Molecular Plant • Volume 6 • Number 6 • Pages 1889–1903 • November 2013 RESEARCH ARTICLE

Characterization and Ectopic Expression of a *Populus* Hydroxyacid Hydroxycinnamoyltransferase

Ai-Xia Cheng[a,b,2], Jin-Ying Gou[a,2], Xiao-Hong Yu[a], Huijun Yang[a], Xin Fang[c], Xiao-Ya Chen[c], and **Chang-Jun Liu[a,1]**

a Biosciences Department, Brookhaven National Laboratory, Upton, NY 11973, USA
b School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China
c Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Shanghai 200032, China

ABSTRACT Cutinized and suberized cell walls in plants constitute physiologically important environment interfaces. They act as barriers limiting the loss of water and nutrients and protecting against radiation and invasion of pathogens. The roles of cutin- and suberin polyesters are often attributed to their dominant aliphatic components, but the contribution of aromatic composition to their physiological function remains unclear. By functionally screening a subset of *Populus trichocarpa* BAHD/HXXXD acyltransferases, we identified a hydroxycinnamoyltransferase that shows specific transacylation activity on ω-hydroxyacids using both feruloyl- and *p*-coumaroyl- CoA as the acyl donors. We named this enzyme *P. trichocarpa* hydroxyacid/fatty alcohol hydroxycinnamoyltransferase 1 (PtFHT1). The ectopic expression of the *PtFHT1* gene in *Arabidopsis* increased the incorporation of ferulate in root and seed suberins and in leaf cutin, but not that of *p*-coumarate, while the aliphatic load in both suberin and cutin polyesters essentially remained unaffected. The overaccumulation of ferulate in lipophilic polyester significantly increased the tolerance of transgenic plants to salt stress treatment; under sub-lethal conditions of salt stress, the ratios of their seed germination and seedling establishment were 50% higher than those of wild-type plants. Our study suggests that, although aromatics are the minor component of polyesters, they play important role in the sealing function of lipidic polymers *in planta*.

Key words: cutin; suberin; ferulate; BAHD; acyltransferase; *Populus*.

INTRODUCTION

Terrestrial plants have adaptively developed specialized surface tissues covering all aerial and the subterraneous root organs to prevent uncontrolled water loss, nutrient depletion, and limit infection by pathogens (Pollard et al., 2008; Schreiber, 2011; Beisson et al., 2012). Characteristically, these encompassing tissues contain substantial amounts of the apoplastic biopolymers, cutin and suberin (Kolattukudy, 1980, 1981; Franke and Schreiber, 2007; Graça and Santos, 2007; Pollard et al., 2008). Cutin covers virtually all the aerial parts of the plant at a primary developmental stage; as one of nature's most abundant lipid matrix polymers, it is a structural component of plant cuticles, which contributes to the barrier function in controlling water and gas exchange, protecting against the invasion of pathogens and exposure to UV radiation, and preventing cell fusing during organogenesis (Kolattukudy, 2001; Pollard et al., 2008; Reina-Pinto and Yephremov, 2009; Schreiber, 2011; Yeats et al., 2012). Suberin is another common lipid biopolymer with similar functions that occurs in the cell walls of the root's hypodermis and endodermis, the outer- and inner-sealing tissues of primary roots of all angiosperms (Franke et al., 2005; Franke and Schreiber, 2007; Graça and Santos, 2007). Furthermore, suberin is present in the cell walls of the periderm, the outer tissues of stems in a secondary growth stage, the endodermal cell walls of the bundle sheaths of grass leaves, and in seed coats (Espelie and Kolattukudy, 1979; Kolattukudy, 1981; Molina et al., 2008). Suberin deposition is also induced by wounding, and in response to pathogen attacks and unfavorable environmental conditions, such as drought and

[1]To whom correspondence should be addressed. Email cliu@bnl.gov, tel. 631-344-2966, fax 631-344-3407.

[2]These authors contributed equally to the work.

doi:10.1093/mp/sst085, Advance Access publication 24 May 2013

Received 20 March 2013; accepted 16 May 2013

salt stress (Cottle and Kolattukudy, 1982a; Bernards and Lewis, 1998; Degenhardt and Gimmler, 2000; Schreiber et al., 2005a). Similarly to cutin, the apoplastic deposition of suberin in the periderms and endoderms of many plant species strongly retards the movement of water, and the dissolved nutrients and ions (Groh et al., 2002; Franke et al., 2005; Franke and Schreiber, 2007; Graça and Santos, 2007). In particular, suberization of the casparian strip in the endodermis of the root enables this inner tissue to control the uptake of water and ions into the stele (Ranathunge and Schreiber, 2011). When suberin is deposited in outer tissues, like the periderm of the root, it also functions as an antimicrobial barrier against pathogens (Lulai and Suttle, 2009; Serra et al., 2009).

Structurally, cutin is predominantly built from C16 and C18 aliphatics either directly linked via ester bonds, typically between the carboxy and ω-hydroxy groups of individual fatty acid derivatives, or through esterification to glycerol (Graça and Santos, 2007; Pollard et al., 2008). Small amounts of phenolics, such as *p*-coumarate and ferulate, are also esterified to cutin (Kolattukudy, 1980, 1981; Graça and Santos, 2007; Graça and Lamosa, 2010). Compared to cutin, suberin is a more complex polyester polymer that is composed of both aromatic and aliphatic domains (Kolattukudy, 2001; Bernards, 2002; Franke and Schreiber, 2007; Graça and Santos, 2007; Serra et al., 2011). The latter constitutes a three-dimensional polyester polymer mainly comprising oxygenated long-chain fatty acids (ω-hydroxyacids and α,ω-diacids) wherein chain-length ranges from C16 to C32, which then partially is cross-linked by glycerol, and with integrated aromatic compounds (Kolattukudy and Dean, 1974; Graça and Pereira, 2000; Franke et al., 2005; Graça and Santos, 2007). Upon *trans*-esterification, the aliphatic suberin releases ω-hydroxyacids, α,ω-diacids, fatty acids, primary alcohols, glycerol and small amounts of hydroxycinnamic acids, mainly ferulic acid (Graça and Pereira, 2000; Schreiber et al., 2005b). The (poly) aromatics, derived from the phenylpropanoid pathway, form a lignin-like polymer mostly containing hydroxycinnamic acids; they are restricted to the primary cell wall, and presumably are involved in cross-linking the aliphatic domain to the cell wall's polysaccharides (Cottle and Kolattukudy, 1982b; Bernards et al., 1995; Bernards and Razem, 2001; Serra et al., 2011).

Recent identifications and characterizations of the genes and enzymes involved in synthesizing the lipid compositions of polyesters, along with studies on the corresponding mutant lines defective in one or more components of lipid polymers, have greatly facilitated our evaluation of the roles of lipid polyesters in water sealing, gas exchange, ion conductance, and resistance to pathogenic diseases (Pollard et al., 2008; Serra et al., 2011; Beisson et al., 2012). Broad explorations have delineated the contributions of the lipidic compositions of cutin and suberin to their functions as transport barriers in ion permeability (Kosma et al., 2009; Panikashvili et al., 2009; Ranathunge and Schreiber, 2011; Yang et al., 2011; Lu et al., 2012). However, the full biological and structural roles of cutin and suberin phenolics remain elusive due to their lesser abundance in the polyester networks, and the lack of adequate molecular tools to modulate their biosynthesis.

Hydroxycinnamoyl-CoA:ω-hydroxyacid/fatty alcohol transferase (HHT) catalyzes the formation of aromatic esters in lipidic polymers. The activity of this enzyme was widely detected in angiosperms and gymnosperms (Lotfy et al., 1995, 1996), and a 52-kD protein was purified from tobacco suspension cells that transfers the feruloyl moiety from the thioester donor to the 16-hydroxyhexadecanoic acid (Lotfy et al., 1995, 1996). An *Arabidopsis* BAHD/HXXXD family acyltransferase member, encoded by At5g41040, was demonstrated with HHT activity (Gou et al., 2009; Molina et al., 2009) that preferentially transfers the feruloyl moiety to the ω-hydroxyl group of long-chain fatty acids specifically responsible for the formation of suberin aromatics (named hydroxycinnamoyl-CoA:hydroxyacid hydroxycinnamoyltransferase, AtHHT1, or aliphatic suberin feruloyltransferase, ASFT). Its T-DNA insertion mutant lines showed a substantial reduction in the ferulate content of seed and root suberins and, consequently, an elevated permeability to ionic dyes (Gou et al., 2009). Suppression of an AtHHT1/AtASFT homolog gene in potato (*Solanum tuberosum*), namely the ω-hydroxyacid/fatty alcohol hydroxycinnamoyltransferase (FHT), reduced both ferulate ester and ω-hydroxy fatty acid contents in suberin and in the related wax fraction, which consequently resulted in a thicker, russet skin of the potato tuber, and increased water loss (Serra et al., 2010). Those data suggest deficit of aromatics of suberin and the related wax directly or indirectly impairs the function of lipid polymers. In contrast, in a study on a mutant deficient in the gene encoding cutin feruloyltransferase (DCF), a distant homolog of AtHHT1/ASFT, in *Arabidopsis*, the loss of cutin ferulate showed no marked physiological effect on the function of plant cuticles in regulating ion permeability, water loss, and pathogen infection compared to the wild-type plants (Rautengarten et al., 2012), suggesting the potential functional disparity of polyester aromatics in cutin and suberin.

In attempting to systematically identify the aromatic acyltransferases responsible for synthesizing the 'wall-bound' phenolic esters and elucidating their biological functions, previously we identified a large family of BAHD/HXXXD acyltransferases from *Populus trichocarpa*, and detailed their tissue-specific expression patterns (Yu et al., 2009). Subsequently, we functionally screened the potential transacylation activities of a set of the selected BAHD acyltransferases using aromatic and aliphatic thioacyl donors. In the present study, we describe our identification of an acyltransferase member, previously named PtACT41 (Yu et al., 2009), that recognizes both *p*-coumaroyl and feruloyl-CoA as thioester donors, and acylates ω-hydroxyacids and fatty alcohols *in vitro*. Ectopically expressing this acyltransferase in *Arabidopsis* results in the increase of phenolic esters in leaf cutin, and in root and seed suberins. More importantly, the overaccumulation of ferulate esters in polyesters confers significantly increased tolerance of plants to salt stress.

RESULTS

Isolation of BAHD/HXXXD Acyltransferase PtACT41 from *P. trichocarpa*

With identification of 94 BAHD/HXXXD family of acyltransferase sequences from *P. trichocarpa* genome and recognition of their tissue-specific expression (Yu et al., 2009), we subcloned 22 putative acyltransferase genes that are preferentially expressed in the stem, leaf, and root tissues of poplar, and produced their corresponding recombinant proteins by expressing them in *Escherichia coli*. Subsequently, we used these recombinant enzymes to screen for their catalytic activities toward particular aromatic- or aliphatic-acyl donors and the corresponding acyl acceptors. By this approach, we revealed an acyltransferase member, previously named PtACT41 (Port ID XP_002298644.1) (Yu et al., 2009), that exhibited considerable activity toward feruloyl-CoA and ω-hydroxyacid. The full-length CDS of PtACT41 contains 1299 nucleotides with an open reading frame (ORF) coding for a polypeptide of 432 amino-acids. The deduced polypeptide of PtACT41 possesses the typical diagnostic sequence signatures of 'HXXXD' and 'DFGWG' of BAHD/HXXXD family members (Supplemental Figure 1). Blast search non-redundant protein sequence database of *P. trichocarpa* in National Center for Biotechnology Information (NCBI) revealed a few close homologs of PtACT41. Phylogenetic analysis (Figure 1) indicated that PtACT41 is close to the potato suberin/wax FHT (ACS70946; Serra et al., 2010) with amino-acid sequence identity of 76.4%; the *Arabidopsis* suberin HHT1/ASFT (At5g41040; Gou et al., 2009; Molina et al., 2009) with 74% identity; *Arabidopsis* fatty alcohol caffeoyltransferase (FACT) (Kosma et al., 2012) with 61.8% identity; and *Arabidopsis* cutin feruloyltransferase DCF (At3g48720; Rautengarten et al., 2012) with 57.6% identity. But it is clearly distant from the predicted *Populus* hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT) that is involved in the synthesis of guaiacyl and syringyl lignin precursors (Shi et al., 2010). These data imply that PtACT41 may have a similar or related function for the formation of polyester aromatics.

Recombinant PtACT41 Functions as ω-Hydroxyacid/Fatty Alcohol Hydroxycinnamoyltransferase *In Vitro*

To fully elucidate the biochemical functions of PtACT41, we examined its substrate specificity and catalytic properties using the purified recombinant protein with different CoA thioester donors and fatty acids/alcohol acceptors. After incubating PtACT41 with feruloyl-CoA and 16-hydroxypalmitic acid, we resolved a dominant product by liquid chromatography–mass spectrometry (LC–MS) (Figure 2A) that exhibits an UV spectrum similar to that of ferulic acid; however, its positive electron spray ionization (ESI)–ion mass spectrum denotes a molecular ion $[M+H]^+$ at a mass-to-charge ratio (*m/z*) of 449, pointing to the conjugation of ferulate with 16-hydroxypalmitic acid (Figure 2B). Further fragmentation of the so-detected main molecular ion generated daughter ions with a fragment mass-to-charge ratio (*m/z*) of 177 in the MS_2 mass spectrum (Figure 2C), further verifying the presence of a feruloyl residue in the product. No product was detected in the control reaction wherein we incubated the heat-inactivated recombinant enzyme with donor- and acceptor-substrates (Figure 2A). Similarly, after incubating PtACT41 with *p*-coumaroyl CoA and ω-hydroxyacid acceptor, we detected a major phenolic conjugate whose UV-absorptive spectrum and mass spectrum suggest the production of alkyl-coumarate (Figure 2D–2F). Under the same reaction conditions, the specific activity of the enzyme using *p*-coumaroyl CoA was about 78% of that of using feruloyl-CoA, but, when using sinapoyl-CoA, the activity was only 6% of that of using feruloyl-CoA (Table 1). These data indicate that the recombinant enzyme of PtACT41 recognizes both feruloyl- and *p*-coumaroyl-CoA as the acyl donors to acylate ω-hydroxyacid.

Using feruloyl-CoA as the acyl donor, we then tested 15 aliphatic substrates as potential acyl acceptors. As listed in Table 1, the highest transacylation activity was found in using 16-hydroxypalmitic acid as the substrate. We registered no activity when β-hydroxypalmitic acid or 12-hydroxystearic acid was used as the potential substrate, indicating a strict regio-selectivity of PtACT41 for ω-hydroxyl of fatty acid. Besides ω-hydroxyacid, the enzyme also showed detectable activity with short- to mid-chain fatty alcohols (1-hexanol, 1-heptanol, 1-decanol, 1-dodecanol, and 1-tetradecanol); however, the overall activity was much lower (about 13~50%) than that for ω-hydroxyacid (Table 1).

Monitoring the catalytic activity of the enzyme at different temperatures and pH values revealed that the optimal temperature for the maximum activity of PtACT41 was 40°C, and the optimum pH value was 7.5. Under such conditions, using 16-hydroxypalmitic acid as acyl acceptor, PtACT41 recombinant enzyme showed an apparent K_m of 14.3 ± 5.2 μM and k_{cat} of 0.04 s^{-1} for feruloyl-CoA, and K_m of 59 ± 10.8 μM and k_{cat} of 0.076 s^{-1} for *p*-coumar oyl-CoA (Table 1), indicating the enzyme has slightly stronger binding affinity thus higher catalytic efficiency to feruloyl-CoA than to *p*-coumaroyl-CoA *in vitro*. At the fixed concentration (~150 μM) of feruloyl-CoA or *p*-coumaroyl-CoA (as the acyl donor), the enzymes, however, showed very similar apparent K_m values and the turnover rates (k_{cat}) for 16-hydroxypalmitic acid; the overall catalytic efficiencies (k_{cat}/K_m) in catalyzing hydroxyacid in using either CoA thioester essentially are the same (Table 1). These data suggest that, under the saturated concentration of CoA thioester, PtACT41 could effectively catalyze either transferuloylation or transcoumaroylation on aliphatic monomers *in vitro*. Accordingly, we denote PtACT41 as *Populus* hydroxyacid/fatty alcohol hydroxycinnamoyltransferase 1 (PtFHT1).

PtFHT1 Is Ubiquitously Expressed in Different Tissues of *P. trichocarpa*

Our previous RT–PCR analysis revealed that *PtFHT1* (*PtACT41*) was among many other BAHD/HXXXD family

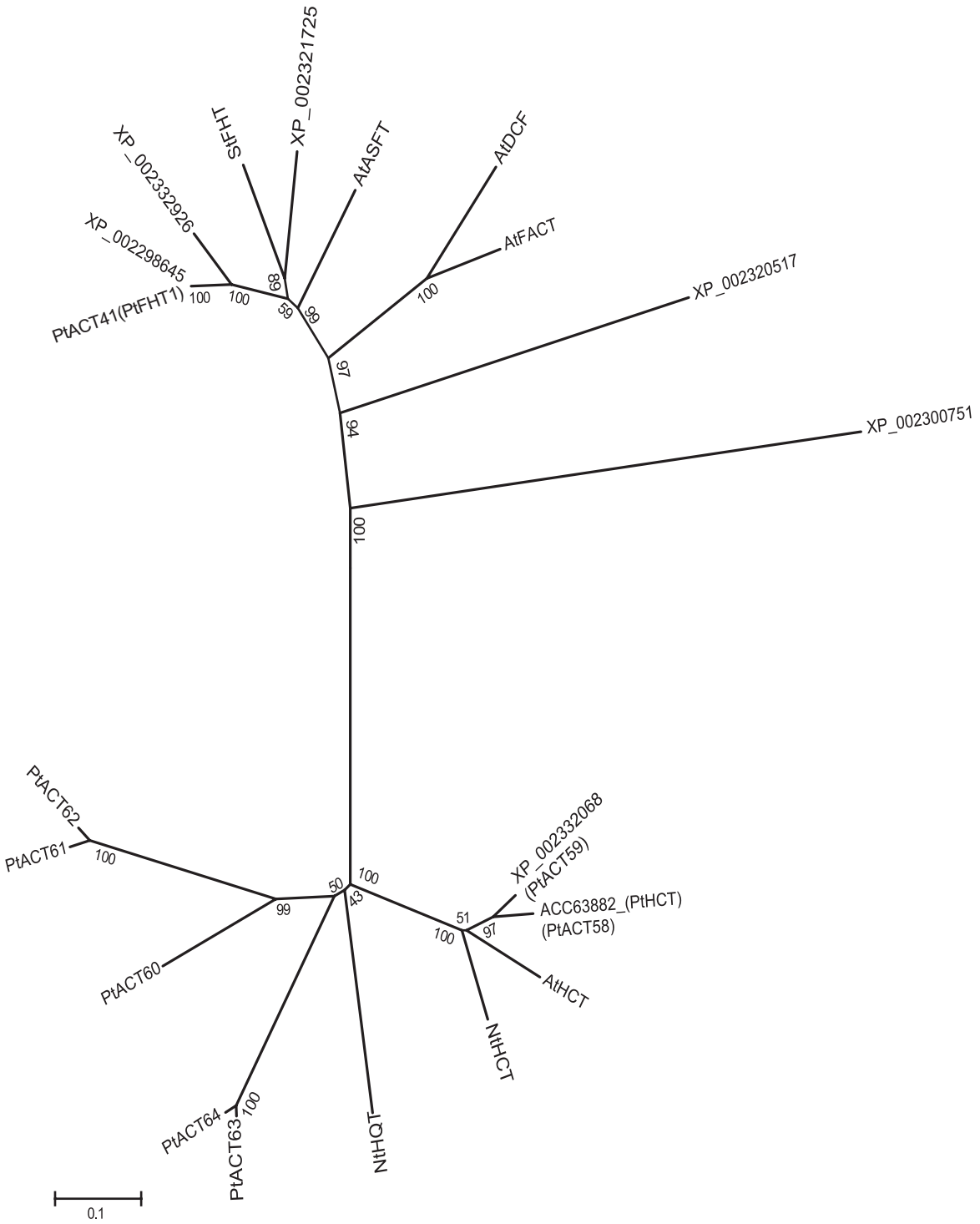

Figure 1. Phylogenetic Tree of PtACT41 (JX515962) with Its Closely Related *Populus* Homologs and the Other Function-Known Hydroxycinnamoyltransferases Responsible for Synthesizing Aromatics of Suberin, Cutin, and Associated Waxes.

The amino-acid sequences of the following enzymes were used in alignment with the CLUSTALW (version 1.83) program: *Arabidopsis* AtHCT (NP_199704), AtHHT1/ASFT (At5g41040), AtFACT (At5g63560), AtDCF (At3G48720); tobacco NtHCT (CAD47830) and NtHQT(AJ582651); and *Solanum tuberosum* FHT (ACS70946), and 7 *Populus* HCT homologs PtACT58-64 as described (Yu et al., 2009). The numbers beside the branches represent bootstrap values based on 1000 replications.

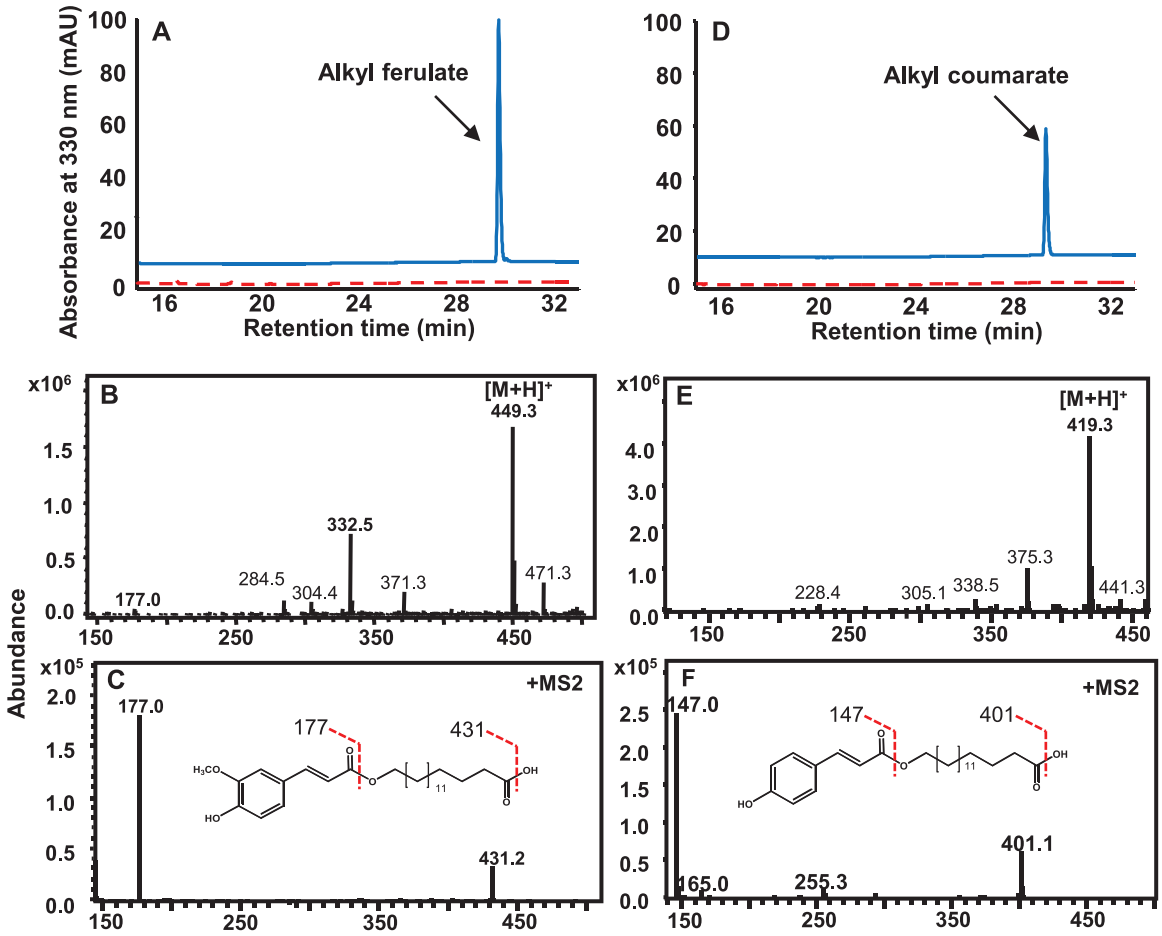

Figure 2. *In Vitro* Activity of PtACT41 Recombinant Enzymes.
(A) HPLC chromatogram of the products in the reactions of recombinant protein (the solid line) or the heat-inactivated protein (dashed line, as the control) incubated with feruloyl-CoA and 16-hydroxypalmitic acid.
(B, C) The MS spectra of the alkyl ferulate product from (A).
(D) HPLC chromatogram of the products in the reactions of recombinant protein (the solid line) or the heat-inactivated protein (dashed line, as the control) incubated with *p*-coumaroyl-CoA and 16-hydroxypalmitic acid.
(E, F) The MS spectra of the alkyl-coumarate product from (D).

acyltransferase genes broadly expressed in the different tissues of *P. trichocarpa* (Yu et al., 2009). To quantitatively re-examine/confirm PtFHT1 gene expression patterns, we performed qRT–PCR analysis using total RNAs from the tissues of root, leaf, stem, apical bud, bark/phloem/cambium, and young wood of *P. trichocarpa*. Technically, the developing poplar plantlets (about 3 months) were collected and sampled as the apical buds (the tips of developing stems), the leaves (the mixture of all the attached leaves on plantlets), stems (with bark), and roots. Some developing stems were also peeled to separate the bark/phloem/cambium and xylem (young wood). The data were normalized with the ubiquitin gene transcripts, and the level of gene expression in young wood (xylem) tissue then was set as 100%. As depicted in Figure 3, the transcripts of *PtFHT1* were ubiquitously present within all these tissues, with the highest expression in the apical bud, implying that PtFHT1 might function broadly in the formation of polyester aromatics in both aerial and underground organs. The high expression of *PtFHT1* in the fast-growing tissues like apical buds, which is reminiscent of the high expression of lignin biosynthetic genes in the developmentally active cambium tissue, may suggest an active build-up of lipophilic polyesters including their aromatic component in the growing tissues.

Overexpression of *PtFHT1* Increases Ferulate Content of Root and Seed Suberins

To further dissect the *in vivo* function of PtFHT1, we subcloned the gene coding region into the binary vector pMDC32 (Curtis and Grossniklaus, 2003) via the gateway cloning technique, then transformed the construct into *Arabidopsis* (Figure 4A). The transcripts of overexpressed *PtFHT1* gene were detected in the resulting T3 generation of transgenic plants (Figure 4A). None of transgenic plants showed discernible morphological

Table 1. Activity and Kinetics of Recombinant PtFHT1 on Different Acyl Donors and Acceptors.

Chemicals	Specific activity (%)	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Acyl donor					
Feruloyl-CoA	100*	14.32 ± 5.2	51.4 ± 5.0	0.04 ± 0.004	2793
p-Coumaroyl-CoA	78 ± 7.6	59.07 ± 10.78	96.5 ± 6.2	0.076 ± 0.005	1287
Sinapyl-CoA	6 ± 0.7				
Acyl acceptor					
16-Hydroxypalmitic acid	100*	60.34 ± 15.98[a]	72.7 ± 7.4[a]	0.058 ± 0.006[a]	961[a]
		48.7 ± 5.8[b]	58.3 ± 2.4[b]	0.046 ± 0.002[b]	944[b]
DL-α-hydroxybehenic acid	n.d.				
12-Hydroxystearic acid	n.d.				
Halmitic acid	n.d.				
DL-β-hydroxypalmitic acid	n.d.				
1-Docosanol	n.d.				
1-Eicosanol	n.d.				
1-Octadecanol	n.d.				
12-Hydroxystearyl alcohol	n.d.				
1-Hexadecanol	n.d.				
1-Tetradecanol	13.1 ± 0.58				
1-Dodecanol	53.1 ± 4.39				
1-Decanol	40.6 ± 1.14				
1-Heptanol	17.7 ± 0.17				
1-Hexanol	32.2 ± 0.6				

* Specific activity with feruloyl-CoA and 16-hydroxypalmitic acid (13.39 nmol mg^{-1} min^{-1}) was taken to be 100%. nd, not detectable.
[a] Reactions were performed using feruloyl-CoA as the acyl donor and 16-hydroxypalmitic acid as the acyl acceptor.
[b] Reactions were performed using *p*-Coumaroyl-CoA as acyl donor and 16-hydroxypalmitic acid as the acyl acceptor.

and developmental abnormalities under the normal growth conditions. Subsequently, we prepared root and seed suberins and analyzed their phenolic esters after BF_3/methanol depolymerization via HPLC. As delineated in Figure 4B–4E, similar HPLC-UV phenolic profiles were resolved from the suberin hydrolysates of the wild-type and T3-generation of transgenic plants; both ferulate- and *p*-coumarate-methyl esters were detected in suberin hydrolysates from the roots (Figure 4B and 4C), while ferulate dominated in the suberin of the seeds from both transgenic and wild-type plants (Figure 4D and 4E). The average level of suberin ferulate detected in the roots increased by about 10–25% in three independent transgenic lines compared to the level in the wild-type plants; in contrast, the *p*-coumarate content essentially remained the same in transgenic and control plants (Figure 4F). Similarly, we observed about a 20–30% increase of ferulates in the suberin hydrolysates of seeds of the transgenic lines compared with wild-type plants (Figure 4G). These data imply that PtFHT1 acts primarily as a feruloyltransferase *in planta*, involved in synthesizing suberin aromatics.

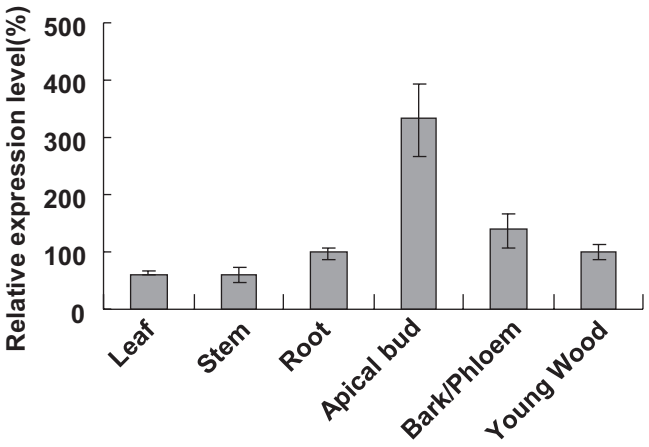

Figure 3. The Relative Expression Level of PtACT41 in Different Tissues of *P. trichocarpa*.
The expression level of gene in young wood tissue was set at 100%.

Overexpression of *PtFHT1* Increases the Level of Cutin Ferulate in Leaves

We collected and prepared cell wall fractions from the 6-week-old rosette leaves of both the transgenic and wild-type plants and depolymerized them by the BF_3/methanol *trans*-esterification method. When the cutin's hydrolysates were resolved by LC–MS, similarly to root suberin, both *p*-coumarate- and ferulate esters were detected (Figure 5A and 5B), but their levels were much smaller than those in the suberin from roots (Figure 5C). While the content of *p*-coumarate in leaf cutin showed no statistically significant difference between wild-type plants and three transgenic lines, the level of ferulate was about 30–45% higher in *PtFHT1* transgenic lines than in

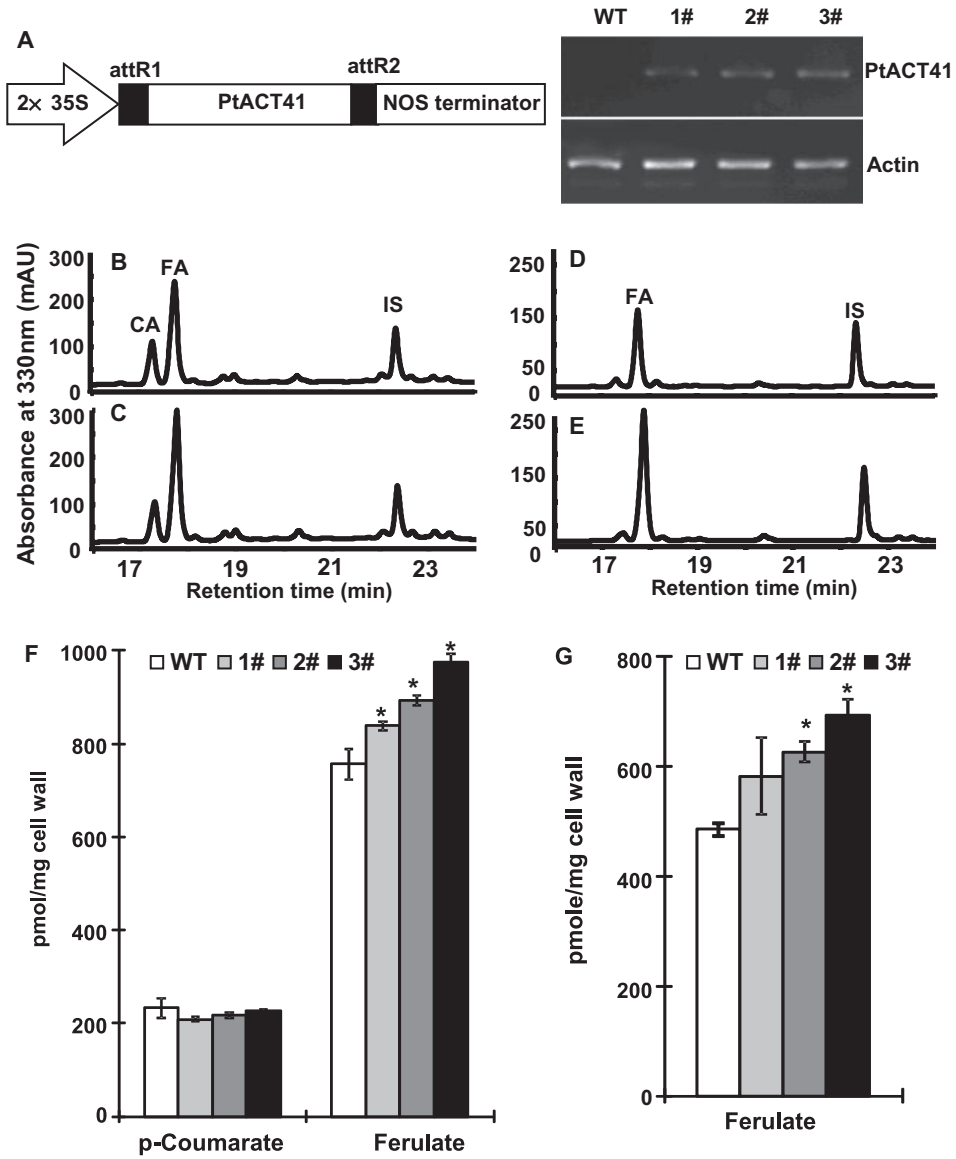

Figure 4. Ectopic Expression of PtFHT1 in *Arabidopsis* and Accumulation of Aromatics in Root and Seed Suberins of the Resulting Transgenic Plants.
(A) Schema of overexpression of PtFHT1 in *Arabidopsis* and the monitoring of transgene expression in three independent T3 generation of transgenic lines by RT–PCR.
(B, C) Representative HPLC profiles of phenolics released upon depolymerization of root extract-free residues by BF_3/methanol of the wild-type (B) and transgenic (C) lines.
(D, E) Representative HPLC profiles of phenolics released upon depolymerization of seed extract-free residues by BF_3/methanol of wild-type (D) and transgenic (E) lines. mAU, milliabsorbance units. CA, *p*-coumarate methylester; FA, ferulate methylester; both compounds were identified based on their identical UV spectra with those of *p*-coumarate and ferulate, respectively, and their corresponding mass spectra; IS: Internal Standard.
(F) Aromatic contents of root suberins of wild-type and three transgenic lines.
(G) Aromatic contents of seed suberins of the wild-type and three transgenic lines. The values represent means ± SD of at least three biological replicates. Statistic analysis was performed with Student's *t*-test. * indicates significant difference at $p < 0.05$.

the wild-type plants (Figure 5C), suggesting that PtFHT1 also functions for synthesizing cutin ferulate *in planta*.

Overexpression of *PtFHT1* Does Not Disturb the Lipid Load of Cutin and Suberin Polymers

Our previous study revealed that a deficit of suberin aromatics disturbs the incorporation of aliphatic monomers in polyesters (Gou et al., 2009). To examine the potential alteration of lipid load in cutin and suberin of PtFHT1 overexpression plants, we analyzed the aliphatic monomer compositions of the root and leaf de-lipidated extract-free cell wall residues by gas chromatography–mass spectrometry (GC–MS) after BF_3/methanol transesterification-mediated depolymerization. Consistently with previous compositional studies

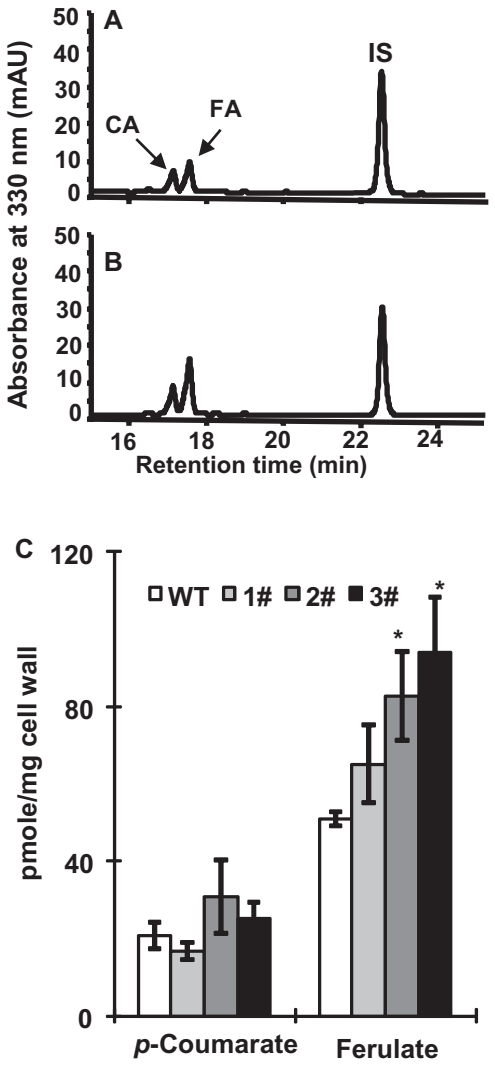

Figure 5. Accumulation of Aromatics in Leaf Cutin of PtFHT1 Overexpression Plants.

(A, B) Representative HPLC profiles of phenolics released upon depolymerization of de-lipidated leaf residues by BF_3/methanol of wild-type (A) and transgenic (B) lines. mAU, Milliabsorbance units; CA, *p*-coumarate methylester; FA, ferulate methylester; IS, Internal Standard.

(C) The accumulation level of the ferulate and *p*-coumarate esters of leaf cutins from wild-type and transgenic lines. Values represent means ± SD of at least three biological replicates. Statistic analysis was performed with Student's *t*-test. * indicates significant difference at $p < 0.05$.

in *Arabidopsis* (Bonaventure et al., 2004; Franke et al., 2005; Molina et al., 2006), ω-hydroxyacid and α,ω-diacid were largely found in the cutin and suberin hydrolysates. Those aliphatic compositions did not vary significantly between transgenic and control root suberins (Figure 6A). Similarly, the lipidic-monomer composition of leaf cutin, including the saturated and unsaturated fatty acids, ω-hydroxyacids, and α,ω-diacids, was essentially unchanged in transgenic lines compared with the wild-type plants (Figure 6B). These results imply that the constitutive expression of *PtFHT1* in *Arabidopsis* does not drastically affect the load of aliphatics of suberin and cutin.

PtFHT1 Overexpression Enhances the Tolerance to Salt Stress

When conventional toluidine blue (Tanaka et al., 2004) and tetrazolium salt (Molina et al., 2008) were used to stain both the mature seeds and seedling roots of transgenic plants, in an attempt to monitor the potential change of their permeability, there was no discernible difference in comparison with the staining on wild-type tissues. In both wild-type and transgenic plant tissues, the dyes were limited to permeate in the tissues even with the prolonged time, suggesting those staining methods are good at distinguishing mutant plants deficient in the lipophic polyesters but limited at discriminating the plants with over-accumulated polyesters. However, when mature seeds of three independent T3 homozygous transgenic lines were incubated on Murashige and Skoog agar medium (Murashige and Skoog, 1962) containing different concentrations of NaCl for 2 weeks, we monitored the obvious differences in the ratios of seed germination (roots punching out) and seedling establishment (plantlet with two green cotyledons). This suggests the changes in permeability of seed coat of transgenic plants to the salt ion. The *PtFHT1* overexpression lines clearly showed the higher ratios of seed germination and establishment than the wild-type plants under the same growth condition (Figure 7A). Approximately 30–50% more transgenic seeds germinated than did the wild-type ones when they were incubated in media containing a 125 or 150 mM sub-lethal concentration of NaCl (Figure 7B). Similarly, more seedlings were established from transgenic seeds than from the wild-type controls, when they grew on plates containing a high concentration of salt; the ratio of seedling establishment of transgenic lines was approximately 30–40% higher than that of the wild-type under 125 mM NaCl (Figure 7C). Furthermore, measuring the length of the roots of established seedlings cultured vertically on plates (Figure 7D), we noted that, while the roots of wild-type and transgenic seedlings elongated similarly on the growth media without addition of NaCl, an increase in NaCl concentration therein notably impaired the root elongation in transgenic and control plants. However, the roots of the transgenic seedlings were always longer than those of the wild-type plants under the same concentration of NaCl (Figure 7D and 7E), indicating that the detrimental effect of salt stress on the growth of the *PtFHT1* expression plants was less. Together, these data imply that the expression of *PtFHT1*, via increasing the aromatics in seed and root suberins, enhances the salinity tolerance of transgenic plants.

DISCUSSION

PtFHT1 Functions as a Feruloyltransferase for Synthesizing Cutin and Suberin Aromatics

Small amounts of phenolic acids, such as ferulic acid and *p*-coumaric acid, are known to covalently attach to the cell wall's lipidic polyesters (Kolattukudy, 1980, 1981; Bernards

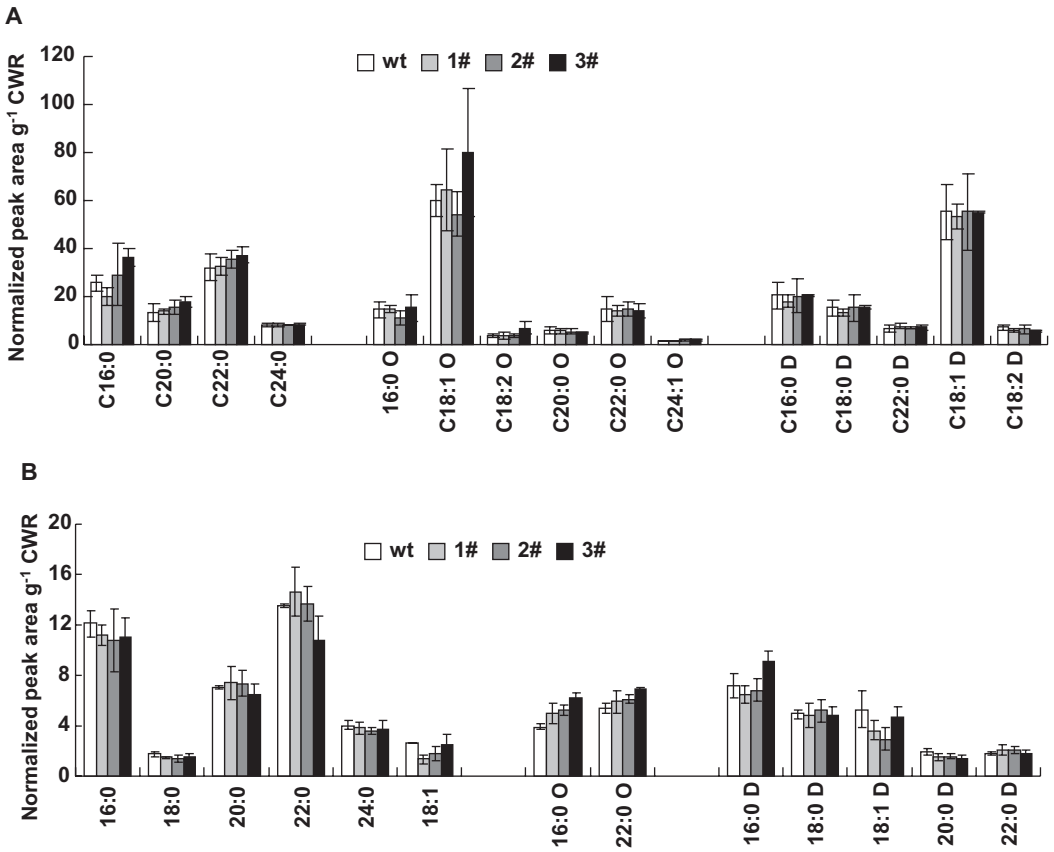

Figure 6. The Aliphatic Monomer Profiles of Lipid Polyesters of the Wild-Type and Transgenic Plant in Roots **(A)** and Leaves **(B)** after BF_3/Methanol Depolymerization and Determined by GC–MS.
The values indicate means of two or three biological replicates ± SD. Statistical analysis via *t*-test found no difference between the wild-type and transgenic plants. CWR, cell wall residues; O, ω-hydroxyacid; D, diacid.

and Razem, 2001; Graça and Santos, 2007; Pollard et al., 2008; Graça and Lamosa, 2010). Purportedly, the alkyl hydroxycinnamates are the biosynthetic precursors for the aromatic esters incorporated in those lipidic polymers (Kolattukudy, 1980, 1981; Graça and Santos, 2007; Graça and Lamosa, 2010). Synthesis of alkyl phenolates requires the activity of hydroxycinnamoyl-CoA-dependent aromatic acyltransferases (Lotfy et al., 1994, 1995; Liu, 2010). Recently, impressive progress was made in characterizing those acyltransferases responsible for the formation of polyester aromatics. They encompass three close *Arabidopsis* homologous enzymes that catalyze transferuloylation or transcaffeoylation for synthesizing the aromatics of cutin, suberin, and related root waxes (Gou et al., 2009; Molina et al., 2009; Kosma et al., 2012; Rautengarten et al., 2012), and one potato hydroxyacid/fatty alcohol feruloyltransferase for forming suberin aromatics of the potato tuber periderm (Serra et al., 2010). The PtFHT1 screened from a subset of *Populus* BAHD/HXXXD family members shares the high identity at amino-acid level with potato and *Arabidopsis* suberin feruloyltransferases (Gou et al., 2009; Molina et al., 2009). Similarly to AtHHT1/ASFT, the PtFHT1 recognizes both feruloyl- and *p*-coumaroyl-CoA as the acyl donor *in vitro*. However, it showed less discrimination for two thioester donors than did AtHHT1/ASFT, and its catalytic efficiencies using either of the thioester donors for transacylation of ω-hydroxyacid are essentially similar (Table 1). Interestingly, when PtFHT1 is constitutively expressed *in planta*, only ferulate esters increased in both suberin and cutin polyesters, while *p*-coumarate that is commonly found in leaf cutin and root suberin showed minimum changes. These data imply that synthesis of alkyl hydroxycinnamate *in vivo* might undergo exquisite spatial or metabolic organization; the availability of thioester donors in the biosynthesis acts as the key determinator, dominating the aromatic composition of lipid polyesters. So far, modulating any characterized hydroxyacid/fatty alcohol hydroxycinnamoyltransferases *in planta* failed to disturb the formation of *p*-coumarate esters. It would be highly interesting to explore how *p*-coumarate is incorporated into the lipid polymers.

In *Arabidopsis*, the genes encoding suberin- or cutin-related hydroxycinnamoyltransferase are differentially expressed in specific tissues with the correspondingly different functions (Gou et al., 2009; Molina et al., 2009; Rautengarten et al.,

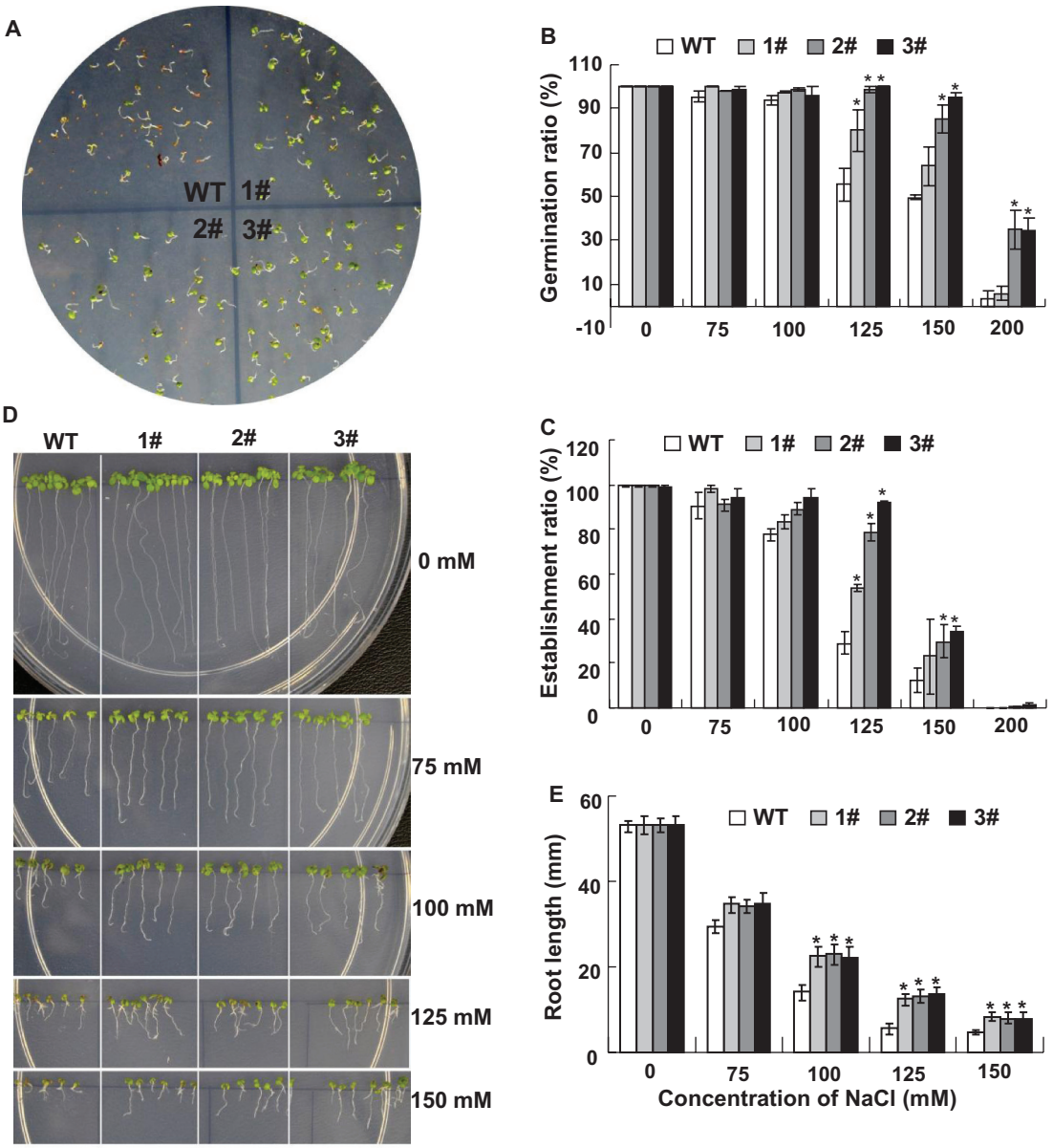

Figure 7. The Responses of the Wild-Type and Transgenic Plants Grown under Salt Stress.
(A) Germination and seedling establishment of the wild-type and transgenic plant seeds grown on the MS medium containing 150 mM NaCl.
(B) The calculated germination ratio (i.e. the ratio of root punching out) of wild-type and PtFHT1 transgenic seeds on MS medium containing increasing concentrations of NaCl after 14-d culture.
(C) The calculated seedling establishment ratio (i.e. the ratio of seedlings with two green cotyledons) under different concentration of NaCl after 14-d culture. Values in (B) and (C) represent means ± SD of at least three replicates, in each 50~100 seeds were scored. * indicates significant difference at $p < 0.05$ under *t*-test.
(D) The root elongation of the vertically cultured seedlings on the MS agar medium supplemented with different concentrations of NaCl, imaged after 14-d growth.
(E) The measured root length of the wild-type and transgenic plants after 14-d growth on the MS agar medium supplemented with different concentrations of NaCl. Values are means of three replicates with six seeds for each. * indicates significant difference at $p < 0.05$.

2012). The DCF involved in the formation of surface polymer cutin was strictly expressed in the aerial tissues (Rautengarten et al., 2012), while the transcripts of AtHHT1/ASFT were predominantly found in the underground roots and mature seeds (Gou et al., 2009; Molina et al., 2009; Rautengarten et al., 2012). The tissue-specific gene expression, and thus the distribution of the encoded enzymes rather than their catalytic activity, apparently determines their biological functions in the formation of polyester aromatics. PtFHT1 was universally expressed in aerial and underground tissues in poplar (Figure 3); its ectopic expression in *Arabidopsis* led to the overaccumulation of phenolic esters in leaf cutin, and in

root and seed suberins (Figures 4 and 5). These data suggest that PtFHT1, differing from functionally specified AtDCF and AtASFT, is involved in synthesizing both cutin and suberin polyesters *in vivo.*

Since PtFHT1 shares certain homology to potato FHT (Figure 1) and disturbing *StFHT* expression in potato tubers affects ferulate accumulation in the suberin-related wax layer, we also examined the potential changes of both aliphatics and aromatics of tap root waxes of *PtFHT1* overexpression *Arabidopsis* following the described procedure (Kosma et al., 2012), but we did not detect substantial change in those wax extracts, suggesting PtFHT1 mainly involves ferulate synthesis in cutin and suberin polyesters when it was ectopically expressed in *Arabidopsis*.

Aromatic Composition Exerts Significant Effects on the Sealing Property of Polyesters

The cutin matrix covering the surfaces of aerial organs, and the suberins deposited in the subterraneous root organs and in the outer integumentary layers of seed coats, together with their associated waxes, form the important lipophilic barrier regulating the plant's impermeability, water loss/uptake, and the transport of solutes in and out of the plants (Schreiber, 2011). Since their predominant composition is aliphatic, the barrier properties of the cutinized and suberized tissues are commonly attributed to the hydrophobic nature of the lipidic components of the biopolymers. Indeed, many studies demonstrated that disruption of the aliphatic load in either cuticle or suberin polyesters, significantly alters the ion permeability of plant cells, increases water loss, and reduces the tolerance to various stresses (Panikashvili et al., 2009). Aromatics, as the minor components of suberin and cutin, approximately account for only 0~10% of the total polyesters; hence, their contributions to polyesters' structure and physiological functions often are overlooked (Serra et al., 2011). In present study, the moderate increase of phenolics in polyesters by ectopic expression of PtFHT1 led to an observable resistance to salt stress (Figure 7). Unlike the reduction of ferulate incorporation in polyesters, which concomitantly altered the deposition of aliphatic lipids (Gou et al., 2009; Rautengarten et al., 2012), excessive accumulation of ferulate esters in the PtFHT1 overexpression *Arabidopsis* did not significantly disturb the incorporation of aliphatic monomers in leaf cutin and in root suberin (Figure 6). Therefore, the resistance of the transgenic seeds and seedlings to the sub-lethal salt stress are directly correlated to the changes in incorporation of aromatics in polyester polymers. Although the exact molecular mechanism in enhancing the plant's resistance to stresses remains unresolved, the incorporation of aromatics in polyesters presumably corroborates the polymer's barrier property, and therefore their impermeability. This assumption is seemingly consistent with our previous observation that deficiency of suberin aromatics in the AtHHT1/ASFT mutant lines enhanced the permeability of their roots and seed coats to ionic dye and, consequently, the seeds of the mutant lines are more sensitive to the salt treatment than the wild-type lines (Gou et al., 2009). It remains of high interest to clarify how moderate changes of minor aromatic composition without affecting aliphatic load would substantially affect the lipophilic polymers' barrier property. Aromatics in both suberin and cutin are documented to act as the structural component interconnecting aliphatics. In suberin polymer, ferulate esters hypothetically are cross-linked to each other to form a polyaromatic domain (Bernards, 2002; Franke and Schreiber, 2007; Graça and Santos, 2007), wherein the aromatic esters may covalently link the aliphatic suberin to this domain or to polysaccharides. Therefore, subtle disturbance of aromatics content or composition may considerably influence the arrangement of aliphatic monomers in polyesters and the microstructure of biopolymers, although such nanostructural changes in the polyesters may not result in the observable anatomic structural changes of polymers as reported in the electron microscopic examination (Serra et al., 2010; Molina et al., 2009). This assumption circumstantially agrees with the study of a suberin mutant *esb* line, in which the elevated load of suberin aliphatics was not associated with a reduction in either water or salt permeability (Ranathunge and Schreiber, 2011), suggesting that the amount of polyesters does not always negatively correlate with permeability; instead, the aliphatic monomer arrangement in the suberin may play a role in forming an apoplastic barrier.

In conclusion, our characterization demonstrates that the *Populus* hydroxyacid/fatty alcohol hydroxycinnamoyltransferase is responsible for both suberin and cutin ferulate formation; its overexpression substantially affects the ion permeability, which potentially provides a valuable tool to engineer plant salt stress resistance.

METHODS

Chemicals

p-Coumaroyl and feruloyl-CoA were synthesized following the method described by Stöckigt and Zenk (1975). Sinapoyl-CoA was purchased from TransMIT (Marburg, Denmark). Acetonitrile was purchased from Fisher (Pittsburgh, USA) and all the other solvents and chemicals were purchased from Sigma-Aldrich (St Louis, USA) unless otherwise stated.

Gene Expression Analysis

RNA was extracted from leaf, stem, root, apical bud, phloem, and young wood tissues of *P. trichocarpa* L. (black cottonwood) plantlets grown in a greenhouse in spring using RNeasy plant mini kit (Qiagen Sciences, Maryland) as described (Yu et al., 2009). Briefly, *P. trichocarpa* plantlets were grown in the greenhouse at Brookhaven National Laboratory. In spring, the newly developed leaves, stems (with the bark) (<3 months), and the newly developed roots were collected. Some newly developed stems were peeled to separate the

portion of bark/phloem/cambium and the xylem (young wood). Apical buds were collected from the tip of developing stems. All materials were ground in liquid N2 and their RNA was isolated immediately.

The RNA samples were treated with DNase I (New England Bio-Labs, Ipswich, MA) following the manufacture's protocol. Reverse transcription was carried out with a SuperScript III First Strand Synthesis System (Invitrogen) according to the user's manual. Real-time PCR was carried out with iQ SYBR Green Supermix (Bio-Rad), with gene-specific primers PtACT41F: 5′-TGGCTCAGGTCACCAAGTTCACAT-3′ and PtACT41R: 5′-AATCTTT GGAGGGTTCCGGGCTTT-3′ in an iCycler real-time PCR machine (Bio-Rad) in triplicate using ubiquitin primers UbiF: 5′-GTTGATTTTTGCTGGGAA GCAG-3′ and UbiR: 5′-GATCTTGGCCTTCACGTTGTCA-3′ as internal standards.

PtACT41 Gene Cloning, Recombinant Protein Expression, and Purification

The RNA samples isolated from *P. trichocarp* were treated with DNase I (New England Bio-Labs, Ipswich, MA) following the manufacture's protocol. 1 µl of reverse transcription product was used as template to amplify the PtPACT41 gene using primers PtPACT41FLF: 5′-GCGTGGATCCATGGAGTACTCTAATGGCAATGTATTT-3′ and PtPACT41FLR: 5′-GAAAGCTGGGTCTAAATCTGTATCAT TTCTTGGAAGGT-3′. The PCR product was further amplified with primers of attB1-thrombin and attB2 and cloned into pDONR207 as described by Yu and Liu (2006) and designated IIIPAT1. After confirmation by sequencing, the resulted IIIPAT1 was then subcloned into the modified gateway cloning compatible pET28(+) vector through LR reaction (Invitrogene). The construct was transferred into *E. coli* BL21 (DE3) competent cells for protein expression. The recombinant protein was produced in BL21 (DE3) by induction with 0.5 mM IPTG at 18°C for 16 h and the recombinant protein was purified as described (Gou et al., 2009).

Enzymatic Assay and Kinetics Determination

The transferase activity assays of the purified recombinant protein were performed at 40°C for 20 min in 50 µl 100 mM Tris buffer (pH 7.5) containing 50 µM *p*-coumaroyl-CoA or feruloyl-CoA, 20 µM acyl acceptor, and 1 µg purified protein. The boiled protein was used as the control. Feruloyl-CoA was used for screening acyl acceptors, and 16-hydroxypalmitic acid for testing thioester donors. The reactions were terminated by adding 50 µl acetonitrile and directly subjected to LC–MS on an Agilent 1100 system (Agilent Technologies, CA), equipped with a multi-wavelength diode array detector and electron spray ionization mass spectrometer. Samples were separated on a reverse-phase C18 column (Luna C18 (2), 5 µm; Phenomenex) at a flow of 0.8 ml min^{-1} and a gradient mobile phase as follows: 0–5 min, 15% solvent B (0.2% acetic acid in acetonitrile) in solvent A (0.2% acetic acid in water); 5–25 min, 15% to 100% B; 25–35 min, 100% B; 35–40 min, 100% to 15% B.

For determining the kinetic parameters on acyl acceptors, represented by 16-hydroxypalmitic acid, the feruloyl- and *p*-coumaroyl-CoA were fixed at 100 and 150 µM, respectively, and 1 µg purified protein was incubated with different concentrations of substrate for 10 min at 40°C. To determine the kinetics of thioester donors, 16-hydroxypalmitic acid was fixed at 100 µM and a series of concentrations of feruloyl- or *p*-coumaroyl-CoA was used in the reactions.

Phylogenetic and Sequence Analysis

Phylogenetic analysis was conducted by using *Populus* nonredundant protein full-length sequences and the function-known hydroxycinnamoyltransferase for synthesizing aromatic of cutin, suberin, and waxes from other species. Full-length amino-acid sequences were first aligned by the integrated CLUSTALW program with default parameters in the Molecular Evolutionary Genetics Analysis (MEGA) package version 5.1 (Kumar et al., 2004). Phylogenetic analyses were conducted using the neighbor-joining (NJ) method (Saitou and Nei, 1987) implemented in MEGA, with the pairwise deletion option for handling alignment gaps, and with the Poisson correction model for computing distance. The final tree graphics were generated using TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Transformation of PtACT41 Gene into *Arabidopsis*

The full-length CDS of PtACT41 in the entry clone pDONRII-IPAT1 was recombined into transgenic vector pMDC32 under 2×35S promoter, based on the gateway cloning technique (Curtis and Grossniklaus, 2003). *Agrobacterium tumefaciens* GV3101 harboring the transgenic vector was used to transform *Arabidopsis thaliana* wild-type (Col 0) through the flower-dip method (Clough and Bent, 1998). Hygromycin-resistant transgenic plants were selected on ½ MS plates. The transgenic plants were grown in a growth chamber at 22°C under a light/dark cycle of 16/8 h. The T3 generation of plants was used for analysis. Expression of the transgenics was confirmed by RT–PCR with RNA extracted from 4-week-old leaves and primers PtACT41F: 5′-TAGACAGGCCACTATCACA-3′; and PtACT41R: 5′-CCCAGATAGAACTGGCT-3′. Actin gene was amplified using primers ActinF: 5′-CGTCTTCCCCTCCATCG-3′ and ActinR: 5′-CTCGTTAATGTCACGCAC-3′ and used as an internal control.

Analysis of Suberin and Cutin Aromatic and Aliphatic Composition

Arabidopsis roots were harvested from 8-week-old plants and rinsed with tap water to remove all soil, and ground into fine powder in liquid nitrogen and sequentially extracted with 70% ethanol, acetone, then dried. The residues were digested with Driselase in 50 mM MES at 37°C for 3 d then centrifuged at 800 *g* for 10 min. The pellets were sequentially washed with 100 mM sodium tetraborate buffer (pH 9.0), water and chloroform:methanol (1:1) with agitation (300 rpm) and then air dried.

Seed samples for suberin analysis were prepared according to Molina et al. (2006) with slight modifications. 0.1 g whole seeds of *A. thaliana* were ground into fine powder in boiling isopropanol (25 ml g^{-1}) with glass homogenizer and heated for 2 h in an 80°C water bath. The residues sequentially were extracted with isopropanol, overnight, $CHCl_3$:MeOH (2:1 v/v), overnight, $CHCl_3$:MeOH (1:2 v/v), overnight, then sequentially washed with MeOH, water, 2 N NaCl, water, and MeOH with each step for 30 min; after that, the residues were then treated with $CHCl_3$:MeOH (1:2 v/v) and $CHCl_3$:MeOH (2:1 v/v), overnight. All extractions were performed in a 300 rpm shaker at room temperature and the residue was separated from the solvent by centrifugation.

Leaves of 6-week-old plants were ground to fine powder in liquid nitrogen, immersed in hot isopropanol, and extracted twice in isopropanol for 30 min. Soluble lipids were sequentially extracted with chloroform:methanol (2:1 v/v) for 2 h, chloroform:methanol (1:2 v/v) overnight, and methanol for 2 h. All steps were performed in 10-ml Teflon tubes on a rocking agitator with 5 ml of solvent. Insoluble residues were dried and used to BF3/methanol depolymerization.

To depolymerize suberin, 10 mg of the dried extract-free cell wall residues from roots, seeds, and 25 mg for leaves were incubated with 1 ml of BF3/methanol (14%) (chrysin and tetracosane were added as the internal standards for HPLC and GCMS analysis, respectively) in a screw-cap Teflon-seal tube at 70°C for 2 h. Then, 2 ml water was added and the hydrolysates were extracted three times with 3 ml of $CHCl_3$. The combined solvent extracts were separated into two portions and dried under a stream of nitrogen. One portion was dissolved in 100 µl methanol for LC–MS analysis with a gradient of solvent B (0.2% acetic acid in acetonitrile) in solvent A (0.2% acetic acid in water) as follows: 0–2 min, 15%; 2–15 min, 50%; 15–35 min, 80%; 35–38 min, 100%; 38–41 min, 100% B; 41–46 min, 5%; at a linear flow rate of 0.8 ml min^{-1} in a reverse-phase C18 column (Luna C18 (2), 5 µm; Phenomenex).

The rest of hydrolysates were derivatized with 50 µl of pyridine and 50 µl MSTFA at 90°C for 40 min. The solvent was dried under a stream of nitrogen and then dissolved in hexane before injecting 1 µl into the GC–MS on a DB-5ms capillary column (Agilent), with the settings detailed by Franke et al. (2005).

To quantify the content of the suberin phenolics, the UV-absorptive area of particular peak from each sample was first normalized with that of the internal standard, then calibrated with the standard curve of ferulate, or *p*-coumarate established in the same HPLC running using a series of concentrations of authentic chemicals.

Salt Stress Treatment

T3 transgenic seeds were sterilized with 70% (v/v) ethanol and 5% NaClO (v/v), followed by several rinses with sterile distilled water. About 100 seeds of each transgenic line and wild-type controls were placed on the MS agar medium (0.9%) supplemented with different concentrations of NaCl (at 75, 100, 125, 150, or 200 mM) and maintained at 4°C in dark for 3 d, and then the plates were transferred to a growth chamber at 22°C with 16/8-h light and dark regime. The percentage of seed germination (i.e. the root punching out seeds) and the percentage of seedling establishment (i.e. seedlings with two green cotyledons) were calculated after 14-d growth. The root length of the vertically grown seedlings on the MS agar medium (0.9%) supplemented with different concentrations of NaCl (75, 100, 125, or 150 mM) was measured after 14-d growth.

Accession Number

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: JX515962 for the identified *P. trichocarpa* hydroxyacid/fatty alcohol hydroxycinnamoyltransferase; At5g41040 for AtHHT1/ASFT, At5g63560 for FACT, At3G48720 for AtCFT, and ACS70946 for *Solanum tuberosum* FHT.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the US Department of Energy (DOE) (grant no. DEAC0298CH10886 to C.J.L.); the transgenic *Arabidopsis* analyses were also partially supported by National Science Foundation (grant no. MCB-1051675) and by the oversea collaborative project of National Science Foundation of China (grant no. 31028003).

ACKNOWLEDGMENTS

We thank Fang Chen in the Samuel Roberts Noble Foundation for discussion and guidance on synthesis of CoA thioesters. No conflict of interest declared.

REFERENCES

Beisson, F., Li-Beisson, Y., and Pollard, M. (2012). Solving the puzzles of cutin and suberin polymer biosynthesis. Curr. Opin. Plant Biol. **15**, 329–337.

Bernards, M.A. (2002). Demystifying suberin. Can. J. Bot. **80**, 227–240.

Bernards, M.A., and Lewis, N.G. (1998). The macromolecular aromatic domain in suberized tissue: a changing paradigm. Phytochemistry. **47**, 915–933.

Bernards, M.A., and Razem, F.A. (2001). The poly(phenolic) domain of potato suberin: a non-lignin cell wall bio-polymer. Phytochemistry. **57**, 1115–1122.

Bernards, M.A., Lopez, M.L., Zajicek, J., and Lewis, N.G. (1995). Hydroxycinnamic acid-derived polymers constitute the polyaromatic domain of suberin. J. Biol. Chem. **270**, 7382–7386.

Bonaventure, G., Beisson, F., Ohlrogge, J., and Pollard, M. (2004). Analysis of the aliphatic monomer composition of polyesters associated with *Arabidopsis* epidermis: occurrence of octadeca-*cis*-6, *cis*-9-diene-1,18-dioate as the major component. Plant J. **40**, 920–930.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**, 735–743.

Cottle, W., and Kolattukudy, P.E. (1982a). Abscisic acid stimulation of suberization induction of enzymes and deposition of polymeric components and associated waxes in tissue cultures of potato tuber. Plant Physiol. **70**, 775–780.

Cottle, W., and Kolattukudy, P.E. (1982b). Biosynthesis, deposition, and partial characterization of potato suberin phenolics. Plant Physiol. **69**, 393–399.

Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. Plant Physiol. **133**, 462–469.

Degenhardt, B., and Gimmler, H. (2000). Cell wall adaptations to multiple environmental stresses in maize roots. J. Exp. Bot. **51**, 595–603.

Espelie, K.E., and Kolattukudy, P.E. (1979). Composition of the aliphatic components of suberin of the endodermal fraction from the first internode of etiolated Sorghum seedlings. Plant Physiol. **63**, 433–435.

Franke, R., and Schreiber, L. (2007). Suberin—a biopolyester forming apoplastic plant interfaces. Curr. Opin. Plant Biol. **10**, 252–259.

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

Gou, J.Y., Yu, X.H., and Liu, C.J. (2009). A hydroxycinnamoyltransferase responsible for synthesizing suberin aromatics in *Arabidopsis*. Proc. Natl Acad. Sci. U S A. **106**, 18855–18860.

Graça, J., and Lamosa, P. (2010). Linear and branched poly(omega-hydroxyacid) esters in plant cutins. J. Agric. Food Chem. **58**, 9666–9674.

Graça, J., and Pereira, H. (2000). Suberin structure in potato periderm: glycerol, long-chain monomers, and glyceryl and feruloyl dimers. J. Agric. Food Chem. **48**, 5476–5483.

Graça, J., and Santos, S. (2007). Suberin: a biopolyester of plants' skin. Macromol. Biosci. **7**, 128–135.

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

Kolattukudy, P.E. (1980). Biopolyester membranes of plants: cutin and suberin. Science. **208**, 990–1000.

Kolattukudy, P.E. (1981). Structure, biosynthesis, and biodegradation of cutin and suberin. Annu. Rev. Plant Physiol. **32**, 539–567.

Kolattukudy, P.E. (2001). Polyesters in higher plants. Adv. Biochem. Eng. Biotechnol. **71**, 1–49.

Kolattukudy, P.E., and Dean, B.B. (1974). Structure, gas chromatographic measurement, and function of suberin synthesized by potato tuber tissue slices. Plant Physiol. **54**, 116–121.

Kosma, D.K., Bourdenx, B., Bernard, A., Parsons, E.P., Lü, S., Joubès, J., Jenks, M.A. (2009). The impact of water deficiency on leaf cuticle lipids of *Arabidopsis*. Plant Physiol. **151**, 1918–1929.

Kosma, D.K., Molina, I., Ohlrogge, J.B., and Pollard, M. (2012). Identification of an *Arabidopsis* fatty alcohol:caffeoyl-coenzyme A acyltransferase required for the synthesis of alkyl hydroxycinnamates in root waxes. Plant Physiol., **160**, 237–248.

Kumar, S., Tamura, K., and Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment briefings. Bioinformatics. **5**, 150–163.

Liu, C.J. (2010). Biosynthesis of hydroxycinnamate conjugates: implications for sustainable biomass and biofuel production. Biofuels. **1**, 745–761.

Lotfy, S., Javelle, F., and Negrel, J. (1995). Distribution of hydroxycinnamoyl-CoA:omega-hydroxypalmitic acid *O*-hydroxycinnamoyltransferase in higher plants. Phytochemistry. **40**, 389–391.

Lotfy, S., Javelle, F., and Negrel, J. (1996). Purification and characterization of hydroxycinnamoyl-Coenzyme A:omega-hydroxypalmitic acid *O*-hydroxycinnamoyltransferase from tobacco (*Nicotiana tabacum* L.) cell-suspension cultures. Planta. **199**, 475–480.

Lotfy, S., Negrel, J., and Javelle, F. (1994). Formation of ω-feruloyloxypalmitic acid by an enzyme from wound-healing potato tuber discs. Phytochemistry. **35**, 1419–1424.

Lu, S., Zhao, H., Des Marais, D.L., Parsons, E.P., Wen, X., Xu, X., Bangarusamy, D.K., Wang, G., Rowland, O., Juenger, T., et al. (2012). *Arabidopsis* ECERIFERUM9 involvement in cuticle formation and maintenance of plant water status. Plant Physiol. **159**, 930–944.

Lulai, E.C., and Suttle, J.C. (2009). Signals involved in tuber wound-healing. Plant Signal. Behav. **4**, 620–622.

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

Molina, I., Li-Beisson, Y., Beisson, F., Ohlrogge, J.B., and Pollard, M. (2009). Identification of an *Arabidopsis* feruloyl-coenzyme A transferase required for suberin synthesis. Plant Physiol. **151**, 1317–1328.

Molina, I., Ohlrogge, J.B., and Pollard, M. (2008). Deposition and localization of lipid polyester in developing seeds of *Brassica napus* and *Arabidopsis thaliana*. Plant J. **53**, 437–449.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**, 473–497.

Panikashvili, D., Shi, J.X., Schreiber, L., and Aharoni, A. (2009). The *Arabidopsis* DCR encoding a soluble BAHD acyltransferase is required for cutin polyester formation and seed hydration properties. Plant Physiol. **151**, 1773–1789.

Pollard, M., Beisson, F., Li, Y., and Ohlrogge, J.B. (2008). Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci. **13**, 236–246.

Ranathunge, K., and Schreiber, L. (2011). Water and solute permeabilities of *Arabidopsis* roots in relation to the amount and composition of aliphatic suberin. J. Exp. Bot. **62**, 1961–1974.

Rautengarten, C., Ebert, B., Ouellet, M., Nafisi, M., Baidoo, E.E., Benke, P., Stranne, M., Mukhopadhyay, A., Keasling, J.D., Sakuragi, Y., et al. (2012). *Arabidopsis* deficient in cutin ferulate encodes a transferase required for feruloylation of ω-hydroxy fatty acids in cutin polyester. Plant Physiol. **158**, 654–665.

Reina-Pinto, J.J., and Yephremov, A. (2009). Surface lipids and plant defenses. Plant Physiol. Biochem. **47**, 540–549.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**, 406–425.

Schreiber, L. (2011). Transport barriers made of cutin, suberin and associated waxes. Trends Plant Sci. **15**, 546–553.

Schreiber, L., Franke, R., and Hartmann, K. (2005a). Wax and suberin development of native and wound periderm of potato (*Solanum tuberosum* L.) and its relation to peridermal transpiration. Planta. **220**, 520–530.

Schreiber, L., Franke, R., Hartmann, K.D., Ranathunge, K., and Steudle, E. (2005b). The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix). J. Exp. Bot. **56**, 1427–1436.

Serra, O., Figueras, M., Franke, R., Prat, S., and Molinas, M. (2011). Unraveling ferulate role in suberin and periderm biology by reverse genetics. Plant Signal Behav. **5**, 953–958.

Serra, O., Hohn, C., Franke, R., Prat, S., Molinas, M., and Figueras, M. (2010). A feruloyl transferase involved in the biosynthesis of suberin and suberin-associated wax is required for maturation and sealing properties of potato periderm. Plant J. **62**, 277–290.

Serra, O., Soler, M., Hohn, C., Sauveplane, V., Pinot, F., Franke, R., Schreiber, L., Prat, S., Molinas, M., and Figueras, M. (2009). CYP86A33-targeted gene silencing in potato tuber alters suberin composition, distorts suberin lamellae, and impairs the periderm's water barrier function. Plant Physiol. **149**, 1050–1060.

Shi, R., Sun, Y.H., Li, Q., Heber, S., Sederoff, R., and Chiang, V.L. (2010). Towards a systems approach for lignin biosynthesis in Populus *trichocarpa*: transcript abundance and specificity of the monolignol biosynthetic genes. Plant Cell Physiol. **51**, 144–163.

Stöckigt, J., and Zenk, M.H. (1975). Chemical syntheses and properties of hydroxycinnamoyl-coenzyme A derivatives. Z. Naturforschung. **30C**, 352–358.

Tanaka, T., Tanaka, H., Machida, C., Watanabe, M., and Machida, Y. (2004). A new method for rapid visualization of defects in leaf cuticle reveals five intrinsic patterns of surface defects in *Arabidopsis*. Plant J. **37**, 139–146.

Yang, J., Isabel Ordiz, M., Jaworski, J.G., and Beachy, R.N. (2011). Induced accumulation of cuticular waxes enhances drought tolerance in *Arabidopsis* by changes in development of stomata. Plant Physiol. Biochem. **49**, 1448–1455.

Yeats, T.H., Martin, L.B., Viart, H.M., Isaacson, T., He, Y., Zhao, L., Matas, A.J., Buda, G.J., Domozych, D.S., Clausen, M.H., et al. (2012). The identification of cutin synthase: formation of the plant polyester cutin. Nat. Chem. Biol. **8**, 609–611.

Yu, X.-H., and Liu, C.-J. (2006). Development of an analytical method for genome-wide functional identification of plant acyl-CoA dependent acyltransferases. Anal. Biochem. **358**, 146–148.

Yu, X.-H., Gou, J.-Y., and Liu, C.J. (2009). BAHD superfamily of acyl-CoA dependent acyltransferases in *Populus* and *Arabidopsis*: bioinformatics and gene expression. Plant Mol. Biol. **70**, 421–442.