The Acyltransferase GPAT5 Is Required for the Synthesis of Suberin in Seed Coat and Root of Arabidopsis

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The Acyltransferase GPAT5 Is Required for the Synthesis of Suberin in Seed Coat and Root of *Arabidopsis* [W][OA]

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Suberin and cutin are fatty acid– and glycerol-based plant polymers that act as pathogen barriers and function in the control of water and solute transport. However, despite important physiological roles, their biosynthetic pathways, including the acyl transfer reactions, remain hypothetical. We report the characterization of two suberin mutants (*gpat5-1* and *gpat5-2*) of *Arabidopsis thaliana GPAT5*, encoding a protein with acyl-CoA:glycerol-3-phosphate acyltransferase activity. RT-PCR and β-glucuronidase–promoter fusion analyses demonstrated *GPAT5* expression in seed coat, root, hypocotyl, and anther. The *gpat5* plants showed a 50% decrease in aliphatic suberin in young roots and produced seed coats with a severalfold reduction in very long chain dicarboxylic acid and ω-hydroxy fatty acids typical of suberin but no change in the composition or content of membrane or storage glycerolipids or surface waxes. Consistent with their altered suberin, seed coats of *gpat5* mutants had a steep increase in permeability to tetrazolium salts compared with wild-type seed coats. Furthermore, the germination rate of *gpat5* seeds under high salt was reduced, and *gpat5* seedlings had lower tolerance to salt stress. These results provide evidence for a critical role of GPAT5 in polyester biogenesis in seed coats and roots and for the importance of lipid polymer structures in the normal function of these organs.

INTRODUCTION

Cutin and suberin are the two major types of lipid polyesters found in plants. They are both fatty acid– and glycerol-based extracellular polymers that are insoluble in water and organic solvents. Although cutin is found mostly at the surface of the epidermis and constitutes the polymer matrix of the hydrophobic cuticle that covers higher plants, suberin is deposited in various inner and outer tissues at specific locations during plant growth (Kolattukudy, 2001; Bernards, 2002; Nawrath, 2002; Kunst et al., 2005; Stark and Tian, 2006). For example, suberin has been chemically identified in seed coat (Espelie et al., 1980; Ryser and Holloway, 1985; Moire et al., 1999), root and stem endodermis (Espelie and Kolattukudy, 1979a; Zeier et al., 1999; Enstone et al., 2003), bundle sheath of monocots (Espelie and Kolattukudy, 1979b; Griffith et al., 1985), and conifer needles (Wu et al., 2003). In addition to this widespread deposition, suberin is also synthesized in response to stress and wounds (Dean and Kolattukudy, 1976). Regarding the subcellular location, cutin abuts the outer face of cell walls, whereas suberin is located within cell walls and also is found between the plasma membrane and the cell wall inner face to which it is attached (Bernards, 2002). Suberin is also distinguished from cutin by its very substantial hydroxycinnamic acid–derived aromatic domain (Bernards et al., 1995; Bernards, 2002) and the longer chain length (>20 carbons) of its fatty alcohol/acid and dicarboxylic acid monomers (Kolattukudy, 2001). The effect of the deposition of suberin and associated waxes is to reduce water and solute transport across and within suberized cell walls (Soliday et al., 1979; Vogt et al., 1983) as well as to provide a barrier to pathogens (Lulai and Corsini, 1998). A well-known example of suberin deposition is the root Casparian bands (Schreiber et al., 1994), which are involved in reducing the flow of water and ions through the apoplast, allowing the root endodermis to control water and ion uptake (Sattelmacher, 2001; Enstone et al., 2003; Ma and Peterson, 2003). Despite differences in subcellular and tissue localization as well as monomer composition, both the cutin-based epidermal cuticle and the suberin-based depositions of cell walls share a similar basic function as resistors/barriers for water, solutes, and pathogens.

Glycerol has been reported to be covalently bound to aliphatic and aromatic suberin domains (Moire et al., 1999; Graça and Pereira, 2000a) and to be esterified to fatty acid monomers in cutin (Graça et al., 2002) and suberin (Graça and Pereira, 2000b). These findings are structurally important because the aliphatic monomers of suberin can by themselves only form linear polyesters, whereas the presence of glycerol allows the formation of a three-dimensional network or cross-linked polymer. Also, the presence of glycerol has begun to shed a different light on the pathways of synthesis, transport, and the assembly of polyester monomers.

The recent successes in the analysis of *Arabidopsis thaliana* polyester monomers (Bonaventure et al., 2004; Xiao et al., 2004; Franke et al., 2005) have opened the way to identifying *Arabidopsis* mutants affected in polyesters. The first mutant demonstrated to be specifically affected in polyester monomer synthesis was *att1*, in which a gene encoding a cytochrome P450

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monooxygenase is altered (Xiao et al., 2004). The involvement of another type of oxidase (Kurdyukov et al., 2006b) and a hydrolase (Kurdyukov et al., 2006a) in cutin metabolism was proposed recently. The *fatB* and *fad2* mutants are affected in polyester composition but are mutants of general fatty acid metabolism (Bonaventure et al., 2004). Other cuticle mutants have not been analyzed for polyester composition, but some are almost certainly (e.g., *lacerata* [Wellesen et al., 2001] and *lacs2* [Schnurr et al., 2004]) or likely (e.g., *wax2* [Chen et al., 2003]) affected in cutin. Mutants such as *elongation defective1* (Cheng et al., 2000) and *scarecrow* (Di Laurenzio et al., 1996) show unusual suberin deposition but are not known to be affected in suberin metabolism. To date, no gene has been identified or characterized as involved in the acyl transfer reactions required to assemble a polyester network.

The identification of glycerol as an important monomer, together with evidence for acyl-CoA incorporation into cutin of broad bean (*Vicia faba*) (Croteau and Kolattukudy, 1974), suggest that acyl-CoA–dependent glycerol acyltransferases may be a type of acyltransferase involved in polyester synthesis. In *Arabidopsis*, at least 30 such potential acyltransferases have been identified through sequence similarity and conserved motif searches (Beisson et al., 2003; The Arabidopsis Lipid Gene Database, http://www.plantbiology.msu.edu/lipids/genesurvey/front_page.htm), but only a few of them have been characterized and matched to major acyltransfer reactions (Nishida et al., 1993; Routaboul et al., 1999; Zou et al., 1999; Zheng et al., 2003; Kim and Huang, 2004; Yu et al., 2004; Kim et al., 2005). Knowledge of the specific functions of each of these might be useful in unraveling the complex movements and fates of acyl chains in plant cells and to help determine whether some are involved in the synthesis and transport of surface lipids. In animals, acyltransferases involved in extracellular lipid synthesis were identified recently. A multifunctional *O*-acyltransferase from skin that synthesizes in vitro acylglycerols, waxes, and retinyl esters (Yen et al., 2005) and a lysophosphatidylcholine acyltransferase from lung thought to be involved in the synthesis of pulmonary surfactant (Chen et al., 2006; Nakanishi et al., 2006) were recently described.

In this study, we report the identification and characterization of *Arabidopsis* knockout mutants for the glycerol-3-phosphate acyltransferase5 gene (*GPAT5*; At3g11430), which belongs to a plant-specific family. We show that the mutants *gpat5-1* and *gpat5-2* are affected in polyester synthesis in seed coats and roots. Several phenotypic characteristics of the *gpat5* mutants, such as enhanced seed coat permeability, decreased seed germination, and abnormal root growth under salt stress conditions, suggest that GPAT5 plays a critical role in suberized cell wall biogenesis in seeds and roots and that this structure is required for normal seed and root function.

RESULTS

Identification of T-DNA Insertional Mutants and *GPAT5* Expression in Organs

Two independent T-DNA insertion lines, SALK_018117 and SALK_142456 (Alonso et al., 2003), were selected and screened for disruption of the *GPAT5* gene using PCR. As shown in Figure 1A, SALK_018117 has a T-DNA insertion in the first exon, whereas SALK_142456 has a T-DNA inserted in the only intron of *GPAT5*. Plants homozygous for the T-DNA insertion in *GPAT5* that were obtained for the independent insertion lines SALK_018117 and SALK_142456 were named *gpat5-1* and *gpat5-2*, respectively. When *GPAT5* transcript was checked by RT-PCR in flowers from homozygous *gpat5-1* and *gpat5-2* plants, it could not be detected (Figure 1B), confirming that both T-DNA insertion lines generated a complete knockout of the *GPAT5* gene. The phenotypes described in this article have been observed in both *gpat5-1* and *gpat5-2* independent alleles and therefore can be attributed to the disruption of the *GPAT5* gene.

RT-PCR analysis showed that in wild-type plants, *GPAT5* mRNA was detected in flowers, roots, and seeds but not in stems and rosette leaves (Figure 1C). To confirm these results and to more precisely identify the tissue specificity of GPAT5 expression, a fragment consisting of 1 kb upstream of the first ATG of the *GPAT5* cDNA together with the first exon (588 bp) of the gene was used to drive the expression of the β-glucuronidase (GUS) reporter gene. Consistent with the RT-PCR analysis, histochemical staining of transgenic plants expressing Pro_{GPAT5}*:GUS* (Figure 2) indicated *GPAT5* expression in roots, flowers, and seeds but not in leaves and stems (Figures 2E and 2I). Dissection of GUS-stained

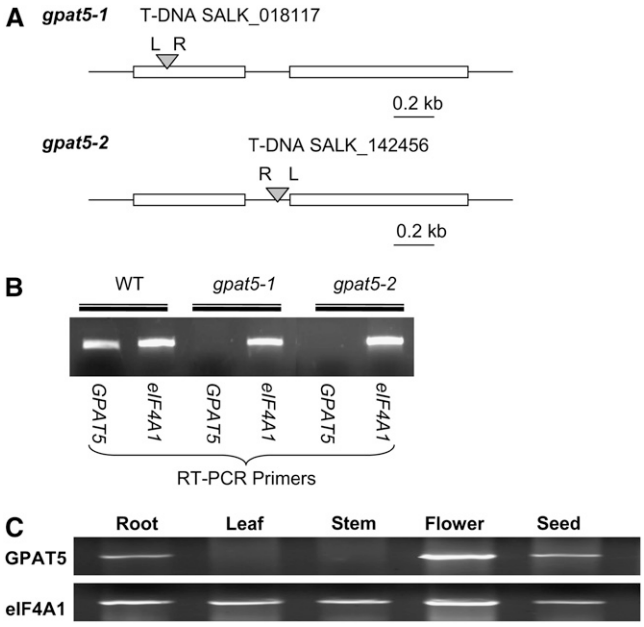

Figure 1. Structure of the *GPAT5* Gene Carrying a T-DNA Insertion, and Analysis of *GPAT5* Expression by RT-PCR.

(A) Genomic organization of the *gpat5-1* and *gpat5-2* loci. Boxes represent exons. The T-DNA insertion point is indicated as a triangle, with L and R indicating left and right borders, respectively.

(B) RT-PCR analysis of the *GPAT5* transcript in wild-type and mutant (*gpat5-1* and *gpat5-2*) flowers. Approximately 0.1 μg of total RNA was used in each PCR, and eIF4A-1 (At3g13920) was used as a control.

(C) RT-PCR analysis of *GPAT5* expression in roots, rosette leaves, stems, open flowers, and developing seeds. Approximately 0.1 μg of total RNA was used in each PCR, and eIF4A-1 (At3g13920) was used as a control.

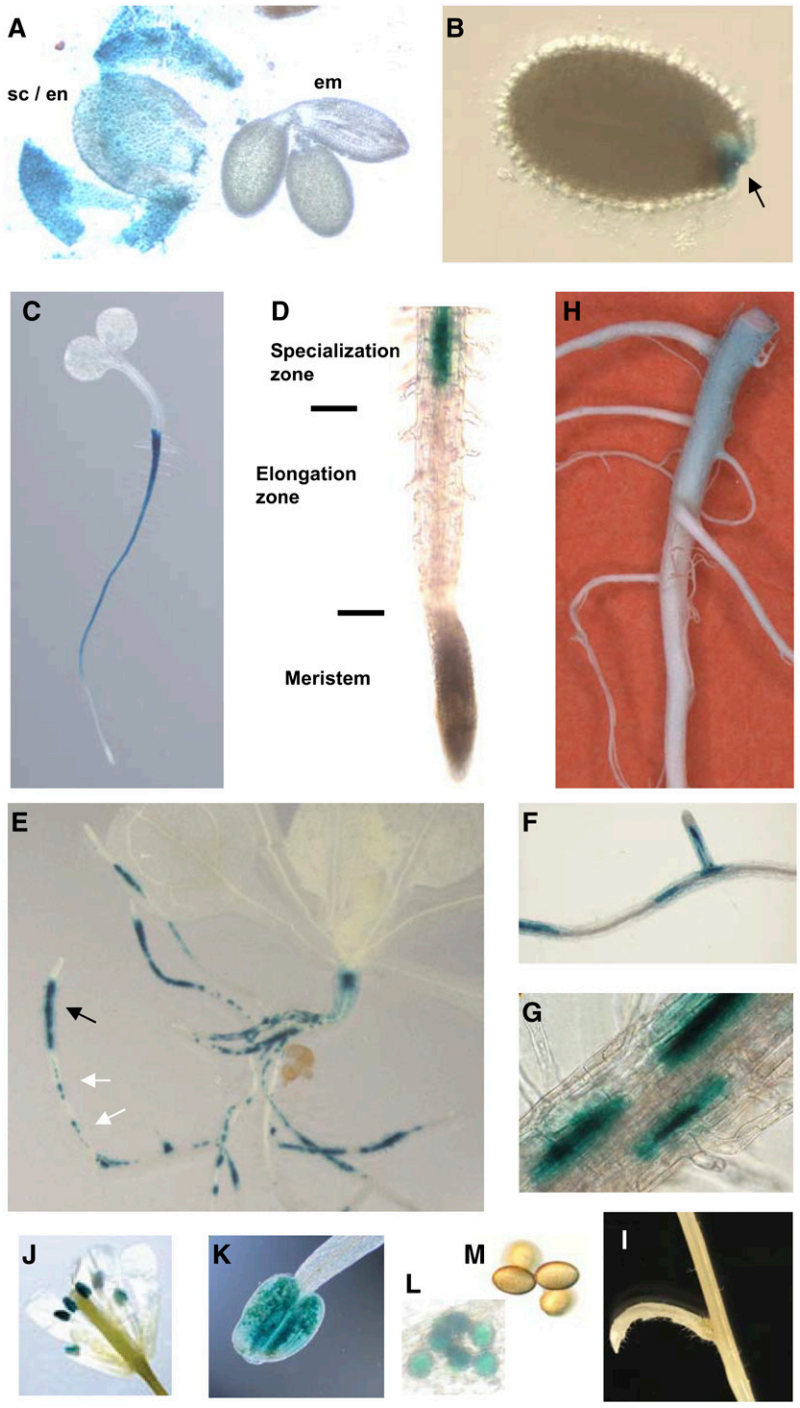

Figure 2. Analysis of *GPAT5* Expression in *Arabidopsis* Wild-Type Plants by Pro_{GPAT5}:*GUS*.

(A) and **(B)** Crushed GUS-stained seeds show that staining is limited to the seed coat/endosperm. The beginning **(A)** and end **(B)** stages of seed desiccation are shown. In **(B)**, staining is limited to the funiculus attachment region only (arrow). em, embryo; en, endosperm; sc, seed coat.
(C) GUS staining in a 4-d-old seedling grown on agar.
(D) GUS staining is detected in the specialization zone but not in the meristem and elongation zone of the root in a 7-d-old seedling.
(E) GUS staining of a 3-week-old seedling. For arrows, see text.
(F) GUS staining in a seminal root and a first-order lateral root of a 3-week-old seedling.
(G) Patchy GUS staining is often observed in roots of 1- to 4-week-old seedlings.
(H) GUS staining is faint in roots of 6-week-old plants on soil and is restricted to the older parts.

developing seeds showed that GUS expression was detectable at the desiccation stage and only in the endosperm/seed coat fraction, not in the embryo (Figures 2A and 2B). At the beginning of the seed desiccation stage, *GPAT5* expression was observed uniformly throughout the seed coat/endosperm fraction (Figure 2A), whereas at the end of the desiccation stage (Figure 2B), GUS staining was greater in the funiculus attachment region and possibly in some endosperm cells of this seed end.

In roots, the GUS staining pattern also varied with development. In the seminal roots of 4- to 7-d-old seedlings grown on agar (Figure 2C), staining was strongest at the junction of roots and hypocotyls, extended all along the differentiated (specialization) zone where suberin deposition is known to occur, but was never present in the elongation zone or the root apical meristem (Figure 2D). In 1- to 4-week-old roots grown on agar, which can still be elongating but whose older parts have entered the secondary state of growth (Dolan and Roberts, 1995; Baum et al., 2002), GUS staining was still present above (but never in) the division/elongation zones of the seminal root and the lateral roots (Figure 2E, black arrow), consistent with the staining in 4-d-old roots. Additional staining was seen in some older parts of the roots (Figure 2E, white arrows) and in hypocotyls (Figure 2E). Junctions to first and second order lateral roots were also almost always stained (Figure 2F). Closer examination of 1- to 4-week-old roots revealed that the additional staining along older parts of seminal and lateral roots was often made of small patches (Figure 2G). In 6-week-old roots, which were grown on soil and clearly in their secondary state of growth, GUS staining was much weaker and observed mostly at the periphery of older parts of the roots (Figure 2H). GUS staining in 1- to 4-week-old roots of control Pro_{35S}*:GUS* plants was not patchy but, as expected, was strong and present all over the root.

In flowers, strong staining of GUS was observed in stamens but not in sepals, petals, and carpels (Figure 2J). Microscopic examination of GUS-stained flowers showed that GUS activity was detected only in the anthers and not in the filaments (Figure 2K). Dissection of anthers showed that GUS staining was present in the developing pollen (Figure 2L). Mature pollen did not show any staining (Figure 2M).

These GUS and RT-PCR expression data are in agreement with AtGenExpress microarray data (Schmid et al., 2005) showing that *GPAT5* mRNA is only significantly expressed in hypocotyls, roots, seeds, and stamens.

gpat5 Mutants Are Affected in the Composition and Amount of Lipid Polyesters but Not in Membrane and Storage Lipids

Homozygous *gpat5-1* and *gpat5-2* mutant plants were morphologically identical throughout development and reached similar sizes compared with wild-type plants. No significant differences in root growth were observed when seeds were germinated on vertical agar plates. The fertility of *gpat5* mutants was not affected (the number of seeds per silique was ~50, similar to that in the wild type). No differences in pollen grain size and shape between the wild type and *gpat5* were observed under scanning electron microscopy. GPAT5 has been shown to have glycerol-3-phosphate acyltransferase activity in vitro (Zheng et al., 2003). Thus, to evaluate whether *gpat5* mutants carried a biochemical defect in some lipids, we analyzed the fatty acids of lipid compounds in organs in which GPAT5 is expressed (seed, root, flower) and, as a control, in leaves. Analyses were performed on intracellular lipids extractable in organic solvents (membrane and storage lipids) and also on lipid polymers, which are nonextractable in organic solvents.

No differences in the amount of fatty acids derived from soluble lipids were detected in seeds, roots, and leaves of the mutants compared with the wild type (see Supplemental Figure 1 online). When soluble lipids of the manually dissected seed coat/endosperm fraction of mature seeds were analyzed, the amount of total fatty acids and the fatty acid composition were found to be the same in the wild type and *gpat5* (Figure 3). The soluble lipids collected at the seed surface by chloroform dipping were typical *Arabidopsis* stem/leaf wax components, including alkane and fatty alcohols (see Supplemental Figure 2 online). The major component identified in seed coats was 29-carbon alkane, which is also the major wax component of *Arabidopsis* stems. Expressed per seed surface area, the wax coverage was ~0.3 $\mu g/cm^2$ for both the wild type and mutants. In contrast with soluble lipids, aliphatic monomers released from insoluble lipid polyesters were on average reduced by twofold in *gpat5* seeds (Table 1). More than 90% of the total insoluble aliphatic polyester monomers found in seeds have been shown to come from the seed coat/endosperm fraction (Molina et al., 2006). The major aliphatic monomer (24:0 ω-hydroxy fatty acid), as well as 22:0 fatty acid, 22:0 ω-hydroxy fatty acid, and 22:0/24:0 dicarboxylic acids, were reduced by at least twofold in the mutants compared with the wild type (Figure 4A). All of these strongly reduced monomers were typical monomers of aliphatic suberin (Kolattukudy, 2001). In contrast with the aliphatic monomers, the amount of aromatic monomers released from the polyester by depolymerization was not different between mutants and the wild type. However, depolymerization by transesterification presumably underestimates total hydroxycinnamate derivatives, which may be cross-linked by nonester bonds (Bernards et al., 1995).

Similar to seed coats, roots of 1-week-old seedlings grown on agar (primary state of growth), showed significant changes in the aliphatic monomers of suberin in *gpat5* compared with the wild type (Figure 4B). As with seeds, the change was not the same for

Figure 2. (continued).

(I) GUS staining is not observed in stems and cauline leaves.
(J) GUS staining in flowers is restricted to the stamen.
(K) GUS staining is observed in anthers but not in filaments of the stamen.
(L) GUS staining is observed in developing pollen.
(M) GUS staining is not observed in mature pollen.

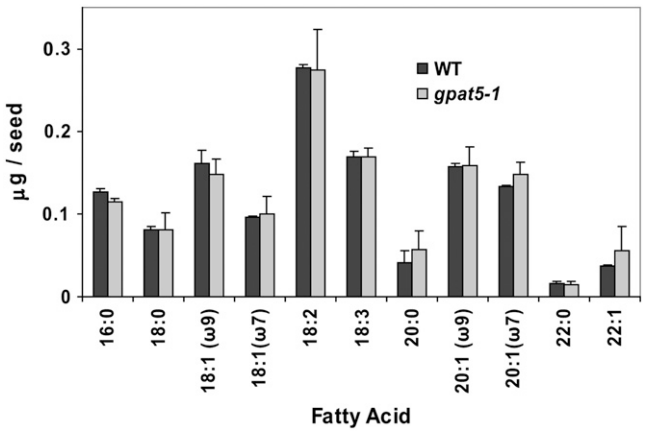

Figure 3. Fatty Acids from the Seed Coat/Endosperm Fraction of the Wild Type and *gpat5* Mutants.

Mature seeds were manually dissected, and total fatty acids of the membrane and storage lipids of the seed coat/endosperm fraction were analyzed as fatty acid methyl esters by gas chromatography. Values are means of six replicates. Error bars denote 95% confidence intervals.

all monomers. A 20 to 50% reduction was observed in C20-C24 fatty acid derivatives, whereas the C16-C18 fatty acid derivatives remained constant or increased. In 3-week-old roots grown on agar (beginning of the secondary state of growth), the suberin composition was different and the mutants showed a global 50% decrease in some monomers, including the 22:0 fatty acid and the major 18:1 ω-hydroxy fatty acid (see Supplemental Figure 3 online). The reduction in total aliphatic suberin monomers in 3-week-old roots was also shown by the reduced staining intensity of some parts of the *gpat5* roots when stained with Sudan black B (Figure 5), a lipophilic dye used for suberin histochemical detection (Robb et al., 1991). In older wild-type roots grown on soil, in which the *GPAT5* gene is weakly expressed (6 weeks old, secondary state of growth), the composition of suberin aliphatic monomers detected was similar to that reported previously (Franke et al., 2005), and no significant difference in the amount of each monomer was found between the wild type and the mutants.

In flowers (Figure 4C), a decrease in some lipid polyester monomers was also detected (e.g., 22:0 fatty acid and 18:2 ω-hydroxy fatty acid) but was globally less strong than in roots and seeds, consistent with the fact that the gene is expressed only in anthers. As expected, no difference in the lipid polyester monomers of leaves was detected (see Supplemental Figure 4 online).

Together, these results show that the *gpat5* mutants are altered (both quantitatively and qualitatively) in the synthesis of insoluble lipid polyesters but are not impaired in the synthesis of the bulk of storage and membrane lipids. A specific role of a GPAT family member in lipid polyester synthesis is strongly supported by other observations, including the fact that two other GPATs are required for lipid polyester synthesis in leaves (see Discussion).

Seed Coats of *gpat5* Mutants Show Increased Permeability to Dyes and Darker Color

The mutant plants produced seeds of the same weight as wild-type plants (17.0 ± 0.7 compared with 16.9 ± 0.1 μg/seed). Closer examination of the seeds via scanning electron microscopy showed that the mutant seeds are in the same range of size, have the same oblong shape, and show the same surface structure, with similar columella heaps, as wild-type seeds (Figure 6A). Mucilage production of the mutant seeds is also the same as that of wild-type seeds, as demonstrated via staining with ruthenium red (Figure 6B).

Permeability properties of the seed coat of the mutants were tested using tetrazolium red, a cationic dye that is normally excluded by the *Arabidopsis* seed coat but that is reduced to red products (formazans) by NADPH-dependent reductases after penetrating the embryo (Debeaujon et al., 2000). As shown in Figures 6C and 6D, after staining for 24 h, *gpat5-1* and *gpat5-2* seed coats were much more permeable to tetrazolium red than were wild-type seed coats, suggesting that the seed coat is indeed affected in the mutants. Shorter incubation times with tetrazolium red showed that the staining first appeared in the region of the hilum and diffused outward from there (Figure 6E). Control staining experiments run on embryos whose seed coats had been manually removed showed that wild-type and mutant embryos had the same kinetics and intensity of staining and that the red products appeared at the same time in all parts of the embryo surface. Therefore, these controls ruled out a possible difference in the capacity to metabolize tetrazolium red between mutant and wild-type embryos or between the parts of the embryo close to the hilum region and other parts. Differences seen in Figures 6C and 6D could be attributed to a difference in the permeability of wild-type and mutant seed coats to tetrazolium red, especially in the hilum region. The hilum is the scar left on the seed coat after detachment from the funiculus. In mature seeds of *Arabidopsis*, it is adjacent to the micropyle (where the radicle will emerge) and faces the chalazal pole. When excited at 365 nm (Figure 6F), seed coats of the mutant revealed a decrease in autofluorescence in the hilum region (arrows), suggesting a decrease in suberin content. Furthermore, the seed coat surface of *gpat5* mutants was clearly less stained in the hilum region (Figure 6G, arrows) when using the lipophilic suberin dye Sudan

Table 1. Quantification of Waxes, Other Soluble Lipids, and Polyesters in Wild-Type and *gpat5-1* Seeds

Seed	Seed Weight (μg/Seed)	Fatty Acid Methyl Esters from Extractable Lipids (ng/Seed)	Surface Waxes (ng/Seed)	Aliphatic Polyester Monomers (ng/Seed)
Wild type	16.9 ± 0.1	5230 ± 80	1.3 ± 0.05	68.9 ± 5.1
gpat5-1	17.0 ± 0.7	4910 ± 370	1.4 ± 0.01	35.2 ± 1.2

Values shown are means ± SE from two independent experiments.

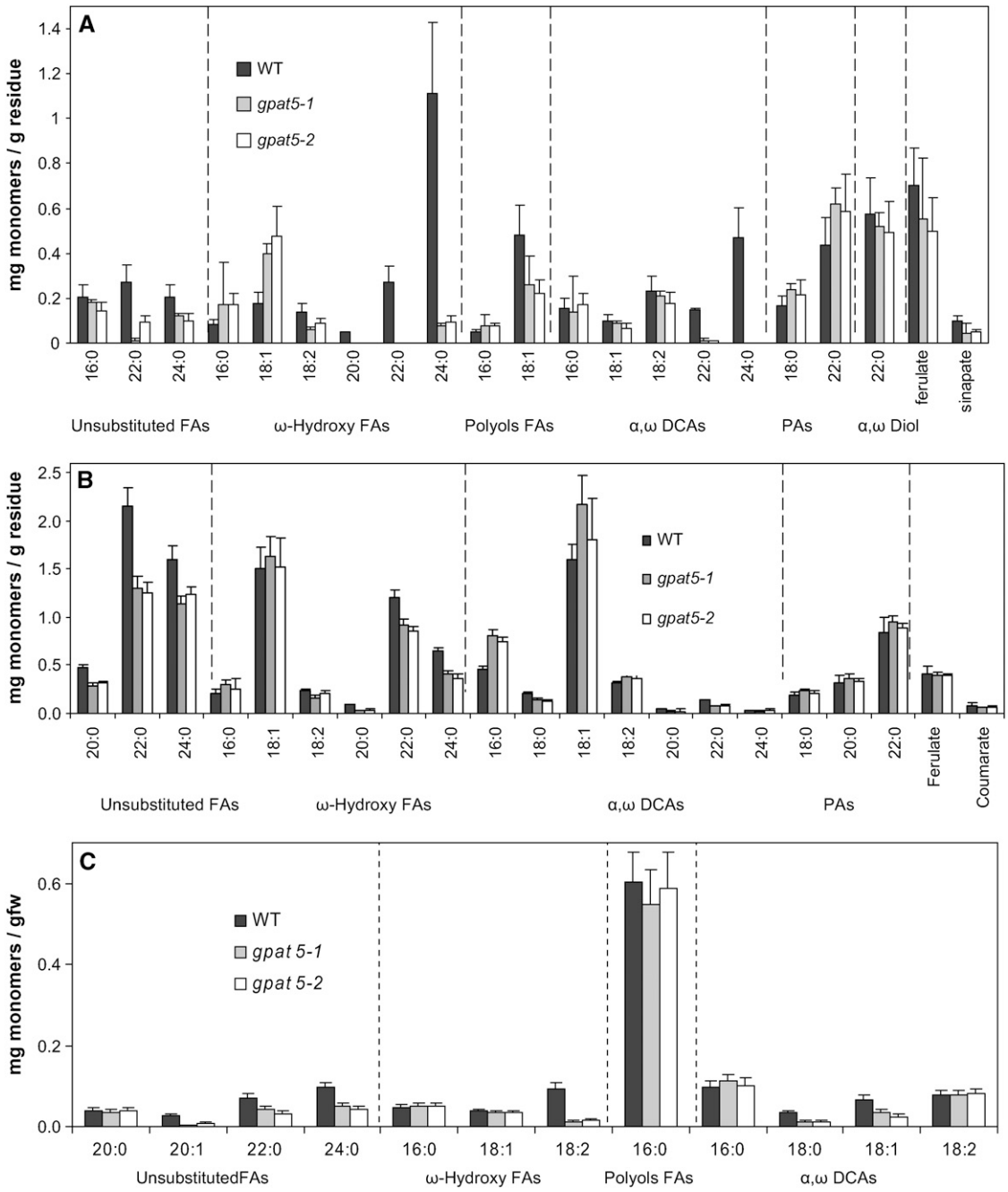

Figure 4. Lipid Polyester Monomers from Seeds, Roots, and Flowers of Wild-Type and *gpat5* Plants.

(A) Polyester monomers from mature seeds.

(B) Polyester monomers from roots of 1-week-old seedlings grown on agar.

(C) Polyester monomers from opened flowers.

The insoluble dry residue obtained after grinding and delipidation of tissues with organic solvents was depolymerized with sodium methoxide, and aliphatic and aromatic monomers released were analyzed by gas chromatography–mass spectrometry. Values are means of six data points (two independent experiments using different biological samples involving triplicate assays for the depolymerization reaction). Error bars denote 95% confidence intervals. DCAs, fatty dicarboxylic acids; FAs, fatty acids; fw, fresh weight; PAs, primary alcohols. The polyol fatty acids are 10,16-hydroxy 16:0 and 9,10,18-hydroxy 18:1.

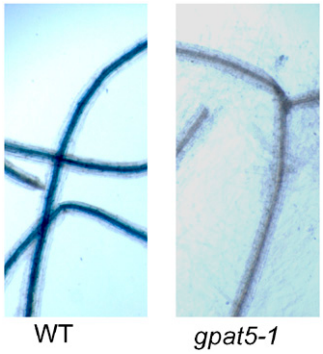

Figure 5. Staining of 3-Week-Old Roots of *gpat5-1* and Wild-Type Plants with the Suberin Dye Sudan Black B.

red 7B (which proved to be more efficient than Sudan black B for staining polyesters of wild-type *Arabidopsis* seed coats). A very localized area of strong Sudan red 7B staining was visible in the wild type, whereas in the mutant the staining was weak or not visible at all in this region. The seed coats of the mutant were more fragile, as they almost always broke when mounted between slide and cover slip. The permeability of wild-type seeds remained unaffected after the removal of surface waxes by chloroform dipping, excluding the possibility that seed surface waxes contribute to seed coat impermeability to tetrazolium salts.

Genetic analysis of the seed coat permeability phenotype in *gpat5* mutants indicated that this phenotype segregated as a single recessive allele and that its inheritance was consistent with the expression of *GPAT5* in the seed coat (tissue of maternal origin). Indeed, F1 seeds (i.e., on the F0 mother plants) were all nonpermeable when wild-type plants were fertilized by pollen from *gpat5-1* knockout plants, whereas they were all permeable when *gpat5-1* knockout plants were fertilized by wild-type pollen (Figure 6H). In addition, analysis of subsequent progeny of the F1 heterozygous plants showed that all F2 seeds were nonpermeable. Segregation analysis of F2 plants demonstrated that the seed permeability phenotype segregated in F3 seeds fitted a 3:1 ratio for a Mendelian single recessive mutation ($\chi^2 = 0.17$; $P = 0.68$). As expected, F2 plants giving permeable F3 seeds were homozygous and the F2 plants giving nonpermeable F3 seeds were either wild type or heterozygous, as determined by PCR. Furthermore, it was observed that *gpat5-1* and *gpat5-2* seeds had a darker appearance than wild-type seeds (Figure 7A) and that in the F1, F2, and F3 seeds this darker seed coat color was always associated with the seed coat permeability phenotype and never with the nonpermeable phenotype. The segregation analysis thus demonstrated that seed coat permeability and color cosegregated with the T-DNA insertions and that these genetic lesions in *GPAT5* segregated as single recessive alleles.

Here, it is important to note that *Arabidopsis* seed coat color is conferred by the brown pigments formed during seed desiccation by the oxidation of colorless proanthocyanidins (PAs; also called condensed tannins) (Lepiniec et al., 2006). Analysis of soluble PAs and the cell wall–bound insoluble PAs by an acid hydrolysis method optimized for *Arabidopsis* seeds (Routaboul et al., 2006) showed that *gpat5* mutant seeds have the same level of both types of PAs as wild-type seeds, ruling out an increase in PA amount (Figure 7B). This result suggests that the darker color may result indirectly from a higher degree of oxidation of PAs, although we cannot exclude other alternative explanations.

gpat5 Mutants Show an Increased After-Ripening Requirement of Seeds and Higher Sensitivity of Seedlings to Salinity

The effect of the knockout of *GPAT5* on seed physiology was further analyzed in terms of dormancy release, germination rate under various conditions, and seedling establishment. Seed dormancy is defined as the temporary failure of an intact viable seed to complete germination under favorable conditions (Bewley, 1997) and is controlled by environmental factors such as light, temperature, oxygen availability, and time of dry storage (after-ripening requirement) as well as by genetic factors (Bentsink and Koornneef, 2002). We compared the dormancy release of mutant seeds and wild-type seeds, which had been harvested at the same time from plants grown together under identical conditions. As shown in Figure 8A, both wild-type and mutant seeds were dormant when harvested immediately after the end of seed maturation (no germination at day 0). Wild-type seeds increased germination to ~60% after 17 d of dry storage; by contrast, *gpat5* germination remained low (<10%). However, with increasing length of postharvest storage, dormancy was gradually released, with 100% germination observed after 30 d of storage for both wild-type and mutant seeds. Thus, the difference between wild-type and mutant seeds is the kinetics of the dormancy release. Germination of dormant mutant seeds in light or dark after cold treatment was almost 100% when scored at day 7 after transfer to a growth chamber. Moreover, the increased after-ripening requirement of the *gpat5* mutants was observed in seeds resulting from the fertilization of *gpat5-1* plants by wild-type pollen but not in the seeds of wild-type plants fertilized by *gpat5-1* pollen, which showed that the phenotype was not attributable to the embryo.

Interestingly, when cold-treated seeds were germinated on Murashige and Skoog (MS) medium with increasing salt concentrations, an average 50% reduction in the rate of germination of *gpat5* seeds compared with wild-type seeds was observed at 150 mM NaCl (Figure 8B). Similar results were obtained with other ions, ruling out a specific effect of sodium or chloride (Figures 8C and 8D). These data show that seed coat control of ion uptake is critical for proper germination.

More than half of the seeds that germinated at 150 mM NaCl showed a development arrest before the establishment of green cotyledons (Figures 9A and 9B). A sensitivity of *gpat5* seedlings to salt (100 to 150 mM) was also observed with KCl and K_2SO_4 (Figures 9C and 9D). To test whether a possible defect in young developing roots could be at least partially responsible for this salt-sensitivity phenotype of the seedlings, seeds were germinated for 3 d on MS plates and transferred to MS plates supplemented with 200 mM NaCl, where they were grown for an additional 5 d. The percentage of *gpat5* seedlings showing bleaching was significantly higher than that of wild-type seedlings after 5 d at 200 mM NaCl, suggesting that the roots of *gpat5* seedlings were severely impaired in their ability to control ion uptake (Figure 10). No significant difference between the wild

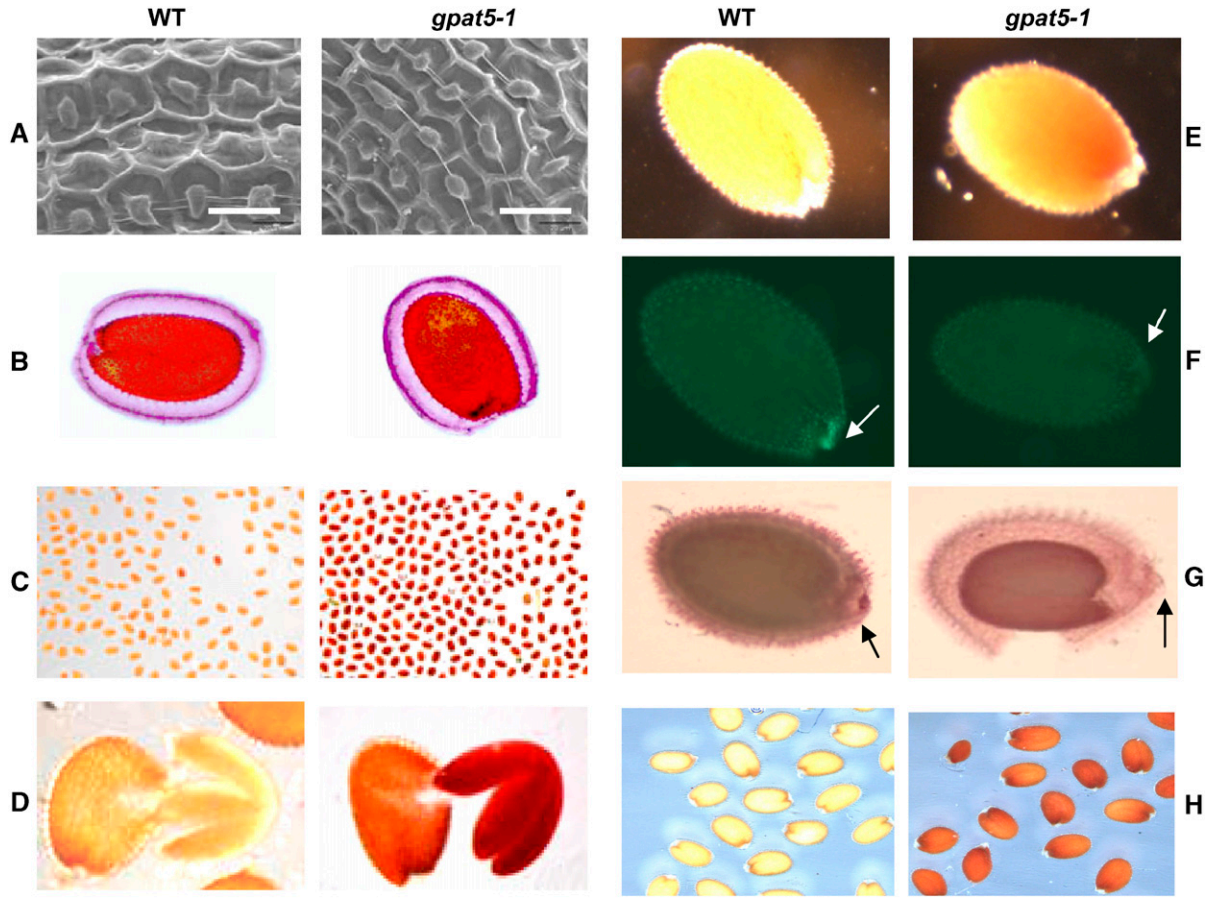

Figure 6. Phenotype of *gpat5* Seeds, and Permeability to Dyes.

Similar results were obtained with *gpat5-2*.
(A) Scanning electron microscopy of the surface of wild-type versus *gpat5-1* seeds. Bars = 40 μm.
(B) Ruthenium red staining of seed mucilage in wild-type versus *gpat5-1* seeds.
(C) Tetrazolium salt staining (24 h) of wild-type versus *gpat5-1* seeds.
(D) Seeds from **(C)** dissected after staining.
(E) Tetrazolium salt staining (4 h) of wild-type versus *gpat5-1* seeds.
(F) Autofluorescence of wild-type versus *gpat5-1* seeds (365-nm excitation).
(G) Sudan red 7B staining of wild-type versus *gpat5-1* seeds.
(H) Tetrazolium salt staining (24 h) of seeds resulting from the fertilization of wild-type plants by *gpat5-1* pollen (left) and of *gpat5-1* plants by wild-type pollen (right).

type and *gpat5* was observed when seeds were germinated and seedlings were grown for 12 d on agar plates supplemented with up to 400 mM of the neutral organic osmoticum polyethylene glycol 8000 or mannitol.

DISCUSSION

The disruption of the *GPAT5* gene caused several biochemical and physiological phenotypes, including reduction in seed and root suberin aliphatic monomer content, increased seed coat permeability, and increased sensitivity of germinating seeds and roots to salts. Here, we will consider the possible molecular functions of GPAT5 and other GPATs in the synthesis of aliphatic polyesters as well as the physiological roles of GPAT5 and aliphatic suberin monomers in the organs affected.

Possible Functions of GPAT5 in Lipid Metabolism

Analysis of the fatty acid derivatives found in seeds, roots, and flowers showed that GPAT5 was essential for the synthesis of lipid polyesters but not for the other lipids analyzed. Moreover, in these organs, the expression of the gene was restricted (e.g., in the root the gene was expressed in the differentiation zone but not in the division and elongation zones that must actively synthesize membrane lipids). These results indicated that *GPAT5* is not likely to be a housekeeping gene of fatty acid metabolism that indirectly affects lipid polyester composition, like, for example, the *FatB* gene (Bonaventure et al., 2004). A specific role of a GPAT family member in lipid polyester synthesis is further supported by two other observations. First, a GPAT (GPAT6; At2g38110) is among the few genes upregulated by the

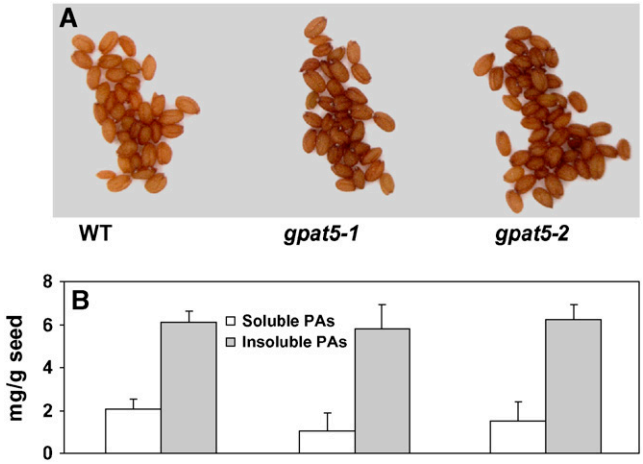

Figure 7. Brown Pigmentation of Wild-Type and *gpat5* Seeds.

(A) Batch color of wild-type versus *gpat5-1* and *gpat5-2* seeds.
(B) Amount of soluble and insoluble PAs in wild-type versus *gpat5-1* and *gpat5-2* seeds. Values are means of six data points (two independent experiments using different seed batches involving triplicate assays for the depolymerization reactions). Error bars denote 95% confidence intervals.

overexpression of the transcription factor WIN1 that is specifically required for cuticle formation (Broun et al., 2004). Second, at least two other GPATs are required for cutin synthesis and normal cuticle impermeability in leaves but are not essential for the synthesis of membrane and storage lipids (Y. Li and F. Beisson, unpublished data). Nevertheless, at this stage, we cannot completely exclude the involvement of GPAT5 in the synthesis of intracellular soluble glycerolipids.

The pathways of the synthesis, transport, and assembly of plant extracellular lipid polymers are poorly understood. Central to polyester synthesis is fatty acid ω-oxidation to form fatty hydroxy and dicarboxylic acids. The reactions of fatty acid oxidation and some partially characterized enzymes (cytochrome P450 monooxygenases, etc.) that may be involved in these oxidation processes have been described (Kolattukudy, 2001), but the in vivo substrates of the oxidation enzymes are not known and might be acyl-CoAs, free fatty acids, etc. After the oxidation steps, the monomers are assembled by the polyester synthase(s)—that is, the enzyme(s) responsible for the synthesis of the primary ester bonds in the polyester chain. The presence of glycerol in cutin and suberin raises the possibility that acylglycerols are substrates for oxidation and/or polymerization reactions. Glycerol 3-phosphate acyltransferase (EC 2.3.1.15) catalyzes the transfer of an acyl group to the *sn*-1 position of glycerol 3-phosphate to form 2-lysophosphatidic acid (Murata and Tasaka, 1997). Because *Arabidopsis* GPATs were isolated by structural similarity to yeast glycerol-phosphate acyltransferases and because most of the members, including GPAT5, have been shown to be able to catalyze the transfer of acyl chains from acyl-CoA to glycerol-3-phosphate to form lysophosphatidic acid (Zheng et al., 2003), it is tempting to conclude that the function of GPAT5 is to provide lysophosphatidic acid for the oxidation or assembly machinery of suberin synthesis. However, because other acyl acceptors than glycerol-3-phosphate and other acyl donors than acyl-CoAs have not been tested for GPAT5, it cannot be excluded that in vivo glycerol could be an acyl acceptor and hydroxy acyl-CoA could be an acyl donor. Direct acylation of glycerol has indeed been observed in animal cells (Hanel and Gelb, 1995; Lee et al., 2001). Thus, the reaction catalyzed by GPAT5 could occur before and/or after the oxidation steps. Other possibilities are more unlikely but cannot be ruled out at this stage (e.g., that GPAT5 is a polyester synthase). Therefore, based on the previous biochemical characterization of GPAT5 and the results described here, the possibilities for the molecular function of GPAT5 can be summarized as follows: (1) formation of unsubstituted fatty acid–containing acylglycerols to feed the oxidation machinery; (2) formation of oxidized fatty acid–containing acylglycerols that will be used as acyl carriers and/or substrates for the polymerization reactions; and (3) addition of acyl chains to a growing glycerol-containing lipid polyester network. These possibilities can be fitted into the tentative metabolic pathway for suberin biosynthesis described by Bernards (2002).

Regarding the possible acyl chain specificity of GPAT5, the common feature of the polyester monomer profiles in seeds, roots, and flowers of the *gpat5* mutants is the strong reduction (at least twofold) observed in 22:0/24:0 fatty acids and derivatives (Figure 4). Therefore, we propose that the role of GPAT5 is to provide the polyester synthesis pathway with acylglycerols containing 22:0 and 24:0 fatty acids (and possibly oxidized derivatives, if oxidation steps precede GPAT5 activity). The hypothetical specificity of GPAT5 for C22-C24 acyl chains is strongly supported by the fact that the ectopic overexpression of GPAT5 in *Arabidopsis* results in the accumulation of saturated very long chain fatty acids attached to glycerol (Y. Li and F. Beisson, unpublished data).

The increase and decrease in various C16 and C18 monomers that were also observed to different degrees in roots, seeds, and flowers of the mutants are not unexpected given the nature of lipid polymers. Indeed, the aliphatic monomers analyzed are not part of independent simple lipid molecules but are structurally linked to each other in a complex manner, possibly a network in which glycerol and other monomers such as dicarboxylic acids allow cross-linking. Therefore, the decrease in very long chain fatty acids resulting from the loss of GPAT5 activity could influence the incorporation of C16 and C18 monomers in a different way in seeds, roots, and flowers, depending on (1) the respective structures and compositions of aliphatic suberin in these organs, (2) the necessity of incorporation of GPAT5-provided long chain monomers before some C16-C18 chain monomers (sequential order requirement), and (3) the overincorporation of C16-C18 monomers wherever possible to maintain some structural features of the polymer (compensatory mechanism). Complex effects on polymer composition involving a sequential order requirement and a compensatory mechanism have been observed in xyloglucan chains of mutants affected in the incorporation of a specific sugar monomer (Madson et al., 2003).

Given the complexity of lipid polyester assembly, the unknown connectivity between monomers in suberin of different organs, and the functional redundancy in the GPAT5 family (see below), more complex interpretations of the polyester monomer profiles of the mutants cannot be excluded. Further experiments will be

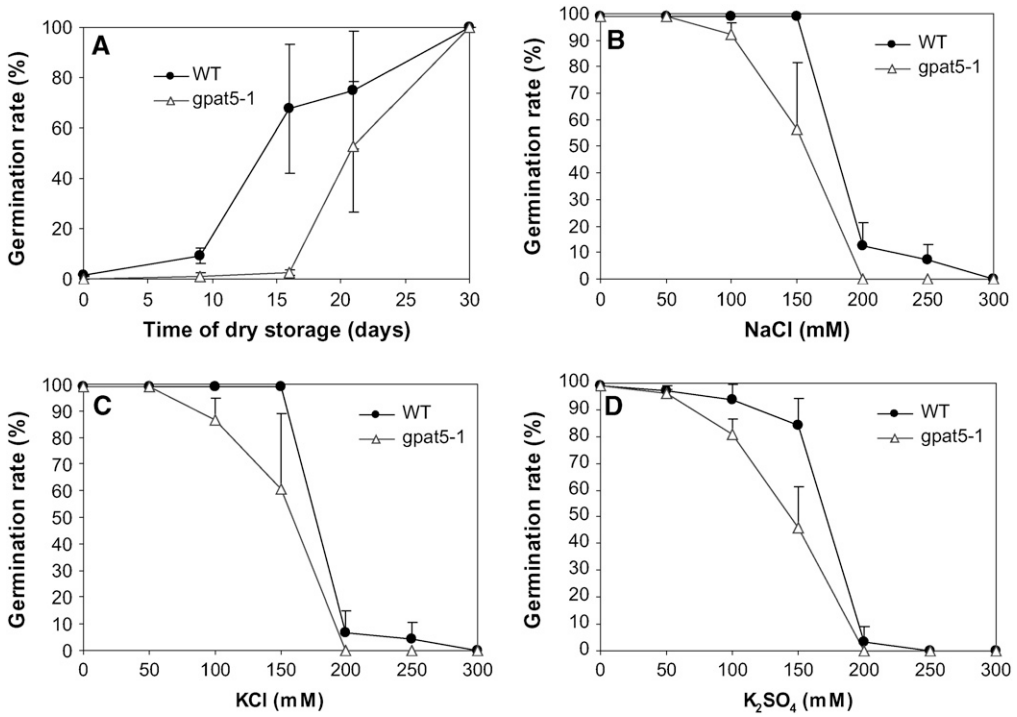

Figure 8. Germination of *gpat5* Seeds under Various Conditions.

(A) Rate of germination after harvest and increasing periods of dry storage.
(B) Rate of germination on MS medium supplemented with increasing NaCl concentrations.
(C) Rate of germination on MS medium supplemented with increasing KCl concentrations.
(D) Rate of germination on MS medium supplemented with increasing K_2SO_4 concentrations.
Seeds were germinated after cold treatment (except in **[A]**). Values are means of 9 data points **(A)** or 12 data points (**[B]** to **[D]**; i.e., from three or four independent experiments, respectively, which use different seed batches and involve three replicate lots of ~100 seeds for each seed batch). Error bars denote 95% confidence intervals. Similar results were obtained with *gpat5-2*.

required to confirm our hypothesis and identify the precise in vivo substrates of GPAT5.

GPAT5 and Seed Coat Permeability

The polyesters of mature seed coats have been shown in several species to consist largely of cutin-type monomers, whereas typical suberin monomers have been found to be of lower abundance and thought to be restricted to a small region of the seed coat (Espelie et al., 1979). At the end of seed maturation, the import of nutrients from the mother plant via the funiculus ceases and the funiculus attachment region is sealed, leaving a scar on the seed coat, the hilum. In grapefruit (*Citrus paradisi*) seeds, it has been shown that the hilum region is indeed sealed by the deposition of aliphatic suberin in several cell layers (Espelie et al., 1980). Unlike *Arabidopsis* seeds, grapefruit seeds can be dissected and enough material from the hilum/chalazal region of the seed coat can be obtained for polyester analysis. The polyester monomers in this seed coat region are typical suberin monomers, with 22- and 24-carbon ω-hydroxy fatty acids and dicarboxylic acids representing ~37 mol% of the total. The rest of the grapefruit inner seed coat is enriched in cutin monomers. Therefore, based on the following lines of evidence, we propose that a major role of GPAT5 in *Arabidopsis* seeds is to seal the hilum region by providing aliphatic monomers for suberin deposition: (1) the typical suberin very long chain monomers found in the grapefruit hilum are abundant in *Arabidopsis* seed coat polyester monomers, and these monomers are the ones that are specifically reduced severalfold in *gpat5* mutants (Figure 4); (2) at the end of the desiccation stage, when suberin deposition in the hilum region is expected to occur, the expression of the *GPAT5* gene is highest in this region (Figure 2B); (3) the kinetics and pattern of appearance of the red staining in the *gpat5* embryo suggest that the tetrazolium salts diffuse mostly or exclusively through the hilum region to the embryo (Figure 6E); and (4) the hilum region of the mutant seed coat had a lower amount of insoluble lipophilic material (Figure 6G).

GPAT5 expression was also detected more broadly throughout the seed coat, especially during early seed desiccation (Figure 2A). This observation suggests a role of GPAT5 in the synthesis of the polyesters in other parts of the seed coat than the hilum region (the extrahilar region), mostly by providing suberin-like monomers. However, the extrahilar region of the seed coat seemed to remain impermeable to tetrazolium salts, which suggests that a defect in GPAT5 may be compensated by functionally redundant GPATs.

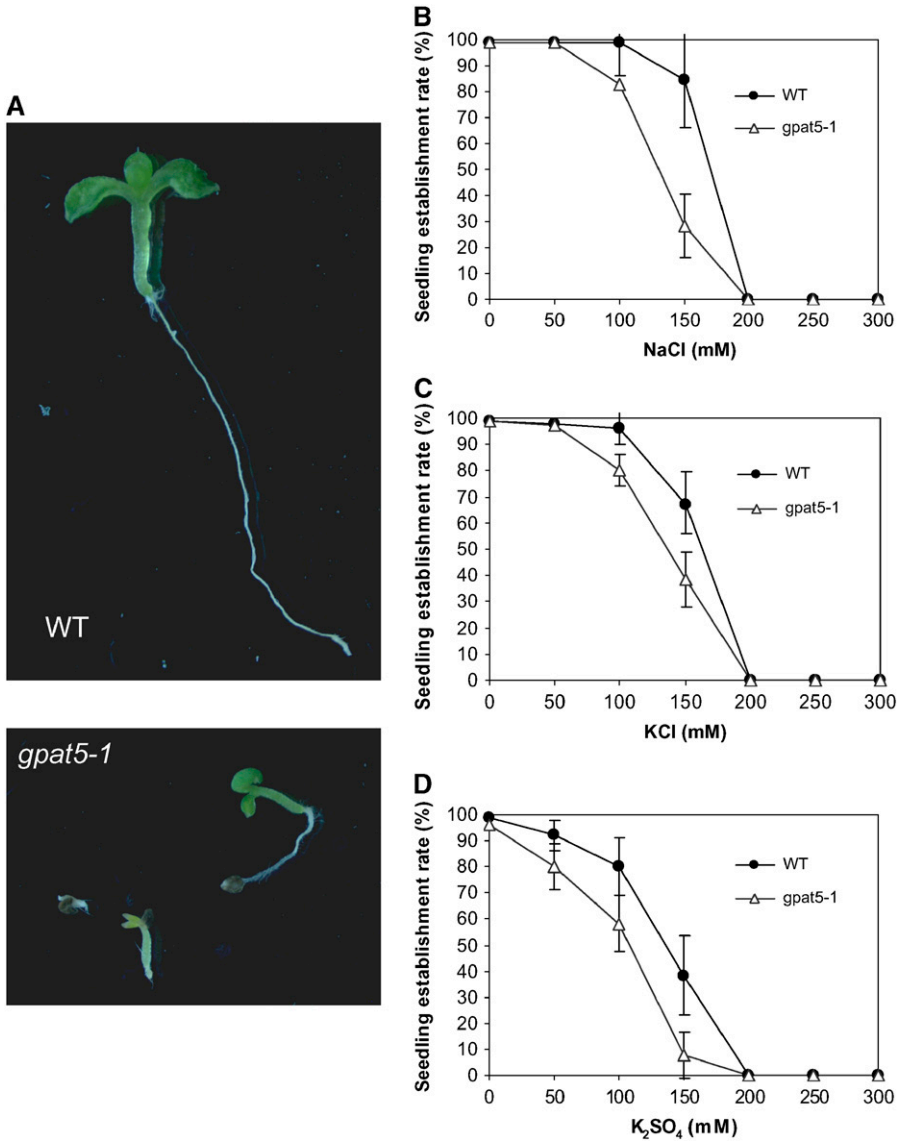

Figure 9. Rate of Seedling Establishment under Increasing Salt Concentrations, and Phenotypes of Seedlings Germinated on 150 mM NaCl.

(A) Seedlings after 12 d at 150 mM NaCl. All seedlings were photographed at the same magnification.
(B) Rate of seedling establishment under increasing NaCl concentrations.
(C) Rate of seedling establishment under increasing KCl concentrations.
(D) Rate of seedling establishment under increasing K_2SO_4 concentrations.
After cold treatment, seeds were germinated for 12 d and seedling establishment was scored. Values are means of 12 data points (four independent experiments using different seed batches involving three replicate lots of ~100 seeds for each seed batch). Error bars denote 95% confidence intervals. Similar results were obtained with *gpat5-2*.

The location of the lipid polyesters in the extrahilar region of *Arabidopsis* is unknown. At maturity, the *Arabidopsis* seed coat consists of dead cells corresponding to the five cell layers of epidermal origin differentiating from the ovule integuments (L1 to L5 from the outermost to the innermost). L1 (epidermis) has a preserved structure with the thick cell walls of the columella and the mucilage, whereas layers 2 to 5 are largely collapsed and crushed together and contain the brown pigments (Haughn and Chaudhury, 2005). In mature *Arabidopsis* seeds, an electron-dense cutin-like layer of polyesters cannot be observed clearly on transmission electron micrographs in the L1 cell layer (epidermis) and may be located in the thick electron-dense crushed cell layers L2 to L5. Interestingly, in developing seeds, an electron-dense layer bordering the inner cell wall of L5 (endothelium), which is in contact with the embryo sac, has been observed. This layer reacts positively with osmium tetroxide and therefore looks like a cuticle that is present throughout seed development until the mature embryo stage (Beeckman et al., 2000).

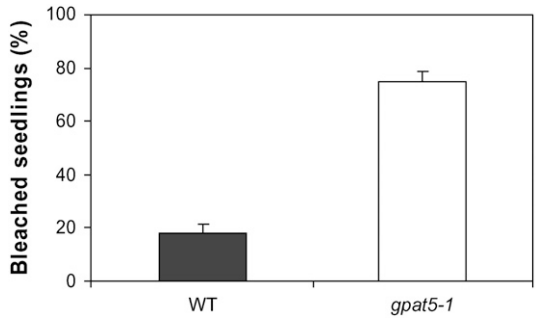

Figure 10. Rate of Seedling Bleaching at 5 d after Transfer on 200 mM NaCl.

Seeds were germinated on MS medium for 3 d and transferred to MS medium supplemented with 200 mM NaCl. Values are means of nine data points (three independent experiments using different seed batches involving three replicate lots of ~100 seeds for each seed batch). Error bars denote 95% confidence intervals.

The increased permeability of the seed coat in the *gpat5* mutants had an effect on the germination rates under increased salt conditions, which could be physiologically important for germination and seedling establishment on various soils.

After-Ripening Requirement and Seed Coat Color in *gpat5* Mutants

The darker color of the *gpat5* mutants is not attributable to an increased amount of PAs (Figure 7B) but may result from a higher visibility of the brown pigments through the seed coat, to a fraction of the brown pigments that cannot be depolymerized or extracted (Routaboul et al., 2006), or from a higher degree of oxidation of PAs. Although, at this time, the connection between seed coat polyesters and seed color is not understood, we speculate that the crushed L2 to L5 layers of the final seed desiccating stage might be composed in part of a polyester network (the cuticle initially bordering L5) in close contact with oxidized polymerized PAs (brown complexes) and that disruption of the polyester network might cause some changes in the formation of these brown complexes or in their visibility.

The dormancy of the mutant seeds was released more slowly, as indicated by the time shift in germination rates measured under normal conditions (Figure 8). The germination rate was always scored 7 d after imbibition, so the difference observed was not attributable to a delay in germination in mutant seeds. Reciprocal crossing of *gpat5* and the wild type showed that the embryo of the mutants was not more dormant. In *Arabidopsis*, seed dormancy has been described as seed coat–enhanced dormancy (Bewley, 1997). The seed coat exerts a germination-restrictive action by being impermeable to water and/or oxygen, by producing germination-inhibiting compounds, and/or by its mechanical resistance to radicle protrusion. The increase in the after-ripening period and the darker seed color we have observed in the *gpat5* mutants are consistent with previous observations in *Arabidopsis*, in which a reduced dormancy phenotype has been observed in several seed coat mutants with reduced brown pigmentation (Leon-Kloosterziel et al., 1994; Debeaujon et al., 2000), and in several other plant seeds in which it has been noted that the darker the seed coat, the more dormant the seeds (Kantar et al., 1996). The underlying mechanism connecting seed coat color and dormancy, however, is unclear, and a direct effect of seed coat polyesters on seed dormancy cannot be completely excluded. Indeed, the reduction in mutant seed coat polyesters could alter the kinetics of critical compounds leaking from the embryo, such as nitric oxide, the accumulation of which is important for the breaking of dormancy (Bethke et al., 2006). Because germination and dormancy are of agronomic significance, the identification of lipid polyesters from the hilum or extrahilar region as a factor regulating seed dormancy may provide additional insights into the control of this process and will be worth further investigation.

GPAT5 and Root Suberin

The chemical analysis of 1- and 3-week-old roots grown on agar clearly shows that GPAT5 is required for suberin synthesis in roots. Thus, the expression of *GPAT5* (as viewed by GUS staining) in the youngest part of the specialization zone (but not in the division and elongation zones) in 4-d-old roots and 1- to 4-week-old roots still elongating is consistent with a role in the initial deposition of Casparian and lamellar suberin, which occurs in the primary state of growth for all roots investigated (Enstone et al., 2003), including *Arabidopsis* (Di Laurenzio et al., 1996; Cheng et al., 2000). A transverse section of the differentiation zone of 4-d-old Pro_{GPAT5}:GUS roots showed that GUS staining was present in all cell layers (data not shown). This pattern of expression of *GPAT5* was also observed in microarray data from root tissues (Birnbaum et al., 2003). Histochemical staining of *Arabidopsis* young roots (Di Laurenzio et al., 1996; Cheng et al., 2000; Franke et al., 2005) indicates an apparent restriction of suberized cell walls to the endodermis. The more widespread expression of the *GPAT5* gene thus suggests that additional depositions of aliphatic lipid polyesters may occur in roots but might remain undetectable by the usual staining procedures, either because of a lower abundance than the endodermal suberin or a difference in structure (the usual dyes are thought to bind the aromatic domain of suberin, but their exact mode of action and their sensitivity/specificity for various lipophilic polymers remain unclear). Another possibility is that GPAT5 produces in the differentiation zone of roots a small pool of soluble glycerolipids.

The patchy expression of GPAT5 in some of the older parts of 1- to 4-week-old roots, in cells in which the initial suberin deposition has already taken place earlier during development, suggests that an additional deposition or a remodeling of suberin composition may occur later in root development before the formation of periderm. In this regard, the composition of aliphatic suberin in 3-week-old roots was changed compared with that in 1-week-old roots grown under the same conditions (Figure 4B; see Supplemental Figure 3 online). The later deposition/remodeling of suberin in apparently specific zones could have several nonexclusive causes. First, in 1- to 4-week-old roots grown on agar, the endodermis divides periclinally according to a complex helicoidal pattern and features of secondary growth are observed, which could also give rise to new suberin deposition

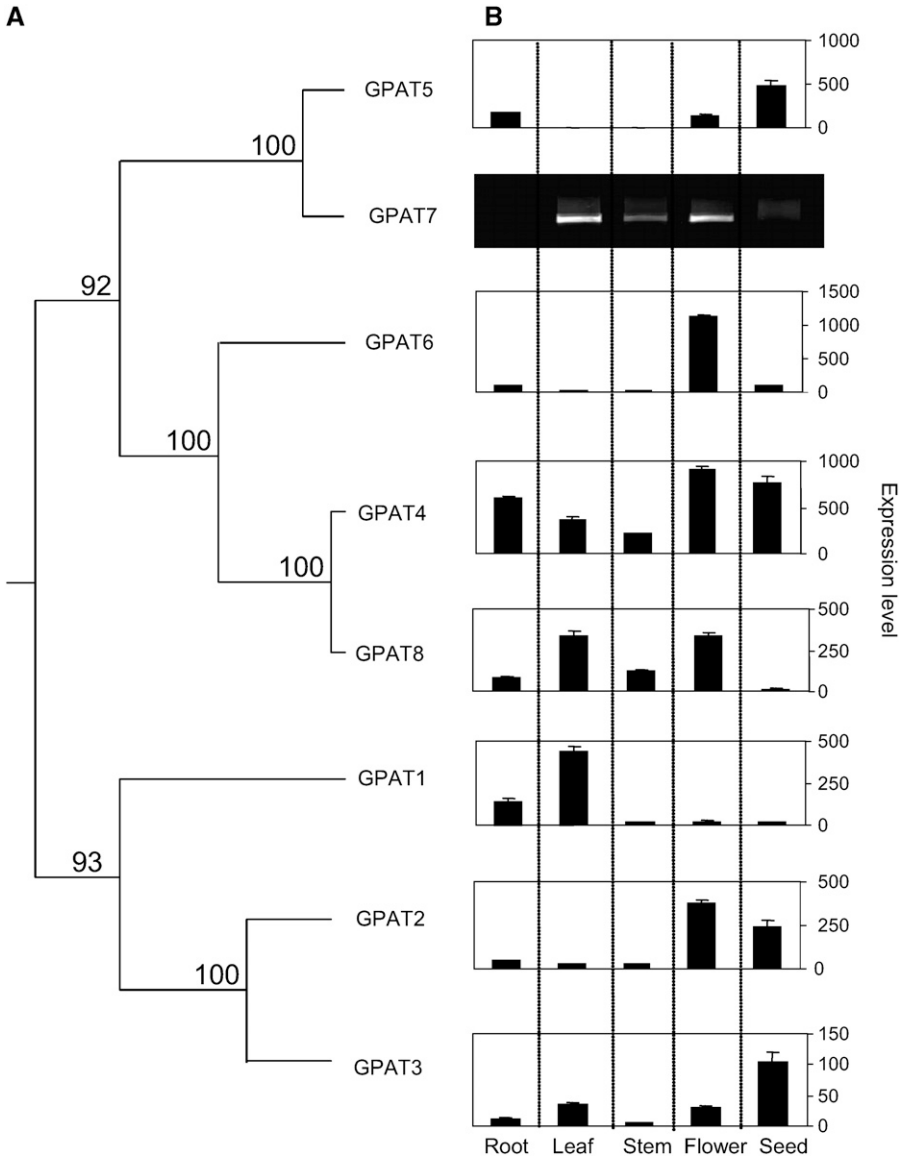

Figure 11. Gene Tree and Gene Expression Profiles of the Eight Putative GPATs of *Arabidopsis*.

(A) The cladogram shows the branching order of *Arabidopsis* GPATs according to a phylogenetic tree of protein sequences of plant acyltransferases (Kim and Huang, 2004). The original tree was built using the neighbor-joining method with 1000 bootstrap replicates. Bootstrap values are percentages.
(B) Microarray expression data derived from AtGenExpress (Schmid et al., 2005). Expression levels in each tissue (root, leaf, stem, flower, and seed) at different developmental stages were averaged (bars represent means ± SE). The expression profile for *GPAT7* was determined in this study via RT-PCR analysis because its expression profile is not available at AtGenExpress.

(Dolan and Roberts, 1995; Baum et al., 2002). Second, there are at least six other GPATs expressed in roots (Figure 11), and they could contribute to this additional suberin deposition/remodeling in other zones than GPAT5 (see below). Third, the formation of lateral roots could require the expression of GPAT5, as suggested by Figure 2F. Fourth, the synthesis of suberin could be induced by environmental conditions. Concerning this latter point, it is not likely that the GPAT5 GUS staining pattern results from induction by pathogens (the roots are grown on sterile medium), but it could result from local gradients of water or nutrients on the agar plates. The understanding of the contribution of GPAT5 and the other GPAT isoforms expressed in roots to the building of the suberin depositions at various stages of *Arabidopsis* root development will require detailed studies of tissue gene expression in conjunction with histochemical studies of the deposition of Casparian and lamellar suberin.

The fact that the transfer of 3-d-old germinated seedlings to a medium supplemented with 200 mM NaCl resulted on average in a fourfold higher rate of bleaching for *gpat5* compared with the wild type (Figure 10) suggests that *gpat5* roots are indeed

affected in their ability to restrict ion movements and prevent a massive entry into the cortex, a role that has been ascribed to suberin depositions of Casparian bands (Sattelmacher, 2001; Enstone et al., 2003; Ma and Peterson, 2003). An effect attributable to osmotic stress could be ruled out by the absence of any difference in seed germination and seedling establishment between wild-type and mutant plants grown with up to 400 mM mannitol or polyethylene glycol 8000. The increased sensitivity to salt stress observed in a mutant affected in root suberin content thus confirms a role previously proposed for suberin in roots and shows that it can be critical for seedling survival at moderate salt concentrations.

Possible Roles of Other Plant GPATs

Among the eight *Arabidopsis* GPATs, GPAT1 has been most fully characterized, and it has been shown to be involved in pollen fertility (Zheng et al., 2003). All *Arabidopsis* GPATs have predicted transmembrane domains (ARAMEMNON database, http://aramemnon.botanik.uni-koeln.de/; Schwacke et al., 2003). Three isoforms (GPAT1 to GPAT3) are predicted to be localized in mitochondria, and uptake of GPAT1 by mitochondria has been demonstrated (Zheng et al., 2003). For GPAT4 to GPAT8, there is no obvious predicted targeting signal. An additional protein (At3g11325) was found to be 57% similar to GPAT5 at the amino acid level. However, this is unlikely to be an active member of the GPAT family, because of the absence of conserved putative catalytic His and Asp residues (Lewin et al., 1999).

Expression data for each gene of the family (Figure 11) were obtained from AtGenExpress (Schmid et al., 2005), except for GPAT7, for which RT-PCR analysis was done. Although overlapping, the pattern of tissue-specific expression of each gene is distinctive and suggests strong candidates for the synthesis of cutin in leaves and stems. The three GPAT genes (GPAT2, GPAT4, and GPAT8) whose expression can be detected in *Arabidopsis* stems using microarrays are all upregulated in the epidermis (Suh et al., 2005). We recently obtained evidence that two GPATs are essential in leaves for cutin synthesis but not for intracellular lipid synthesis (Y. Li and F. Beisson, unpublished data). We are currently investigating the function of all *Arabidopsis* GPATs.

Homologs of *Arabidopsis* GPATs can be found in rice (*Oryza sativa*), poplar (*Populus* spp), maize (*Zea mays*), lettuce (*Lactuca sativa*), and other species, yet not in other nonplant organisms, suggesting that GPATs are plant-specific, which is consistent with a role in the formation of lipid polyesters unique to plants. In addition, a phylogenetic tree constructed with the 8 *Arabidopsis* and 16 rice GPAT sequences (Horan et al., 2005; http://bioweb.ucr.edu/databaseWeb/index.jsp) indicates that for each *Arabidopsis* GPAT group (e.g., GPAT5,7 group), there is at least one rice GPAT that clusters with it. Therefore, the subfamily organization of the different GPAT members observed in *Arabidopsis* is also found in rice, suggesting that the functional differences of various GPAT subfamilies have been conserved over 100 million years.

The identification of acyltransferases involved in suberin and cutin synthesis is an important step toward understanding the biosynthesis of surface lipid polymers in plants and obtaining transgenic plants with a modified cuticle that may confer more resistance to pests or stresses. In addition, the main experimental model to date to study suberin deposition has been the potato (*Solanum tuberosum*) wounding system, and GPAT5 should provide a new molecular tool to dissect the pathway of synthesis and assembly.

METHODS

Plant Materials and Growth Conditions

All *Arabidopsis thaliana* plants (ecotype Columbia-0 and *gpat5* T-DNA knockout lines in the Columbia-0 background) were grown in pots on a soil mixture (1:1:1 mixture of peat moss–enriched soil:vermiculite:perlite) or on solidified agar sucrose medium containing MS salts (Murashige and Skoog, 1962), 1% (w/v) sucrose, and 0.8% (w/v) Phytablend agar (Caisson Laboratories) adjusted to pH 5.7 using KOH. Seeds grown on agar sucrose plates were surface-sterilized for 5 min in 70% (v/v) ethanol and rinsed three times with sterile water. Unless indicated otherwise, seeds were cold-treated for 3 d at 4°C in the dark before being transferred to a controlled growth chamber (at 21 to 22°C, 40 to 60% RH, a 16-/8-h light/dark cycle, and a light intensity of 80 to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by fluorescent bulbs). For observation of root growth, seeds were grown on vertical agar plates.

Mutant Isolation

T-DNA insertional lines (SALK_018117 and SALK_142456) were identified using the SIGnAL T-DNA Express Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) provided by the Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003). SALK_018117 has a T-DNA insertion at the first exon, with the left border of the T-DNA pointing toward the 5′ end of the gene; SALK_142456 has a T-DNA insertion in the first and only intron, with the T-DNA left border pointing to the 3′ end of the gene. Individual seeds for these lines were obtained from the ABRC at Ohio State University. Plants were grown from these seeds, and for each line DNA was prepared and used for genotype screening. The gene-specific primers used for the screening of insertions into the *GPAT5* gene were 5′-GCTATTTTTCCATTTGCAGATACGT-3′ (forward) and 5′-ACATCTCGGATTCTTGTCAATC-3′ (reverse) for SALK_018117 and 5′-CTAAGGAGCATCTTAGAGCAGATGA-3′ (forward) and 5′-TCCAGCGAGAACCCTATACTTATCT-3′ (reverse) for SALK_142456. These primers were used together with the T-DNA left border primer LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) to check for the presence of a wild-type or T-DNA mutant allele, respectively.

RNA Isolation and Gene Transcript Analysis by RT-PCR

Total RNA was prepared from rosette leaves, stems, open flowers, roots, and developing seeds of *Arabidopsis* plants by grinding these tissues in liquid nitrogen followed by RNA extraction with the Plant RNeasy Mini kit from Qiagen. Extracted RNA was quantified by spectrophotometer, and its integrity was verified by separating 1 μg of total RNA on a denaturing formaldehyde gel. RNA was stored at −80°C until use in reverse transcription reactions. To eliminate any contaminating DNA, RNA preparations were treated with DNase using the DNA-free kit from Ambion, and the treated RNA (1 to 5 μg) was subjected to reverse transcription using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). The *GPAT5* transcript was amplified using the gene-specific primers 5′-CTAAGGAGCATCTTAGAGCAGATGA-3′ (forward) and 5′-TCCAGCGAGAACCCTATACTTATCT-3′ (reverse), and the *GPAT7* transcript was amplified using the gene-specific primers 5′-CCTTCGCCTACTTCATGCTC-3′ (forward) and 5′-GGTCTCGGGTTCATGAAAAA-3′ (reverse), with the initiation factor eIF4A-1 (At3g13920) as a control (forward, 5′-CCAGAAGGCACACAGTTTGATGCA-3′; reverse, 5′-TCATCATCACGGGTCACGAAATTG-3′).

Promoter–GUS Fusion Analysis

A 1-kb sequence upstream of the first ATG in the *GPAT5* cDNA, and 588 bp of the *GPAT5* coding region, were cloned in-frame as a *Hin*dIII-*Xba*I fragment into the binary vector pBI101.1 carrying the GUS gene downstream of the inserted promoter. The primers used for amplification of the fragment from genomic DNA were 5′-CACACAAGCTTAAAAAAGCGTTTTAATTAG-3′ (forward) and 5′-CACACTCTAGACTCACATAACGATAAGAA-3′ (reverse) (inserted restriction sites are underlined). The construct (*Pro$_{GPAT5}$:GUS*) was used to transform *Arabidopsis* wild-type plants by *Agrobacterium tumefaciens* vacuum infiltration (Bechtold et al., 1993). T1 seeds of *Pro$_{GPAT5}$:GUS* transformants were selected on sterile plates (50 μg/mL kanamycin) from surface-sterilized seeds. Resistant seedlings were transferred to soil for continued growth and seed collection. The T3 progeny from several individual kanamycin-resistant plants were analyzed for GUS gene expression. Briefly, fresh tissues were suspended and vacuum-infiltrated in a staining solution (0.1 M NaH_2PO_4, pH 7.0, 10 mM Na_2-EDTA, 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 1.0 mM X-glucuronide prepared freshly each time in DMSO, and 0.1% Triton X-100 for 30 min to 1 h depending on the tissue) (Jefferson, 1987). The samples were then incubated at 37°C for 2 to 18 h until staining was visible. Stained tissues were fixed in a mixture of ethanol:acetic acid:formaldehyde (50:5:3.7, v/v) for 10 min at 65°C and destained in 80% (v/v) ethanol until removal of chlorophyll. Images of whole mount tissues were taken with a Leica MZ 12.5 microscope coupled with a digital camera.

Seed Germination and Dormancy Tests

Germination rates of wild-type and *gpat5* seeds were compared for both soil-grown seeds and seeds grown on agar plates supplemented with increasing concentrations of salts. Plates were transferred to a controlled growth chamber (see Plant Materials and Growth Conditions) after cold treatment in the dark for 3 d at 4°C. Germination was scored at 7 d after transfer to the growth chamber (12 d in the case of added salts). Seeds that showed penetration of the radicle through the seed coat were counted as germinated seeds. Seedling establishment was scored by looking for the presence of two green cotyledons. The tests were repeated on at least three different batches of seeds harvested from plants grown at different times.

For dormancy release experiments, wild-type and *gpat5* seeds harvested immediately after maturity from plants of the same age and grown under the same conditions were tested over a period of dry storage (0, 10, 17, 21, and 30 d at room temperature) (Bentsink and Koornneef, 2002). Mature seeds were sown in triplicate in Petri dishes (100 to 150 seeds per plate), incubated in a controlled growth chamber, and scored for germination as described above. The tests were repeated on three different batches of seeds harvested from plants grown at different times.

Staining Procedures

For seed coat permeability tests, tetrazolium red assays were used (Debeaujon et al., 2000). Briefly, *Arabidopsis* dry seeds were incubated in the dark in an aqueous solution of 1% (w/v) tetrazolium red (2,3,5-triphenyltetrazolium) at 30°C for 4 to 48 h. Mucilage of *Arabidopsis* mature seeds was stained in an aqueous solution of 0.03% (w/v) ruthenium red for 15 min at room temperature (Western et al., 2000). Seeds were rinsed in water before imaging.

For lipid polyester staining of roots, a solution of 1% (w/v) Sudan black in 75% ethanol was used. Roots were placed into this solution for 1 h, rinsed briefly in water, and imaged.

For lipid polyesters of seed coats, mature seeds were incubated for 24 h at room temperature in water containing 0.01% (w/v) Triton X-100 and 10% (v/v) commercial bleach to fade the seed coat pigments. After rinsing successively with distilled water and 100% ethanol, seeds were incubated for 30 min with chloroform:methanol (2:1, v/v), rinsed with 100% ethanol, and air-dried. Seeds were finally incubated at room temperature for 1 to 4 h with a solution of Sudan red 7B in polyethylene glycol 400:glycerol:water prepared as described by Bundrett et al. (1991), rinsed in water, mounted between slide and cover slip, and observed with a Leica MZ12.5 light microscope coupled to a digital camera.

Analysis of Fatty Acids and Cuticular Waxes

Total fatty acid content and composition in *Arabidopsis* seeds were quantified according to Li et al. (2006). The same method of direct methylation was used for total fatty acids of leaf, root, and flower. For cuticular waxes, the leaf tissue (1 g from 5-week-old plants) was dipped in chloroform (20 mL) for 30 s, with the addition of internal standards (20 μg/g *n*-octacosane, 10 μg/g docosanoic acid, and 10 μg/g fresh leaf 1-tricosanol). The epicuticular waxes were silylated to convert free alcohols and carboxylic acids to their trimethylsilyl ethers and esters, respectively, by heating the sample at 110°C for 10 min in 100 μL of pyridine and 100 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide. After cooling, the solvent was evaporated under nitrogen and the product was resuspended in heptane:toluene (1:1, v/v) for gas chromatography–mass spectrometry analysis (Bonaventure et al., 2003). For seed surface wax analysis, mature seeds (100 mg) were soaked in chloroform for 2 min, and internal standards (5 μg/g *n*-octacosane, 5 μg/g docosanoic acid, and 5 μg/g seed 1-tricosanol) were added and processed as described above for leaf cuticular wax analysis. Seed surface areas were calculated assuming a prolate spheroid geometry and using semiaxes estimated from scanning electron microscopy images of seeds.

Analysis of Lipid Polyesters

Polyester monomers were obtained and analyzed according to Bonaventure et al. (2004). Briefly, the sodium methoxide depolymerization method was used with slight modifications described by Suh et al. (2005). Polyester monomers were separated, identified, and quantified by gas chromatography–mass spectrometry. Splitless injection was used, and the mass spectrometer was run in scan mode over 40 to 500 atomic mass units (electron impact ionization), with peaks quantified on the basis of their total ion current. Polyester monomer amounts are expressed per surface area for seeds and per gram of dry residue depolymerized for roots. Further details of aliphatic and aromatic monomer identifications, analytical methods, and seed coat and embryo polyester monomer localization experiments are presented by Molina et al. (2006).

Analysis of PAs

Analysis was performed on samples of 15 mg of mature seeds using acid-catalyzed depolymerization and spectrophotometric quantification as described previously (Routaboul et al., 2006).

Scanning Electron Microscopy and Fluorescence Microscopy

Arabidopsis pollen and mature seeds were coated with gold particles during 3 min at a coating rate of ~7 nm/min using an EMSCOPE SC500 sputter coater (Ashford). Scanning electron microscopy images were taken with a JEOL JSM-6400V microscope. Assuming prolate spheroid seed geometry, semiaxes were estimated from scanning electron microscopy images using Analysis PRO software version 3.2 (Soft Imaging System).

For seed coat autofluorescence, mature seeds were illuminated and observed with a Zeiss Axiophot microscope using epifluorescence interference and absorption filters (excitation filter, 365 nm; dichromatic beam splitter, 395 nm; long-pass emission filter, 435 nm) coupled to a digital camera.

Accession Numbers

Arabidopsis Genome Initiative locus codes are as follows: GPAT1, At1g06520; GPAT2, At1g02390; GPAT3, At4g01950; GPAT4, At1g01610; GPAT5, At3g11430; GPAT6, At2g38110; GPAT7, At5g06090; and GPAT8, At4g00400.

Supplemental Data

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

Supplemental Figure 1. Fatty Acids from Intracellular Lipids.

Supplemental Figure 2. Wax Composition of the *Arabidopsis* Seed Surface.

Supplemental Figure 3. Lipid Polyester Monomers from Roots of 3-Week-Old Seedlings Grown on Agar.

Supplemental Figure 4. Lipid Polyester Monomers from Leaves of Wild-Type and *gpat5* Mutant Plants.

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