Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis

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Plants synthesize numerous secondary metabolites that are used as developmental signals or as defense against pathogens. Tryptophan (Trp)-derived secondary metabolites include camalexin, indole glucosinolates, and indole-3-acetic acid (IAA); however, the steps in their synthesis from Trp or its precursors remain unclear. We have identified two Arabidopsis cytochrome P450s (CYP79B2 and CYP79B3) that can convert Trp to indole-3-acetaldoxime (IAOx), a precursor to IAA and indole glucosinolates.

We describe here the isolation and characterization of two Arabidopsis genes, CYP79B2 and CYP79B3, that encode cytochrome P450s predicted to N-hydroxylate amino acids. These two genes, when expressed in Escherichia coli, can specifically convert Trp to IAOx in vitro. We also show that CYP79B2, when overexpressed in Arabidopsis or yeast, confers resistance to toxic analogs of Trp. In addition, CYP79B2 is expressed in response to bacterial pathogens in a manner similar to Trp biosynthetic genes. Taken together, our data suggest a model in which CYP79B2 and CYP79B3 metabolize Trp to IAOx that can be used for either IAA or indole glucosinolate biosynthesis.

Materials and Methods

Selection of Arabidopsis cDNAs in Yeast and Plasmid Construction. Unless noted, all yeast and E. coli growth and molecular biological techniques followed standard methods (14). Restriction enzymes were purchased from New England Biolabs; [α-32P]dCTP was from NEN; oligonucleotides were from Genemed Biotechnologies (South San Francisco, CA); chemicals were purchased from Sigma. E. coli strains expressing CYP79B2 or CYP79B3 were grown at 30°C to maintain plasmid stability. CYP79B2 was isolated as follows. An Arabidopsis cDNA expression library (15) was transformed into a diploid yeast strain constructed by crossing L5140 × L5141 (a gift from G. R. Fink, Whitehead Institute for Biomedical Research, Cambridge, MA). L5140 = MATa ura3–52 leu2–3,112 his3Δ200 trp1 Δ63 ade2 GAL. L5141 = MATa ura3–52 leu2–3,112 his3–11,15 lys2 Δ 201 ade2 GAL. Approximately 200,000 transformants were screened on complete minimal medium lacking uracil and containing 2% galactose, 200 μM 5-fluorodole (SFI), and 2 mg/liter Trp (instead of the standard 20 mg/liter Trp). SFI-resistant clones, which conferred galactose-dependent SFI-resistant growth in yeast, were recovered by transformation into E. coli strain DH5α. cDNA clones were subcloned with EcoRI into pBluescript KS(+) (Stratagene), sequenced, and determined to be identical to each other and to CYP79B2 (GenBank accession no. AT5S171.120). One of these subclones was in frame with the lacZ gene carried on pBluescript KS(+) and was used for isopropyl β-d-thiogalactoside (IPTG)-inducible expression in E. coli and assayed for Trp-metabolizing activity in Fig. 2C. CYP79B3 cDNAs were cloned as follows. Oligonucleotide primers AO18 (5’-ACGACCAAGTCAAGTCTCGGAATG-3’) and AO19 (5’-ACAGCAATTTGACCAAGTCAAGTCTC-3’) were designed to anneal approximately 200 bp of the 5’ end of the cDNA, but do not overlap the reading frame. These primers were used with PCR4Sk+ vector and pBluecript KS(+) to generate pBluescript KS(+) containing the entire open reading frame of CYP79B3. These cDNAs were then ligated into yeast expression vectors containing the GAL1 promoter, and the resulting plasmids were transformed into yeast L5140-Δ63 strain. The resulting strains were tested for Trp reductase activity. CYP79B3 construct was found to confer resistance to 2 mg/liter Trp. CYP79B2 and CYP79B3 transformants were then screened for increased resistance to Trp analogs (15).

Abbreviations: Trp, tryptophan; IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; SFI, 5-fluorodole; SFT, 5-fluorothiogalactoside; SFA, S-methylanthranilate; SMT, methyltryptophan.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AT5S171.120 and AC006592.4).

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3') and AO19 (5'-GGATACGTTGATTCTTTCTGAC-3') were used in a PCR on Arabidopsis cDNAs to create a 286-bp fragment identical to the 5' end of CYP79B3 (GenBank accession no. AC006592.4). This fragment was used to screen an Arabidopsis λ YES cDNA library (15). Five positive clones were isolated, and the two longest clones were subcloned with EcoRI into pBluescript KS(+). One of these contained the entire coding region whereas the other lacked the first 8 bp. The incomplete cDNA was in frame with the lacZ gene of pBluescript KS(+) and was used for IPTG-inducible expression in E. coli and assayed for Trp-metabolizing activity in Fig. 2C.

Modification of the 5' end of the CYP79B2 cDNA was performed by replacing the first 90 bp of the coding region with a sequence encoding the first 14 aa of CYP17A1 (16). This sequence was encoded by the complementary oligonucleotides AO10 (5'-TCGAGCATATGCTCTGTTATTAGCAGTTTTTCAT-3') and AO11 (5'-AGCTTGAAACACTGCTATAACAGACCATATG-3'). These oligonucleotides, after annealing, create an XhoI site at the 5' end of CYP79B2 and a HindIII site at the 3' end. The annealed oligonucleotides were cloned into the 5' end of CYP79B2 carried in pBluescript KS(+) by using XhoI and HindIII. CYP79B2mod then was subcloned into pCWori+ by using the NdeI site (contained within the annealed oligonucleotides) and an XbaI site at the 3' end of the gene.

For expression in plants, the CYP79B2 cDNA was subcloned by using EcoRI into the pBICA MV expression vector. pBICA MV is derived from pBI121 (CLONTECH) in which the original EcoRI site was destroyed and the annealed oligonucleotides (5'-CCGTTACGGAATTCGAGCT-3' and 5'-GAATTCGTTAC-3') were cloned into the XmaI and SacI sites.

Assay for Trp-Metabolizing Activity. Expression of CYP79B2mod is under the control of the tac promoter in the plasmid pCWori+ and is inducible by IPTG (17). Membrane extracts were prepared from E. coli expressing either CYP79B2mod or a non-P450 cDNA as follows. An E. coli culture was grown overnight in LB containing 100 μg/ml Ap, diluted 1:100 into Terrific Broth containing 100 μg/ml Ap, and grown at 37°C to 225 rpm. The culture was grown to an A550 of 0.9, 1 mM IPTG and 80 μg/ml 3-aminolevulonic acid was added, and incubation was continued at 30°C and 190 rpm for 72 h. The cells were collected by centrifugation in a Sorvall SH3000 rotor at 4,000 rpm for 15 min at 4°C, washed twice with 4°C Mops buffer (100 mM Mops, pH 7.3), and resuspended in Mops buffer before sonication on ice in a Fisher Scientific 550 Sonic Dismembrator for 30 × 2-s bursts on setting 3. The membranes were collected by centrifugation at 31,000 rpm in a Beckman Ti60 rotor for 30 min at 4°C, and the membrane proteins were solubilized in 4°C Mops buffer containing 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) by slow rocking on ice for 2 h. The sample was centrifuged again at 31,000 rpm in a Beckman Ti60 rotor for 30 min at 4°C, and the supernatant containing the solubilized membranes was frozen in aliquots. For assays, thawed extracts then were reconstituted with dialyzed phosphatidylcholine, mixed with purified rat NADPH-cytochrome P450 reductase, NADPH, and 750 μM [14C]-l-Trp (side chain 3-14C, 300,000 dpm; American Radiolabeled Chemicals, St. Louis) (Fig. 2A) or 750 μM nonradioactive Trp (Fig. 2 B and C), and incubated at 30°C for various times. The indolic compounds were extracted with ethyl acetate, evaporated, and applied onto a silica gel 60 F254 TLC plate. The compounds were separated in ethyl acetate/chloroform/formic acid (55:35:10). Plates were air-dried and compounds were visualized by autoradiography (Fig. 2A) by using a Bio-Rad Molecular Imager or by UV epifluorescence (Fig. 2B and C) by using a Bio-Rad Fluor-S imager. IAOx was synthesized as described previously (18, 19). GC-MS analysis of the synthesized IAOx confirmed its identity as represented by ions at m/z 174 and 130 (20).

Transgenic Plants Expressing CYP79B2. Arabidopsis (Col-0 ecotype) were transformed (21) with pBICA MV carrying the CYP79B2 cDNA (see above). Two homozygous transgenic lines were selected that showed the highest level of CYP79B2 mRNA expression (data not shown).

Northern Blot Analysis. RNA samples prepared at the times indicated from Pseudomonas syringae pv maculicola (ES4326)-infected Arabidopsis were described previously (22). Ten micrograms of total RNA per sample was used to prepare a Northern blot that was probed with either a 32P-labeled CYP79B2 cDNA fragment or an Arabidopsis β-tubulin gene (14).

Results

Isolation of CYP79B2. IAA and indole glucosinolates are both derived from Trp or its immediate precursors. However, no gene has been cloned from Arabidopsis thaliana whose product can convert Trp or indole to any of the proposed intermediates in IAA or indole glucosinolate biosynthesis. Therefore, we designed a yeast screen to identify Arabidopsis cDNAs encoding enzymes capable of indole or Trp metabolism. This screen took advantage of the toxicity to yeast of an analog of indole, 5FI. In yeast, 5FI is converted to 5-fluorotryptophan (5FT) by tryptophan synthase β. 5FT is toxic to yeast for two reasons: first, 5FT gets incorporated into proteins and disrupts protein function, and second, 5FT mimics Trp in its ability to feedback-inhibit anthranilate synthase, thus shutting down further Trp biosynthesis (23).

We screened ~200,000 Arabidopsis cDNAs and identified one clone that conferred 5FI resistance upon retransformation into yeast (Fig. L4). DNA sequencing revealed that this cDNA was identical in part to a previously described cDNA fragment called CYP79B2 based on its similarity to the Sorghum cytochrome P450, CYP79A1 (25). A comparison of the deduced amino acid sequence of the newly isolated CYP79B2 clone and CYP79A1 is shown in Fig. 1B. Our functionally isolated CYP79B2 cDNA is full-length based on comparison with the recently sequenced CYP79B2 genomic region (Arabidopsis Genome Initiative, http://www.arabidopsis.org/agi.html) and with CYP79A1 and other cytochrome P450 sequences. CYP79A1 and CYP79B2 are approximately 49% identical and both contain a predicted N-terminal transmembrane domain found in all cytochrome P450s. However, based on the amino acid composition of the N-terminus of 22 aa, CYP79B2 contains a chloroplast transit peptide (26) that is not found in CYP79A1 (27).

CYP79B2 Metabolizes Trp to IAOx in Vitro. CYP79A1 is the cytochrome P450 most similar to CYP79B2 whose activity is known. CYP79A1 converts tyrosine to p-hydroxyphenylacetaldoxime (27). However, we hypothesized that CYP79B2 would use tryptophan (not tyrosine) as a substrate for the following reasons. First, a trp1 auxotrophic yeast strain expressing CYP79B2 requires more exogenous Trp than does the same yeast strain carrying the vector without the CYP79B2 gene (data not shown). This suggests that CYP79B2 depletes Trp away from its use in protein synthesis. Second, CYP79B2 expression in yeast does not confer resistance to m-fluorotyrosine (data not shown), suggesting that tyrosine is not a substrate for CYP79B2.

To identify the substrate for CYP79B2 biochemically, we tested the in vitro activity of CYP79B2 by expressing an N-terminally modified form of CYP79B2 (CYP79B2mod) in E. coli. This modification removes the predicted chloroplast transit peptide and substitutes a different transmembrane domain for the putative transmembrane domain of CYP79B2. This modification enables efficient expression of P450s in E. coli (16).

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Membrane extracts prepared from *E. coli* expressing either CYP79B2mod or a non-P450 cDNA were assayed for Trp-metabolizing activity, and indolic compounds were separated by TLC. As seen in Fig. 2A, a novel product accumulates in a time-dependent manner only in the reactions containing the CYP79B2mod extract. When extracts were prepared from uninduced *E. coli* carrying CYP79B2mod, no products were detected (data not shown). In addition, when [14C]tyrosine or [14C]indole was substituted for [14C]tryptophan, no novel spots were detected (data not shown). These results suggest that CYP79B2 is specific for Trp; strict substrate specificity also has been observed for CYP79A1 (28). Two lines of evidence suggest that the product generated by CYP79B2 in *E. coli* extracts is IAOx. First, the *Rf* value of the product is identical to the previously identified sequence for IAOx (29). Second, indolic compounds comigrated with the IAOx standard. The reactions were visualized as in B. Reaction products are indicated by an arrow, and reaction times are indicated in minutes.
published value for IAOx (data not shown; ref. 19). Second, the product comigrates with partially purified IAOx (Fig. 2B).

Based on the structure of other cytochrome P450 proteins, the conserved cysteine residue required for heme binding and P450 function is cysteine 477 (C477) (29). To confirm that the CYP79B2 cytochrome P450 activity observed in E. coli extracts is responsible for the 5FI resistance conferred by CYP79B2 expressed in yeast, we created a mutant form of CYP79B2mod in which C477 was changed to an alanine (data not shown). This mutant allele of CYP79B2 showed no activity when expressed in E. coli and failed to confer 5FI resistance in yeast (data not shown). This result indicates that the P450 activity responsible for Trp metabolism in vitro is also responsible for the 5FI resistance observed in yeast.

A Second Arabidopsis Gene also Converts Trp to IAOx. A database search revealed that Arabidopsis contains a second sequence predicted to encode a protein with 85% amino acid identity to CYP79B2 called CYP79B3 (Fig. 1B). We cloned a CYP79B3 cDNA and expressed CYP79B3 in E. coli. The extract then was assayed for IAOx-forming activity as before except nonradioactive Trp was used in the assay. As seen in Fig. 2C, CYP79B3 also can catalyze the conversion of Trp to IAOx.

CYP79B2 Overexpression in Plants Confers Resistance to Toxic Trp Pathway Analogs. Because CYP79B2 expression in yeast confers resistance to 5FI and 5FT, we predicted that overexpression of CYP79B2 in plants would make plants resistant to these and other Trp pathway analogs. Therefore, we constructed Columbia ecotype (Col-0) Arabidopsis overexpressing CYP79B2 from the cauliflower mosaic virus 35S promoter. Two independently derived transgenic lines with increased CYP79B2 mRNA levels (data not shown) were tested for their sensitivity to toxic analogs of Trp [5-methyltryptophan (5MT)] or of the Trp biosynthetic intermediates anthranilate [5-methylanthranilate (5MA)] and indole (5FI). Fig. 3 shows the growth of one of these lines compared with Col-0. When grown on unsupplemented medium (Fig. 3A), Col-0 and the 35S-CYP79B2 plants grew similarly. However, 35S-CYP79B2 plants were less sensitive than Col-0 to the toxic effects of 5MA, 5MT, or 5FI (Fig. 3B–D).

CYP79B2 Expression Is Pathogen-Induced. Because genes involved in Trp biosynthesis and its secondary metabolites are induced in response to pathogens (22, 31, 32), we tested whether CYP79B2 mRNA expression is induced in response to bacterial infection. A Northern blot was prepared from Col-0 Arabidopsis leaves that were infected for various times with either the virulent strain P. syringae pv. maculicola (ES4326) or the avirulent strain P. syringae pv. tomato (MM1065) (22). We observed induction of the CYP79B2 mRNA levels 12.5 h after infection by virulent strain (Fig. 4), but not in a mock infection or with the avirulent strain (data not shown). In the mock infection and avirulent strain infection, CYP79B2 mRNA was not detectable at any time point. The increase in expression after 12.5 h of infection with the virulent strain is similar to the induction seen for the ASA1 and ASB1 genes of Arabidopsis (22). These genes encode, respectively, α- and β-subunits of Arabidopsis anthranilate synthase, the first committed step in Trp biosynthesis. In the analysis of ASA1 and ASB1, a modest induction was seen after infection by an avirulent strain (22). We did not see induction of CYP79B2 under this condition; however, this could be because CYP79B2 expression was below the level of detection. Nonetheless, that CYP79B2 expression parallels that of ASA1 and ASB1 suggests that there is coordinate regulation between Trp biosynthetic genes and genes that encode Trp-metabolizing enzymes.

Discussion

We have cloned and characterized two Arabidopsis genes, CYP79B2 and CYP79B3, that encode cytochrome P450s capable
of converting Trp to IAOx. IAOx has been proposed as an intermediate for both IAA and indole glucosinolate biosynthesis (see below). Previous reports suggested that in Chinese Cabbage (also a member of the Brassicaceae), the conversion of Trp to IAOx is catalyzed by a plasma membrane-associated peroxidase (20), although earlier reports suggested a cytochrome P450-like activity (33). Whether a peroxidase capable of converting Trp to IAOx exists in Arabidopsis is in addition to CYP79B2 and CYP79B3 is unknown. Besides an in vitro Trp-metabolizing activity, several lines of evidence are consistent with CYP79B2 and CYP79B3 having a role in Trp metabolism in plants. First, CYP79B2, when overexpressed in plants, confers resistance to toxic Trp pathway analogs (discussed below). Second, CYP79B2 mRNA expression is induced in response to pathogens in a manner similar to other Trp biosynthetic genes (22). Third, both proteins are predicted to be targeted to the chloroplast, which is the site of Trp biosynthesis (25, 34).

Why does CYP79B2 confer resistance to toxic analogs of Trp when expressed in yeast or when overexpressed in plants? Trp analogs such as 5FT are toxic because they disrupt protein function and feedback-inhibit anthranilate synthase (23). Therefore, any mechanism that increases the ratio of Trp:5FT should relieve toxicity. For example, Arabidopsis strains carrying feedback-resistant alleles of anthranilate synthase or mutants that cause increased transcription of the ASA1 gene confer resistance to toxic Trp analogs (35, 36). From our in vitro data, CYP79B2 can metabolize Trp and Trp analogs to IAOx or IAOx analogs (Fig. 2A and data not shown). Therefore, overexpression of CYP79B2 in plants or yeast is predicted to result in a decrease in the concentrations of both Trp and the toxic analogs of Trp and reduce feedback inhibition of anthranilate synthase. An increase in anthranilate synthase activity would cause increased production of Trp resulting in a higher ratio of Trp:5FT than found in wild-type Arabidopsis or yeast. Thus, increased conversion of Trp to IAOx by CYP79B2 should result in resistance to toxic Trp pathway analogs.

What is the role of IAOx in plants? In Brassicaceae, IAOx has been proposed as an intermediate for Trp-dependent IAA biosynthesis via the IAN pathway (Fig. 5). CYP79B2 and CYP79B3 therefore are prime candidates for the enzymes that catalyze the first step in this pathway in Arabidopsis. Although there is contradictory evidence as to whether IAN is derived from Trp (37, 38), discovery of CYP79B2 and CYP79B3 suggests that at least a portion of IAN could be Trp-derived via an IAOx intermediate. In addition, the CYP79B2 and CYP79B3 genes will be useful in clarifying the roles of the four Arabidopsis nitrilase genes (Fig. 5; refs. 31 and 39), whose in vivo role in IAA biosynthesis remains unclear (35, 40).

IAOx is also the likely intermediate for indole glucosinolates (Fig. 5). Glucosinolates are derived from oxime derivatives of amino acids; therefore, indole glucosinolates should be derived from Trp via IAOx (10). Thus, CYP79B2 and CYP79B3 encode enzymes that make IAOx, which could be used for IAA and/or indole glucosinolate biosynthesis, suggesting a common mode of regulation for these two pathways.

The elucidation of the different IAA biosynthetic pathways is critical to understanding the roles of IAA in plant development. In addition, glucosinolates have been implicated in plant/ pathogen interactions, but their function remains unclear. The identification of CYP79B2 and CYP79B3 now provides tools to examine the biosynthesis and functions of these two important Trp secondary metabolites.

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