are listed in Supplemental Table S2. *Pseudomonas putida* CYP-cam served as an outgroup. F3'H, Flavonoid 3'-hydroxylase; F3',5'H, flavonoid 3',5'-hydroxylase; NMC3'H, N-methylcoclaurine 3'-hydroxylase; BS, berbamunine synthase; CS, corytuberine synthase; FS, flavone synthase; IFS, isoflavone synthase; 2HIS, 2-hydroxyisoflavanone synthase; C4H, cinnamate 4-hydroxylase; I3'H, isoflavone 3'-hydroxylase; I2'H, isoflavone 2'-hydroxylase.
SaB4H exhibits absolute specificity for 3-hydroxy-5-methoxybiphenyl. Discrimination of 3,5-dihydroxybiphenyl and 3,5-dimethoxybiphenyl as substrates confirms the sequence of the aucuparin biosynthetic steps, which were recently detected in cell-free extracts from elicitor-treated S. aucuparia cell cultures (Khalil et al., 2013). Furthermore, discrimination of 2-hydroxy-4-methoxydibenzofuran indicates that this compound is not related to eriobofuran formation, which implies a revision of the previously proposed pathway (Hüttner et al., 2010). The 4-hydroxylation reaction is framed by the two methylation steps, which are catalyzed by distinct O-methyltransferases. OMT1 and OMT2 catalyze the methylation of the 5- and 3-hydroxy groups, respectively. SaB4H does not accept stilbenes, which have an additional double bond, and one-ring compounds. Nor does the enzyme convert cinnamic acid derivatives, such as ferulic acid and coniferaldehyde, which are related to lignin biosynthesis. In a phylogenetic tree, B4Hs are closest to CASH enzymes, which catalyze the formation of 5-hydroxyconiferaldehyde. O-Methylation then yields sinapaldehyde as a precursor of syringyl monolignols (Chiang, 2006; Bonawitz and Chapple, 2010). The 5-hydroxylase of the phenylpropanoid pathway was initially considered a ferulate 5-hydroxylase (Grand, 1984; Meyer et al., 1996); however, coniferaldehyde and coniferyl alcohol were later found to be the preferred substrates, implying that the enzyme should be referred to as CASH (Humphreys et al., 1999; Osakabe et al., 1999). The CASH gene, CYP84, is present in angiosperms but not in moss or gymnosperms, which thus lack syringyl lignin monomers (Nelson et al., 2008). Phylogenetic reconstruction suggests that B4H and CASH may arise from a common ancestor. They share around 40% amino acid sequence identity. After duplication of an ancestral gene, one copy retained the essential CA5H function, whereas the second gene copy has diverged. Mutations led to its neofunctionalization and recruitment into the aucuparin biosynthetic pathway of the Malinae. The new gene product still catalyzed a hydroxylation reaction, but on 3-hydroxy-5-methoxybiphenyl rather than coniferaldehyde.

The properties of a CYP may be affected by interaction with a heterologous CPR (Davin et al., 1997; Pauli and Kutchan, 1998). For this reason, a cDNA encoding rowan class II CPR was isolated and allowed interaction of this protein with SaB4H. CPR catalyzes the transfer of two electrons from NADPH via FAD and FMN, as electron acceptor and donor flavins, respectively, to the prosthetic heme group of diverse CYP enzymes (Porter, 2004; Jensen and Møller, 2010). In contrast to animals, which contain a single CPR (Porter et al., 1990), plants have multiple CPR isoforms that fall into two classes structurally distinguishable by their different N termini (Urban et al., 1997; Ro et al., 2002). In Arabidopsis and Centaurium erythraea, the expression of class II CPR is induced by wounding, light treatment, and elicitation, whereas class I CPR is constitutively regulated (Nisimoto, 1986; Meijer et al., 1993; Schwarz et al., 2009). Consistently, the transcript for rowan class II CPR, which contained all essential structural motifs, could be isolated from cell cultures after the addition of elicitor.

Aucuparin biosynthesis is initiated by BIS (Liu et al., 2004, 2007). In apple, BIS is encoded by a gene family consisting of four differentially regulated subfamilies (Chizzali et al., 2012a). MdBIS3 is expressed in stems upon fire blight infection. Inoculation of shoots with the fire blight bacterium E. amylovora leads to the formation of a transition zone between the necrotic and the healthy stem segments. Recently, an E. amylovora mutant that was deficient in producing the highly potent cytotoxin 6-thioguanine was found to lack the capacity of inducing disease symptoms in cv Holsteiner Cox, which is indicative of the crucial role of the antimetabolite in the development of the fire blight disease (Coyne et al., 2013, 2014). The transition zone gradually advances from the inoculated shoot tip down the stem with increasing postinoculation time, as was observed here with cv Golden Delicious and previously with cv Holsteiner Cox (Chizzali et al., 2012b, 2013). The Md(HC)/BIS3 transcript level in the transition zone of cv Holsteiner Cox was approximately 4,000 times that in the respective stem segment of mock-inoculated control shoots and somewhat lower in the healthy stem segment. A similar expression profile was found for Md(GD)/B4H in cv Golden Delicious. The maximum expression rate was observed in the transition zone, whereas the healthy tissue contained a slightly reduced transcript level. In accordance with these BIS3 and B4H expression data, phytoalexins accumulate in the transition zone, and the flanking stem segments are devoid of detectable phytoalexin amounts. While diseased cv Golden Delicious mainly contains noraucuparin and 2'-hydroxyaucuparin, cv Holsteiner Cox primarily accumulates noraucuparin and aucuparin and, in addition, the dibenzofurans noreriobioburan and eriobofuran (Chizzali et al., 2012b, 2013). Using a BLASTP analysis against the genome of cv Golden Delicious (GDR), Md(GD) B4H was located at the lower part of linkage group 11 between the two single-nucleotide polymorphism markers GDSnp02800 (50.04 centimorgan [cM]) and GDSnp01705 (51.4 cM). Recently, a number of Diversity Arrays Technology markers correlating with resistance to fire blight were identified in the genome of the crabapple Malus fusca MAL0045 (Emeriwenn et al., 2014). After DNA sequencing, two of them (970048 and 969599) could be aligned to linkage group 11 of cv Golden Delicious at positions 49.11 and 49.12 cM, respectively. In addition, a minor quantitative trait locus (QTL) for resistance to fire blight bacterium E. amylovora was recently described on linkage group 11 of M. × robusta 5 (Wöhner et al., 2014). This QTL is linked to marker CH04a12. Using the apple integrated map (Velasco et al., 2010), CH04a12 was located at position 34.3 cM, about 16 cM from marker GDSnp02800. However, whether Md(GD) B4H contributes to this minor QTL remains unknown.
Biphenyls and dibenzofurans are derivatives of benzoic acid, the biosynthesis of which branches from the general phenylpropanoid pathway, initiated by PAL. The fast increase in the SaPAL transcript level in rowan cell cultures in response to V. inaequalis treatment indicates a rapid redirection of carbon flow from primary to secondary metabolism. The basal SaPAL transcript level observed before and after the transient primary to secondary metabolism. The basal SaPAL indicates a rapid redirection of carbon flow from the general phenylpropanoid pathway to benzenoid metabolism and further to aucuparin biosynthesis. In rowan cell cultures, aucuparin accumulation starts 9 h after the addition of V. inaequalis extract (Khalil et al., 2013). It is not only preceded by a fast increase in the SaPAL mRNA level but also by coordinated, albeit somewhat delayed, increases in the SaBIS1 and SaB4H transcript levels.

SaB4H was localized to the ER upon transient expression of a reporter gene construct in N. benthamiana leaf epidermis cells. The majority of eukaryotic CYPs are cotranslationally inserted into the ER membrane and remain anchored by an N-terminal hydrophobic helix (Chappelle, 1998). The N-terminal leader includes the transmembrane domain (Johnson and Stout, 2013). However, CYPs are not restricted exclusively to this subcellular location. In gibberellin biosynthesis, CYP701A, ent-kaurene oxidase, is present on the outer surface of the outer chloroplast envelope, thereby linking the plastid- and ER-located steps of this pathway (Helliwell et al., 2001). The same location was found for CYP68B1, which catalyzes the ω-hydroxylation of long-chain fatty acids in suberin biosynthesis (Watson et al., 2001; Compagnon et al., 2009). To supply the CYPs with electrons, a CPR isoform was always found for an CYP74s, which are involved in oxylipin (e.g. jasmonic acid) metabolism (Hughes et al., 2009). Tomato (Solanum lycopersicum) CYP74A and CYP74B were localized to the inner chloroplast membrane facing the stroma and the outer chloroplast membrane facing the space between the two envelope membranes, respectively (Froehlich et al., 2001). Different locations were also reported for the CYP74C and CYP74D proteins, such as cytosol, tonoplast, and amyloplasts and leucoplasts (Hughes et al., 2009). However, it should be noted that CYP74s are atypical members of the superfamily, because they do not require molecular oxygen and electron donors and they exhibit unusually high turnover rates (Hughes et al., 2009). In animals, mitochondria contain steroid biosynthetic- and xenobiotic-metabolizing CYPs, which utilize adrenodoxin and a NADPH-dependent adrenodoxin reductase for their electron transfer (Guzov et al., 1998).

CONCLUSION

B4H is an ER-anchored CYP that is involved in aucuparin biosynthesis initiated by BIS. Recently, MdBIS3 was immunochemically localized to the junctions between neighboring cortical parenchyma cells in the transition zone of fire blight-infected apple stems, suggesting an association of the enzyme with plasmodesmata in primary pit fields (Chizzali et al., 2012a, 2013). Thus, it is tempting to speculate that the aucuparin biosynthetic enzymes may be assembled into a metabolon, which allows for an efficient transfer of biosynthetic intermediates between the active sites in close proximity (Winkel, 2004; Jørgensen et al., 2005). Protein complexes are commonly associated with structural elements within the cell, such as the ER and microtubules (Zajchowski and Robbins, 2002; Chuong et al., 2004). B4H may act as an anchor inside the plasmodesmata for assembling the upstream and downstream aucuparin biosynthetic enzymes. Which role the products of this metabolon might play in preventing an infection from spreading from cell to cell via the symplastic connections remains unknown.

MATERIALS AND METHODS

Chemicals

3,5-Dihydroxybiphenyl, 3-hydroxy-5-methoxybiphenyl, 3,5-dimethoxybiphenyl, 3,4,5-trihydroxybiphenyl, 2',3',5-trihydroxybiphenyl, noraucuparin, 2,4-dihydroxydibenzoferan, 2-hydroxy-4-methoxydibenzoferan, and 4-hydroxy-2-methoxydibenzoferan were synthesized as described previously (Liu et al., 2004; Hüttner et al., 2010; Chizzali et al., 2012b; Khalil et al., 2013). 2,5-Dihydroxybiphenyl, benzoic acid, 3,5-dihydroxybenzoic acid, cinnamic acid, pinosylvin, orcinol, and their monomethyl ethers were purchased from Sigma-Aldrich (www.sigmaaldrich.com).

Plant Material and Treatments

Rowan (Sorbus aucuparia) cell cultures were grown and treated with Venturia inaequalis elicitor, as described previously (Liu et al., 2004; Khalil et al., 2013). Greenhouse-grown grafted shoots of apple (Malus × domestica ‘Golden Delicious’ and ‘Holstein Cox’) were inoculated with the Erwinia amylovora strain 222 gfp (Plachowsky et al., 2008), as described previously (Chizzali et al., 2012b).

Extraction and Analysis of Phytoalexins

Freeze-dried stem segments (0.5 g) were extracted and analyzed as described previously (Hüttner et al., 2010; Chizzali et al., 2012a).

Construction of a Subtracted cDNA Library

An SSH library was constructed as described previously (Gaid et al., 2012), except that poly(A) RNA was isolated from chitosan-treated (9 h after the onset of elicitation) and control rowan cell cultures and cDNA synthesis was started with 1 μg of mRNA from each.

Identification and Cloning of Rowan cDNAs Encoding B4H and CPR

The genome sequence of cv Golden Delicious (apple genome version 0.1 contigs) was searched for putative B4H sequences via the BLASTN server of the GDR (www.rosaceae.org), using selected CYP contigs from the SSH cDNA library as queries (Supplemental Table S1). Downloaded sequences were filtered against CYP data bank entries related to the modification of
products of the shikimate pathway (http://metabolomics.jp/wiki/Indecx:
P450). MDP0000152900 and closely related apple unigenes as the most promising candidates for hydroxylation in biphenyl metabolism served to derive 5' and 3' primers for the amplification of the cds of the putative rowan B4H cDNA (Supplemental Table S3). To clone a CPR transcript from rowan cell cultures, the poplar (Populus trichocarpa) CPR3 sequence (AF502498.1), which encodes a class II CPR (Ro et al., 2002), was used as a query to search the GDR via the BLASTN server. The apple unigene MDP0000167955, which shared high similarity with the only CPR transcript fragment in the rowan SSH cDNA library, was selected as a candidate CPR gene, from which 5' and 3' primers were derived for the amplification of the CPR cds.

The coding sequences for SaB4H and SaCPR were PCR amplified from a rowan cDNA pool prepared 4 h post elicitation using Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific; www.thermoscientific.com). The SaB4H cds was digested using forward primer 1 (with an EcoRI restriction site) and reverse primer 2 (with a PciI restriction site; Supplemental Table S3). The PCR conditions were 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 63°C for 30 s, and 72°C for 70 s, and a final extension of 10 min at 72°C.

The gene-specific forward primer 3 was used for 3' RACE to obtain the 3' UTR. The 5' UTR was obtained by amplifying a fragment of genomic DNA, which was prepared using the DNeasy Mini Kit (Qiagen; www.qiagen.com) and which was prepared using the forward primer 6 and reverse primer 7, which introduced Sfl and HindIII restriction sites, respectively. PCR conditions were identical to those used for the SaB4H sequence. The PCR product of the expected size was excised from a 1% (w/v) agarose gel and purified using the innuPREP DOUBLEPure Extraction Kit (Analytik Jena; www. analytik-jena.de).

The Md(G)B4H and Md(H)C4H coding sequences of cv Golden Delicious and cv Holstein Cox, respectively, were amplified from reverse-transcribed RNA isolated from the transition zones of fire blister-infected stems.

Expression of B4H and CPR cDNAs in Yeast

The PCR products for apple and rowan B4Hs were digested with EcoRI and PciI and inserted into the MULTICLONING SITE1 (MCS1) of the pESC-Ura expression construct was transferred to competent yeast cells (INVSc1) using the EasyComp Transformation Kit (Life Technologies; www.lifetechnologies.com) and an oligo(dT) primer.

Enzyme Assays

The standard B4H assay consisted of 10 μl 3-hydroxy-5-methoxibiphenyl, 1 mM NADPH, and 10 pmol of recombinant CYP in a final assay volume of 200 μL adjusted with 100 mM potassium phosphate buffer, pH 7.5. The reaction was started by the addition of NADPH, incubated at 30°C for 30 min, and stopped by the addition of 20 μL of ice-cold stopping mixture (acetic acid: methanol, 1:1), followed by two extractions with 250 μL of ethyl acetate. The combined extract was evaporated to dryness, resuspended in 100 μL of methanol, and analyzed by HPLC. Arrays of related compounds, as listed in Table I, were tested as potential B4H substrates at final concentrations of 10 μM. For enzyme characterization, pH values ranged from 5.5 to 10.5, temperatures from 15°C to 60°C, incubation times from 5 to 120 min, and CYP concentrations from 1 to 50 pmol per assay. The Km value for 3-hydroxy-5-methoxybiphenyl was determined using concentrations from 0.2 to 30 μM while keeping NADPH at 1 mM. To measure the Km value for NADPH, its concentration varied from 1 to 1,000 μM, keeping the concentration of 3-hydroxy-5-methoxibiphenyl constant at 10 μM. Enzyme concentration (10 pmol per assay) and incubation time (30 min) were selected to get a linear reaction velocity during the assay period. All kinetic analyses were carried out in triplicate. The kinetic parameters Km and Vmax were determined by the Hanes method using the Hyper 32 software (http://www.plantphysiol.org on November 11, 2017 - Published by Downloaded from www.plantphysiol.org).
www.applediosystems.com) using 5X HOT FIREPol EvaGreen qPCR Mix Plus (ROX; Solis BioDyne; www.sbd.ee). We followed the procedure outlined in the manufacturer’s instructions with small modification: 45 cycles at 95°C for 30 s and 61°C for 1 min. Gene-specific amplification was evaluated by melt-curve analysis. Amplification and correlation efficiencies of each PCR were determined using six serial dilutions of cDNA from all samples. The PCR efficiency was used to transform the cycle threshold values into raw data for relative quantification. Expression of the Md(GD)B4H gene was evaluated using the gene-specific primers listed in Supplemental Table S3 [primers 16 forward and 17 reverse for Md(GD)B4H and primers 18 forward and 19 reverse for Actin]. Quantitative PCR primer pairs for Md(GD)B4H were designed based on the sequence of unigene MDP0000152900. All samples were normalized using mRNA of the reference gene, MdActin, as an internal control sample for each line (Paris et al., 2009). Scaling of Md(GD)B4H expression was performed in relation to the respective mRNA expression levels in mock-inoculated stems, which were set to 1. All reactions were performed in technical triplicates. Estimations of efficiency and expression level were based on the mathematical model of Pfaffl (2001).

Phylogenetic Reconstruction

A number of functional CYP sequences, which are related to hydroxylation reactions in natural product biosynthesis, were used. The accession numbers and the CYP nomenclature are listed in Supplemental Table S2. The phylogenetic tree was constructed by the neighbor-joining method using MEGA 5.05 with 1,000 bootstrap support (Tamura et al., 2011). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in units of the number of amino acid substitutions per site. The pairwise deletion method was used to nullify gaps and missing data.

Agrobacterium tumefaciens-Mediated Transient Expression and Subcellular Localization

Expression constructs were generated using the Gateway cloning technology according to the manufacturer’s instructions (Life Technologies; www.lifetechnologies.com). Three pairs of Gateway-compatible attB primers (Supplemental Table S3) were used to amplify (1) the complete cds of SaB4H (SaB4H; 1,497 bp; primer pair forward 20 and reverse 21), (2) the N-terminal anchor sequence (SIG; 81 bp; primer pair forward 20 and reverse 22), and (3) the truncated sequence that lacks the 30 N-terminal amino acids including the membrane anchor sequence (TRUNC; 1,407 bp; primer pair forward 23 and reverse 21). All reverse primers lacked a stop codon to enable C-terminal fusion with Venus. The modified attB PCR products (SaB4H, SIG, and TRUNC) were cloned into the donor vector pDONR/Zeo (Life Technologies) using the Gateway BP (Life Technologies) reaction to create entry vectors without a restriction site. The pairwise deletion method was used to nullify gaps and missing data.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Proposed functions of unigenes in a subtracted cDNA library.

Supplemental Figure S2. Alignment of CYP amino acid sequences.

Supplemental Figure S3. Carbon monoxide difference spectrum.

Supplemental Figure S4. Product analysis by HPLC-DAD.

Supplemental Figure S5. Transient SaB4H expression in N. benthamiana leaves.

Supplemental Figure S6. Detection of phytoalexins in cv Golden Delicious.

Supplemental Table S1. CYP transcript contigs in the subtracted cDNA library.

Supplemental Table S2. Accession numbers and CYP nomenclature for the phylogenetic tree.

Supplemental Table S3. Primers used.

ACKNOWLEDGMENTS

We thank Dr. David R. Nelson (University of Tennessee) for naming the rowan and apple B4H sequences according to the standardized CYP nomenclature system and Dr. Boris Voigt (University of Bonn) for providing the SaB4H-Venus::HDEL-DsRED construct.

Received January 19, 2015; accepted April 9, 2015; published April 10, 2015.

LITERATURE CITED

Biphenyl 4-Hydroxylases Are CYP736A Proteins


Copyright © 2015 American Society of Plant Biologists. All rights reserved.