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RESEARCH PAPER

The *Arabidopsis* cytochrome P450 *CYP86A1* encodes a fatty acid ω-hydroxylase involved in suberin monomer biosynthesis

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Abstract

The lipophilic biopolyester suberin forms important boundaries to protect the plant from its surrounding environment or to separate different tissues within the plant. In roots, suberin can be found in the cell walls of the endodermis and the hypodermis or periderm. Apoplastic barriers composed of suberin accomplish the challenge to restrict water and nutrient loss and prevent the invasion of pathogens. Despite the physiological importance of suberin and the knowledge of the suberin composition of many plants, very little is known about its biosynthesis and the genes involved. Here, a detailed analysis of the *Arabidopsis* aliphatic suberin in roots at different developmental stages is presented. This study demonstrates some variability in suberin amount and composition along the root axis and indicates the importance of ω-hydroxylation for suberin biosynthesis. Using reverse genetics, the cytochrome P450 fatty acid ω-hydroxylase CYP86A1 (At5g58860) has been identified as a key enzyme for aliphatic root suberin biosynthesis in *Arabidopsis*. The corresponding *horst* mutants show a substantial reduction in ω-hydroxyacids with a chain length $<C_{20}$, demonstrating that CYP86A1 functions as a hydroxylase of root suberized tissue. Detailed expression studies revealed a strong root specificity and a localized expression in the root endodermis. Transgenic expression of CYP86A1 fused to GFP distributed CYP86A1 to the endoplasmic reticulum, indicating that suberin monomer biosynthesis takes place in this sub-cellular compartment before intermediates are exported in the apoplast.

Key words: Cell wall, cytochrome P450, root, suberin, ω-hydroxyacids.

Introduction

A fundamental development for plants to colonize the terrestrial habitat was the evolution of protective surface tissues deposited in the cell walls, the polyesters cutin and suberin. These aliphatic biopolymers prevent the uncontrolled loss of water and nutrients from plants and infection by pathogens. Cutin covers all aerial parts of the plant in a primary developmental stage, whereas suberin depositions are more variable. Suberin can be found in the aerial part of a plant in the cell walls of bark tissues, bundle sheath cells of grasses, conifer needles, and seeds (reviewed in Kolattukudy, 1981, 2001; Bernards, 2002). Subterrestrial suberin is located in the cell walls of the root endodermis and periderm of all angiosperm species, including *Arabidopsis* (Ma and Peterson, 2003; Franke *et al*., 2005) and in rhizodermal and hypodermal cell walls of primary roots (Perumalla *et al*., 1990). For periderms of many species, it was shown that the apoplastic deposition of suberin acts as a barrier that strongly reduces the movement of water and dissolved nutrients and ions (Schönherr and Ziegler, 1980; Vogt *et al*., 1983; Groh *et al*., 2002). Similarly, suberized root cell walls prevent the movement of water and ions. A good example is the suberized Casparian strip in the endodermis of the root (Schreiber *et al*., 1994) that enables this inner plant tissue to control water and ion uptake into the stele (Sattelmacher *et al*., 1998; Ma and Peterson, 2003). When suberin is formed in outer tissues like the periderm of the root, it also

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acts as an antimicrobial barrier against pathogens (Lulai and Corsini, 1998). Additional suberin depositions can occur in response to wounding and pathogen attacks (Agrios, 1997) and in response to unfavourable environmental conditions such as drought and salt stress (North and Nobel, 1994; Reinhardt and Rost, 1995; Steudle and Peterson, 1998). This increased suberization, and similarly the induction of a suberized exodermis in aeroponically grown corn roots, led to an increased resistance to water movement in roots (Zimmermann *et al*., 2000). This indicated that 'root hydraulics' can be modulated by the deposition of the aliphatic polymer suberin.

To understand the property of suberin to act as an apoplastic barrier in multiple ways, the composition of suberin must be considered. The macromolecule suberin is composed of a polyaromatic domain and a polyaliphatic domain (Kolattukudy, 2001; Bernards, 2002). The polyaromatic domain, which is derived from the phenylpropanoid pathway, is restricted to the primary cell wall and is covalently attached to cell wall carbohydrate units. The polyaliphatic domain is a three-dimensional polyester network mainly composed of oxygenated long-chain fatty acids, partially cross-linked by glycerol and with integrated aromatic compounds. The aliphatic domain is supposed to be the main reason for the physiological important water-sealing properties of suberin and was therefore the focus of the present studies. Aliphatic compounds in *Arabidopsis* root suberin are mainly ω-hydroxyacids and α,ω-diacids of carbon chain-length C_{16}–C_{24}, with lower amounts of very long-chain fatty acids (VLCFA) (C_{16}–C_{24}), primary alcohols (C_{16}–C_{20}), and 2-hydroxyacid (C_{24}) (Franke *et al*., 2005; Beisson *et al*., 2007) comparable to the suberin composition of other species (Holloway, 1983; Matzke and Riederer, 1991, Schreiber *et al*., 1999).

Based on carbon flux studies in wound-healing potato tuber tissue (Yang and Bernards, 2006) and the chemical composition of suberin from *Arabidopsis* and other species (Franke and Schreiber, 2007) two main metabolic pathways have been suggested to be characteristic for aliphatic suberin biosynthesis. One is the elongation of C_{16} and C_{18} fatty acid precursors to C_{20}–C_{32} VLCFA (in *Arabidopsis* up to C_{24}); the other pathway consists of ω-oxygenation reactions needed for the transformation into fatty acid derivatives such as ω-hydroxyacids and α,ω-diacids. Fatty acid elongation (FAE) is a four-step reaction sequence involving endoplasmic reticulum (ER)-localized multienzyme complexes that are also required in the production of VLCFA for seed storage lipids (James *et al*., 1995), waxes (Millar *et al*., 1999; Todd *et al*., 1999), and membrane lipids (Zheng *et al*., 2005). By contrast, fatty acid ω-oxygenation in plants has mostly been implicated in cell wall polyester formation, such as cutin synthesis, although hydroxy fatty acids have recently been reported to be involved in fertilization and signalling processes (Kandel *et al*., 2006).

The oxygenation of fatty acids can be performed by NADPH-dependent cytochrome-P450 monooxygenases (P450), catalysing the insertion of one of the atoms from molecular oxygen into a substrate (Werck-Reichhart and Feyereisen, 2000). In plants this has been demonstrated in biochemical studies showing that P450-containing microsomes from *Vicia sativa* and *Pisum* are capable of catalysing the formation of ω-hydroxyacids (Soliday and Kolattukudy, 1977; Benveniste *et al*., 1982; Pinot *et al*., 1992, 1993). Subsequent molecular approaches led to the isolation and cloning of *CYP94A1* from *Vicia sativa* the first characterized plant ω-hydroxylase (Tijet *et al*., 1998). Shortly after, CYP86A1, cloned from *Arabidopsis*, was found to catalyse the ω-hydroxylation of saturated and unsaturated fatty acids in microsomal preparations from yeast, heterologously expressing the encoded P450 (Benveniste *et al*., 1998). Furthermore CYP86A8 from *Arabidopsis* and several CYP94 family members were biochemically characterized as ω-hydroxylases (Wellesen *et al*., 2001; Benveniste *et al*., 2006), leading to the ontological classification of P450 genes from the CYP86 clan, especially the CYP86 and CYP94 subfamilies, as fatty acid ω-hydroxylases.

The most prominent substance class in *Arabidopsis* aliphatic root suberin are ω-hydroxyacids. Although to date many P450 ω-hydroxylases have been functionally characterized, the biological process they act in, is mostly unknown and no P450 gene that is directly involved in the synthesis of these predominant suberin monomers could be identified. Instead, the recent characterization of mutants in a putative glycerol-3-phosphate acyltransferase (*GPAT5*) identified the first gene involved in suberin biosynthesis, probably involved in the formation of fatty acid-containing acylglycerol precursors for suberin deposition (Beisson *et al*., 2007). In the past three decades, fatty acid ω-hydroxylases have mostly been implicated in cutin biosynthesis. This was supported by the isolation of *Arabidopsis* mutants in *CYP86A2* and *CYP86A8*, both characterized by defects in cuticle formation (Wellesen *et al*., 2001; Xiao *et al*., 2004). Similar to CYP86A1, fatty acid ω-hydroxylase activity was demonstrated biochemically for CYP86A8, whereas the involvement of *CYP86A2* in ω-hydroxylation was indicated by the strong reduction in ω-oxygenated fatty acids in stem cutin of corresponding mutants. Based on these investigations, members of the CYP86A subfamily are also potential candidates for enzymes involved in the ω-hydroxylation of aliphatic suberin monomers. This is supported by P450-specific expression studies revealing root expression for some of the members of the CYP86A subfamily. Of these, *CYP86A1* is specifically expressed in roots (Duan and Schuler, 2005). In addition, transcriptom analysis of developing phelloderm in the suberin model *Quercus suber* identified a P450 with closest similarity to the *Arabidopsis* homologue CYP86A1 and was suggested to

be involved in suberin formation (Soler *et al*., 2007). Altogether, CYP86A1 seemed to be a good candidate for an involvement in suberin biosynthesis.

Here the characterization of T-DNA insertion mutants in the *Arabidopsis CYP86A1* gene is reported. The *in vivo* role of CYP86A1 as a hydroxylase of root suberized tissue (*HORST*) is demonstrated. The *horst* mutants exhibit a highly reduced aliphatic root suberin. Furthermore the monomer composition of the aliphatic root suberin is altered in a way that is consistent with the biochemical *in vitro* characterization of CYP86A1 as an ω-hydroxylase.

Materials and methods

Plant material and growth conditions

All plants used are in the *Arabidopsis thaliana* L. Heynh. ecotype Columbia background. Plants were either cultivated in Floradur potting mix (Floraguard, Germany), or on agar sucrose medium [1.5% (w/v) sucrose, 0.7% (w/v) agar agar] containing MS salts (Murashige and Skoog, 1962) adjusted to pH 5.7 using KOH. When grown on agar sucrose medium, seeds where surface-sterilized by shaking for 15 min in sterilization-solution [50% (v/v) EtOH, 1% (v/v) NaHClO], rinsed three times with 100% EtOH, and dried. Plants were grown in a growth chamber at 22 °C with a photoperiod of 16 h/8 h light/dark cycle and 100 μE m^{-2} s^{-1} light intensity.

Mutant isolation

The T-DNA insertion mutant lines SALK_107454 and SALK_104083 (Alonso *et al*., 2003) were obtained from the Nottingham *Arabidopsis* Stock Centre. For the genotype screening to isolate homozygous mutant alleles, the T-DNA left border primer LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′) has been used together with the gene-specific primers 5′-AAGAACCAGCTCAAGGCCACC-3′ (forward) and 5′-AGCAAAAAGCCTAAACCGGGA-3′ (reverse) for line SALK_107454 and 5′-AACGAGTTTCTTGAGCCTCAAG-3′ (forward) and 5′-ACCAGGATTTCAAATACGTCG-3′ (reverse) for line SALK_104083. Homozygous T-DNA insertion mutants were verified by RT-PCR analysis (see below) of 10-d-old seedlings cultivated on MS-agar and examined for the presence or absence of a gene transcript of *CYP86A1* (At5g58860).

Generation of plant transformation vectors and transgenic Arabidopsis

For cloning and generation of expression constructs, the Gateway® Cloning Technology (Hartley *et al*., 2000) was used according to the manufacture's suggestions (Invitrogen, Karlsruhe, Germany). After PCR amplification of the desired fragment, the amplicon was cloned by homologous recombination using Gateway® BP Clonase™ II Enzyme Mix and the pDONR/Zeo entry vector (Invitrogen). The generated entry clones were recombined with selected binary destination vectors (pMDC) provided by Curtis and Grossniklaus (2003). The correctness of all vectors was confirmed by sequencing. For cloning of the putative *CYP86A1*-promoter, the 1426 bp upstream region of At5g58860 was amplified from genomic DNA using the Gateway-compatible primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTACGTGTTGATTATGTTGATGATGCTGAG-3′ (forward; LS248) and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGGTTTAGGCTTTTTGCTTTTGTTCTGT-3′ (reverse) and inserted in the pDONR/Zeo entry vector yielding plasmid p176. LR recombination of p176 with destination vector pMDC162 yielded the promoter reporter gene (β-glucoronidase; GUS) fusion $Prom_{CYP86A1}$*:GUS* for expression studies. Similarly, the coding sequence of At5g58860 was amplified from cDNA with the primer pair 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTACAGAACAAAAGCAAAAAGCCTAAACC-3′ (forward) and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTGCAAGCACCTCACCACGAG-3′ (reverse; LS298) to generate the entry clone p161. The C-terminal fusion to GFP (CYP86A1:GFP) was obtained by recombination of p161 with destination vector pMCD84. The 3.4 kb genomic DNA fragment for complementation analysis was generated by PCR using primers LS248 and LS298 amplifying the promoter of At5g58860 together with the genomic sequence. After cloning in entry vector p166, the genomic fragment was inserted in pMDC99 for mutant complementation. Transgenic plants were generated by introduction of the plant expression constructs into *Agrobacterium tumefaciens* (strain GV3101) and subsequent floral dip transformation as described previously (Clough and Bent, 1998).

Semi-quantitative RT-PCR analysis

The RNA for organ-specific expression studies was extracted from 5-week-old soil-grown *Arabidopsis* plants using hot phenol according to De Vries *et al*. (1988). For expression studies confirming the knock-out or complementation, the total RNA was isolated from 10-d-old seedlings with TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the instructions of the manufacturer. The RT-PCR was performed using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen) with each reaction containing 200 ng RNA. *ACTIN* was used as a control. The primer sequences and predicted amplicon sizes were 5′-ACAGAACAAAAGCAAAAAGCCTAAACC-3′ (forward) and 5′-TGCAAGCACCTCACCACGAG-3′ (reverse) for *CYP86A1 (*1572 bp) and 5′-GTGATGATGCCCCGAGAGC-3′ (forward) and 5′-GACCCGCAAGATCAAGACGA-3′ (reverse) for *ACTIN* (At5g09810) (480 bp). RT-PCR conditions were 30 min at 50 °C followed by 25–40 cycles of 30 s at 94 °C, 30 s at 57 °C and 90 s at 68 °C, and finally 5 min at 68 °C.

GUS-staining procedure

Transgenic T1 and T2 plants containing the $Prom_{CYP86A1}$*:GUS* construct were selected on agar sucrose medium containing hygromycin (50 μg ml^{-1}). Hygromycin-resistant seedlings were either transferred to Floradur potting mix (Floraguard) and grown to maturity, or directly stained. All samples were stained according to De Block and Debrouwer (1992) with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (1 mM) in staining buffer solution [10^{-1} M sodium phosphate buffer (pH 7.0), 10^{-2} M EDTA, 0.5 mM $FeK_3(CN)_6$, 0.1% (v/v) Triton X-100] at 37 °C for 2–18 h. To stop staining and remove chlorophyll in above-ground organs, samples were transferred to 70% ethanol with two or three changes. Specimens were examined using an Olympus SZ61 binocular (Olympus, Japan) or fixed for light microscopy.

Transient expression of GFP-fusions in Nicotiana benthamiana

Agrobacterium tumefaciens strain GV3101 containing the CYP86A1:GFP construct under the control of the CaMV35S-promoter (35S) were grown in 2 ml LB medium containing the appropriate antibiotics. Following overnight growth at 28 °C, bacteria were pelleted from 500 μl culture by centrifugation and resuspended in 2 ml infiltration medium (20 mM citric acid, 2% sucrose, 200 μM Acetosyringon; adjusted to pH 5.2) according to Wydro *et al*. (2006) with minor modifications. Bacteria were

resuspended in this medium. Cultures were incubated at room temperature for 3 h before infiltration. Bacterial suspensions were infiltrated into young but fully expanded leaves of 6- to 8-week-old *N. benthamiana* plants using a needle-less syringe. After infiltration, plants were placed at 20 °C with a photoperiod of 16 h/8 h light/dark. After 40–48 h, GFP fluorescence was monitored by confocal laser scanning microscopy. As a positive control for co-localization studies, a similar 35S-driven fusion construct, HDEL:DsRED, possesing the ER resident protein retention signal HDEL (Haseloff *et al*., 1997) fused to the *Discosoma* sp. red fluorescing protein (DsRed; Jach *et al*., 2001) was co-infiltrated in *N. benthamiana* leaves.

Transfected *N. benthamiana* leaves were examined with an Olympus FV 1000 confocal microscope (Olympus, Germany) using a ×63 water-immersion objective. GFP fluorescence was imaged using excitation with the 488 nm line of an argon/krypton laser and a 530 nm band pass emission filter. Serial confocal optical sections were taken at different step sizes. Projections of serial confocal sections were done using Olympus FV100 software.

Light microscopic and histochemical techniques

Whole root bundles or root parts were washed with water and fixed in a mixture of formaldehyde (3.7%; v/v) in phosphate buffer saline (10^{-2} M $NaPO_4$, 0.137 M NaCl, 27×10^{-4} M KCl, adjusted to pH 7.4) for at least 24 h. The specimens were embedded in frozen section medium (Neg-50; Richard-Allan Scientific, USA) and frozen at –25 °C. Sections (20 μm thick) were prepared using a cryo microtome (Cryostat H 500). Sections were transferred to microscope slides, embedded in glycerol:water (1:1; v/v) and examined with an Axioplan microscope (Zeiss, Germany) with bright field illumination. Sudan staining was performed according to Brundrett *et al*. (1991) with Sudan red 7B (0.1%; w/v) in 50% (v/v) PEG-400, 45% (v/v) glycerol, 5% (v/v) H_2O at room temperature for 1 h. After staining, sections were rinsed with 1% SDS, washed thoroughly with water and mounted in glycerol:water (1:1; v/v) before microscopic examination.

Analytical techniques

Isolation and depolymerization of suberized root cell wall material: As described previously (Franke *et al*., 2005) one to five roots of 5-week-old plants were carefully dug out from the potting mix, washed with water and incubated in polysaccharide hydrolases [1% v/v cellulase (Celluclast, Novo Nordisc, Denmark), 1% v/v pectinase (Trenolin, Germany), 10^{-3} M NaN_3 in 10^{-2} M citric buffer pH 3] for 2 weeks with one exchange of enzyme solution. The remaining cell wall materials were washed with borate buffer (10^{-2} M, pH 9) and distilled water. Finally samples were copiously extracted with chloroform:methanol (1:1; v/v), dried, weighed, and used for depolymerization reaction. The samples were depolymerized by transesterification with 2 ml 1 M MeOH/HCl (Supelco, USA) for 2 h at 80 °C. After addition of 2 ml saturated NaCl/H_2O and 10 μg dotriacontane as internal standard, aliphatic monomers were gradually extracted (three times with 1 ml) in hexane. The combined organic phase was evaporated in a steam of nitrogen to a volume of ~100 μl before analysis using gas-liquid chromatography (GC) and mass spectrometry (MS).

GC and GC-MS analysis: All samples were treated with bis-(*N*,*N*,-trimethylsilyl)-tri-fluoroacetamide (BSTFA; Macherey-Nagel, Germany) for 40 min at 70 °C to convert free hydroxyl and carboxyl groups into their corresponding trimethylsilyl (TMS) ethers and esters. Monomers were identified on the basis of their electron-impact MS spectra (70 eV, m/z 50–700) after capillary GC (DB-1, 30 m, 0.32 mm, 0.1 μm) on an Agilent 6890N gas chromatograph combined with an Agilent 5973N quadrupole mass-selective detector (Agilent Technologies, Germany). The depolymerization products were separated by on-column-injection at 50 °C, 2 min at 50 °C, 10 °C/min to 150 °C, 1 min at 150 °C, 3 °C/min to 310 °C, 15 min at 310 °C. Quantitative determination of the components was carried out with an identical GC system coupled with a flame ionization detector based on the internal standard. All analyses are presented as means ±standard deviation of three to five replicates.

Leaf wax extraction and polyester analysis from leaf and seed coat

Leaf wax and leaf polyester analysis was basically performed as described by Kurdyukov *et al*. (2006). For wax extraction, 6–10 rosette leaves from 35-d-old plants were harvested, individually dipped in chloroform for 10 s and scanned to determine the leaf surface area using imaging software. The chloroform extract was spiked with 10 μg of tetracosan (Fluka, Germany) as internal standard and evaporated under a steam of nitrogen to a volume of ~100 μl prior to GC and GC-MS analysis. Following BSTFA derivatization, wax extracts were separated by the GC system described above applying the following temperature gradient: on-column-injection at 50 °C, 2 min at 50 °C, 40 °C/min to 200 °C, 2 min at 200 °C, 3 °C/min to 310 °C, 30 min at 310 °C.

Leaf polyesters were analysed from totally extracted, delipidated leaves. After collecting and scanning 15–20 rosette leaves from 5-week-old plants, leaves were completely extracted in chloroform:-methanol (1:1; v/v). The solvent was changed five times within 2 weeks. Samples were dried and used for depolymerization reaction as outlined above (Franke *et al*., 2005).

Adapted from the protocols of Molina *et al*. (2006) for seed coat polyester analysis, at least 15 μg of fully developed and dried seeds were crushed in liquid nitrogen using a mortar and pestle. The resulting powder was exhaustively extracted in chloroform:methanol (1:1; v/v) for 2 weeks with five changes of solvent. After drying, the residue was used for depolymerization reaction.

Results

ω-Hydroxyacid suberin monomers increase during root development

To gain more insights into the biochemical reactions involved in the formation of aliphatic root suberin, suberin compositional changes in the course of root development were studied in more detail. Roots of 5-week-old, fully developed *Arabidopsis* plants were dissected in three zones along the root axis for histochemical and chemical analysis (Fig. 1A). The basal zone ranged from the root base 15 mm towards the tip. The middle zone contained the tissue of the next consecutive 25 mm and the apical root zone is represented by the remaining distal tissue including the root tip. Representative cross-sections were microscopically examined and histochemically analysed using Sudan 7B, a lipophilic dye commonly used to stain suberin depositions. The basal root zone is mainly represented by roots in a secondary developmental stage, characterized by suberin depositions in the cell walls of the outermost peridermal cell layer (Fig. 1B). Similar suberized periderms were observed in cross-sections from the middle root zone (Fig. 1C). The apical root zone was

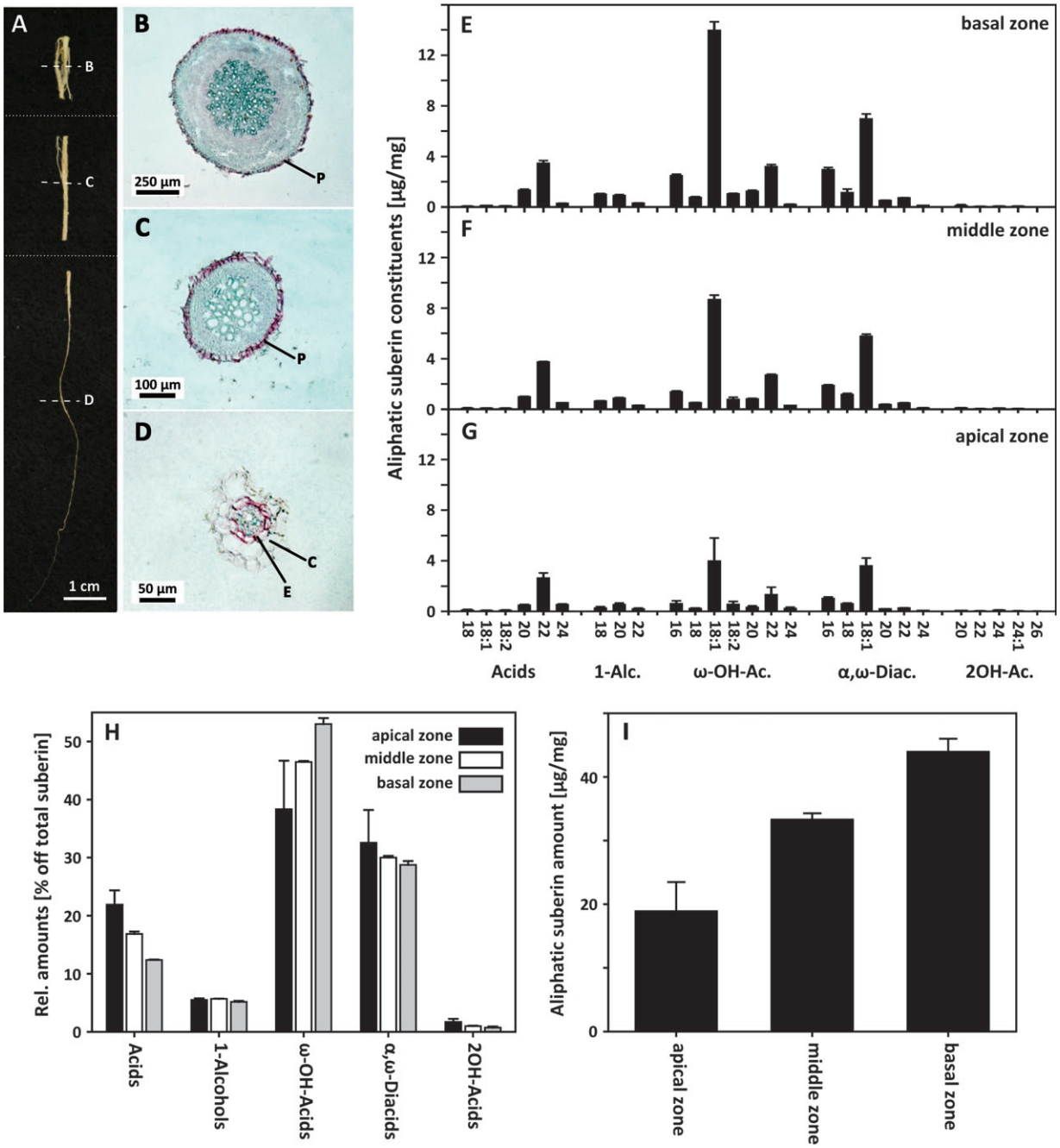

Fig. 1. Tissue distribution and suberin composition in different developmental stages of the *Arabidopsis* root. (A) *Arabidopsis* root sectioned in the three zones investigated. The positions of the cross-sections from B, C, and D are indicated by dashed lines. (B–D) Bright field microscopic picture of representative cross-sections of the *Arabidopsis* root stained with the lipophilic dye Sudan 7B. The red-stained suberin is deposited in the periderm of the roots in a secondary developmental stage (B, C) or in the endodermal cell walls of the roots in a primary developmental stage (D). (E–I) Aliphatic suberin composition and amount in the different developmental stages of the *Arabidopsis* root. (E–G) Suberin monomer composition in µg mg^{-1} dry weight in the basal (E), middle (F), and apical (G) root zone. (H) Substance class distribution in the three root zones in relative amounts. (I) Total aliphatic suberin amount in µg mg^{-1} dry weight in the three root zones. Values 1-Alc., 1-Alcohols; ω-OH-Ac., ω-hydroxyacids; α,ω-Diac., α,ω-diacids; 2OH-Ac., 2-hydroxyacid are given as mean ±SD of three replicates, each containing 2–8 mg isolated suberized root cell wall material. P, Periderm; E, endodermis; C, cortex.

dominated by roots in a primary developmental stage characterized by the presence of an endodermis with apoplastic suberin depositions (Fig. 1D).

To analyse the quantity and composition of the aliphatic root suberin, root tissue of the different root zones was subjected to depolymerization and subsequently analysed using GC and GC-MS. The same monomers were detected in the suberin hydrolysate of all three root zones: unsubstituted fatty acids ranging in chain length from C_{18} to C_{24}, primary alcohols (C_{18}–C_{22}), ω-hydroxyacids

(C_{16}–C_{24}), α,ω-diacids (C_{16}–C_{24}), and 2-hydroxyacids (C_{20}–C_{26}) (Fig. 1E–G). The most prominent substance classes in all root zones are ω-hydroxyacids (37–52%) and α,ω-diacids (28–31%) (Fig. 1H). Along the root axis all aliphatic suberin monomers increase from the tip to the base. The total aliphatic suberin amount in the basal root part is more than twice the amount in the apical part (Fig. 1I). In the substance class composition, the relative amounts of α,ω-diacids and alcohols did not change. However, significant changes in the relative amounts of ω-hydroxyacids and fatty acids were detected among the different root zones. Comparing the apical and the basal root zones, the relative amount of ω-hydroxyacids increased >40% in the basal zone. By contrast the relative amount of fatty acids is significantly reduced in this zone; 12% compared with 21% in the apical zone. A reduction from the tip to the base was also observed in the minor compounds of the 2-hydroxyacid class.

horst knock-out mutants have a highly modified and reduced root suberin

The abundance of ω-hydroxyacids and α,ω-diacids in aliphatic root suberin in *Arabidopsis* and the significant increase in ω-hydroxyacids as suberin formation goes on during root development indicates that ω-hydroxylases are required for suberin monomer biosynthesis. Of the known fatty acid ω-hydroxylases, CYP86A1 is preferentially expressed in roots. Therefore CYP86A1 seemed to be a good candidate for an involvement in suberin biosynthesis. To investigate the *in vivo* function of CYP86A1, a reverse genetics approach was chosen. Two allelic T-DNA insertion mutant lines in *CYP86A1* were obtained from the Nottingham Arabidopsis Stock Centre and further characterized. Since it was supposed that *CYP86A1* encodes a ω-hydroxylase in suberin formation the mutant lines were named *horst-1* (SALK_107454) and *horst-2* (SALK_104083) for hydroxylase of root suberized tissue. PCR amplification of T-DNA flanking genomic sequences showed that *horst-1* carries the T-DNA insertion in the first exon of *CYP86A1*, whereas in *horst-2* the T-DNA insertion is located in the second exon (Fig. 2A). The T3 progeny of the SALK insertion lines were genotyped using PCR, and homozygous T4 *horst-1* and *horst-2* plants were used to determine the *CYP86A1* transcript levels by semi-quantitative RT-PCR (Fig. 2B). In contrast to wild type, no *CYP86A1* transcript could be detected in total RNA from *horst-1* and *horst-2* seedlings. No obvious phenotypes could be observed when *horst-1* and *horst-2* mutant lines were grown together and compared with wild-type plants.

To investigate a potential involvement of *CYP86A1* in suberin biosynthesis, a qualitative and quantitative root suberin analysis of *horst* mutants was performed by GC and GC-MS and compared with the corresponding wild type. *horst-1* and *horst-2* aliphatic suberin exhibited significant reductions in the amount of specific monomers of all compound classes except alcohols (Fig. 3A, B). The most pronounced reductions were detected in ω-hydroxyacids of chain length C_{16} and C_{18} and α,ω-diacids with chain length C_{16}–C_{20} (Fig. 3A). However, significant reductions could also be detected in C_{22} and C_{24} fatty acids and significant but less pronounced reductions can be detected in 2-hydroxyacids of *horst-1* and *horst-2* suberin. In total, *horst-1* and *horst-2* aliphatic root suberin amount is reduced by 60.86% (±7.96) and 63.4% (±10.61), respectively, compared with the wild type (Fig. 3B).

CYP86A1 is required for the biosynthesis of suberin in Arabidopsis roots

To verify that the suberin phenotypes determined in *horst* mutant lines are attributed to the knockout of *CYP86A1*, *horst-1* plants were transformed with a wild-type genomic fragment comprising the 1.4 kb 5′ region upstream of the predicted *CYP86A1* start codon and the *CYP86A1* encoding genomic sequence. RT-PCR analysis on total RNA

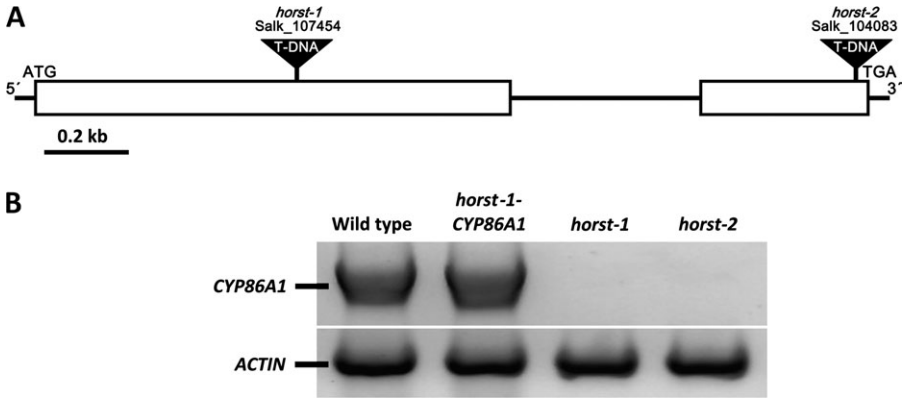

Fig. 2. *CYP86A1* locus and mutant verification. (A) Structure of the *CYP86A1* gene and designated locations of the *horst* alleles. (B) RT-PCR analysis of *CYP86A1* expression in 10-d-old seedlings of wild-type, *horst-1*, *horst-2*, and *horst1-CYP86A1* complemented plants.

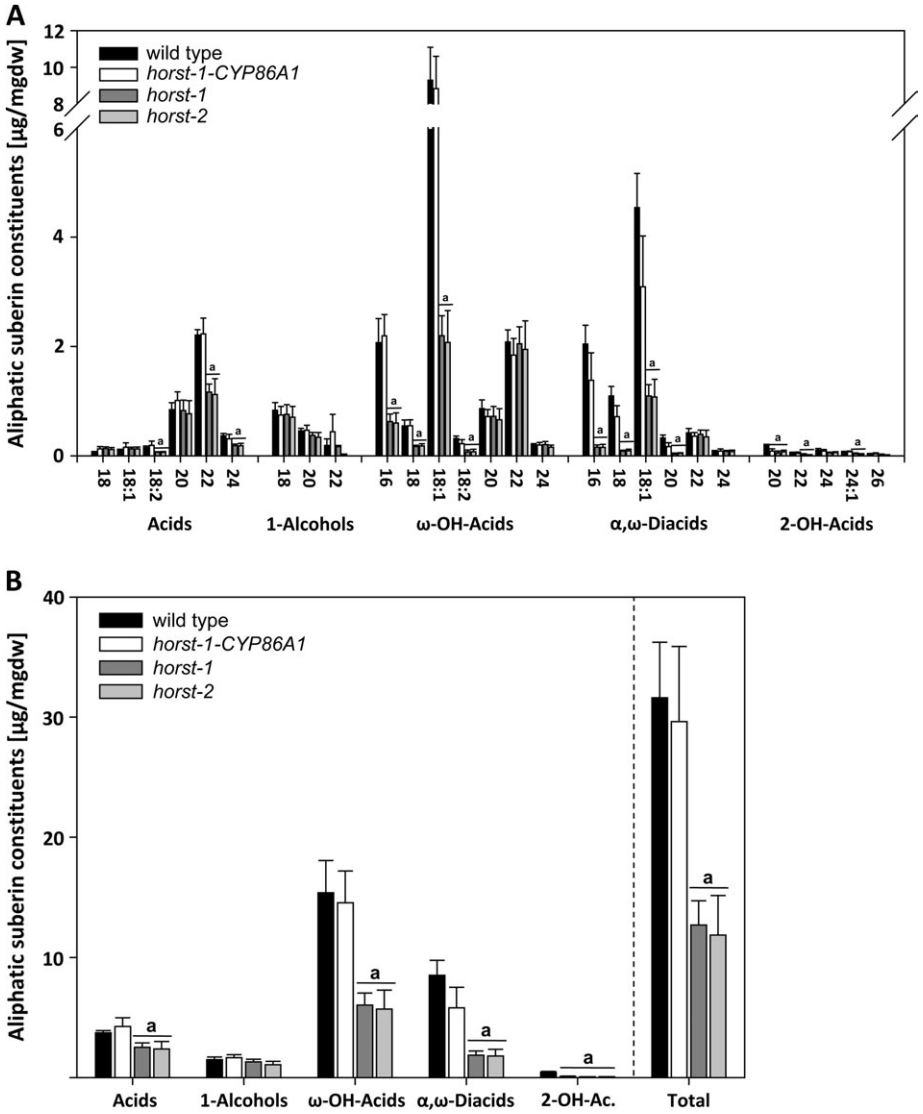

Fig. 3. Aliphatic suberin composition in wild type and *horst* mutant plants. (A) Suberin monomer composition. (B) Total amount of each substance class and total aliphatic suberin amount. Suberin analysis of 5-week-old plants in µg mg^{-1} dry weight ±SD of four or five replicates, each containing one or two roots. Statistically significant changes are indicated at ⩾99%.

from hygromycin-resistant seedlings (*horst-1-CYP86A1*) confirmed the successful reintegration and expression of wild-type *CYP86A1*. Wild-type levels of *CYP86A1* intron-excised transcript were detected in *horst-1-CYP86A1* transgenic plants (Fig. 2B). Root suberin analysis of the complemented *CYP86A1*-transformed *horst-1* plants revealed a quantitative and qualitative aliphatic suberin composition similar to the wild type (Fig. 3).

Since ω-hydroxyacids and α,ω-diacids, the most strongly affected compounds in the *horst* mutant suberin, are also abundant monomers in the biopolyesters of leaves and seeds, these tissues were also analysed for compositional changes in their long-chain aliphatic compounds. No significant differences could be detected in the leaf and seed polyester composition of the wild-type and *horst-1* mutant plants (Fig. 4A, B). Other significantly reduced monomers in *horst* suberin are unsubstituted fatty acids. However, the amount of long-chain fatty acids as part of leaf waxes and other fatty acid-derived wax monomers is not modified in *horst-1* mutant plants (Fig. 4C).

The suberin biosynthetic gene CYP86A1 is expressed in roots

To investigate the expression pattern of *CYP86A1* in more detail on the organ and tissue level, experiments using RT-PCR and GUS reporter gene constructs were performed. RT-PCR analysis on total RNA preparations from different organs resulted in no detectable *CYP86A1* transcript levels in above-ground organs such as leaves, stems, flowers, and siliques (Fig. 5). In roots, high

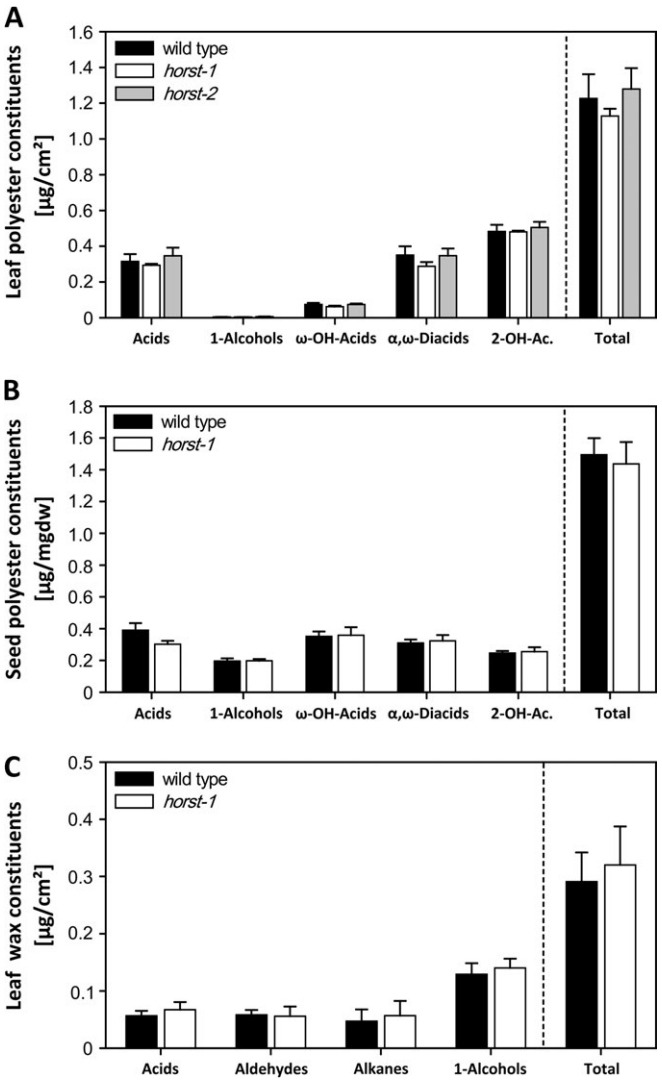

Fig. 4. Cutin, seed coat, and wax analysis in wild type and *horst* mutant plants. (A) Leaf cutin substance class composition and total amount in µg cm^{-2} ±SD of three replicates, each containing 15–20 leaves. (B) Seed coat substance class composition and total amount in µg mg^{-1} dry weight ±SD of five replicates, each containing at least 16 µg seeds. (C) Leaf wax substance class composition and total amount in µg cm^{-2} ±SD of five replicates, each containing seven leaves.

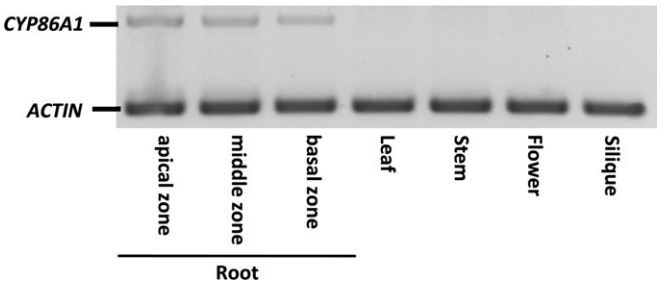

Fig. 5. Organ-specific expression pattern of *CYP86A1*. Expression determined by RT-PCR of *CYP86A1* transcript levels in three different root zones, leaves, stems, flowers, and siliques of 5-week-old fully developed *Arabidopsis* plants. In each reaction, 0.2 µg of total RNA was used. *ACTIN* was used as a control.

expression levels for *CYP86A1* were detected in all three root zones from the tip to the base.

For expression analysis of *CYP86A1* on the tissue level the 1.4 kb putative promoter region upstream of the predicted *CYP86A1* start codon was fused in frame to the β-glucuronidase (*GUS*) gene and used to transform wild-type *Arabidopsis*. Several independent $Prom_{CYP86A1}$*:GUS* transgenic plant lines were investigated. GUS staining was observed in the root of all $Prom_{CYP86A1}$*:GUS* seedlings, while GUS activity was not found in the hypocotyl or cotyledons (Fig. 6A). Microscopic examination of the intact seedling roots showed GUS staining in the centre of the root, inside cortical tissue (Fig. 6B). In cross-sections of GUS-stained $Prom_{CYP86A1}$*:GUS* roots, GUS protein activity could be located to the endodermis (Fig. 6C). Similar to seedlings, no GUS staining could be observed under these conditions in above-ground organs like leaves or stems of mature plants.

The CYP86A1:GFP protein is targeted to the ER

In order to examine the sub-cellular protein localization of CYP86A1, the green-fluorescent-protein (GFP) was fused to the 3′ end of the coding sequence of *CYP86A1*. Employment of the native promoter to drive the expression of this construct in transgenic *Arabidopsis* plants resulted in a weak GFP fluorescence in roots, only slightly above background (data not shown). The weak fluorescence signal did not allow the GFP localization inside the root tissue with a resolution high enough for sub-cellular localization in endodermal tissue. Therefore, we focused on heterologous expression and transiently expressed this construct (*CYP86A1:GFP*) under the control of the CaMV35S-promoter (35S) in the epidermis of *Nicotiana benthamiana* leaves. Using confocal laser scanning microscopy, intense GFP fluorescence was observed in reticulate structures characteristic for ER (Fig. 6D). Continuous observation of the GFP-tagged CYP86A1 revealed the movement of these membranous structures, typical of ER in vital cells (Fig. 6E; Video S1 in Supplementary material available at *JXB* online). Co-expression with an ER marker construct containing the HDEL retention signal for ER fused to DsRed (HDEL:DsRed) revealed it co-localized with CYP86A1:GFP (Fig. 6F–H).

Discussion

Root suberin analysis in many species has usually been applied to enzymatically isolated endodermal and hypodermal/peridermal cell walls (Schreiber *et al.*, 1999, 2007). However, dissection and isolation of suberized tissue from the frail *Arabidopsis* root is not feasible. Therefore previous analyses of *Arabidopsis* root suberin have been carried out with whole root samples (Franke

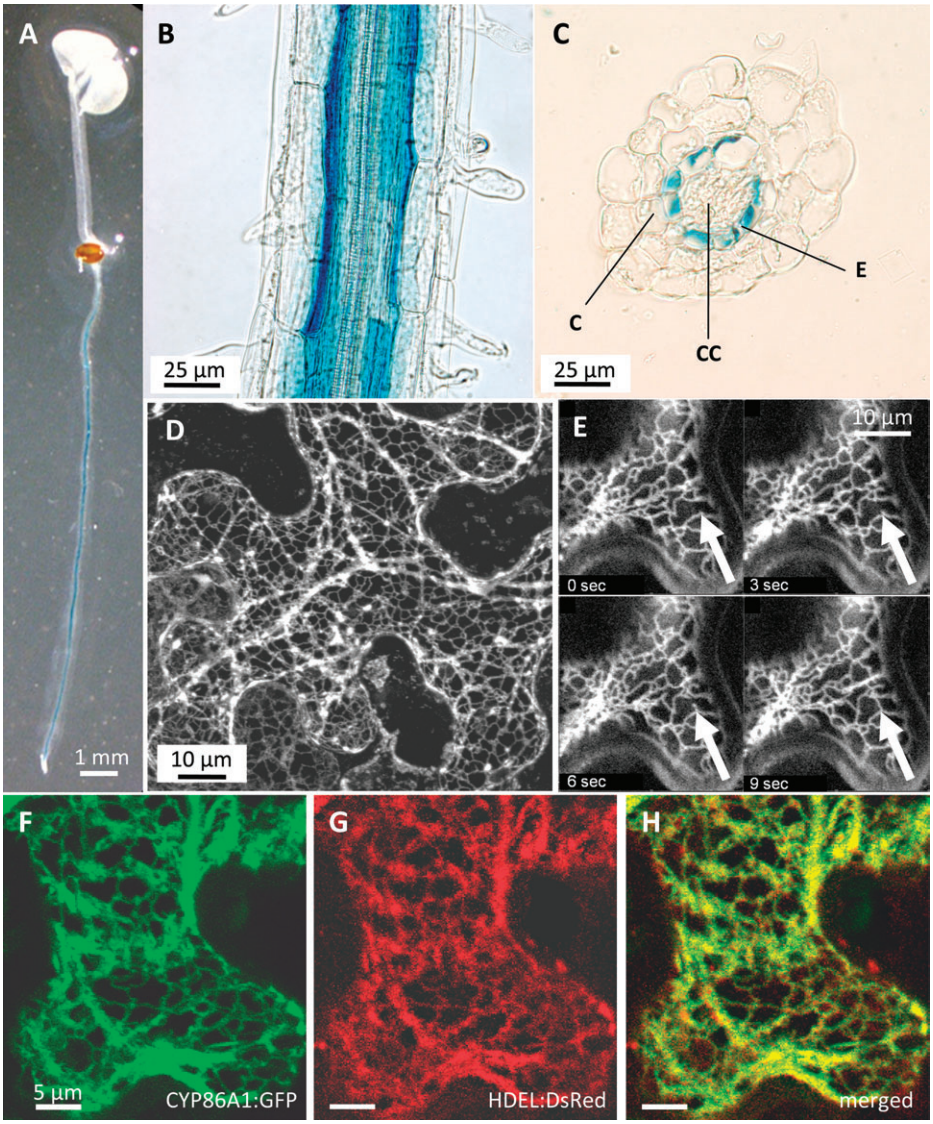

Fig. 6. Tissue expression pattern and sub-cellular protein localization. (A–C) GUS staining in *Arabidopsis* transformed with a $Prom_{CYP86A1}$*:GUS* construct: (A) in a 7-d-old seedling GUS staining is only observed in the root; (B) close-up view of the intact seedling root with a GUS staining limited to the centre of the root; (C) cross-section through the seedling root showing GUS staining restricted to the endodermis. (D–H) Confocal microscope pictures of transiently expressed green fluorescent protein (GFP) and red fluorescent protein (DsRED) tagged peptides in epidermis of tobacco (*N. benthamiana*) leaves: (D) projection of serial confocal optical sections through an epidermis cell expressing a CYP86A1:GFP fusion protein, showing reticulate ER-like structures (brightness indicates GFP fluorescence); (E) close-up view showing the vitality of the cell by movement of the ER-like structures over the time indicated by an arrow (also see Video S1 in Supplementary material available at *JXB* online); (F–H) co-localization of CYP86A1:GFP (green, F) and HDEL:DsRed (red, G) in ER network, shown in the merged image (yellow, H). CC, Central cylinder; E, endodermis; C, cortex.

et al., 2005; Beisson *et al*., 2007). These analyses represented a 'mixed' suberin composition as the whole roots comprised both root tissues in a primary and secondary developmental stage. The allocation of fully developed *Arabidopsis* roots into three segments along the root axis, enabled the generation of root tissue for comparative compositional suberin analysis in root zones of different developmental stages. As demonstrated in the histochemical analysis of representative cross-sections (Fig. 1B, D), the analysis of the basal root zone sample represents a suberin predominantly derived from peridermal cell walls of a root in a secondary developmental stage, whereas suberin in the apical zone is representative of endodermal suberin from roots in a primary developmental stage. The middle zone mostly comprises roots in a secondary developmental stage (Fig. 1C). This differentiation pattern and the peridermal origin are consistent with previous solely microscopic studies of 4-week-old *Arabidopsis* roots. Baum *et al*. (2002) observed first periclinal divisions of the pericycle 35 mm from the tip and a phellogen was detected 20 mm from the base. The development of a periderm and therefore the transition

from a primary to a secondary developmental stage probably takes place between the distal part of the middle root zone and the proximal region of the apical root zone.

The chemical composition determined for the aliphatic suberin of the three root zones is comparable to previous analysis in *Arabidopsis* roots (Franke *et al*., 2005; Beisson *et al*., 2007). Independent of the zonal origin of the root samples, the same aliphatic suberin monomers could be identified in all three root zones (Fig. 1E–G). However, pronounced quantitative and qualitative differences could be detected in the suberin of the three root zones. The total amount of aliphatic suberin in the basal root part is more than twice the amount in the apical root part (Fig. 1,I). This is the result of a substantial increase in root diameter from the tip to the base. In addition, the proportion of suberized tissue layers increases during root development: in the primary developmental stage suberin depositions occur only in an inner cell layer, the endodermis. By contrast the suberized tissue of the root in the secondary developmental stage, the periderm, forms the outermost cell layer of the root. Compared with the whole root surface the endodermis represents an inner ring of a lower circumference compared with the area covered by the periderm.

The most obvious compositional changes along the root zones were observed in ω-hydroxyacids and primary fatty acids (Fig. 1H). From the tip to the base the amount of ω-hydroxyacids, characteristic suberin monomers, increases substantially, and this increase correlates with a decrease in primary fatty acids. Similarly, in suberin of endodermal and rhizodermal/hypodermal cell walls of corn roots, primary fatty acids decrease in sub-apical root zones whereas ω-hydroxyacids increase (Zeier *et al*., 1999). Although these changes were most pronounced between the very apical zone (0–80 mm from the tip), characterized by Casparian strip suberin, and the basal zones, characterized by suberin lamellae around the cell wall, the increase in ω-hydroxyacids on the expense of primary fatty acids continued over the next two root zones (80–160 mm and 160–240 mm from the tip, respectively) towards the base. The compositional changes observed in suberin along the axis of corn and *Arabidopsis* roots point out the importance of ω-hydroxylation in the suberization of cell walls in general and might indicate an increase in the activity of fatty acid ω-hydroxylation during development.

Due to the two opposite functional groups, ω-hydroxyacids are chemically ideally suited to form a polyester. Whether the increase in ω-hydroxyacids is associated with physiological or functional properties of suberized barriers in an endodermis or in a periderm still remains to be established.

The majority of fatty acid hydroxylation reactions in plants is catalysed by cytochrome P450 of the CYP86 clan, especially the CYP86 and CYP94 families (Duan and Schuler, 2005; Kandel *et al*., 2006). Although many CYP86 and CYP94 have been characterized biochemically, their physiological function in a biological process is mostly unknown. *horst-1* and *horst-2*, two allelic *Arabidopsis* mutants carrying a T-DNA insertion in *At5g58860* were used to study the *in vivo* function of CYP86A1. As a consequence of the transcriptional knockout of *CYP86A1* major suberin monomers are significantly reduced resulting in a >60% reduced amount of total aliphatic suberin (Fig. 3B). Wild-type levels of both total aliphatic root suberin amount and amounts of single suberin components were restored by complementation with a genomic DNA fragment spanning the *CYP86A1* locus-including promoter region (Fig. 3A, B). Taken together, the analysis of the aliphatic root suberin composition of the two allelic *horst-1 and horst-2* mutants and complemented plants provides the genetic evidence for the involvement of CYP86A1 in suberin biosynthesis. This is further supported by very similar suberin monomer profiles from two other allelic *cyp86A1* insertion mutants, reported by Li *et al*. (2007) during the preparation of this paper.

Recent P450 protein sequence analysis has set CYP86A1 on a single clade within the CYP86A subfamily in *Arabidopsis* (Nelson *et al*., 2004; Duan and Schuler, 2005), probably indicative of unique functional properties. Therefore, the detailed monomer composition obtained by GC-MS of *horst* mutant suberin is also strongly indicative for the substrate specificity of CYP86A1 *in vivo*, although some overlap in catalytic properties with other ω-hydroxylases probably exists (see below). The strong reduction in suberin ω-hydroxyacids $<C_{20}$ in *horst* plants compared with the wild-type plants is essentially in agreement with the *in vitro* catalytic activity of CYP86A1. Microsomal preparations from yeast expressing *CYP86A1*, actively metabolized $C_{16}>C_{18:1}>C_{18:2}>C_{14}>C_{12}$ fatty acids to the corresponding ω-hydroxyacids with highest activities towards C_{16} and $C_{18:1}$ fatty acids (Benveniste *et al*., 1998). Similarly, CYP86A1 from baculovirus-infected insect cells has been shown to bind these substrates (Rupasinghe *et al*., 2007). However, the *CYP86A1*-mutant suberin analysis not only confirms these studies, it also provides new information, as substrates longer than C_{18} have not been tested in *in vitro* studies. The fact that the content of C_{20}, C_{22}, and C_{24} ω-hydroxyacids is not affected by the *horst* mutation indicates a strong chain-length specificity of CYP86A1 catalysed ω-hydroxylation. Most likely fatty acids longer than C_{20} do not act as substrates for CYP86A1 *in vivo*. Inconsistent with the inability of heterologously expressed *CYP86A1* to efficiently bind or use stearic acid ($C_{18:0}$) as a substrate *in vitro* (Benveniste *et al*., 1998; Rupasinghe *et al*., 2007), the *horst* aliphatic root suberin is also significantly reduced in saturated C_{18} ω-hydroxyacid. Although some deficiencies of the heterologous *in vitro*

systems cannot be excluded (e.g. endogenous yeast reactions compete for the stearic acid), this might indicate that *in situ* additional factors affect CYP86A1 substrate specificity, enabling the conversion of C_{18} fatty acid to C_{18} ω-hydroxyacid. An alternative explanation would be that in wild-type plants an elongation of ω-hydroxyacids from C_{16} to C_{18} could occur, but only the CoA-activation, required for fatty acid elongation (FAE), has been demonstrated for C_{16} ω-hydroxyacid (Schnurr *et al*., 2004).

As C_{16} and C_{18} ω-hydroxyacids are not entirely omitted in the *horst* mutant's suberin, other ω-hydroxylases must be encoded in the genome which provide a functional redundancy, at least partially compensating the knock-out of *CYP86A1*. In plants, fatty acid ω-hydroxylation activity has been demonstrated for P450 of the CYP78, CYP92, CYP96, and many members of the CYP86 and CYP94 families (Kandel *et al*., 2006). In *Arabidopsis*, four members of the CYP94 family (CYP94B1, CYP94B2, CYP94B3, and CYP94C1) were shown to be ω-hydroxylases after heterologous expression in yeast (Benveniste *et al*., 2006). Similar to CYP86A8 (Wellesen, *et al*., 2001), all 4 CYP94 ω-hydroxylated C12:0, C14:0, C16:0, and C18:1 fatty acids. Recently, the biochemically less-characterized CYP86A members in *Arabidopsis*, CYP86A2, CYP86A4, and CYP86A7, have been shown to ω-hydroxylate C18:1 fatty acid (Rupasinghe *et al*., 2007). Of these P450 with ω-hydroxylase activity, the corresponding genes for *CYP94B1*, *CYP94B3*, *CYP86A2*, *CYP86A4*, and *CYP86A8* have been reported to be also expressed in roots (Wellesen *et al*., 2001; Duan and Schuler, 2005; Schuler *et al*., 2006). Although not preferentially expressed in suberizing tissue, residual root expression in one or more of these ω-hydroxylases could result in ω-hydroxylation activity partially compensating the lack of CYP86A1 activity.

In addition to the strong reduction in ω-hydroxyacids $<C_{20}$, specific suberin monomers in the compound classes of α,ω-diacids, acids and 2-hydroxyacids are also significantly reduced in the *horst* mutant suberin (Fig. 3). According to the biochemical pathways suggested for ω-oxidation in apoplastic polyester biosynthesis, α,ω-diacid formation can be performed in two different ways (Franke *et al*., 2005; Kurdyukov *et al*., 2006). One requires two sequential dehydrogenase reactions on ω-hydroxyacids as initial substrates. These reactions were initially demonstrated in suberizing potato tuber tissue (Agrawal and Kolattukudy, 1978) and are supposed to involve *HOTHEAD*-like oxydoreductases. A mutation in *HOTHEAD* (*HTH*) leads to an increase in ω-hydroxyacids in cutin polyester (Kurdyukov *et al*., 2006). Therefore the strong reduction in C_{16}–C_{18} α,ω-diacids is most likely the result of a depletion in ω-hydroxyacids, the substrate for the dehydrogenase activity of *HTH*-like oxidoreductase. In this context, it is interesting to note that the *HTH* gene is also expressed in roots. In a second pathway to α,ω-diacids, CYP86A1 could act as a multifunctional ω-hydroxylase, similar to CYP94A5 and CYP94C1 which catalyse the multi-step oxidation of fatty acids to the corresponding ω-hydroxyacid and α,ω-diacids (Le Bouquin *et al*., 2001; Kandel *et al*., 2007). However, for CYP86A1 this multifunctional activity has not been demonstrated *in vitro* under conditions successfully applied to other multifunctional ω-hydroxylases.

Interestingly, the supposed substrates of the C_{16} and C_{18} ω-hydroxylation catalysed by CYP86A1, mainly medium chain fatty acids, do not accumulate in the mutant suberin. Instead long-chain fatty acids and 2-hydroxy fatty acids, aliphatic suberin monomers that are not direct or downstream products of fatty acid ω-oxidation, are affected by the mutation in *CYP86A1* (Fig. 3). This indicates significant interaction between metabolic branches in aliphatic suberin biosynthesis and maybe other lipid biosynthetic pathways. So far undiscovered, complex regulatory mechanisms in suberin biosynthetic pathways lead to more or less specific secondary effects on suberin composition by 'down-regulation' of upstream reactions in suberin biosynthetic pathways. A very specific effect can be observed on the chain length distribution of fatty acids. C_{22} and C_{24} fatty acids are significantly reduced in the *horst* suberin, indicating that the expression or activity of enzymes involved in C_{20} FAE is affected by the *CYP86A1* knock out. FAE has been suggested as a major metabolic activity in aliphatic suberin biosynthesis in potato and *Arabidopsis* (Franke *et al*., 2005; Yang and Bernards, 2006). Of the 21 *Arabidopsis* β-ketoacyl-CoA synthases (FAE-KCS), which catalyse the rate-limiting and product-determining step in FAE (Millar and Kunst, 1997), at least six FAE-KCS have been shown to have the catalytic properties to provide C_{22} or C_{24} fatty acids (Trenkamp *et al*., 2004; Blacklock *et al*., 2006; Paul *et al*., 2006). Studying these *FAE-KCS* in the *horst* mutants could provide a first insight in a coordinated regulation or 'cross talk' between FAE and fatty acid hydroxylation.

Similarly, other metabolic fates, e.g. 2-hydroxylation, seem to be secondarily affected by an unknown inhibition mechanism. The reduction in C_{20} α,ω-diacids also seems to be a secondary effect as the supposed precursor, C_{20} ω-hydroxyacid, is not reduced in *horst* suberin. Maybe, *HTH*-like oxidoreductases, metabolizing C_{20} ω-hydroxyacid, are inhibited or depressed in the *horst* mutant.

The suberin analysis of the *horst* mutant and complemented lines demonstrated that *CYP86A1* is required for suberin biosynthesis. In agreement with the tissue distribution of suberin depositions in roots in a primary developmental stage (Fig. 1D), GUS activity in plants transformed with a $Prom_{CYP86A1}$*:GUS* construct was preferentially observed in the endodermis (Fig. 6C), indicating the role of CYP86A1 in early deposition of suberin. In peridermal tissue of secondary roots,

CYP86A1-promoter driven GUS expression could not be detected under the applied conditions (data not shown). However, consistent with the predominance of ω-hydroxyacids in apical, middle, and basal root zone suberin, RT-PCR analysis revealed that *CYP86A1* is expressed throughout root development (Fig. 5). Furthermore, *CYP86A1* is also specifically root expressed with no detectable expression levels in above-ground organs. These results are in agreement with predictions for gene expression (Zimmermann *et al*., 2004) and previous whole-organ expression studies (Duan and Schuler, 2005). Consistent with the lack of expression in above-ground organs, aliphatic cell wall depositions such as wax or ω-hydroxyacid-containing polyesters in above-ground organs, such as cutin and the seed coat, are not altered in the substance class composition (Fig. 4). This emphasizes the specific involvement of CYP86A1 in root suberization.

At the sub-cellular level, visualization of the CYP86A1–GFP fusion protein (Fig. 6D, E) strongly indicates an ER localization of CYP86A1. Although, heterologous overexpression may eventually lead to artificial ER retention, the localization of CYP86A1 to the ER was supported by co-localization studies with an ER reporter construct (HDEL:DsRed protein), when simultaneously expressed in *N. benthamiana* epidermis cells (Fig. 6F–H). An ER localization of the fatty acid ω-hydroxylase CYP86A1 is also in agreement with studies from decades ago showing that P450-containing plant microsomes can catalyse the production of ω-hydroxyacids (Benveniste *et al*., 1982; Pinot *et al*., 1992; 1993). Current gene ontology predicts CYP86A1 to have an N-terminal hydrophobic transmembrane domain and is targeted to the endomembrane system (Schwacke *et al*. 2003). Furthermore, the ER localization of the GFP-tagged CYP86A1 refines biochemical studies that assigned CYP86A1 activity to endomembrane compartments due to the successful employment of microsomal preparations from *CYP86A1*-expressing yeast to determine the catalytic properties of CYP86A1 (Benveniste *et al*., 1998). In this context, it is interesting to note that FAE, the other key reaction in aliphatic suberin biosynthesis, has been demonstrated in microsomes of suberizing corn root tissue (Schreiber *et al*., 2005). The FAE activity in different root zones correlated with the demand in suberin constituents. In addition, similar GFP-tagging approaches successfully determined the ER localization of reaction steps in FAE (Kunst and Samuels, 2003; Zheng *et al*., 2005). Together, these results suggest that core reactions of the suberin biosynthetic machinery take place at the ER. It remains to be determined if the ER-produced aliphatic suberin constituents are exported to the apoplast as monomers or preformed esters.

In summary, CYP86A1 has been identified as a key enzyme for aliphatic suberin biosynthesis in *Arabidopsis* roots. Corresponding mutants exhibiting a 60% reduction in aliphatic suberin do not show any phenotypic variation, indicating that small amounts of aliphatic suberin are sufficient to provide the physiological function, at least under the optimized laboratory culture conditions. Further experiments on the behaviour of *hors*t to different abiotic and biotic stresses might shed light on the physiological consequence of an altered suberin substance class composition and total aliphatic suberin amount during the plant life cycle. Mutants with reduced and modified suberin content are now available to test the barrier properties in relation to the chemical composition when tests have been developed for *Arabidopsis*.

Supplementary material

Video S1: Dynamics of CYP86A1:GFP in ER-like reticulate structures

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References

Agrawal VP, Kolattukudy PE. 1978. Purification and characterization of a wound-induced ω-hydroxyfatty acid:NADP oxidoreductase from potato tuber disks (*Solanum tuberosum* L). *Archives of Biochemistry and Biophysics* **191,** 452–465.

Agrios GN. 1997. *Plant pathology*. San Diego: Academic Press.

Alonso JM, Stepanova AN, Leisse TJ, *et al*. 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301,** 653–657.

Baum SF, Dubrovsky JG, Rost TL. 2002. Apical organization and maturation of the cortex and vascular cylinder in *Arabidopsis thaliana* (Brassicaceae) roots. *American Journal of Botany* **89,** 908–920.

Beisson F, Yonghua L, Bonaventure G, Pollard M, Ohlrogge JB. 2007. The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of *Arabidopsis*. *The Plant Cell* **19,** 351–368.

Benveniste I, Saito T, Wang Y, Kandel S, Huang H, Pinot F, Kahn R, Salaün JP, Shimoji M. 2006. Evolutionary relationship and substrate specificity of *Arabidopsis thaliana* fatty acid hydroxylase. *Plant Sciences* **170,** 326–338.

Benveniste I, Salaün JP, Simon A, Reichhart D, Durst F. 1982. Cytochrome P-450- dependent ω-hydroxylation of lauric acid by microsomes from pea seedlings. *Plant Physiology* **70,** 122–126.

Benveniste I, Tijet N, Adas F, Philipps G, Salaün JP, Durst F. 1998. CYP86A1 from *Arabidopsis thaliana* encodes a cytochrome P450-dependent fatty acid omega-hydroxylase. *Biochemical and Biophysical Research Communications* **243,** 688–693.

Bernards MA. 2002. Demystifying suberin. *Canadian Journal of Botany* **80,** 227–240.

Blacklock BJ, Jaworski JG. 2006. Substrate specificity of *Arabidopsis* 3-ketoacyl-CoA synthases. *Biochemical and Biophysical Research Communications* **346,** 583–590.

Brundrett MC, Kendrick B, Peterson CA. 1991. Efficient lipid staining in plant-material with Sudan Red 7B or Fluorol Yellow-088 in polyethylene glycol-glycerol. *Biotechnic and Histochemistry* **66,** 111–116.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in plants. *Plant Physiology* **133,** 462–469.

De Block M, Debrouwer D. 1992. *In-situ* enzyme histochemistry on plastic-embedded plant material: the development of an artefact-free β-glucoronidase assay. *The Plant Journal* **2,** 261–266.

De Vries S, Hoge H, Bisseling T. 1988. Isolation of total and polysomal RNA from plant tissues. In: Gelvin SB, Schilperoot RA, eds. *Plant molecular biology*. Dordrecht: Kluwer Academic Publishers, 1–5.

Duan H, Schuler MA. 2005. Differential expression and evolution of the *Arabidopsis* CYP86A subfamily. *Plant Physiology* **137,** 1067–1081.

Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Schreiber L. 2005. Apoplastic polyesters in *Arabidopsis* surface tissues: a typical suberin and a particular cutin. *Phytochemistry* **66,** 2643–2658.

Franke R, Schreiber L. 2007. Suberin – a biopolyester forming apoplastic plant interfaces. *Current Opinion in Plant Biology* **10,** 252–259.

Groh B, Hubner C, Lendzian KJ. 2002. Water and oxygen permeance of phellems isolated from trees: the role of waxes and lenticels. *Planta* **215,** 794–801.

Hartley JL, Temple GF, Brasch MA. 2000. DNA cloning using *in vitro* site-specific recombination. *Genome Research* **10,** 1788–1795.

Haseloff J, Siemering KR, Prasher DC, Hodge S. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proceedings of the National Academy of Sciences, USA* **94,** 2122–2127.

Holloway PJ. 1983. Some variations in the composition of suberin from the cork layers of higher plants. *Phytochemistry* **22,** 495–502.

Jach G, Binot E, Frings S, Luxa K, Schell J. 2001. Use of red fluorescent protein from *Discosoma sp*. (dsRED) as a reporter for plant gene expression. *The Plant Journal* **28,** 483–491.

James DW, Lim E, Keller J, Plooy I, Ralston E, Dooner HK. 1995. Directed tagging of the *Arabidopsis FATTY ACID ELONGATION1 (FAE1)* gene with the maize transposon activator. *The Plant Cell* **7,** 309–319.

Kandel S, Sauveplane V, Compagnon V, Franke R, Millet Y, Schreiber L, Werck-Reichhart D, Pinot F. 2007. Characterization of a methyl jasmonate and wounding-responsive cytochrome P450 of *Arabidopsis thaliana* catalyzing dicarboxylic fatty acid formation *in vitro*. *FEBS Journal* **274,** 5116–5127.

Kandel S, Sauveplane V, Olry A, Diss L, Benveniste I, Pinot F. 2006. Cytochrome P450-dependent fatty acids hydroxylases in plants. *Phytochemistry Reviews* **5,** 359–372.

Kolattukudy PE. 1981. Structure, biosynthesis, and biodegradation of cutin and suberin. *Annual Review of Plant Physiology and Plant Molecular Biology* **32,** 539–567.

Kolattukudy PE. 2001. Suberin from plants. In: Doi Y, Steinbuechel A, eds. Biopolymers I: *Biological systems and biotechnological production* 3. Münster: Wiley–VCH, 41–68.

Kunst L, Samuels AL. 2003. Biosynthesis and secretion of plant cuticular wax. *Progress in Lipid Research* **42,** 51–80.

Kurdyukov S, Faust A, Trenkamp S, Bär S, Franke R, Efremova N, Tiedjen K, Schreiber L, Saedler H, Yephremov A. 2006. Genetic and biochemical evidence for involvement of *HOTHEAD* in the biosynthesis of long-chain α-,ω-dicarboxylic fatty acids and formation of extracellular matrix. *Planta* **224,** 315–329.

Le Bouquin R, Skrabs M, Kahn R, Benveniste I, Salaün JP, Schreiber L, Durst F, Pinot F. 2001. CYP94A5, a new cytochrome P450 from *Nicotiana tabacum* is able to catalyze the oxidation of fatty acids to the ω-alcohol and to the corresponding diacid. *European Journal of Biochemistry* **268,** 3083–3090.

Li Y, Beisson F, Koo AJK, Molina I, Pollard M, Ohlrogge J. 2007. Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. *Proceedings of the National Academy of Sciences, USA* **104,** 18339–18344.

Lulai EC, Corsini DL. 1998. Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum tuberosum* L.) wound-healing. *Physiological and Molecular Plant Pathology* **53,** 209–222.

Ma FS, Peterson CA. 2003. Current insights into the development, structure, and chemistry of the endodermis and exodermis of roots. *Canadian Journal of Botany* **81,** 405–421.

Matzke K, Riederer M. 1991. A comparative study into the chemical constitution of cutins and suberins from *Picea abies* (L.) Karst., *Quercus robur* L., and *Fagus sylvatica* L. *Planta* **185,** 233–245.

Millar AA, Clemens S, Zachgo S, Giblin EM, Taylor DC, Kunst L. 1999. *CUT1*, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *The Plant Cell* **11,** 825–838.

Millar AA, Kunst L. 1997. Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *The Plant Journal* **12,** 121–131.

Molina I, Bonaventure G, Ohlrogge J, Pollard M. 2006. The lipid polyester composition of *Arabidopsis thaliana* and *Brassica napus* seeds. *Phytochemistry* **67,** 2597–2610.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Nelson DR, Schuler MA, Paquette SM, Werck-Reichhart D, Bak S. 2004. Comparative genomics of rice and *Arabidopsis*: analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. *Plant Physiology* **135,** 756–772.

North GB, Nobel PS. 1994. Changes in root hydraulic conductivity for two tropical epiphytic cacti as soil-moisture varies. *American Journal of Botany* **81,** 46–53.

Paul S, Gable K, Beaudoin F, Cahoon E, Jaworski J, Napier JA, Dunn TM. 2006. Members of the *Arabidopsis* FAE1-like 3-ketoacyl-CoA synthase gene family substitute for the Elop proteins of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **281,** 9018–9029.

Perumalla CJ, Peterson CA, Enstone DE. 1990. A survey of angiosperm species to detect hypodermal casparian bands. I. Roots with a uniseriate hypodermis and epidermis. *Botanical Journal of the Linnean Society* **103,** 93–112.

Pinot F, Bosch H, Alayrac C, Mioskowski C, Vendais A, Durst F, Salaün JP. 1993. ω-Hydroxylation of oleic acid in *Vicia sativa* microsomes. *Plant Physiology* **102,** 1313–1318.

Pinot F, Salaün JP, Bosch H, Lesot A, Mioskowsky C, Durst F. 1992. ω-Hydroxylation of Z9-octadecenoic, Z9,10-epoxystearic and 9,10-dihydroxystearic acids by microsomal cytochrome P450 systems from *Vicia sativa*. *Biochemical and Biophysical Research Communications* **184,** 183–193.

Reinhardt DH, Rost TL. 1995. Salinity accelerates endodermal development and induces an exodermis in cotton seedling roots. *Environmental and Experimental Botany* **35,** 563–574.

Rupasinghe SG, Duan H, Schuler MA. 2007. Molecular definitions of fatty acid hydroxylases in *Arabidopsis thaliana*. *PROTEINS: Structure, Function, and Bioinformatics* **68,** 279–293.

Sattelmacher B, Muhling KH, Pennewiss K. 1998. The apoplast – its significance for the nutrition of higher plants. *Zeitschrift fur Pflanzenernährung und Bodenkunde* **161,** 485–498.

Schnurr J, Shockey J, Browse J. 2004. The acyl-CoA synthetase encoded by *LACS2* is essential for normal cuticle development in *Arabidopsis*. *The Plant Cell* **16,** 629–642.

Schönherr J, Ziegler H. 1980. Water permeability of *Betula* periderm. *Planta* **147,** 345–354.

Schreiber L, Breiner HW, Riederer M, Duggelin M, Guggenheim R. 1994. The casparian strip of *Clivia miniata* Reg. roots – isolation, fine structure and chemical nature. *Botanica Acta* **107,** 353–361.

Schreiber L, Franke R, Hartmann K. 2007. Chemical composition of apoplastic transport barriers in roots: quantification of suberin depositions in endodermal and hypodermal root cell walls. In: Sattelmacher B, Horst WJ, eds. *The apoplast of higher plants: compartment of storage, transport and reactions*. Heidelberg: Springer, 109–118.

Schreiber L, Franke R, Lessire R. 2005. Biochemical characterisation of elongase activity in corn (*Zea mays* L.) roots. *Phytochemistry* **66,** 131–138.

Schreiber L, Hartmann K, Skrabs M, Zeier J. 1999. Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. *Journal of Experimental Botany* **50,** 1267–1280.

Schuler MA, Duan H, Bilgin M, Ali S. 2006. *Arabidopsis* cytochrome P450s through the looking glass: a window on plant biochemistry. *Phytochemistry Reviews* **5,** 205–237.

Schwacke R, Schneider A, Van Der Graaff E, Fischer K, Catoni E, Desimone M, Frommer WB, Flügge UI, Kunze R. 2003. ARAMEMNON, a novel database for *Arabidopsis* integral membrane proteins. *Plant Physiology* **131,** 16–26.

Soler M, Serra O, Molinas M, Huguet G, Fluch S, Figueras M. 2007. A genomic approach to suberin biosynthesis and cork differentiation. *Plant Physiology* **144,** 419–431.

Soliday CL, Kolattukudy PE. 1977. Biosynthesis of cutin: ω-hydroxylation of fatty acids by a microsomal preparation from germinating *Vicia faba*. *Plant Physiology* **59,** 1116–1121.

Steudle E, Peterson CA. 1998. How does water get through roots? *Journal of Experimental Botany* **49,** 775–788.

Tijet N, Helvig C, Pinot F, Le Bouquin R, Lesot A, Durst F, Salaün JP, Benveniste I. 1998. Functional expression in yeast and characterization of a clofibrate-inducible plant cytochrome P-450 (CYP94A1) involved in cutin monomers synthesis. *The Biochemical Journal* **332,** 583–589.

Todd J, Post-Beittenmiller D, Jaworski JG. 1999. *KCS1* encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. *The Plant Journal* **17,** 119–130.

Trenkamp S Martin W, Tietjen K. 2004. Specific and differential inhibition of very-long-chain fatty acid elongases from *Arabidopsis thaliana* by different herbicides. *Proceedings of the National Academy of Sciences, USA* **101,** 11903–11908.

Vogt E, Schönherr J, Schmidt HW. 1983. Water permeability of periderm membranes isolated enzymatically from potato tubers (*Solanum tuberosum* L.). *Planta* **158,** 294–301.

Wellesen K, Durst F, Pinot F, Benveniste I, Nettesheim K, Wisman E, Steiner-Lange S, Saedler H, Yephremov A. 2001. Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid ω-hydroxylation in development. *Proceedings of the National Academy of Sciences, USA* **98,** 9694–9699.

Werck-Reichhart D, Feyereisen R. 2000. Cytochrome P450: a success story. *Genome Biology* **1,** reviews3003.1-3003.9.

Wydro M, Kozubek E, Lehman P. 2006. Optimization of transient *Agrobacterium*-mediated gene expression system in leaves of *Nicotioana benthamiana*. *Acta Biochimica Polonica* **53,** 289–298.

Xiao FM, Goodwin SM, Xiao YM, Sun ZY, Baker D, Tang XY, Jenks MA, Zhou JM. 2004. *Arabidopsis* CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. *EMBO Journal* **23,** 2903–2913.

Yang W-L, Bernards MA. 2006. Wound induced metabolism in potato (*Solanum tuberosum*) tubers: biosynthesis of aliphatic domain monomers. *Plant Signaling and Behavior* **1,** 59–66.

Zeier J, Ruel K, Ryser U, Schreiber L. 1999. Chemical analysis and immunolocalisation of lignin and suberin in endodermal and hypodermal/rhizodermal cell walls of developing maize (*Zea mays* L.) primary roots. *Planta* **209,** 1–12.

Zheng HQ, Rowland O, Kunst L. 2005. Disruptions of the *Arabidopsis* enoyl-CoA reductase gene reveal an essential role for very-long-chain fatty acid synthesis in cell expansion during plant morphogenesis. *The Plant Cell* **17,** 1467–1481.

Zimmermann HM, Hartmann K, Schreiber L, Steudle E. 2000. Chemical composition of apoplastic transport barriers in relation to radial hydraulic conductivity of corn roots (*Zea mays* L.). *Planta* **210,** 302–311.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiology* **136,** 2621–2632.