Differential Expression and Evolution of the Arabidopsis CYP86A Subfamily[1][w]

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Some members of the Arabidopsis (*Arabidopsis thaliana*) CYP86A and CYP94B cytochrome P450 monooxygenase subfamilies, which share some sequence homology with the animal and fungal fatty acid hydroxylases, have been functionally defined as fatty acid ω-hydroxylases. With these activities, these and other fatty acid hydroxylases have potential roles in the synthesis of cutin, production of signaling molecules, and prevention of accumulation of toxic levels of free fatty acids. The constitutive and stress-inducible patterns of the five Arabidopsis CYP86A subfamily members have been defined in 7-d-old seedlings and 1-month-old plant tissues grown under normal conditions, and 7-d-old seedlings treated with different hormones (indole-3-acetic acid, abscisic acid, gibberellin, methyl jasmonic acid, brassinosteroid, salicylic acid), chemicals (clofibrate, 1-amino-cyclopropane-1 carboxylic acid), or environmental stresses (cold, wounding, drought, mannitol, etiolation). Very distinct expression patterns exist for each of these fatty acid hydroxylases under normal growth conditions and in response to environmental and chemical stresses. Analysis of the promoter sequences for each of these genes with their expression patterns has highlighted a number of elements in current databases that potentially correlate with the responses of individual genes.

Multiple forms of cytochrome P450-dependent monooxygenases (P450s) catalyze in-chain hydroxylations, end-terminal hydroxylations, and epoxidations of medium- and long-chain fatty acids (Salaün and Helvig, 1995; Scheller et al., 1996; Simpson, 1997). Evidence exists that some of these enzymes have distinct substrate preferences and regiospecificities, with some modifying terminal positions on medium-chain fatty acids and others modifying internal positions on long-chain fatty acids. In plants, fatty acid hydroxylases are particularly important in the synthesis of plant cuticles and signaling molecules derived from fatty acids (Kolattukudy, 1980; Schweizer et al., 1996a; Fauth et al., 1998; Pinot et al., 1999). They also represent the primary enzymes responsible for preventing the accumulation of high, possibly toxic, concentrations of free fatty acids in the plant cells liberated by phospholipases in the early stress responses (Kahn and Durst, 2000). Because the fatty acid profiles of many crop plants are not ideal, there is much potential for the genetic manipulation of fatty acid hydroxylases aimed at improving commercial properties of crop plants.

In the broad set of activities catalyzed by fatty acid hydroxylases, those that catalyze the hydroxylation of the terminal methyl group on aliphatic fatty acid chains are designated as ω-hydroxylases, falling within many divergent P450 families and subfamilies. In mammalian systems that contain relatively large numbers of P450 genes, these P450s included in the CYP4 family are induced by groups of compounds known to induce peroxisome proliferation (Gibson, 1989; Johnson et al., 1996; Simpson, 1997). In yeast (*Saccharomyces cerevisiae*) strains that contain a very limited number of P450s, these P450s included in the CYP52 family are induced by aliphatic hydrocarbons (Seghezzi et al., 1991; Scheller et al., 1996, 1998; Ohkuma et al., 1998). Based on sequence conservations among these mammalian and yeast hydroxylases, the first plant fatty acid ω-hydroxylase designated CYP86A1 was cloned from Arabidopsis (*Arabidopsis thaliana*; Benveniste et al., 1998). Subsequent ω-hydroxylase clones have included CYP86A8 (Arabidopsis; Wellesen et al., 2001), CYP94A1 (*Vicia sativa*; Tijet et al., 1998), CYP94A2 (Vicia; LeBouquin et al., 1999), CYP94A3 (Vicia; GenBank accession no. AF092914), and CYP94A5 (*Nicotiana tabacum*; LeBouquin et al., 2001).

Heterologous expressions of these fatty acid hydroxylases have indicated that each has distinct substrate specificities. For example, CYP94A1 ω-hydroxylates saturated and unsaturated fatty acids with aliphatic chains ranging from C10 to C18 as well as 9,10-epoxystearic acid and 9,10-dihydroxysteric acid (Tijet et al., 1998; Pinot et al., 1999). CYP94A2 preferentially ω-hydroxylates short-chain fatty acids such as lauric acid (C12:0), and in-chain hydroxylates myristic acid (C14:0) and palmitic acid (C16:0) with varying proportions of the alternate activity on each of these substrates (Kahn et al., 2001). CYP94A5, CYP86A1, and CYP86A8 ω-hydroxylate saturated and unsaturated fatty acids with aliphatic chains ranging from C12 to C18 (Benveniste et al., 1998; LeBouquin et al., 2001; Wellesen et al., 2001). In contrast with the broad specificities defined for these ω-hydroxylases, the less

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extensively characterized CYP92B1 (*Petunia hybrida*) ω-hydroxylates one saturated fatty acid (lauric) and in-chain hydroxylates two unsaturated fatty acids (linoleic [C18:2] and linolenic [C18:3]; Petkova-Andonova et al., 2002). Two others that have not been extensively characterized (*Zea mays* CYP78A1 [Imaishi et al., 2000] and Petunia CYP703A1 [Imaishi et al., 1999]) have been reported to ω-hydroxylate lauric acid.

Given the involvement of many P450 families in mediating fatty acid hydroxylations occurring in different plants and animals, it is clear that P450s metabolizing these types of endogenous compounds are among the earliest and most diversified of the P450s that exist in eukaryotes. But, because of the multiplicity of related P450 genes in various plant species with potentially overlapping functions and the dearth of information on their expression patterns, the physiological significance of many of these enzymes has still not been completely established.

Among their many potential roles, fatty acid hydroxylases are important in the production of cutin in the aerial parts of plants and suberin in the roots of plants, which serve as structural components of the permeability barrier protecting plants against water loss and pathogen, insect, and mechanical damage. Derived from cellular lipids, cutin and suberin are polymeric networks of oxygenated C16 and C18 fatty acids cross-linked by ester bonds, such that the carboxyl group of one fatty acid is linked to a primary or secondary hydroxyl group of another (Kolattukudy, 2001). Characterization of the Arabidopsis *lcr* (*cyp86a8*) mutant has implicated the CYP86A8 protein in the synthesis of epidermal cutin (Wellesen et al., 2001). Characterization of the Arabidopsis *att1* (*cyp86a2*) mutant has directly indicated that the CYP86A2 protein is involved in cutin synthesis with the *att1* mutant containing only 30% of the cutin content present in wild-type plants (Xiao et al., 2004).

Once incorporated into elongated cutin, cutin polymers and monomers released from it play important roles in plant development and pathogen defense mechanisms. Cutin monomers released by fungal cutinase in the course of pathogenic infections are perceived as signals enhancing fungal cutinase expression and formation of fungal appressorial structures (Francis et al., 1996; Gilbert et al., 1996), as well as plant resistance components (Schweizer et al., 1996a, 1996b) and H_2O_2 production (Fauth et al., 1998). Induction of CYP94A1 transcript levels in Vicia plants in response to jasmonate (a defense hormone) and clofibrate (an inducer of mammalian peroxisome proliferation) indicates that plants amplify their responses to the initial levels of released cutin monomers by inducing expression of P450 proteins capable of generating additional cutin monomers (Pinot et al., 1998, 1999).

Against this background of plant-fungal interactions, it is now evident that fatty acid hydroxylases play important roles in determining plant-bacterial interactions. Inactivation of the CYP86A2 protein in the *att1* ethyl methanesulfonate mutant and the enhanced pathogenicity of *Pseudomonas syringae* associated with this mutation directly implicate cutin polymers derived from this P450 in establishing resistance to bacterial pathogens (Xiao et al., 2004). Cutin polymers also play important roles in plant development. Evidence of this is derived from studies of transgenic Arabidopsis plants overexpressing fungal cutinase, in which disruption of cuticular ultrastructures coincides with postgenital organ fusions (Sieberer et al., 2000), and from studies of the Arabidopsis *lcr* mutant expressing inactive CYP86A8 protein, in which disruption of cuticular structures coincides with the loss of apical dominance, delayed senescence, and abnormal trichome differentiation (Wellesen et al., 2001).

In addition to CYP86A1 and CYP86A8, whose fatty acid hydroxylase activities on various substrates have been functionally defined in heterologous expression systems, and CYP86A2, whose fatty acid hydroxylase activities have been inferred from mutational analysis, two additional *CYP86A* genes (CYP86A4 and CYP86A7) exist in the Arabidopsis genome (http://www.p450.kvl.dk/p450.shtml; http://Arabidopsis-P450.biotec.uiuc.edu). All five of these CYP86A sequences are typical non-A P450s with significant sequence homology to the fatty acid- and alkane-metabolizing CYP4 proteins and CYP52 proteins previously mentioned from mammals and fungi (Durst and Nelson, 1995; Werck-Reichhart et al., 2002). The degree of sequence conservation that exists within this P450 subfamily has suggested that CYP86A proteins mediate some conserved enzymatic functions, a prediction that has been borne out by our biochemical analysis. Given the importance of fatty acid ω-hydroxylases in cutin synthesis, plant-pathogen interactions and development, and the lack of information on their regulation, we have examined the tissue specificity and stress inducibility of all five members of the CYP86A subfamily. The results provide insight into the cytological locations and stress inductions of these fatty acid hydroxylases.

RESULTS

Comparison of the CYP86A Coding Sequences

The five members of the CYP86A subfamily are scattered over four of the five Arabidopsis chromosomes. As indicated by their genomic locus numbers (At5g58860, At4g00360, At1g01600, At1g63710, At2g45970), CYP86A1 is located on the lower arm of chromosome 5, CYP86A2 is located on the very top of chromosome 2, CYP86A4 is located on the very top of chromosome 1, CYP86A7 is located on the lower arm of chromosome 1, and CYP86A8 is located very close to the bottom of chromosome 2 (http://www.p450.kvl.dk/p450.shtml).

Supported by the existence of full-length cDNA clones for each of these five loci (http://Arabidopsis-P450.biotec.uiuc.edu), comparisons conducted at the

nucleotide level indicate that these genes have very different organizations, with CYP86A1 having a single intron interrupting the coding sequence at position 1,140 nt (relative to ATG), CYP86A2 and CYP86A4 having a single intron interrupting the coding sequence at position 422 (relative to ATG), and CYP86A7 and CYP86A8 having no introns. The intron of CYP86A1 is relatively large (457 nt) compared to the smaller conserved introns of CYP86A2 (166 nt) and CYP86A4 (130 nt) that are 51.9% identical to one another. Except for the splice-site junctions, no significant matches exist between the CYP86A1 intron and the CYP86A2 and CYP86A4 introns. Pairwise comparisons between the five Arabidopsis CYP86A sequences indicate that, as shown in Table I, CYP86A2 and CYP86A4 have the highest degree of nucleotide identity within their coding sequence (82.3%). CYP86A2 and CYP86A4 have similarly high degrees of identity with CYP86A8 (73.6% and 73.3%, respectively). CYP86A7 shares comparably high identity with CYP86A8 (70.1%), CYP86A4 (69.8%), and CYP86A2 (68.4%). CYP86A1 has distinctly lower identity with the other four members of this P450 subfamily (62.3%–64.5%).

Pairwise comparisons conducted at the protein level indicate that these proteins are well conserved throughout most of their coding sequence and display the same derived relationships as seen at the nucleotide level (Table II). CYP86A2 and CYP86A4 have the highest degree of sequence identity (87%), with both equally close to CYP86A8 (75% and 76%, respectively) and, to a lesser degree, to CYP86A7 (70% and 68%, respectively). CYP86A1 has lower similarity with the other four members of the CYP86A subfamily (61%–62%). Quite unusually, these P450s differ in their amino acid lengths from 513 (CYP86A1), 524 (CYP86A7), 537 (CYP86A8), and 553 (CYP86A2), to 557 (CYP86A4). Alignments at the primary sequence level indicate that nearly all of these length differences exist at the C terminus, with CYP86A7 extending 11 residues, CYP86A8 extending 22 residues, CYP86A4 extending 36 residues, and CYP86A2 extending 37 residues beyond the termination point of CYP86A1 (Fig. 1). Analysis of the sequence composition in the extended tails of CYP86A2 and CYP86A4 shows a disproportionate number of nonpolar Val, Ala, and Gly residues (67% and 58%, respectively).

Molecular models have been developed for these five proteins using molecular operating environment (MOE, Chemical Computing Group, Montreal) programs as detailed by Rupasinghe et al. (2004) for other Arabidopsis P450s. Based on these structural predictions, assignments of the substrate recognition site (SRS; Gotoh, 1992) regions have indicated that all five P450s are nearly identical in SRS1, more variable in SRS4 and SRS5, and most variable in SRS2, SRS3, and SRS6 (Fig. 1; Supplemental Fig. 1). The identities in these regions, which are thought to contribute to defining catalytic site specificity, are striking given the level of overall sequence divergence in these proteins. Even more striking is the degree to which the backbones predicted for these proteins overlay one another (data not shown), with all displaying a longer J-K loop or β-pleated sheet structure compared with the crystal structure determined for bacterial CYP102 (Ravichandran et al., 1993; Li and Poulos, 1994, 1997), and all except CYP86A2 displaying a longer F-G loop (SRS2). Among the few more divergent regions that do exist, CYP86A2 and CYP86A8 have longer G-H loops, potentially affecting interactions with P450 reductase; CYP86A2 and CYP86A4 have longer B′-C loops containing SRS1; and CYP86A2, CYP86A4, and CYP86A8 have longer exterior surface loops between β-sheets 3-2 and 4-2. While this relatively small number of backbone differences between these proteins suggests that their catalytic sites have similar configurations, amino acid variations in SRS4, SRS5, and SRS6 suggest that these P450s have the potential to metabolize different ranges of fatty acids.

Table I. *Pairwise nucleotide comparisons of CYP86A proteins*

	CYP86A2	CYP86A4	CYP86A7	CYP86A8
CYP86A1	63.0%	62.8%	62.5%	62.3%
CYP86A2		82.3%	68.4%	73.6%
CYP86A4			69.8%	73.3%
CYP86A7				70.1%

Table II. *Pairwise amino acid comparisons of CYP86A proteins*

	CYP86A2	CYP86A4	CYP86A7	CYP86A8
CYP86A1	72.3%/ 62.0%[a]	71.2%/ 60.9%	70.9%/ 60.9%	71.1%/ 60.7%
CYP86A2		91.4%/ 87.1%	81.4%/ 69.6%	84.1%/ 74.5%
CYP86A4			80.4%/ 68.4%	85.3%/ 75.7%
CYP86A7				80.8%/ 70.7%

[a]Percent similarities/percent identities.

Phylogeny

An unrooted phylogenetic tree of potentially orthologous fatty acid ω-hydroxylases from different organisms was constructed based on multiple sequence alignments performed using ClustalW. Included in this comparison were all fatty acid ω-hydroxylases known to exist in plants, some representative fatty acid ω-hydroxylases from mammalian (CYP4 family) and yeast (CYP52 family) systems, a bacterial fatty acid hydroxylase (CYP102), as well as four A-type plant P450s, which are CYP73A5 (*t*-cinnamic acid hydroxylase), CYP83A1 (indole-3-acetyldoxime *N*-hydroxylase), CYP84A1 (ferulate 5-hydroxylase), and CYP98A3 (*p*-coumaryl shikimic/quinic acid 3′-hydroxylase). Different algorithms, including neighbor joining, maximum parsimony, and maximum

Figure 1. SRS regions of Arabidopsis CYP86A proteins. SRS regions, defined on the basis of molecular models built for each of these proteins, are aligned with red designating residues conserved in five sequences, green designating residues conserved in four sequences, blue designating residues conserved in three sequences, and black designating residues conserved in only two sequences. The SRS6 regions of these proteins are followed by the variable-length C-terminal sequences present on each of these proteins.

evolution within the MEGA program, generated the same phylogenetic tree whether nucleotide or amino acid sequences were used. The organization of the CYP86A clade is completely concordant with the paralogous relationships determined by our pairwise comparisons. Taking into account the positions of introns in these various genes, it appears that, after the first gene duplication within the CYP86A clade, one copy of the ancestral sequence evolved into the present-day *CYP86A1* gene, and another served as the common ancestor for the other four *CYP86A* genes. After the loss of an intron from this second ancestral sequence, the *CYP86A7* gene evolved separately after a second duplication, the *CYP86A8* gene evolved separately after a third duplication, and the most recent *CYP86A4* and *CYP86A2* genes duplicated and evolved after an intron was inserted at an alternate position in the coding sequence (Fig. 2B). Interestingly, the closest relatives of the *CYP86A1* gene in Arabidopsis are the *CYP86B1* and *CYP86B2* genes (43%–45% overall identity), which both contain a single intron interrupting the coding sequence at the position only 12 nt downstream from the single intron in the *CYP86A1* gene. The SRS regions of these two proteins share significant identity with the SRS1, SRS4, and SRS5 regions of the CYP86A1 protein, and limited identity with its SRS2, SRS3, and SRS6 regions.

From the unrooted tree (Fig. 2A), it is apparent that, except for the less well-characterized CYP78A1, CYP92B1, and CYP703A1 proteins, all fatty acid ω-hydroxylases exist in one phylogenetic group, regardless of the fact that they are present in different species and even different kingdoms. The fact that CYP703A1, CYP92B1, and CYP78A1 show up in the plant-specific A-type group, represented by the CYP73A5 and three others, suggests that conserved activities, such as fatty acid hydroxylation normally mediated by non-A-type P450s found in a variety of organisms, can be mediated at some marginal level by A-type P450s.

Fatty Acid Metabolism by CYP86A2, CYP86A4, and CYP86A7

As noted previously, CYP86A1 and CYP86A8 have been functionally defined as ω-hydroxylases by heterologous expression in yeast systems (Benveniste et al., 1998; Wellesen et al., 2001). To determine whether other members of this subfamily are also capable of metabolizing short-chain fatty acids, such as lauric acid (C12:0), all five CYP86A proteins were heterologously expressed in insect cells using baculovirus-mediated expression systems. As demonstrated by metabolism of [1-^{14}C]lauric acid in the presence and absence of NADPH followed by thin-layer chromatography (TLC) analysis of the radioactive products, all five of these CYP86A proteins produce ω-hydroxylauric acid (designated M1 in Fig. 3). The second metabolite, designated M2 in Figure 3, is a metabolite of [1-^{14}C]lauric acid generated in the absence of NADPH in control Sf9 cell microsomes and likely represents the product of an endogenous Sf9 cell fatty acid desaturase.

Tissue Profiling of CYP86A Transcripts

To elucidate the physiological functions of different members within this subfamily, we next defined the constitutive expression levels of the CYP86A transcripts in different tissues of Arabidopsis seedlings and mature plants. For this, RNA samples from 7-d-old shoots and roots, 3-week-old rosettes, and 1-month-old mature leaves, roots, siliques, and

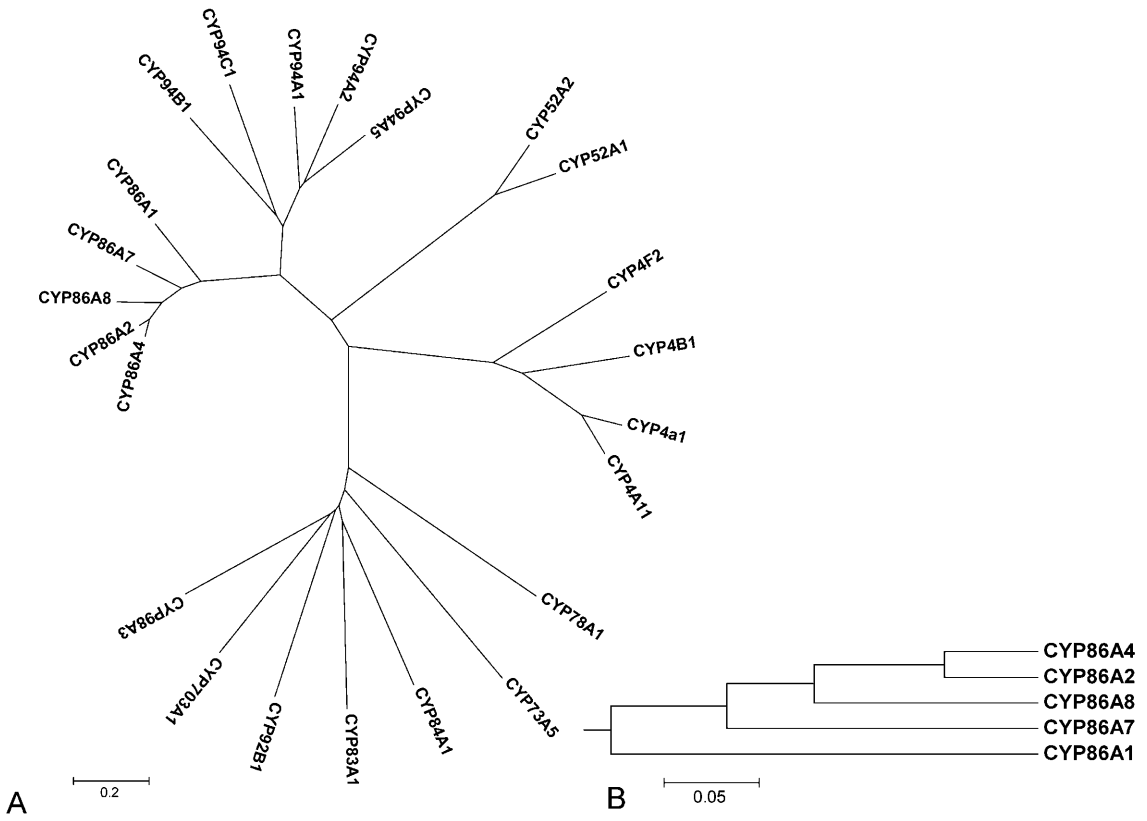

Figure 2. Phylogenetic trees for fatty acid ω-hydroxylases. A, An unrooted phylogenetic tree, developed using MEGA version 2.1 (Kumar et al., 2001), includes the fatty acid ω-hydroxylases from Arabidopsis (CYP94B1, CYP94C1, CYP86A subfamily), *V. sativa* (CYP94A1, CYP94A2), *N. tabacum* (CYP94A5), *P. hybrida* (CYP92B1, CYP703A1), *Z. mays* (CYP78A1), *Candida tropicalis* (CYP52A1, CYP52A2), rat (CYP4a1), human (CYP4A11, CYP4B1, CYP4F2), and a number of Arabidopsis P450s not known for metabolizing fatty acids (CYP73A5, CYP84A1, CYP83A1, CYP98A3). B, The phylogenetic tree for the CYP86A subfamily is drawn proportional to branch lengths calculated using maximum parsimony analysis with the proportional bootstrap value shown below.

flowers were reverse transcription (RT)-PCR amplified using 5′ gene-specific primers and a 3′ primer complementary to the poly(A) tract present on mature mRNAs, and varying PCR cycle numbers determined to quantitatively amplify each transcript. RT-PCR products were subsequently gel blotted, hybridized with probes corresponding to the gene-specific microarray elements, and quantified by phosphor imager analysis. Normalization of each total RNA sample against its level of constitutive elongation factor EF-lα transcript and subsequently against the level of P450 transcript present in seedling shoots was performed to allow for comparisons between tissues and with microarray data described elsewhere (http://Arabidopsis-P450.biotec.uiuc.edu). As shown in Figure 4, A to C, RT-PCR analyses show strong tissue-specific patterns of expression for these genes. CYP86A1 transcripts are 17-fold more abundant in seedling roots than in seedling shoots, and 20-fold more abundant in mature roots (Fig. 4A). Contrasting with this, CYP86A8 transcripts are abundant in seedling shoots, 2-fold more abundant in flowers than in seedling shoots, and less abundant in all other tissues than in seedling shoots. CYP86A2 transcripts are similarly abundant in seedling shoots and most other tissues analyzed, and 2- to 10-fold less abundant in seedling roots, mature roots, and flowers. CYP86A4 and CYP86A7 transcripts display significantly more tissue specificity, with both expressed at their highest levels in flowers and at lower levels in stems and siliques, and CYP86A4 transcripts also expressed in seedling shoots and roots and older rosettes. These tissue fluctuations are in strong agreement with microarray analysis (Fig. 4, B and C), using P450 gene-specific microarrays constructed with 3′ untranslated region sequences (http://Arabidopsis-P450.biotec.uiuc.edu). Together, these data highlight the tissue-specific expression patterns of genes categorized within the same P450 subfamily and support the tissue profiles obtained by microarray analysis.

Stress Profiling of CYP86A Transcripts

To further elucidate the inducibilities of transcripts in this P450 subfamily, the inducible transcript levels were defined by RT-PCR gel-blot analysis, with RNAs isolated from 7-d-old seedlings treated with different hormones such as indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellin (GA), methyl jasmonic acid

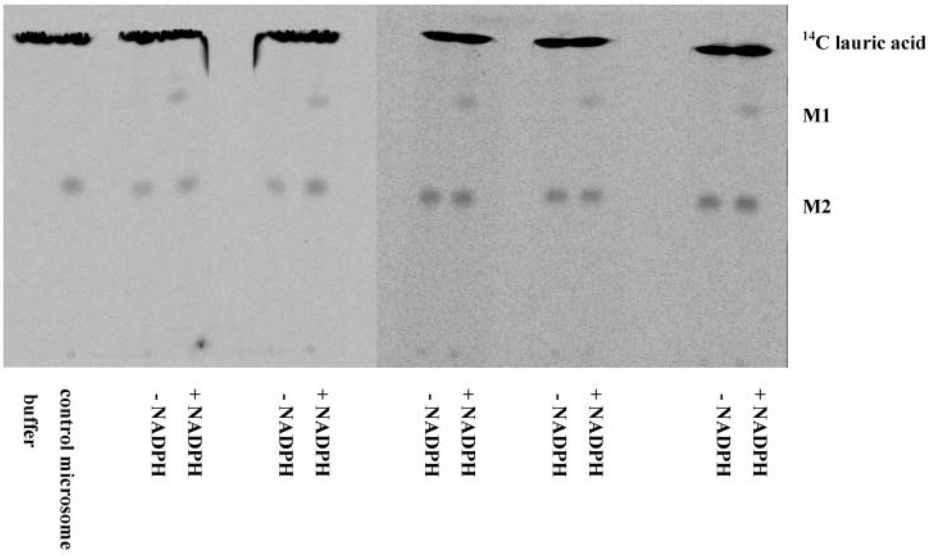

Figure 3. TLC analyses of [1-^{14}C]lauric acid and its metabolites. The metabolism of lauric acid was assayed according to the method described in "Materials and Methods." Positions of unmetabolized substrate and metabolites (M1 and M2) are labeled. Microsomes containing individual heterologously expressed CYP86A proteins (lanes 3 and 4, CYP86A1; lanes 5 and 6, CYP86A2; lanes 7 and 8, CYP86A4; lanes 9 and 10, CYP86A7; lanes 11 and 12, CYP86A8) were incubated with substrate in the presence or absence of NADPH. Samples in the left two lanes were incubated with buffer (lane 1) or control microsomes obtained from insect cells infected with nonrecombinant virus (lane 2).

(MeJA), brassinosteroid (BR), and salicylic acid (SA) or chemicals such as clofibrate and 1-aminocyclopropane-1-carboxylic acid (ACC), for varying times, or stressed with cold, wounding, drought, mannitol, or etiolation. Normalization of each total RNA sample was done against its level of constitutive EF-lα transcript, and induction levels were recorded against the transcript levels in the corresponding control samples (0.1% ethanol for IAA, ABA, JA, SA, BR; sterile water for ACC, mannitol, clofibrate; unstressed light-grown tissue for cold, wounding, drought, etiolated, dark adapted). As shown in Figure 5, CYP86A1 transcripts were transiently induced 2.7-fold by 3 h of ABA treatment and 2.5-fold by 3 h of ACC treatment, more continuously induced 1.7- to 1.8-fold by 3 and 27 h of mannitol treatment and 1.6- to 1.9-fold by 3 and 27 h of clofibrate treatment, and more slowly induced 1.9-fold by 27 h of cold treatment and 1.6-fold by 27 h of BR treatment. CYP86A1 transcripts were repressed 2.4-fold by 27 h of IAA treatment and 5.9-fold by 27 h of SA treatment. CYP86A2 transcripts were transiently induced 2.5-fold by 3 h of wounding and ABA treatment, 2.3-fold by 3 h of mannitol treatment, 1.7-fold by 3 h of IAA treatment, 1.6-fold by 3 h of clofibrate treatment, more continuously induced 2.0- and 1.9-fold by 30 and 120 min of drought treatment, and induced 1.9- to 2.3-fold in etiolated and dark-adapted seedlings compared to light-grown seedlings of the same age. CYP86A2 transcripts were not significantly repressed under any of the conditions tested here. CYP86A4 transcripts were transiently induced 1.9-fold and 3.0-fold by 3 h of ABA and IAA treatment, respectively, and more continuously induced 1.9- to 1.7-fold by 3 and 27 h of cold treatment. In contradiction to the transient responses of other CYP86A transcripts, CYP86A4 transcripts were repressed 9.0-fold by short-term ACC treatment and induced 1.9-fold by long-term ACC treatment. CYP86A4 transcripts were also repressed 3.6-fold by 3 h of wounding and 2.4-fold in etiolated seedlings. CYP86A7 transcripts were transiently induced 1.9-fold by 3 h of clofibrate treatment, more continuously induced 1.6- and 1.5-fold by 3 and 27 h of MeJA treatment, and slowly induced 1.7-fold by 27 h ABA treatment. CYP86A7 transcripts were significantly repressed by 2 h of drought treatment, 3 h of SA and wounding treatments, 3 and 27 h of ACC and mannitol treatments, and in both etiolated and dark-adapted seedlings. CYP86A8 transcripts, encoding the last member of this subfamily, were induced only by 3-h IAA and ABA treatments and repressed by 30 min of wounding. Notably, most of these genes did not respond significantly to MeJA or BR treatment. Only CYP86A7 was moderately increased by MeJA, and CYP86A1 was moderately increased by BR.

Promoter Analysis

Searches for identifiable promoter elements in sequences 2 kb upstream of the translation start site were performed using two databases, Arabidopsis Gene Regulatory Information Server (AGRIS; http://Arabidopsis.med.ohio-state.edu) and PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE). The first one contains cis-elements characterized only in Arabidopsis studies, and the second one contains cis-elements characterized in a variety of different plant species. Elements from the AGRIS database found in the *CYP86A* promoters and 5′ untranslated region are listed in Table III (under Elements Found in AGRIS) and a partial list of elements from the PlantCARE database is included in Table III (under Elements Found in PlantCARE). The chemical/stress treatments inducing transcript accumulation at least 1.5-fold in our RT-PCR analyses are listed below each column, with an asterisk indicating the existence of a known cis-element for a particular chemical/environmental inducer. Among the elements identified in the first Arabidopsis-specific database, there is good agreement

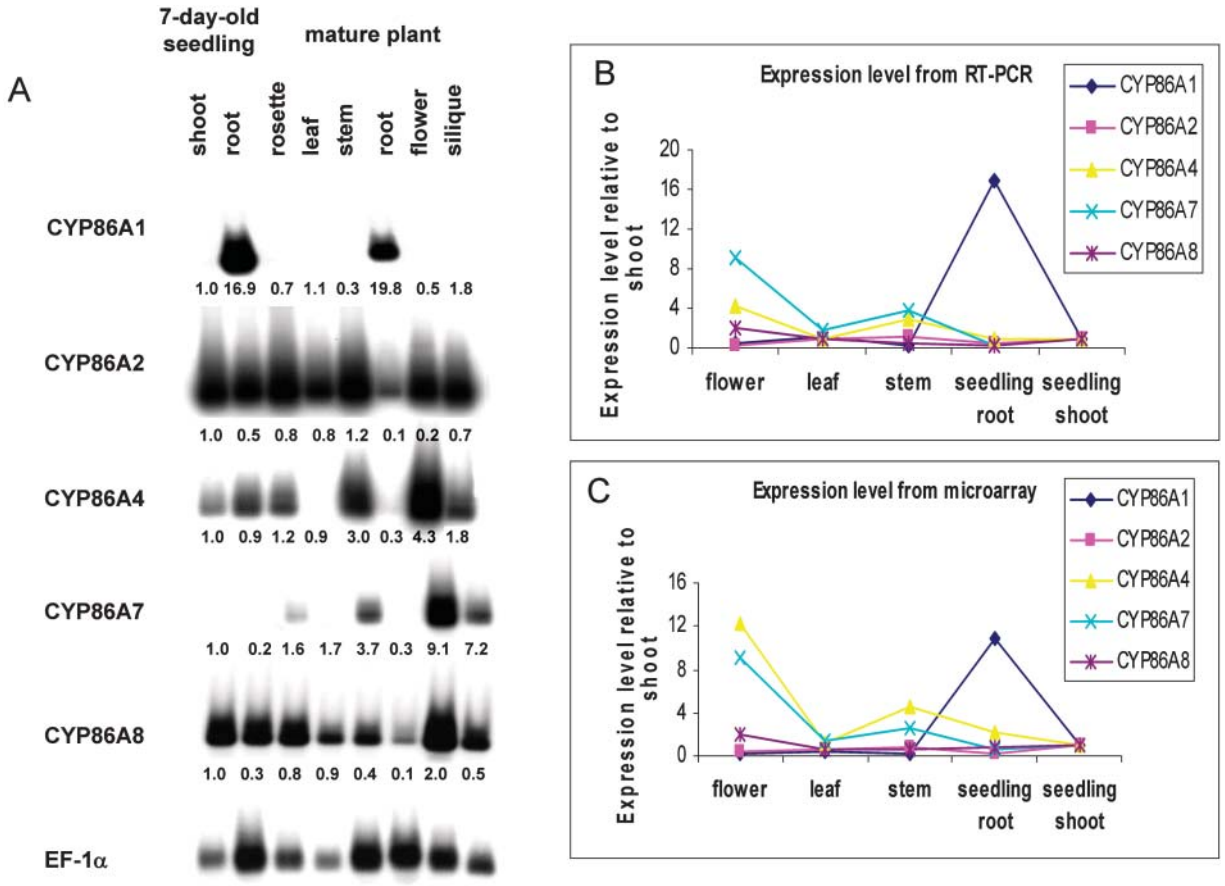

Figure 4. Tissue profiling of CYP86A transcripts. A, Total RNAs isolated from 7-d-old seedling shoots (lane 1) or roots (lane 2), 3-week-old rosettes (lane 3), 1-month-old plant leaves (lane 4), stems (lane 5), roots (lane 6), flowers (lane 6), and green siliques (lane 7) were RT-PCR amplified as outlined in "Materials and Methods," using gene-specific primers for each of the CYP86A transcripts or the EF-1α transcript shown at the left of each image. The RT-PCR products were electrophoresed on 1.0% agarose gels, blotted to Hybond-N nylon membranes, and probed with ^{32}P-labeled gene-specific fragments. The induction level for each transcript calculated after normalization to the amount of EF-1α transcript is recorded below each lane relative to the transcript level in seedling shoots. B and C, The relative expression levels defined from RT-PCR analysis in A are compared with those defined by microarray analysis on P450-specific microarrays (http://Arabidopsis-P450.biotec.uiuc.edu).

between the cis-element search and RT-PCR analysis of responses to ABA for all five *CYP86A* genes, as well as response to IAA for CYP86A4. Likewise, the CYP86A1 and CYP86A2 responses to cold/mannitol and drought/mannitol treatments correlate with the presence of ABA response element (ABRE)-like elements (dehydration/low temperature) and/or AtMYC2 elements (drought/ABA) in their promoters. However, stress-specific elements correlating with the CYP86A4 response to cold at 3 and 27 h, the CYP86A7 response to MeJA at 3 and 27 h, the CYP86A2 response to wounding at 3 h, and the CYP86A1/CYP86A4 responses to ethylene do not exist either due to the existence of novel elements in these promoters or to the high stringency of search functions in this database. In the second plant-specific database, multiple cis-elements not highlighted in the first search correlate with the responses of individual genes. Additional elements identified in this search include some correlating with the CYP86A1/CYP86A4 responses to ACC, the CYP86A2 response to wounding, the CYP86A4 response to cold, and the CYP86A7 response to MeJA. But, in three of these cases (ACC, wounding, MeJA), the additional elements identified in this search exist in multiple copies in promoters not responding to these stresses. In addition, emphasizing the differences between the array of consensus sequences present in these two promoter search sites, the second broader search identified no auxin response elements putatively mediating the CYP86A2 and CYP86A4 response to IAA. Based on these comparisons, the Arabidopsis-specific promoter search process more accurately highlights elements that may be involved in the responses of individual promoters, although, because of the stringencies applied, there is potential to miss response elements.

Because both databases lack elements known to mediate responses to some of these chemicals, these promoters were searched for novel sequence identities using the PromoterWise program. The initial results

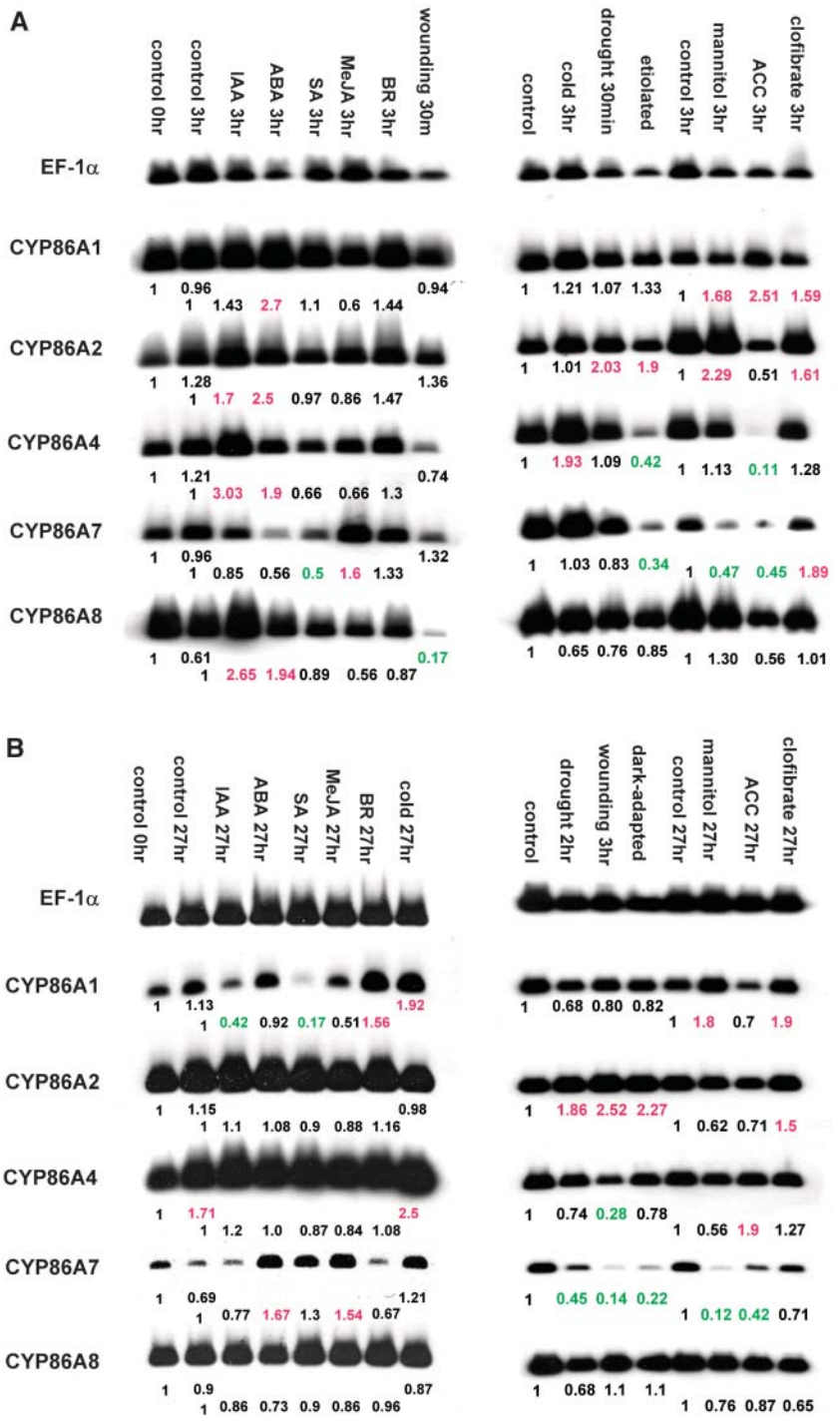

Figure 5. Stress profiling of CYP86A transcripts. Total RNAs isolated from intact 7-d-old seedlings treated with 0.1% ethanol (controls in left image) or water (control in right image), 100 μM IAA, 50 μM ABA, 1 mM SA, 100 μM MeJA, 1 μM BR, 500 mM mannitol, 1 μM ACC, or 1 mM clofibrate, stressed with cold, drought, or wounding for the times indicated, were RT-PCR amplified with gene-specific primers for the CYP86A transcripts and EF-1α transcripts shown at the left. The RT-PCR products were electrophoresed on 1.0% agarose gels, blotted to Hybond-N nylon membranes, and probed with ^{32}P-labeled gene-specific fragments. The induction level for each transcript calculated after normalization to the amount of EF-1α transcript is recorded below each lane relative to the transcript level in its matched control sample. Red designates normalized ratios induced more than 1.5-fold, and green designates normalized ratios repressed more than 2.0-fold.

from this pairwise comparison were manually checked to eliminate direct and inverted matches having less than seven contiguous nucleotides within aligned regions and less than two guanosines or cytosines. The final compilation of conserved elements, shown in Table IV, indicates that the *CYP86A2/CYP86A8* and *CYP86A4/CYP86A8* promoter pairs contain the greatest number of extended sequence elements (nine total), with many in the *CYP86A4/CYP86A8* alignment occurring in the same orientation (designated D in Table IV). The *CYP86A1/CYP86A2* and *CYP86A1/CYP86A4* promoter pairs contain slightly fewer shared sequences with seven and eight extended sequence elements, respectively. The *CYP86A2/CYP86A4* promoter pair has the fewest extended sequence elements (three total), but two of these are the longest ones (14 and 25 nt) identified in this search process. Interestingly, pairwise alignments between the *CYP86A2* and *CYP86A4*

Table III. *Analysis of CYP86A promoters*

*, Indicates agreement between our RT-PCR results and existence of a known cis-element for the same inducer.

Element	Function	CYP86A1	CYP86A2	CYP86A4	CYP86A7	CYP86A8
Elements Found in AGRIS						
ABF/ABRE	ABA	1*				
ABFs	ABA	1*				
ABRE-like	Dehydration/low temperature	1*				
AG in SUP/AP1 in AP3	MADS box domain		1			1
ARF/ARF1	Auxin			3*		
ATHB2	Homeobox domain	1				
ATHB6	ABA			1*		
AtMYC2 in RD22	Drought/ABA	1*	2*	1*	3*	1*
Box II	Core		2	3	3	1
CArG	Flowering		1			2
CCA1	Phytochrome	1	1			1
DPBF1 and 2	ABA/embryo	2*	4*	3*	3*	2*
Evening element	Circadian	1				
GATA	Light response	4	5	5	6	7
G box	Core	1				
HSEs	Heat stress		1			
I box	Light	3				
L1 box	L1 cell of shoot apices	1				
LTRE	Low temperature					1
MYB	Flowering/phenylpropanoid			3		1
MYB1	G-protein binding site					1
MYB4	Stresses		2	4	2	4
RAV1-A	Plant specific	9	4	2	8	4
RAV1-B	Plant specific					1
SV40 core	Core		1			
T box	Elements in GAPB gene	2	1	2		2
TELO box	Root		1			2
W box	Disease	2	4	4		2
Z box	Elements in CAB1 gene		1			
		ABA*	ABA*	ABA*	ABA*	ABA*
		ACC	Clofibrate	ACC	MeJA	IAA
		Clofibrate	Drought*	Cold	Clofibrate	
		Cold*	Etiolation	IAA*		
		BR	Dark adaptation			
		Mannitol*	IAA			
			Mannitol*			
			Wounding			
Elements Found in PlantCARE						
ABRE (from multiple species)	ABA	8*	7*	2*	5*	3*
AUXRE	Auxin					1*
AUXRE core	Auxin	2			1	1*
CGTCA	MeJA				1*	2
ERE	Ethylene	9*	5	3*	2	8
GC motif	Anoxic	3			6	1
HSE	Heat stress	12	15	25	16	15
LTR	Low temperature	1*		1*		1
MBS	Drought	1*	2*			
P box	GA	1	6	7	3	2
TATC box	GA	2	1	1	1	3
TCA element	SA	3	6	10	3	5
TGA box	Auxin					1*
TGACG motif	MeJA				1*	2
TGA-1	Auxin	2				
WUN motif	Wounding	7	21*	14	19	15
		ABA*	ABA*	ABA*	ABA*	ABA*
		ACC*	Clofibrate	ACC*	MeJA*	IAA*
		Clofibrate	Drought*	Cold*	Clofibrate	
		Cold*	Etiolation	IAA		
		BR	Dark adaptation			
		Mannitol*	IAA			
			Mannitol*			
			Wounding*			

Table IV. *Direct and inverted elements conserved between CYP86A promoters*

*, Numbers in parentheses are positions of the elements relative to ATG start codon. D, Same element is in same direction on two promoters; I, same element is in inverted direction on two promoters.

	CYP86A2	CYP86A4	CYP86A7	CYP86A8
CYP86A1	GAAGATTAAAT (A1-180 A2-839 I)* AAGTACA (A1-686 A2-1205 D) AACAATGTTT (A1-837 A2-1720 D) TCTCAACAAT (A1-890 A2-217 I) TTATTTGTG (A1-1062 A2-273 D) AGTCAAA (A1-1104 A2-338 D) AAGAGAA (A1-1255 A2-362 D)	ATTTTGTTTCA (A1-150 A4-668 I) AAAACAC (A1-698 A4-922 I) AAAACGCAA (A1-731 A4-1312 I) ATCAGAAAA (A1-924 A4-1376 D) CTGATTTTTTT (A-962 A4-1369 I) CACAAAC (A1-1162 A4-631 I) AACCAAAA (A1-1289 A4-621 I) TTTGCAG (A1-1891 A4-108 D)	TGTTCTGTG (A1-26 A7-1878 I) GAGAGTAAAC (A1-342 A7-1730 I) CACAATATCA (A1-948 A7-515 I) AATACAATTG (A1-1873 A71346 I)	TATTTTGTTTCAA (A1-149 A8-1976 D) TGATATGAAATA (A1-530 A8-965 I) ATCAGAAATT (A1-924 A8-1647 D) ATAGTCT (A1-996 A8-908 D) TATGTGAA (A1-1457 A8-326 D)
CYP86A2		CAAAATGT (A2-1025 A4-1408 I) GTTCTCAACTAAT-CTCATTTAATT (A2-503 A4-427 D) CATTAATGAGGGTT (A2-200 A4-168 D)	TTCTTCAAA (A2-1820 A7-956 D) ATGTTATTGCT (A2-1292 A7-690 D) TTTGATC (A2-810 A7-1967 D) TTAACCC (A2-618 A7-823 D) TTAATGCC (A2-147 A7-1170 D)	CTCCGAT (A2-1627 A8-1681 D) GTACTATA (A2-1271 A8-1053 I) CATGAATGCATTG (A-1120 A8-1791 I) AAAGAGAGAGAGA (A2-1010 A8-143 I) CTCTTTAACACTTC (A2-927 A8-135 D) TCCCTCA (A2-870 A8-34 I) AAACCAA (A2-845 A8-1949 D) ATCATTGGT (A2-760 A8-1238 D) TTCTCTATAT (A2-115 A8-267 I)
CYP86A4			GAAGAAACT (A4-1097 A7-1935 I) ATAGAT TAAAAAA (A4-755 A7-99 I) TTAATTCACC (A4-423 A7-298 I) AAAAGAG (A4-4 A7-1322 I)	ATAGATGT (A4-1860 A8-826 I) ATTTTCTGATA (A4-1458 A8-1645 D) TGAGAAGAAGA (A4-1101 A8-921 D) CTTCCTTCA (A4-1058 A8-1737 D) CCTTTAT (A4-950 A8-420 D) ACCAAACC (A4-625 A8-1285 D) ACCCTAAA (A4-524 A8-318 D) CAAAAATCA (A4-44 A8-1772 D) GAAACAA (A4-15 A8-1920 I)
CYP86A7				AAGAAGAGAATT (A7-39 A8-916 I) AAAAGAAAAG (A7-136 A8-1966 I) GCGTGATC (A7-755 A8-1376 D) ACCTTTATA (A7-940 A8-419 I) TAAAACC (A7-1652 A8-158 I)

promoters indicate that they have high degrees of identity, with 52.1% identity in 1.5 kb upstream from the translation start site decreasing only slightly to 50.5% identity in 2.0 kb upstream from the translation start site. Notably, the region from −442 to −373 (Fig. 6) has more than 88% (61/69) identity. Pairwise alignments of the other *CYP86A* promoters indicated no other significant identities.

DISCUSSION

Current data indicate that plants have different types of enzymes responsible for fatty acid hydroxylations (Blee and Schuber, 1993; van De Loo et al., 1993; Cabello-Hurtado et al., 1998), but among these, only P450-dependent enzymes are responsible for hydroxylations at the ω-position (Wellesen et al., 2001). Multiple P450 families appear to mediate this hydroxylation with the plant CYP86 and CYP94 families representing two major groups of fatty acid ω-hydroxylases (Benveniste et al., 1998; Tijet et al., 1998; Pinot et al., 1999; Kahn et al., 2001; LeBouquin et al., 2001; Wellesen et al., 2001; Petkova-Andonova et al., 2002). In the CYP86A subfamily of Arabidopsis, which contains five different members that function as fatty acid ω-hydroxylases, CYP86A1 is the oldest member and CYP86A2 and CYP86A4 are the most recently duplicated members. Compared to other plant P450 subfamilies, these five CYP86A proteins share high degrees of primary sequence identity (between 62% and 82%) despite very different lengths (between 513 and 557 amino acids). Nearly all of the length differences are attributable to C-terminal extensions on these proteins, with CYP86A2 and CYP86A4 having 14 to 15 more C-terminal amino acids than CYP86A8, 25 to 27 more amino acids than CYP86A7, and 36 to 37 more amino acids than CYP86A1. Because these additional amino acids lie outside presumed SRSs, it is not known whether they affect the biochemical activities and/or physiological functions of the CYP86A proteins. Molecular modeling of these C-terminal extensions, which contain an unusually high number of nonpolar Val, Ala, and Gly residues, predicts that they form random coil structures extending from the external surface of these proteins.

Phylogenetic analysis of all functionally defined plant, mammalian, and fungal fatty acid ω-hydroxylases indicate that plant fatty acid ω-hydroxylases can be divided into two groups. One well-characterized group (CYP86A, CYP94A, CYP94B) shares a common ancestor with mammalian and fungal sequences, and the other, less well-characterized group (CYP78A, CYP92B, CYP703A) shares a common ancestor with the plant-specific group involved in the synthesis and metabolism of many secondary metabolites. These groups are consistent with the enumerations of plant P450s (http://drnelson.utmem.edu/Arablinks.html) as well as the functional analysis of CYP86A and CYP94B proteins, showing that they are involved in the synthesis and metabolism of fatty acids common to all organisms. Analysis of the more divergent CYP78A1, CYP92B1, and CYP703A1 proteins indicate that, although they have lauric acid ω-hydroxylase activity, their activities are generally very low for this substrate, possibly because they have other physiological substrates.

Phylogenetic comparisons at the gene level indicate that the Arabidopsis *CYP86A* sequences, which contain introns at different positions, arose either from the divergence of a common ancestral gene by a complicated series of intron deletions and reinsertions or from the convergence of several ancestral genes for a common function. Alignments of the intron-containing *CYP86A1*, *CYP86A2*, and *CYP86A4* genes with one another indicate that introns occur at different positions in the *CYP86A1* and *CYP86A2/CYP86A4* genes, suggesting that the single intron in the *CYP86A1* gene was lost during the evolution of these genes and that the reinserted intron in the *CYP86A2/CYP86A4* genes is not related to original CYP86A1 intron. The alignment of the intron in the *CYP86A1* gene with single introns in the *CYP86B1* and *CYP86B2* genes in Arabidopsis supports this hypothesis of intron loss from the ancestral *CYP86A* gene existing in dicots. Phylogenetic comparisons of the Arabidopsis *CYP86A* genes with the recently identified three *CYP86A* genes and six *CYP86A* pseudogenes in the *Oryza sativa* genome (Nelson et al., 2004) have begun to resolve this evolutionary history by showing that, at the protein level, Arabidopsis CYP86A1 is most closely related to Oryza CYP86A9, Arabidopsis CYP86A2 and CYP86A4 are most closely related to Oryza CYP86A10 and CYP86A11, and the remaining Arabidopsis CYP86A7 and CYP86A8 are most closely related to Oryza pseudogenes. Notably, none of these rice *CYP86A* genes contain introns.

```
                        -442                                                -392
Promoter_for_CYP86A2(-571) TACTTACTTCTTCTTATTCCCATCCATATTTTTCTGTGCCTTTAGGTTCT
Promoter_for_CYP86A4(-494) TACTTATTTCTTCTTCTTCCCCACCATCTTTTTTTGTGC-TCTATGTTCT
          Consensus(-442) TACTTA TTCTTCTT TTCCC  CCAT TTTTT TGTGC T TA GTTCT
                        -391            -373
Promoter_for_CYP86A2(-521) CAACTAATCTCATTTAATT
Promoter_for_CYP86A4(-444) CAACTAATCTCATTTAATT
          Consensus(-391) CAACTAATCTCATTTAATT
```

Figure 6. Comparison of the *CYP86A2* and *CYP86A4* promoters. Absolute conservations in the *CYP86A2* and *CYP86A4* promoters identified using the PromoterWise program (http://www.ebi.ac.uk/Wise2/promoterwise.html) are shaded.

The significant conservation that these five CYP86A proteins share at the primary sequence level becomes even more significant when one examines the very limited set of amino acid variations that occur in the six putative SRSs. Based on this higher level of primary sequence conservation and molecular modeling of these proteins, it appears that they might mediate some redundant substrate specificities. Our data support this notion in showing that all five of these proteins metabolize various lengths of saturated and unsaturated fatty acids and evolve it in showing that these proteins have different substrate preferences for long-chain (C18) fatty acids (H. Duan and M.A. Schuler, unpublished data). Importantly, these redundant in vitro catalytic activities do not necessarily translate into redundant in vivo functions since transcript analysis shows that each *CYP86A* gene is regulated in its own tissue-specific manner. The nonredundant nature of these genes is obvious in the case of CYP86A8 and CYP86A2, where *cyp86a8* mutant plants show severe pleiotropic phenotypes associated with developmental cuticular deficiencies under normal growth conditions (Wellesen et al., 2001), and *cyp86a2* mutant plants display severe phenotypic changes only in response to bacterial pathogens (Xiao et al., 2004).

Comparison of transcript profiles in various tissues by both microarray and RT-PCR analysis indicates that members of the CYP86A subfamily are expressed at quite different constitutive levels. CYP86A1 transcripts are expressed significantly only in root tissue, CYP86A4 and CYP86A7 transcripts are expressed at their highest levels in mature stems and flowers, and CYP86A2 and CYP86A8 transcripts are expressed at moderate levels in most tissues analyzed. The very high levels of CYP86A4 and CYP86A7 transcripts in reproductive tissues suggest that they may play roles in the recognition of the stigma by pollen and/or in the process of pollen tube growth, both processes that have substantial requirements for hydroxylated fatty acids (Koiwai and Matsuzaki, 1988; Wolters-Arts et al., 1998). The high levels of CYP86A8 transcript in flower tissues as well as many other tissues are consistent with its roles in preventing postgenital organ fusion and in synthesizing epidermal cutin (Wellesen et al., 2001). The high levels of CYP86A1 transcripts selectively expressed in root tissue coupled with the broad range of fatty acids metabolized by this P450 (Benveniste et al., 1998) suggest that this P450 helps generate the large quantities of fatty acids needed for root growth, elongation, and/or other root-specific functions. Transcript profiling alongside biochemical analysis of different tissues is clearly needed to tease physiological functions apart from biochemical functions.

Just as these *CYP86A* genes show different tissue profiles, they also display different responses to chemical and environmental stresses. This is not unexpected, given the putative roles of CYP86A2, CYP86A8, and CYP94A1 in epidermal cutin and suberin synthesis (Pinot et al., 1999; Wellesen et al., 2001) and suggestions that hydroxylated fatty acids released from cutin are involved in stress signaling and physiological responses (Schweizer et al., 1996a, 1996b; Fauth et al., 1998; Kauss et al., 1999). In analysis of plant defense signaling molecules, it is clear that MeJA induces accumulation of spectrophotometrically detectable P450 levels, CYP94A1 transcripts, and lauric acid ω-hydroxylase activity in *V. sativa* (Pinot et al., 1998). This study presents the responses of the CYP86A subfamily with their potential roles in plant defense and stress responses. Testing the effects of several plant stress- and defense-related hormones (ABA, ethylene, MeJA, SA), other signaling molecules (IAA, BR), and mammalian activators of peroxisome proliferation (clofibrate) on the expression patterns of all *CYP86A* genes, our results indicate that the stress-related hormone ABA induces the expression of all five CYP86A transcripts, with CYP86A7 responding more slowly. Short-term application of IAA or clofibrate induces different sets of three CYP86A transcripts, short-term application of ACC induces CYP86A1 transcripts, whereas long-term application of ACC induces CYP86A4 transcripts. Drought, wounding, etiolation, and dark adaptation selectively induce CYP86A2 transcripts, osmotic treatment induces CYP86A2 and CYP86A1 transcripts, and cold stress induces CYP86A4 and CYP86A1 transcripts. Coupled with the importance of cutin in preventing water loss, our results suggest that expression of CYP86A2 is important for drought resistance. Consistent with this observation is the fact that *cyp86a2* mutants are more water permeable and drought sensitive (Xiao et al., 2004). The notable absence of CYP86A responses to signaling molecules such as MeJA and SA, which have significant roles in plant defense, suggests that activation of these *CYP86A* genes does not involve SA- and MeJA-dependent pathways even though CYP86A2 is clearly involved in bacterial pathogen resistance (Xiao et al., 2004). The observed patterns of inducible expression suggest that, in addition to their roles in normal growth and development, each of these P450s has a particular role in stress response.

The array of elements in each of these promoters picked up with available promoter search programs suggests that the Arabidopsis-specific promoter search identifies sets of putative regulatory elements that for ABA, IAA, and cold correlate more closely with the chemical and environmental inducers of individual genes. Correlations on the regulatory elements for clofibrate, BR, and wounding cannot be drawn since consensus elements for these stresses do not exist in this database. The failure to detect elements for the ethylene responses of CYP86A1/CYP86A4, the MeJA response of CYP86A7, and the root-specific expression of CYP86A1 suggests that these responses are mediated by novel elements or indirectly by other signaling cascades and/or by more divergent elements. Deletion and mutational analysis of some of the novel elements identified in our promoter comparisons will determine the extent to which any one of these is involved in stress signaling.

MATERIALS AND METHODS

Chemicals

Plant hormone and growth regulators, including IAA, ABA, MeJA, SA, ACC, BR, and clofibrate, were obtained from Sigma (St. Louis). NADPH, Glc-6-P, and Glc-6-P dehydrogenase were also obtained from Sigma, and [1-^{14}C]lauric acid was obtained from Amersham Biosciences (Piscataway, NJ).

Growth Conditions and Treatments

Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) seeds were surface sterilized and grown on half-strength Murashige and Skoog-agar plates in a 22°C to 24°C growth room, with a 16-h-light/8-h-dark cycle. For IAA, ABA, MeJA, SA, and BR treatments, chemical compounds were dissolved in final 0.1% ethanol at the following concentrations: 100 μM (IAA, MeJA), 50 μM (ABA), 1 mM (SA), and 1 μM (BR). For ACC, clofibrate, and mannitol treatments, chemicals were dissolved in sterile water at the following concentrations: 1 μM (ACC), 1 mM (clofibrate), and 500 mM (mannitol). At the beginning of each treatment, 50 mL of each solution were poured onto the plates to cover the 7-d-old seedlings, and the plates were grown horizontally for the remainder of the treatment period. At the end of each treatment, whole seedlings were harvested directly from the Murashige and Skoog-agar plates, washed thoroughly with sterile water, and frozen in liquid nitrogen. For wounding treatment, 7-d-old seedlings were chopped with a sharp razor blade and left under a dim light for the indicated time period. For drought treatment, 7-d-old seedlings were placed on a piece of Whatman 3MM filter paper (Clifton, NJ) inside a chemical hood under dim light for the indicated time period. For etiolation, seeds were either germinated and grown in the dark for a total of 10 d (etiolated) or germinated under an 8-h-light/16-h-dark cycle for 3 d and then placed in the dark for an additional 4 d (dark adapted). Total RNA was isolated from each of the treatment and matched control samples (0.1% ethanol for IAA, ABA, JA, SA, BR; sterile water for ACC, mannitol, clofibrate) using TRIzol reagent (Invitrogen, Carlsbad, CA). For these, 1 g of tissue was ground in a mortar and pestle with 12 mL of TRIzol, transferred to a 15-mL Falcon tube, extracted with 3 mL of chloroform, incubated for 5 min at room temperature, and centrifuged at 5,000*g* at 4°C for 15 min. The supernatant was transferred to a fresh tube, and nucleic acids were precipitated by adding one-half volume of isopropanol, gently inverting the mixture, incubating at room temperature for 10 min, and centrifuging at 10,000*g* at 4°C for 10 min. Nucleic acids were redissolved in 500 μL of RNase-free water (Invitrogen), digested with 5 units of RQ DNase at 37°C for 1 h, reextracted with the same amount of phenol:chloroform (1:1) and then chloroform, reprecipitated with one-tenth volume of 3 M sodium acetate, pH 5.0, and two volumes of ethanol for 20 min at −20°C, centrifuged at 13,000*g* for 10 min at 4°C, dried, resuspended in 300 μL of sterile RNase-free water, and stored at −80°C.

RNA Quantitation

Quantitative RT-PCR gel-blot analysis of individual P450 transcripts was carried out by amplifying approximately 0.1 μg of total RNA of each sample in one-step RT-PCR reactions containing 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 200 μM each dNTP, 200 μg/mL gelatin, 40 pmol of a 5′ gene-specific primer, 40 pmol of a 3′ oligo(dT)$_{17}$-*Eco*RI primer, 4 units of AMV reverse transcriptase (Promega, Madison, WI), 20 units of RNasin, and 2.5 units of Taq polymerase (Gibco-BRL, Cleveland). First-strand cDNAs were synthesized for 40 min at 42°C and subsequently PCR amplified for 18 to 22 cycles, with each cycle consisting of denaturation at 95°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 2.5 min, followed by a final extension step of 72°C for 10 min. The number of PCR cycles used for each transcript (20 for CYP86A1, CYP86A8, CYP86A2, and CYP86A4; 22 for CYP86A7; 18 for EF-lα) was determined to be within the linear PCR amplification range for each transcript. PCR products were fractionated on 1.0% agarose gels, transferred to Hybond-N (Amersham-Pharmacia Biotech, Uppsala), and probed with random hexamer ^{32}P-labeled probes corresponding to approximately 400 nt derived from the 3′ end of each P450 gene or to an Arabidopsis EF-1α cDNA clone. After hybridization, the blots were scanned using a Typhoon 8600 variable model imager (Amersham-Pharmacia Biotech) and quantified by ImageQuant 5.1 software. For comparisons between treatments and controls, hybridization signals were corrected for the level of background hybridization and normalized to the level of the EF-1α RT-PCR product. The gene-specific primers used in this analysis were: 86A1 5′, 5′CTTTTCATGAAGAACGGTTT3′; 86A2 5′, 5′TACCCAAAGAGAGAAACGAC3′; 86A4 5′, 5′GGTGTCTCTAACGGTCAATG3′; 86A7 5′, 5′TTGACGTTGTTCATGAAGTT3′; 86A8 5′, 5′GCGGATCTACGAGAGTGTAA3′; oligo(dT), 5′CGGAATTCTTTTTTTTTTTTTTTT3′; EF-1α 5′, 5′ACCACCAAGTACTACTGCAC3′; and EF-1α 3′, 5′GACCTCTCAATCATGTTGTC3′. Probe sequences for each of the CYP86A transcripts are as follows: CYP86A1, 60 nt upstream to 392 nt downstream from the stop codon of the At5g58860 locus; CYP86A2, 101 nt upstream to 299 nt downstream from the stop codon of the At4g00360 locus; CYP86A4, 94 nt upstream to 306 nt downstream from the stop codon of the At1g01600; CYP86A7, 99 nt upstream to 384 nt downstream from the stop codon of the At1g63710 locus; and CYP86A8, 66 nt upstream to 390 nt downstream from the stop codon of the At2g45970 locus.

TLC Assay for Fatty Acid Hydroxylase Activity

Recombinant viruses containing each of the CYP86A subfamily members were constructed by RT-PCR strategies and expressed in Sf9 insect cells as described previously (Duan et al., 2004). Microsomal fractions were prepared from packed Sf9 cells, and the metabolism of lauric acid was assayed in a total volume of 200 μL containing 16× phosphate-buffered saline, pH 7.4, 25 μM [1-^{14}C]lauric acid, 50 pmol P450 (as defined by carbon monoxide difference analysis; Omura and Sato, 1964), 6.7 mM Glc-6-P, and 1 unit Glc-6-P dehydrogenase. After incubation at 30°C for 5 min, the reaction was initiated by the addition of NADPH to a final concentration of 1 mM. After 90-min incubations, reactions were stopped by the addition of 20 μL of 6 N HCl, directly spotted onto silica-gel TLC plates (EMD Chemicals, Gibbstown, NJ), and developed using a mixture of diethyl ether:petroleum ether:formic acid (70:30:1). TLC plates were exposed and quantified on a Typhoon 8600 phosphor imager (Amersham Biosciences).

Sequence Alignment and Promoter Analysis

Amino acid and nucleotide sequence alignments were performed using Vector NTI software version 8.0 (InforMax, Bethesda, MD) and ClustalW version 1.6 (Thompson et al., 1994). SRS domains were determined by aligning the five CYP86A protein sequences to the structure-defined CYP102 (BM3) template using MOE programs (Chemical Computing Group, Montreal) and manually adjusting according to secondary structure predictions. Phylogenetic analyses were conducted using MEGA version 2.1 (Kumar et al., 2001). Promoter searches for cis-elements were done using the Ohio State University Web site (http://Arabidopsis.med.ohio-state.edu; Davuluri et al., 2003) and the PlantCARE Web site (http://intra.psb.ugent.be:8080/PlantCARE; Lescot et al., 2002), with sequences extending 2,000 nt upstream from the translation start site in each CYP86A gene. Pairwise comparisons between promoters were done using the PromoterWise program from European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/Wise2/promoterwise.html), using the default settings. Direct and inverted matches containing two or more guanosines or cytosines in a continuous region of seven or more nucleotides are selected as conserved elements.

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