CYP86B1 Is Required for Very Long Chain ω-Hydroxyacid and α,ω-Dicarboxylic Acid Synthesis in Root and Seed Suberin Polyester1[W][OA]

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Suberin composition of various plants including Arabidopsis (Arabidopsis thaliana) has shown the presence of very long chain fatty acid derivatives C20 in addition to the C16 and C18 series. Phylogenetic studies and plant genome mining have led to the identification of putative aliphatic hydroxylases belonging to the CYP86B subfamily of cytochrome P450 monoxygenases. In Arabidopsis, this subfamily is represented by CYP86B1 and CYP86B2, which share about 45% identity with CYP86A1, a fatty acid ω-hydroxylase implicated in root suberin monomer synthesis. Here, we show that CYP86B1 is located to the endoplasmic reticulum and is highly expressed in roots. Indeed, CYP86B1 promoter-driven β-glucuronidase expression indicated strong reporter activities at known sites of suberin production such as the endodermis. These observations, together with the fact that proteins of the CYP86B type are widespread among plant species, suggested a role of CYP86B1 in suberin biogenesis. To investigate the involvement of CYP86B1 in suberin biogenesis, we characterized an allelic series of cyp86B1 mutants of which two strong alleles were knockouts and two weak ones were RNA interference-silenced lines. These root aliphatic plant hydroxylase lines had a root and a seed coat aliphatic polyester composition in which C22- and C24-hydroxyacids and α,ω-dicarboxylic acids were strongly reduced. However, these changes did not affect seed coat permeability and ion content in leaves. The presumed precursors, C22 and C24 fatty acids, accumulated in the suberin polyester. These results demonstrate that CYP86B1 is a very long chain fatty acid hydroxylase specifically involved in polyester monomer biosynthesis during the course of plant development.

Plants are subjected to physical, chemical, and biological stresses such as UV light, exposure to the negative water potential of the environment, atmospheric pollutants, wounding (i.e. by herbivores), and pathogenic infections. These sessile organisms developed as first physical defense barrier cuticular layers and epicuticular waxes that isolate them from the outer environment. Cutin is associated with the aerial parts of plants, in the cell wall of bark tissues, bundle sheath cells of grasses, conifer needles, and seeds (Kolattukudy, 2001; Bernards, 2002). Suberin is also associated with underground parts of plants. In the hypodermis and endodermis, the outer and inner sealing tissues of primary roots, suberin is deposited as lamellae inside the primary cell wall close to the plasma membrane (Wilson and Peterson, 1983; Nawrath, 2002; Pollard et al., 2008). In addition, suberin occurs in the Casparian band, a specific cell wall modification localized in radial walls of the root endodermis (Schreiber et al., 1994). The role of suberin in root tissue is to control water and solute uptake and to prevent their leak in the rhizosphere (Sattelmacher et al., 1998; Ma and Peterson, 2003). The suberin of the Casparian band is usually more densely packed than suberin from peridermal cells, and this increases the impermeability to solutes (Enstone et al., 2003). The wound-induced suberization of potato (Solanum tuberosum) periderm used as a model for studies of suberin synthesis (Kolattukudy, 2001) illustrates the role of suberin in plant defense. Using this same model, Lulai and Corsini (1998) demonstrated the role of suberin as a barrier against pathogens. In the context of plant resistance, enhancement of suberin synthesis has been shown to occur in response to drought and other plant stresses (North and Nobel, 1994; Reinhardt and Rost, 1995; Steudle and Peterson, 1998).

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Suberin results from the association of a polyaliphatic domain, located between the cell wall and the plasma membrane, with a polyphenolic domain, linked to the cell wall (Bernards, 2002). The latter is synthesized via the general phenylpropanoid pathway initiated by the enzyme Phe ammonia-lyase (Kolattukudy, 1981). Similar to cutin, in the suberin polyaliphatic domain, fatty acids are associated in a three-dimensional network via ester bonds involving carboxyl and \( \omega \)-hydroxyl groups (Kolattukudy, 1981). Glycerol, a further component of the suberin (Moire et al., 1999), has been suggested to play a role as a cross-linker between aliphatic monomers (Graça and Pereira, 2000a, 2000b) and between aliphatics and phenolics (Moire et al., 1999). Here, it is worth mentioning that the first reported suberin biochemical mutant was impaired in \( GPAT5 \), a gene encoding a glycerol-3-phosphate acyltransferase (Beisson et al., 2007). Compared with the wild type, \( gpat5 \) plants showed a reduced amount of suberin in young roots and a modification of aliphatic monomers in seed coat suberin. Such novel plant lines represent valuable tools for the identification of key enzymes involved in suberin biosynthesis. However, a comprehensive analysis of the biogenesis (i.e. the identification of all enzymes involved) and of the molecular regulation of the assembly of the monomers and the deposition of the polymers is still needed.

Fatty acid \( \omega \)-hydroxylases have a key role to play in the biosynthesis of cutin and of the aliphatic domain of suberin due to the predominance of \( \omega \)-hydroxylated fatty acid derivatives in these polyesters. By introducing the terminal hydroxyl function, they allow the condensation reaction for the expansion of the biopolymer to occur. Biochemical investigations from our laboratory demonstrated the presence of and characterized \( P450 \)-dependent \( \omega \)-hydroxylases oxidizing C10 to C18 fatty acids in pea (\( Pisum sativum \)) and \( Vicia sativa \) (Benveniste et al., 1982; Salaün et al., 1986; Pinot et al., 1992, 1993). A strategy based on the use of a radiolabeled suicide substrate allowed us to isolate the gene encoding the plant fatty acid \( \omega \)-hydroxylase \( CYP94A1 \) from \( V. sativa \). Based on sequence homology with fatty acid \( \omega \)-hydroxylases from mammals and \( Candida \), the Arabidopsis \( (Arabidopsis thaliana) \) \( CYP86A1 \) encoding a fatty acid \( \omega \)-hydroxylase was then identified and characterized (Benveniste et al., 1998). Reverse genetics clearly demonstrates the involvement of fatty acid \( \omega \)-hydroxylases in cutin synthesis. Indeed, modification in the coding sequences of two fatty acid \( \omega \)-hydroxylases, \( CYP86A8 \) and \( CYP86A2 \), resulted in drastic modification of cuticle in Arabidopsis (Wellesen et al., 2001; Xiao et al., 2004). The same approach was recently applied to suberin biosynthesis. A mutant named \( hori \) (for \( \omega \)-hydroxylase of root suberized tissue) affected in \( CYP86A1 \) exhibited a 60% reduced amount of total aliphatic suberin, mainly resulting from a strong reduction in C16 and C18 \( \omega \)-hydroxycarids and \( \alpha,\omega \)-dicarboxylic acids (Li et al., 2007; Höfer et al., 2008). The study of this mutant confirmed the key role of fatty acid \( \omega \)-hydroxylases in suberin synthesis.

Besides the presence of phenolics, suberin differs from cutin mainly by the chain length of aliphatic compounds, which can range from C16 to C32 (Schreiber et al., 1999). The compositional analysis of suberin in root and in the seed coat of Arabidopsis showed the presence of \( \omega \)-hydroxyacids of chain length C22 and C24 and the presence of the corresponding \( \alpha,\omega \)-dicarboxylic fatty acids (Franke et al., 2005; Molina et al., 2006; Fig. 1). However, these very long chain fatty acid (VLCFA) derivatives were not affected by the \( hori \) mutation (Li et al., 2007; Höfer et al., 2008). So far, no enzymatic system able to \( \omega \)-hydroxylate VLCFAs has been identified in plants.

\( CYP86B1 \) belongs together with \( CYP86B2 \) to a second \( CYP86 \) subfamily. No catalytic function for a member of this subfamily has been described yet. In silico gene expression analysis of the tissue specificity of \( CYP86B1 \) and its coexpression with suberin biosynthetic genes suggested a potential role in suberin synthesis. In this work, we have studied T-DNA insertion and RNA interference (RNAi) mutants of the Arabidopsis \( CYP86B1 \) gene. Involvement of the corresponding protein in root and seed coat suberin synthesis is demonstrated here. Furthermore, the analysis of suberin monomer composition of mutant and wild-type plants strongly suggests that \( CYP86B1 \) prefers the VLCFA C22 and C24 as substrates.

**RESULTS**

**AtCYP86B1 Upstream Sequence Analysis**

Transcriptome microarray data analysis indicated that \( CYP86B1 \) was highly expressed in roots and that an elevated expression signal could also be detected in developing seeds (http://www.genevestigator.ethz.ch). Moreover, these data indicated that \( CYP86B1 \) was coregulated with \( CYP86A1 \) (http://genecat.mpg.de, http://atted.jp/). These observations, together with the facts that proteins of the \( CYP86B \) type are widespread among plant species (Fig. 2) and that \( CYP86B1 \)

![Figure 1](image-url) Alternative enzymatic routes proposed for VLCFA \( \omega \)-oxidation in the Arabidopsis suberin biosynthetic pathway. \( n = 3 \) to 5.
(and CYP86B2) share about 45% identity with CYP86A1, could suggest an involvement of CYP86B1 in the same metabolic pathway as CYP86A1. In order to further examine the gene sequence of CYP86B1 prior to a functional analysis, we carried out a search for putative cis-acting regulatory elements implicated in the expression of CYP86B1. In order to facilitate this approach, we did the analysis for the aligned promoter sequences of CYP86B1 and CYP86B2 using MEME software (http://meme.nbcr.net; Bailey and Elkan, 1994). Three conserved motifs were found within the 500-bp region upstream of the ATG codon. The first motif of 42 bp, located 83 bp upstream of the transcriptional start site, contains a putative EIRE element (Supplemental Fig. S1A) known to be implicated in a biotic interaction characterized in tobacco (Nicotiana tabacum; Shah and Klessig, 1996). The second motif of 20 bp, located 4 bp upstream of the TATA box, contains an ACACGAG element reminiscent of the abscisic acid response elements implicated in abscisic acid signaling (Zhang et al., 2005). The third conserved motif was found 12 bp downstream of the transcription start site. To our knowledge, this sequence does not correspond to any reported cis-acting element. We searched for similarities between the three CYP86B1 putative cis-regulatory sequences and promoter sequences of orthologous genes using a database of orthologous promoters (Barta et al., 2005). Interestingly, the third motif, which we named CYP86B-BOX, was also found in the promoter sequences of Populus trichocarpa CYP86B and of Oryza sativa CYP86B3. The presence of this motif in different plant species might indicate its conserved fundamental role in the regulation of CYP86B gene expression. It is worth noting that a putative plastid-targeting N-terminal peptide (Supplemental Fig. S1A) was identified in CYP86B1. In fact, putative plastid-targeting sequences in more than 40 P450 enzymes have been recorded in a survey of the Arabidopsis genome (Schuler et al., 2006). This particular group of P450s included, for instance, CYP97C1, a carotenoid hydroxylase (Tian et al., 2004) that has a hydrophobic N terminus of 50 residues, of which 28% are Ser or Thr. In the case of CYP86B1, the N-terminal sequence of the same length had 18% Ser or Thr.

Subcellular and Cellular Localizations: CYP86B1-YFP Fusion Protein and ProCYP86B1::GUS Expression Patterns

To determine the subcellular localization of CYP86B1, we constructed a fusion protein with CYP86B1 comprising at the C terminus the yellow fluorescent protein (YFP) marker (Supplemental Fig. S1B). The expression of the cDNA coding for this reporter protein was under the control of the 35S promoter in a transient expression assay in Nicotiana benthamiana leaves. Confocal microscopic observations clearly showed fluorescence associated with the reticulations of the endoplasmic reticulum (ER) in a very typical way (Fig. 3A). Besides this observation of a predominant localization of CYP86B1-YFP in the ER, we were able to observe at 7 d after inoculation a strong fluorescence associated with the chloroplasts of guard cells in the epidermis (Fig. 3B). Interestingly, this strong fluorescence was also localized toward the stomatal pore surface (Fig. 3, B and C). A series of z-stack images (Fig. 3, D–H) were taken in order to scrutinize thoroughly the localization of our reporter: the fluorescent signal was only detected in the ER or the chloroplasts of guard cells.
ProCYP86B1::GUS transgenic plants from five lines chosen for the analysis showed highly comparable patterns of GUS activity when compared with each other. Seedlings grown in vitro showed a root-specific GUS activity at the edge of the central cortex (Fig. 4A). Hypocotyls, cotyledons, and first leaf pairs did not show any expression of the reporter gene. The analysis of greenhouse-grown plants confirmed the localization of GUS activity in roots. In addition, GUS activities were detected in the anthers (Fig. 4B) and stigma (data not shown), in stomata of young pedicels of inflorescences (Fig. 4C), in the placenta region of siliques, and in mature seeds at the level of the hilum (Fig. 4D). These observations are in agreement with the expression data provided at http://wwwgenevestigator.ethz.ch. A thorough histochemical analysis of CYP86B1::GUS plants was then carried out. Roots of germinating seedlings displayed a GUS signal in an internal layer of eight cells appearing as a typical endodermis (Fig. 4G) among four concentric layers of epidermis, central cortex, endodermis, and pericycle (Fig. 4, E and F). Developing root systems of plants grown in hydroponic conditions were also observed: GUS activities were detected throughout root development (Compagnon, 2006). Next, we focused our observations on the endodermis and on specific areas of GUS activities such as the silique-pedicel junction (Fig. 4J), the nectaries (Fig. 4K), and the region of seed attachment to the funiculus of the placenta (Fig. 4, M and N). GUS expression patterns in these latter areas and also in roots were observed in parallel with a search for Sudan IV staining of neutral lipids. In fact, this series of observations indicated that the expression of CYP86B1 (Fig. 4, H, K, and N) was associated with an intense neutral lipid staining (Fig. 4, I, L, and O).

Loss-of-Function cyp86B1/ralph Mutants Display a Novel Suberin Monomer Composition

In order to demonstrate genetically the role of CYP86B1 in the hydroxylation of fatty acids in the process of suberin biogenesis, we analyzed publicly available insertional cyp86B1 mutants (Supplemental Fig. S2A) and furthermore constructed transgenic lines carrying a CYP86B1 RNAi construct. In the insertion mutant lines cyp86b1-1 and cyp86b1-2, which we named ralph1 (for root aliphatic plant hydroxylase1) and ralph2, respectively, transcript levels were at the detection limit when analyzed by real-time reverse transcription (RT)-PCR (Supplemental Fig. S2B) as well as by RT-PCR (data not shown). Transgenic lines ralph3 and ralph4 expressing the RNAi constructs showed significant down-regulation of CYP86B1 transcript levels when analyzed by real-time RT-PCR (Supplemental Fig. S2B).

We next analyzed the composition and content of lipid polyester in ralph lines compared with the wild type. In a first series of experiments, seeds of ralph2, ralph3, and ralph4 were germinated in vitro, then plantlets were grown in hydropony. Polyester analysis of roots show a slight accumulation of fatty acids in ralph2 compared with the wild type (Supplemental...
This was not observed in ralph3 and ralph4. No differences in the amounts of other substances (primary alcohols, ω-hydroxyacids, and α,ω-dicarboxylic acids) between the wild-type and ralph lines were seen in this series of measurements (Supplemental Table S1). However, a detailed comparison of the

Figure 4. GUS activities in transgenic ProCYP86B1::GUS Arabidopsis. A GUS signal was detected in the stele-width area of primary roots (A), in anthers (mainly tapetal cells; B), in guard cells from stomata of pedicels (C), and at the hilum of mature seeds (D). At a smaller observation scale, GUS activities were detected in the endodermis of growing young roots (G) and in a cell layer clearly identified in cross sections of roots stained with toluidine blue at a distance of 1 mm from the root tip (E and F). GUS activities in the endodermis of ProCYP86B1::GUS roots (H) are coincident with the Sudan IV histochemical staining of lipids in identical wild-type tissues (I). Further detailed observations of GUS activities revealed positive signals at the junction of siliques to pedicels where abscission of floral parts takes place (J) and in nectary glands (K) located at the basis of stamens, areas that also displayed the presence of neutral lipids (L) shown by staining with Sudan IV. GUS activities were shown to appear in the funiculus (M), which attaches the seed to the placenta. The concomitant GUS activity (N) and neutral lipid accumulation shown by Sudan IV staining (O) at the hilum of mature seeds is further illustrated. c, Cortex; en, endodermis; ep, epidermis; p, periderm.
different fatty acids and fatty acid-derived polyester monomers revealed strong differences between *ralph* lines and the wild type. An increase of 30% and 50% in the proportion of C22 saturated fatty acids in *ralph3* and *ralph2*, respectively, was determined (Fig. 5A). Likewise, a substantial decrease of 44% and 95% in polyester-derived C22 ω-hydroxyacid was measured in *ralph3* and *ralph2*, respectively, compared with the wild type. The presence of the corresponding C22 α,ω-dicarboxylic acid could not be detected in the *ralph2* suberin (Fig. 5A). The modulation of the suberin-derived C22 monomer in the RNAi lines was in fact weaker than that found in the transcriptional CYP86B1 knockout line *ralph2*, in agreement with the extent of CYP86B1 gene expression decrease. In the case of *ralph1*, which we analyzed in a second series of experiments in which roots were taken from 5-week soil-grown plants, the lack of CYP86B1 mRNA (Supplemental Fig. S2B) resulted, as was the case for *ralph2*, in an almost complete absence of C22 and C24 ω-hydroxyacids in the root suberin (Fig. 5B; Supplemental Table S2). In addition, α,ω-dicarboxylic acids of the same chain length were also strongly reduced in *ralph1* suberin. Similar to the suberin monomer composition in CYP86B1-silenced *ralph3* and *ralph4* lines, unsubstituted, saturated C22 fatty acids accumulated significantly in the suberin of the strong alleles.

As the strongest effects on *ralph* root suberin were observed on C22 and C24 fatty acid derivatives, which are otherwise the major constituents of the Arabidopsis seed coat, seed coat polyester was also analyzed. Similar to the root suberin, CYP86B1 down-regulation had no significant effect on the total amount of seed coat polyester (wild type, 2.58 ± 0.98 μg mg⁻¹; *ralph1*, 2.92 ± 0.30 μg mg⁻¹; *ralph2*, 2.76 ± 0.45 μg mg⁻¹; *ralph3*, 2.83 ± 0.50 μg mg⁻¹; *ralph4*, 3.05 ± 0.47 μg mg⁻¹). However, compound class-specific effects included a 65% to 80% reduction in ω-hydroxyacids and α,ω-dicarboxylic acids in the *ralph* knockout mutant lines accompanied by a 440% to 510% increase in fatty acids. The complete knockout of CYP86B1 (Supplemental Fig. S2) in *ralph1* and *ralph2* led to an almost complete lack of C22 and C24 ω-hydroxyacids and α,ω-dicarboxylic fatty acids in the seed coat polyester.

Figure 5. Relative composition of suberin monomers in the wild type (WT) and *ralph2*, *ralph3*, and *ralph4* mutants (A) and in the wild type and the *ralph1* mutant (B).
(Fig. 6; Supplemental Table S3). This reduction was also accompanied by a strong increase in C22 and C24 unsubstituted, saturated fatty acids.

Barrier Functions of Suberin Are Not Affected in cyp86B1/ralph Mutants

Wild-type and ralph mutant plants had a similar morphological phenotype and furthermore had identical developmental phenotypes when grown under standard greenhouse conditions. In order to determine whether the chemical modification of the suberin monomers measured in ralph roots had an effect on the physiological properties of the suberin as a root barrier to solute transport and particularly in the ion uptake and translocation through the endodermis (Schreiber et al., 1994; Sattelmacher et al., 1998), we measured the total ion content in whole rosettes taken from plants grown in hydropony using inductively coupled plasma/atomic emission spectroscopy (ICP/AES). We used as a reference line presenting a highly modified suberin content the Arabidopsis gpat5-1 mutant described by Beisson et al. (2007). In addition, we assessed the salt permeability property of the seed coat of ralph mutants using tetrazolium red (Debeaujon et al., 2000), a dye to which gpat5-1 seed coats are permeable compared with wild-type seed coats, which are not (Beisson et al., 2007). We show that the total calcium (Ca), magnesium (Mg), phosphorus (P), potassium (K), and sodium (Na) amounts were similar in the wild-type and ralph rosettes, whereas the amount of Ca, Mg, and K was reduced in gpat5-1 rosettes (Fig. 7A). Likewise, ralph seed coats behaved identically to wild-type ones with respect to permeability to tetrazolium red: whereas gpat5-1 seeds were stained by the salt transformed in a red dye by the embryo, as expected, ralph seeds remained unstained (Fig. 7B).

The two bioassays implemented here suggest that in ralph roots compared with wild-type roots, the specific modification of the ratio of C22 fatty acids to C22 \( \omega \)-hydroxyacids (Fig. 5) is not sufficient to trigger physiological consequences upon suberin barrier function, as is the case for gpat5-1. Along the same lines, the modified distribution of C22 and C24 fatty acids, C22 and C24 \( \omega \)-hydroxyacids, and C22 and C24 \( \alpha,\omega \)-dicarboxylic fatty acids in ralph seeds compared with the wild-type polyester monomers (Fig. 6) is not sufficient to drastically increase the salt permeability, as seen with gpat5-1, a mutant characterized by a reduced seed polyester content.

DISCUSSION

Plants are sessile organisms that conquered land 400 million years ago. Resistance to different stresses and uptake of water and nutrients from soil were among the main problems they had to deal with. To face these problems, they developed specialized structures: cutin covering aerial parts of plants, and suberin mainly located in the aerial peridermal tissues, the root, and the seed coat.

A major compositional difference between cutin and suberin is the presence of VLCFA derivatives in suberin (Schreiber et al., 1999; Pollard et al., 2008). To date, no plant cytochrome P450-dependent \( \omega \)-hydroxylase of VLCFAs has been described. In addition, no biological activity has been assigned to members of the CYP86B subfamily yet. In silico analysis revealed a high expression of CYP86B1 (http://www.genevestigator.ethz.ch) in root and developing seed, both rich in suberin. Furthermore, CYP86B1 is the cytochrome P450 with the best correlation in a search for coexpressed genes with CYP86A1 (http://atted.jp/), a major actor in suberin synthesis (Höfer et al., 2008). Altogether, these data gave a potential role for CYP86B1 in suberin synthesis.

Transgenic expression of CYP86B1 promoter-GUS constructs revealed an expression pattern that colocalizes with the tissue distribution of suberin deposits. In primary roots, GUS activity was specifically localized to the endodermis (Fig. 4, G and H), of which the cell walls are characterized by the occurrence of suberin lamella (Franke et al., 2005). The tissue-specific...
expression in the chalaza-micropyle region of mature seed (Fig. 4, D and N) also colocalizes with a positive Sudan staining in that region (Fig. 4O) and has also been observed for the two other suberin-involved genes identified from Arabidopsis to date: GPAT5 (Beisson et al., 2007) and DAISY (Franke et al., 2009). Similarly, CYP86B1 promoter-driven GUS activity in the floral receptacle colocalizes with suberin deposits (Fig. 4, J and L), as has been reported for the DAISY fatty acid elongase-condensing enzyme (Franke et al., 2009). Finally, GUS activities observed in tapetal cells of anthers and in guard cells of pedicel epidermis could be in agreement with the presence of aliphatic polyester deposited at specific sites of these structures (i.e. surface pollen polymers and cuticule of guard cells that close the ostiole), although chemical analyses of these structures in the ralph series have not been done in the frame of this work due to the lack of methods to isolate sufficient amounts for compositional analysis.

Recently, horst, a suberin mutant affected in the expression of a fatty acid ω-hydroxylase, was described from two groups independently (Li et al., 2007; Höfer et al., 2008). In this plant, the disruption of the coding sequence of CYP86A1 led to an approximately 60% reduction in root suberin amount compared with the wild type. The most strongly affected components of the root suberin were C16 and C18 oxygenated fatty acid derivatives. This was in perfect agreement with the substrate specificity of the enzyme determined in vitro (Benveniste et al., 1998). However, no significant differences in the content of oxygenated VLCFAs (C22 and C24) were determined in this CYP86A1 suberin mutant compared with the wild type. Here, we show that root and seed coat suberin analysis of the Arabidopsis loss-of-function cyp86b1 mutant exhibited a specific decrease in C22 and C24 oxygenated fatty acids, while the content of shorter aliphatic monomers remained unchanged compared with the wild type. This indicates a strong chain length specificity of these ω-hydroxylases. These biochemical suberin phenotypes also suggest the existence of at least two pathways in ω-hydroxylation of fatty acids for suberin: one specific for C16 and C18, and one specific for VLCFAs. It also illustrates the complexity of the suberin biosynthetic network, which was previously proposed from a genomic approach in Quercus suber, to possibly involve more than 60 enzymes displaying various functionalities (Soler et al., 2007).

The strong reduction of C22 and C24 ω-hydroxyacids in the suberin composition of cyp86b1 (or ralph) mutants compared with the wild type was accompanied by a reduction of the corresponding ω,ω-dicarboxylic acids. This decrease in ω,ω-dicarboxylic fatty acid content is likely due to a depletion of ω-hydroxyacids, the presumed precursors of ω,ω-dicarboxylic fatty acids. The enzyme(s) responsible for the oxidation steps leading from an ω-hydroxyacid to an ω,ω-dicarboxylic fatty acid remains to be identified. At least two pathways can be postulated (Fig. 1). In the first pathway, the ω-hydroxyacid can be further oxidized by a multifunctional cytochrome P450-dependent fatty acid hydroxylase, which also catalyzes the formation of ω-hydroxyacid from the unsubstituted fatty acid precursor. Such enzymes have been identified and characterized in plants: CYP94A5 from tobacco (Le Bouquin et al., 2001) and CYP94C1 from Arabidopsis (Kandel et al., 2007) can catalyze the complete set of
reactions oxidizing a terminal methyl to the corresponding carboxyl. The capacities of CYP86B1 to catalyze such reactions remain to be established. The AT11 gene encoding CYP86A2 was recently proved to be also involved in the biosynthesis of \( \alpha,\omega \)-dicarboxylic fatty acids in seed coat polyester (Molina et al., 2008). However, if the att1 mutation resulted in a 70% reduction of C18:1 and C18:2 \( \alpha,\omega \)-dicarboxylic fatty acids, it had only a minor impact on the C22 and C24 \( \alpha,\omega \)-dicarboxylic fatty acid content in seed coat suberin. This suggests that CYP86A2 does not account for the formation of very long chain \( \alpha,\omega \)-dicarboxylic fatty acids. It is noteworthy that the only two fatty acid \( \omega \)-hydroxylases capable of \( \alpha,\omega \)-dicarboxylic fatty acid production described so far belong to the CYP94 family. A second pathway would require reactions catalyzed by alcohol and aldehyde dehydrogenases, starting with \( \omega \)-hydroxyacid as initial substrate. Agrawal and Kolattukudy (1978) showed the presence of such enzymes in suberizing potato tissues. More recently, Kurdyukov et al. (2006) described the Arabidopsis hothead mutant, which exhibited a decrease in \( \alpha,\omega \)-dicarboxylic fatty acid content in leaf. HOTHEAD gene product showed sequence similarities to fatty acid \( \omega \)-hydroxyl dehydrogenases, and it is striking that HOTHEAD is also expressed in roots and siliques (Krolkowski et al., 2003; Kurdyukov et al., 2006). However, the role of HOTHEAD-like genes in the formation of \( \alpha,\omega \)-dicarboxylic acids in root and/or seed coat polyester remains to be investigated.

The suberin monomer analysis in roots and seed coat of cyp86b1 mutants revealed another interesting biochemical phenotype in addition to the modified amounts of oxidized fatty acids. Compared with the wild type, unsubstituted C22 and C24 fatty acids accumulate.

Although major functionalized monomers of the suberin polyester, C16 and C18 \( \omega \)-hydroxyacids and diacids, do not change substantially in the mutant suberin, the increase in carboxylic acids in the polyester raises the question about the availability of hydroxyl groups. A potential hydroxyl deficit, caused by a reduction in very long chain \( \omega \)-hydroxyacids, might be compensated by additional linkages to the polyol glycerol. However, further investigation and method developments to determine the molecular interlinkages and glycerol content are required.

This C22 and C24 fatty acid accumulation also suggests that C22 and C24 fatty acids represent the direct precursor for the CYP86B1-catalyzed \( \omega \)-hydroxylation in the course of suberin biosynthesis. Abstracting the suberin biosynthetic network further, the production of low-\( M_r \) suberin units (e.g. \( \omega \)-hydroxyacylglycerol, a putative structural element of the lipid polymer) requires fatty acid \( \omega \)-hydroxylation, fatty acid activation, and fatty acid transfer to glycerol (Graça and Pereira, 2000a, 2000b). Because the in vivo substrate (i.e. free fatty acid, CoA ester, or fatty acid bound to glycerol) of fatty acid \( \omega \)-hydroxylase is unknown, the order of these events remains to be established. Free fatty acids have been used to study the majority of plant fatty acid \( \omega \)-hydroxylases that have been characterized in heterologous systems (Kandel et al., 2006). We used a protocol already improved in the laboratory (Benveniste et al., 1998; Tijet et al., 1998; Le Bouquin et al., 2001; Kandel et al., 2007) to heterologously express CYP86B1 in a yeast system (data not shown). After microsomal incubation of C22 or C24 fatty acids, we did not detect any metabolite production (data not shown). This could be due to a problem of substrate solubility; however, it also suggests that CYP86B1 does not oxidize free fatty acid but rather fatty acid activated or complexed to a glycerol moiety. In this context, it is noteworthy that Cahoon et al. (2002) identified in Euphorbia lagascae seed a cytochrome P450-dependent fatty acid oxidase classified as CYP72A1 able to convert C18:2 into 12,13-epoxy-octadeca-9-enoic acid (vernolic acid). This enzyme metabolizes C18:2 already complexed to phosphatidyl choline (Bafor et al., 1993; Cahoon et al., 2002). Concerning the origin of VLCFAs incorporated in suberin, two pathways can be postulated: VLCFAs (or derivatives) themselves can be \( \omega \)-hydroxylated, or shorter fatty acids (or derivatives) can be \( \omega \)-hydroxylated and then further elongated before incorporation in suberin. The fact that the horst mutant lacking CYP86A1 was not affected in VLCFA content in the suberin (Höfer et al., 2008), together with the accumulation of C22 and C24 fatty acids in suberin of the cyp86b1 mutant, shows that the pool of substrates of CYP86B1 contains already elongated fatty acids. Characterization of different Arabidopsis mutants for GPAT genes clearly identified these enzymes as partners for fatty acid \( \omega \)-hydroxylases in cutin (Li et al., 2007) and suberin synthesis (Beisson et al., 2007; Molina et al., 2008). The main characteristic of the gpat5 mutant is a drastic and specific reduction in content of C22 and C24 monomers in suberin, in addition to a reduction of the quantity of the total aliphatic polyester monomers. The modification of the VLCFA monomers in both cyp86b1 and gpat5 strongly suggests that both enzymes work in close interaction in the same pathway leading specifically to VLCFA incorporation in suberin.

We did not see any morphological change in cyp86b1 mutants compared with the wild type. Therefore, we tried to detect physiological alterations of the barrier function of suberin. For this, we measured the total ion content of rosettes from plants grown in hydropony and monitored seed coat permeability to salts. In these assays, we used the gpat5-1 mutant as a reference. Indeed, the importance of suberin deposition at the hilum for seed permeability has been discussed (Beisson et al., 2007). We did not see marked differences in ion content and salt permeability between the wild type and cyp86b1 mutants, whereas gpat5-1 exhibited an apparent lower efficiency to translocate Ca, Mg, and K from the nutritive solution to the rosette and, as expected, had an enhanced capacity to incorporate tetrazolium red. These results suggest that the chemical modifications that we show for raph suberin are...
not sufficient to modify its physiological properties, whereas gpat5-1 had a 50% reduction of total suberin content, a phenotype consistent with the observed effects on the barrier function of such suberin.

To get a better insight of the physiological role of CYP86B1, we conducted an intracellular localization study in N. benthamiana leaf cells expressing a CYP86B1 fused at its C terminus with the YFP marker. The protein localization to ER-characteristic reticulate structures is in agreement with the expected membrane localization of P450-dependent ω-hydroxylases (Kandel et al., 2006). It is also similar to the experimentally demonstrated localization of CYP86A1 in the ER (Höfer et al., 2008). This strongly supports the hypothesis that the main metabolic routes in suberin monomer biosynthesis are compartmentalized to the ER. However, after prolonged inspection of N. benthamiana epidermis cells expressing the CYP86B1-YFP fusion, we were able to detect, in addition to the ER-localized signal, a fluorescence associated with chloroplasts of guard cells (Fig. 3B). Different cytochrome P450s predicted to be localized in plastid by the ChloroP program have actually been shown to be present in chloroplast (for review, see Schuler et al., 2006). A plastid localization of CYP86B1 has also been predicted from sequence information (www.armemnon.botanik.uni-koeln.de) and was observed previously in in vitro biochemical studies. Indeed, a study based on 3S-labeled CYP86B1 importation assays in spinach (Spinacia oleracea) isolated plastids led to the conclusion that CYP86B1 was attached to the cytosolic side of the plastid envelope (Watson et al., 2001). In this study, isolated chloroplasts were used, and CYP86B1 import in ER fractions was not specifically investigated. The ER is a major location for VLCFA metabolism because it contains in particular all of the enzymatic machinery required for elongation of fatty acids produced from chloroplasts (Schreiber et al., 2005; Samuels et al., 2008), and based on the predominant YFP signal in the reticulate ER-like structures, the localization that we see with the ER is most likely. We further assayed CYP86B1-YFP transgenic plants expressing the fusion protein under control of the native promoter. This material did not provide sufficient YFP signal for subcellular localization (data not shown). We cannot exclude yet that heterologously expressed (and overabundant) proteins as in biochemical studies were “mislocated” to plastids. Maybe an “imperfect” signal peptide sequence could lead to that additional localization after prolonged expression. The specificity of N-terminal sequences of plastid-predicted P450 was also under debate in a genome-wide survey (Schuler et al., 2006). Alternatively, our observations could indicate a specificity of CYP86B1 in guard cell biochemistry (e.g. for a suberin-like polyester formation implicated in stomata properties). The polar localization of the CYP86B1-YFP fluorescence toward the stomatal pore surface could indicate a specific polyester composition (including very long chain ω-oxygenated fatty acids) in stoma ledge cell walls. Likewise, it cannot be excluded that the chloroplast localization observed after prolonged expression in leaf tissue and in the import studies by Watson et al. (2001) is at least in part a result of the firm attachment of ER membranes to isolated plastids, as demonstrated in Arabidopsis leaf chloroplasts (Andersson et al., 2007).

To conclude, this work assigned a function to a member of the CYP86B subfamily uncharacterized, to our knowledge, until now. In silico data mining suggested a possible role for CYP86B1 in suberin synthesis. Studies of the corresponding Arabidopsis mutants confirmed the role of CYP86B1 in root as well as in seed coat suberin synthesis. Furthermore, a detailed analysis of suberin composition of both organs demonstrated a specific involvement of CYP86B1 in a pathway leading to oxygenated VLCFA (C22 and C24) incorporation. Finally, CYP86B1 represents a thus far unidentified plant cytochrome P450 involved in VLCFA ω-hydroxylation.

MATERIALS AND METHODS

Plant Growth and Transformation Conditions

Arabidopsis (Arabidopsis thaliana) transgenic lines described here are in the Columbia-0 background. Plants were grown in a controlled growth chamber in standard horticultural soil with a 12-h-light/12-h-dark regime at 21°C during the light period and 19°C during the dark period. Relative humidity was measured at 60% to 80% and photon fluence rate from white fluorescent tubes at 60 μmol m⁻² s⁻¹ at the level of rosettes. To generate large quantities of root material, plants were grown in a hydroponic system as described (Tocquin et al., 2003). For axenic cultures of Arabidopsis, seeds were wetted with 70% ethanol, surface sterilized with a 25% commercial solution of sodium hypochlorite, rinsed three times with sterile water, and sown on Murashige and Skoog (MS) salts medium containing 1% Suc. Seeds were sown as batches or seed by seed using low-gelling agarose (type VII Sigma) depending on the purpose of a given experiment. Plates were stored for 48 h at 4°C in the dark for stratification and then transferred in a controlled-growth chamber with a 16-h-light/8-h-dark regime at 23°C during the light period and 21°C during the dark period. Agrobacterium tumefaciens-mediated transformation of wild-type Arabidopsis plants by a floral dip method was as described (Clough and Bent, 1998).

Nicotiana benthamiana wild-type plants were grown in a controlled growth chamber in standard horticultural soil with a 16-h-light/8-h-dark regime at 24°C during the light period and 20°C during the dark period. Relative humidity was measured at 70% to 90% and photon fluence rate from white fluorescent tubes at 60 μmol m⁻² s⁻¹ at the level of bottom leaves. In transient transformation assays, A. tumefaciens suspensions were infiltrated with a needleless 2.5-μL syringe in leaves of young N. benthamiana plantlets bearing four to six expanded leaves.

Insertional Mutant Isolation

The Arabidopsis transposon insertion line SM.37066 (renamed cyp86B1-1 mutant allele or ralph1) was identified in the John Innes Centre Sylvestre Marienmet line collection (Tissier et al., 1999), and the T-DNA insertion mutant line SALK_130265 (renamed cyp86B1-2 mutant allele or ralph2) was identified at http://signal.salk.edu/cgi-bin/tdnaexpress (Alonso et al., 2003) and obtained from the Nottingham Arabidopsis Stock Centre. Individual seeds were grown in vitro as described above in order to assess their genotype at the AtEG23190 locus according to standard genomic PCR procedures. Oligonucleotides used to prime the PCR in this genotype screen are given in Supplemental Table S4. The gpat5-1 mutant (At3g11430; SALK_092050) was described by Beisson et al. (2007) was obtained from the Nottingham Arabidopsis Stock Centre, and its genotype was verified with the primers given in Supplemental Table S2.
Involvement of CYP86B1 in Suberin Synthesis

Semiquantitative and Quantitative Real-Time RT-PCR Analysis

Total root RNA samples from plants grown for 2 weeks on MS-Suc medium or for 6 weeks in hydronic conditions were isolated with TRIzol reagent (Life Technologies) according to the technical specifications given by the manufacturer. The semiquantitative RT-PCR analyses used in a preliminary verification of the knockout mutant lines were performed using the SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen) with 200 ng of RNA in each reaction. Primer sequences for CYP86B1 and ACTIN2 (A5f098t10) amplifications are listed in Supplemental Table S4. RT-PCR conditions were 30 min at 50°C followed by 25 to 40 cycles of 30 s at 94°C, 30 s at 57°C, and 90 s at 68°C, followed by 5 min at 68°C. The RT reaction prior to real-time quantitative PCR analysis of gene expression was done with the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen) using 1 μg of total RNA and 100 ng of random hexamers (Boehringer) following instructions provided by the manufacturer. Real-time PCR measurements were done with a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Primers were designed with Primer Express software (Applied Biosystems), and their specificity was verified with a BLAST analysis with the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen) with 200 ng of RNA in each reaction. Primer sequences for CYP86B1 and ACTIN2 (A5f098t10) are listed in Supplemental Table S4. PCRs were done in a final volume of 25 μL.

Subcellular Localization by Transient Expression of Fusion CYP86B1-YFP

The CYP86B1 coding region was amplified from a cloned cDNA (Watson et al., 2001; AF345898) using primers CV86BF and CV86BR given in Supplemental Table S2. These oligonucleotides were designed to provide USER cloning sites (Nour-Eldin et al., 2006). The fragments were inserted into the unique USER site of pCAMBIA230053 in frame with the downstream eYFP (F. Duval, unpublished data). A. tumefaciens LBA4404 was transformed by heat shock and selected for resistance to kanamycin (50 μg mL⁻¹). For transient transformation of N. benthamiana, the bacteria were grown overnight in Luria-Bertani medium containing 10 μg MES and 20 μg acetylsyringone. The bacterial cells were harvested by centrifugation and resuspended in a buffer containing 10 μs MES, 10 μs MgCl₂, and 100 μs acetylsyringone (optical density at 600 nm = 0.05) and allowed to stand at room temperature for 2 to 3 h. Observations of leaf sections were performed 48 to 72 h after infiltration. The confocal laser scanning microscope (Zeiss LSM 510) was used to sequentially monitor YFP signals.

Light Microscopic Observations and Histological Procedures

Staining procedures for the observation of GUS activities were essentially as described (Jefferson et al., 1987). Briefly, tissue samples were washed with 50 μs NaHPO₄/Na₂HPO₄ phosphate buffer at pH 7 and then infiltrated under partial vacuum in a staining solution containing 50 μs phosphate buffer, 0.1% Triton X-100, 0.5 μs K-ferricyanide, 0.5 μs K-ferricyanide, and 0.3 μg mL⁻¹ X-glucuronide. Certain samples were suspended in 90% cold acetone for 20 min prior to the staining procedure (Rodrigues-Pousada et al., 1993). Samples were incubated in the staining solution for 8 to 16 h at 37°C and then submitted to successive washes in 20% to 70% ethanol in order to stop staining and eventually remove chlorophylls. Samples were stored in 70% ethanol at 4°C in glass vials. For certain observations, a clearing step with chloral hydrate was finally added. Samples of organs or tissues were observed with a Nikon binocular or a Leitz microscope with bright-field illumination. Images were taken with a Nikon Coolpix 4500 camera coupled to the microscope.

Lipid staining of roots and other tissues was done with Sudan IV. Samples were treated freshly, suspended in a 70% ethanol solution containing 1% (w/v) dye, and observed with a Leitz microscope with bright-field illumination. Seed coat permeability assays were done with dry seeds incubated in the dark in an aqueous solution of 1% (w/v) tetracyclazole red at 30°C overnight (Beisson et al., 2007).

Compositional Analysis of Root Suberin and Seed Coat Polyester

Depolymerization and subsequent gas chromatographic (GC) analysis of root suberin and the seed coat material was performed as described previously (Hofer et al., 2008; Franke et al., 2009). Briefly, 3 to 5 mg of roots grown in hydronypos or in soil was washed with water and then incubated in a cellulose and pectinase solution (1% each in 10 mM citric buffer, pH 3, 10 μs NaNO₃) to remove unmodified cell wall carbohydrates. The remaining suberin-enriched cell wall material was depolymerized by transesterification in 1 μs methanolic HCl, 2 h at 80°C, containing dropricotane as an internal standard. Aliphatic suberin constituents in the methanolyte were subsequently extracted in hexane. For seed coat polyester, 10 to 15 mg of dried mature seeds was crushed in an agate stone mortar. Soluble lipids were extracted by a 1-week incubation in chloroform:methanol (1:1, v/v) with daily change of solvent. The remaining seed coats were depolymerized as described above. Hexane-extracted aliphatic suberin and seed coat constituents were derivatized with
bise-(N,N-trimethylsilyl)-tri-fluoroacetamide (Macherey-Nagel) prior to GC analysis as detailed previously (Franke et al., 2005). The quantity of all compounds was calculated based on the internal standard dotriacontane after analysis by GC with flame ionization detection.

Atomic Emission Spectroscopy

ICP/AES was done at the Centre d’Analyse et de Recherche, Département Hydrologie et Environnement (Illkirch, France) according to standard procedure NF EN ISO 11885. For each replicate and plant line, whole flowering plants were collected and homogenized 8 weeks after the start of germination in hydropony. Samples of 300 mg of lyophilized material were taken for further analysis in triplicate.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF345998.

Supplemental Data

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

Supplemental Figure S1. AICYP86B1 5’ sequence peculiarities (A) and reporter protein construct (B).

Supplemental Figure S2. Isolation of loss-of-function mutants for CYP86B1.

Supplemental Table S1. Substance class composition of aliphatic suberin in the wild type and ralph-2, ralph-3, and ralph-4 mutants (total amount of each compound class).

Supplemental Table S2. Substance class composition of aliphatic suberin in the wild-type and the ralph1 mutant (total amount of each compound class).

Supplemental Table S3. Substance class composition (relative amounts) of mature seed coat polyester in the wild type and ralph1, ralph-2, ralph-3, and ralph-4 mutants.

Supplemental Table S4. Primers used in this study.

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