Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers

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Cutin and suberin are the two major lipid-based polymers of plants. Cutin is the structural polymer of the epidermal cuticle, the waterproof layer covering primary aerial organs and which is often the structure first encountered by phytopathogens. Suberin contributes to the control of diffusion of water and solutes across internal root tissues and in periderms. The enzymes responsible for assembly of the cutin polymer are largely unknown. We have identified two *Arabidopsis* acyltransferases essential for cutin biosynthesis, glycerol-3-phosphate acyltransferase (GPAT) 4 and GPAT8. Double knockouts *gpat4/gpat8* were strongly reduced in cutin and were less resistant to desiccation and to infection by the fungus *Alternaria brassicicola*. They also showed striking defects in stomata structure including a lack of cuticular ledges between guard cells, highlighting the importance of cutin in stomatal biology. Overexpression of GPAT4 or GPAT8 in *Arabidopsis* increased the content of C16 and C18 cutin monomers in leaves and stems by 80%. In order to modify cutin composition, the acyltransferase GPAT5 and the cytochrome P450-dependent fatty acyl oxidase CYP86A1, two enzymes associated with suberin biosynthesis, were overexpressed. When both enzymes were overexpressed together the epidermal polyesters accumulated new C20 and C22 ω-hydroxyacids and α,ω-diacids typical of suberin, and the fine structure and water-barrier function of the cuticle were altered. These results identify GPATs as partners of fatty acyl oxidases in lipid polyester synthesis and indicate that their cooverexpression provides a strategy to probe the role of cutin composition and quantity in the function of plant cuticles.

Plant surface lipids fulfill critical roles in the control of water and gas exchange, as protection from pathogens and UV radiation, as structural components, and to prevent cell fusions during organogenesis (1–3). These lipids are organized into the cuticle, a complex hydrophobic layer that covers the epidermis of plant leaves and other aerial organs and therefore is one of the largest biological interfaces in nature. The framework of the cuticle layer is provided by cutin, a plant-specific polyester composed of omega-substituted fatty acids and glycerol monomers (1, 4, 5). This insoluble polyester matrix is embedded and covered with waxes, a mixture of fatty acid derivatives that is easily extractable in organic solvents and has thus been more amenable to study than cutin (2, 6). Suberin is another type of cell-wall-associated lipid polymer, the most well known form being cork. It is composed of aliphatic and aromatic domains and is found in roots, the periderm of stems, and other tissues where it functions to restrict movement of water and ions across cell walls (1, 7, 8). Suberin also differs from cutin in that it is usually deposited abutting the inner face of the primary cell wall, whereas cutin is deposited at the outer face.

Although cutin, one of the most abundant lipid polymers of nature, is the structural polymer of the plant cuticle and as such contributes greatly to the barrier functions of plant surfaces, very little is known about its biosynthesis. Methods to analyze the very thin cutin layer of the model plant *Arabidopsis* (9, 10) and the description of the first cutin mutants have been reported only recently (3). *Arabidopsis* and *Brassica* cutins contain ω-hydroxy-fatty acids, but are rich in α,ω-dicarboxylic acids, particularly that derived from linoleic acid, and thus have a monomer composition diverging from cutins reported in the literature. Genes encoding enzymes involved in activation (11, 12) and ω-oxidation of acyl chains (13) have been identified in *Arabidopsis*, but none of the steps involved in acyl transfer in cutin biosynthesis are known. In addition, no genes encoding enzymes of suberin biosynthesis were known until the recent identification of a suberin mutant, deficient in the acyltransferase glycerol-3-phosphate acyltransferase (GPAT) 5 (14). The search for genes encoding biosynthetic enzymes of polyester assembly has been given additional impetus by transcript profiling of the *Arabidopsis* epidermis (15) for cutin, and recently the cork phelloderm (16) for suberin. Major issues of cutin research include the identification of enzymes for biosynthesis and assembly, the site of polymerization of monomers (intracellular and/or extracellular) and the mechanism of transport of cutin monomers, oligomers, or polymers through the outer epidermal cell wall. To address these issues, gain-of-function strategies that allow modification of the amount and composition of cutin would be an ideal complement to loss-of-function approaches and could help address questions about how the structure of cutin influences the functional properties of plant cuticles. Moreover, the cutin biosynthesis machinery in plants is a widespread source of enzymes producing α,ω-bifunctional fatty acids, which are biomolecules with potential to replace petroleum for the synthesis of polymers and specialty chemicals. Identifying enzymes of cutin biosynthesis and testing their effect by overexpression and inactivation is thus a major goal of research on plant lipid polyesters. Below, we describe the first acyltransferase enzymes known to be involved in cutin assembly and show that cutin amounts can be increased or decreased by manipulating their expression. We also demonstrate that novel monomers typical of aliphatic suberin can be produced in cutin by overexpressing a related suberin-associated acyltransferase in combination with a fatty acid ω-oxidase.

Results

GPAT4 and GPAT8 Acyltransferases Are Involved in Cutin Formation.
Arabidopsis expresses a family of eight GPAT acyltransferases, of which several members were shown to have GPAT activity when
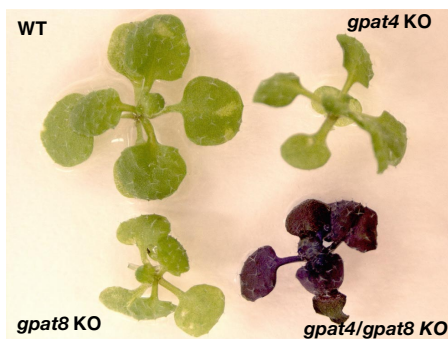
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Fig. 1. Permeability of cuticles of GPAT knockouts to toluidine blue. Seedlings were immersed for 2 min in 0.05% toluidine blue-O and rinsed with water.

expressed in yeast (17). We have recently identified GPAT5 (At3g11430) as a glycerol acyltransferase required for synthesis of suberin in roots and seed coats (14, 18). The presence of ω-oxidized fatty acids and glycerol (4, 19) in both cutin and suberin prompted us to investigate the involvement of other GPAT members in the synthesis of cutin in leaves. Among these, *GPAT4* (At1g01610) and *GPAT8* (At4g00400) were previously found by microarray analysis to be strongly up-regulated in stem epidermis (15). GPAT4 promotor YFP fusions confirmed that this gene was specifically expressed in the epidermis of WT stems and leaves (data not shown). Analysis of the leaf cuticle of single mutants *gpat4* or *gpat8* did not reveal any obvious cuticle defect (Fig. 1) or cutin content phenotype (Fig. 2). However, the toluidine blue dye method (20) revealed that the double knockout *gpat4/gpat8* had a strong increase in cuticle permeability (Fig. 1), suggesting that the two genes were essential for cuticle function but had redundant or largely overlapping functions. Analyses of lipids of *gpat4/gpat8* stems indicated the fatty acid content of the membrane and storage lipid fractions was not changed [supporting information (SI) Fig. 6] and the cuticular

wax load and composition showed only minor changes (SI Fig. 7). However a 60–70% overall decrease in cutin aliphatic monomer content was observed in *gpat4/gpat8* stems (Fig. 2A and SI Fig. 8). The decrease was more pronounced in the major monomer (18:2 dicarboxylic acid) than in other constituents including other dicarboxylic acids or other 18-carbon monomers. Similar observations were made for rosette leaves (data not shown). The reduction in cutin was also clearly revealed by microscopic observation of pavement cells of *gpat4/gpat8* stem epidermis (Fig. 3A). The outermost electron-dense layer of the epidermis that is typically seen in transmission electron micrography (TEM) of *Arabidopsis* WT was absent or greatly reduced. Taken together, these results identify GPAT4 and GPAT8 acyltransferases as essential for the biosynthesis of the cutin polymer in stems and leaves.

gpat4/gpat8 Cutin Mutants Show Increased Water Loss, Susceptibility to Pathogens, and Altered Stomata Structure. The *gpat4/gpat8* plants grew more slowly than WT plants (SI Fig. 9), and both initial (5–30 min) and steady-state (>30 min) water-loss rates (21) for rosettes were 4-fold greater for *gpat4/gpat8* compared with WT (Fig. 4). Because the load and composition of total cuticular waxes were similar between mutant and WT (SI Fig. 7), the increased water loss in the *gpat4/gpat8* plants cannot be attributed to waxes *per se*. Also, *gpat4/gpat8* displayed an increased sensitivity to the necrotrophic fungal pathogen *Alternaria brassicicola* (Fig. 5).

Reduced or increased sensitivity of cuticle mutants to water losses and pathogens are often interpreted in terms of thickness or structure of the cuticle of pavement cells (21). However, stomatal pores, controlled by guard cells, are essential not only for CO_2 uptake and control of water loss (22) but are also sites of entry for bacterial pathogens (23) and some fungi (24). To further evaluate how changes in cuticular lipids affect water and barrier functions of the epidermis, we examined the structure of guard cells in *gpat4/gpat8* mutants. Scanning electron microscopy (SEM) and confocal images of the stem and leaf surface of
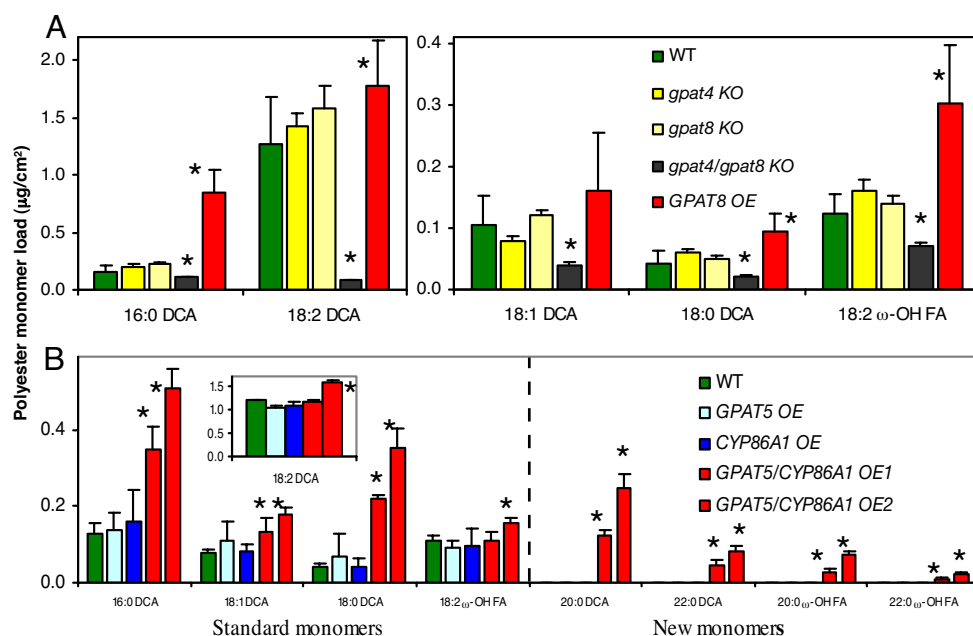

Fig. 2. Analysis of the lipid polyester monomer content of 7-week-old *Arabidopsis* plants. (*A*) Effect of knocking out GPAT4 and GPAT8 or overexpressing GPAT8 on major stem cutin monomers. Overexpression of GPAT4 (data not shown) gave similar results to GPAT8. (*B*) Effect of overexpressing the suberin-associated GPAT5 and/or the fatty acyl oxidase CYP86A1 on major stem cutin monomers. Additional lipid polyester compositions are presented in SI Fig. 8. Values are means from four experiments with 95% confidence intervals. Asterisks denote a significant difference with WT (*t* test, two-sided $P < 0.05$ at least).
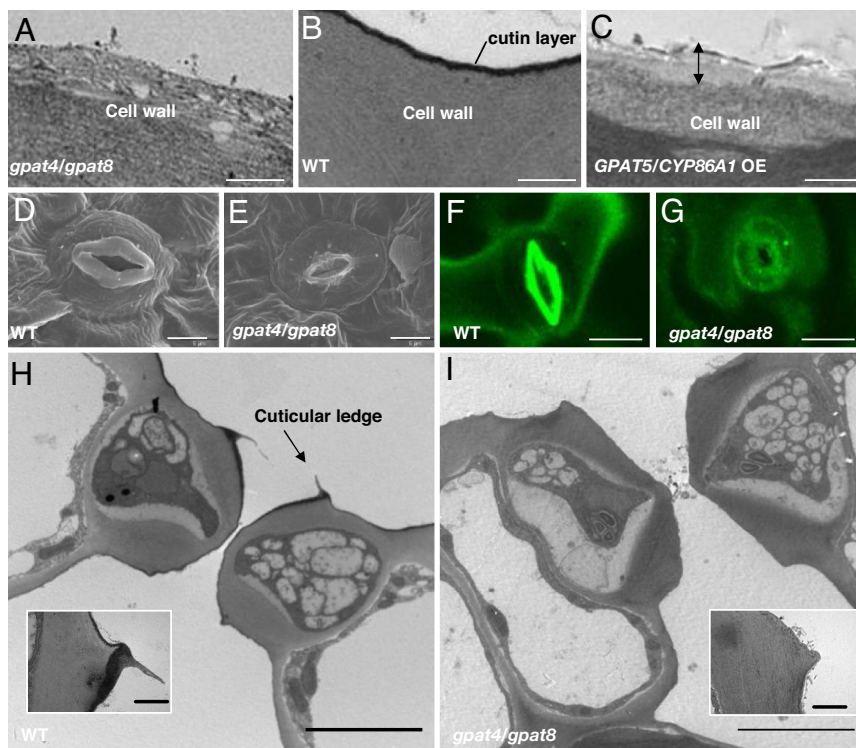
Fig. 3. Features of epidermal cells of WT and *gpat* mutant and overexpressor. (*A–C*) Cuticle of pavement cells of stems as seen by TEM. (Scale bars, 0.5 μm.) (*D* and *E*) SEM images of stomata from adaxial surface. (Scale bars, 5 μm.) (*F* and *G*) Confocal laser scanning microscopy images of stomata from abaxial surface of 5-week-old leaves stained with Nile red (one single optical section at the stomata surface, fluorescence of dye is represented in green). (Scale bars, 10 μm.) (*H* and *I*) TEM images of transdermal section of stem guard cells. (Scale bars, 5 μm.) (*Insets*) Magnified images of outer cuticular ledges. (Scale bars, 0.5 μm.)

gpat4/gpat8 mutants showed alterations in epidermal pavement cells (SI Fig. 10) and a strikingly different stomatal structure (Fig. 3 *D* and *E* and SI Fig. 11). Most notably, the cuticular projections (ledges) surrounding the stomatal pore were absent. In leaves, the cuticular ledges were strongly reduced in all stomata, and $\approx$30% of guard cells had a round shape rarely observed in the WT or the single GPAT mutants. These features of leaf stomata were easily visualized and confirmed on intact leaves by confocal microscopy after staining with the lipophilic fluorescent dye Nile red (Fig. 3 *F* and *G*).

The occurrence of major structural changes in guard cells of the *gpat4/gpat8* mutant was further investigated by TEM. It was obvious that the reduction of the cutin layer seen in the pavement cells of stems (Fig. 3 *A–C*) extended to the surface of guard cells and also to the substomatal chamber. Again, the cuticular ledges that normally extend over the stomatal aperture were clearly absent (Fig. 3 *H* and *I*). These cuticular projections are a conserved feature of almost all dicotyledon guard cells and are believed critical to prevent water penetration into substomatal chambers (25), but their composition is uncertain. The absence of these projections in *gpat4/gpat8* provides direct evidence that cutin is an essential component for their formation. Despite the structural alterations shown in Fig. 3, the stomata of *gpat4/gpat8* leaves can open and close in response to white light or abscisic acid in a way similar to stomata of WT leaves (data not shown), although whether mutant stomata can reach a fully closed state ensuring complete absence of water loss through the pores remains to be determined.
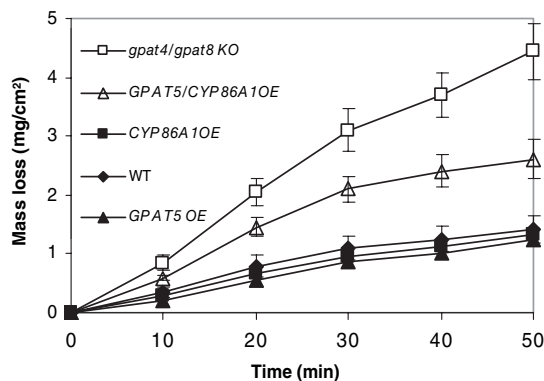
Overexpression of GPAT4 or GPAT8 Increases Cutin Content. Methods to increase cutin loads or to modify cutin composition may be valuable both for better understanding of cutin function and for altering the barrier properties of plant surfaces. To determine whether GPAT4 and GPAT8 activities were limiting in the cutin biosynthesis pathway, gain-of-function experiments were performed. Expression of either the GPAT4 or GPAT8 gene under control of the 35S promoter in *Arabidopsis* resulted in an overall 80% increase in the typical C16 and C18 cutin monomers in stems (Fig. 2*A*). Similar results were obtained in leaves (data not shown). The fact that a similar cutin composition was obtained upon overexpression of either gene confirmed their functional redundancy. The increase in cutin loads indicated that acyl transfer to a glycerol-based acceptor limits or colimits the accumulation of cutin in *Arabidopsis*. By contrast, ectopic overexpression of ATT1 (CYP86A2), a P450-dependent fatty acyl

Fig. 4. Water loss of excised rosettes in the dark. Rosettes were excised in the middle of the night period and immediately weighed in the dark every 10 min. Values are means for six rosettes. Error bars are 95% confidence intervals. Experiment was repeated twice with different overexpressor lines and gave similar results.
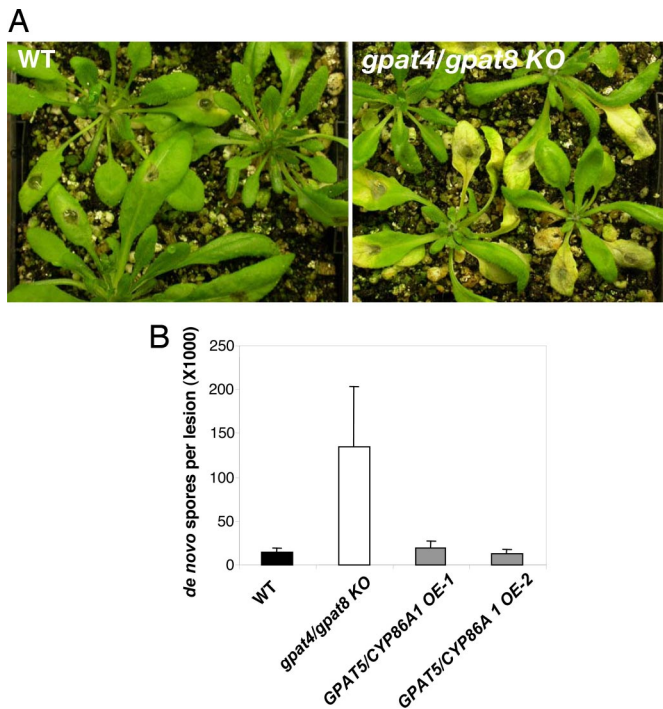
A

WT | gpat4/gpat8 KO

B

Fig. 5. Susceptibility of GPAT KO and overexpressors to *A. brassicicola*. (*A*) Symptom developments in the WT and *gpat4/gpat8* KO rosette leaves 3 days after inoculation. (*B*) *In planta*-formed spores were counted 7 days after inoculation. Three batches of spores from 10 lesions were counted (mean ± SD).

oxidase (26) known to be required for cutin synthesis (13), did not result in significant changes in cutin load or composition in leaves and stems (data not shown).

Cooverexpression of GPAT5 and CYP86A1 Produces Typical Suberin Monomers in Epidermis. To further modify cutin composition, we investigated the possibility that new acyl chains could be incorporated into cutin by overexpressing enzymes of suberin biosynthesis. Previous work showed that ectopic overexpression of the suberin-associated GPAT5 acyltransferase alone in *Arabidopsis* stems and leaves did not produce changes in cutin but instead caused accumulation of very long-chain fatty acid-containing monoacylglycerols in the cuticular waxes (18). The novel surface monoacylglycerols formed by GPAT5 ectopic expression might be potential components of cutin polyesters except that they lack the ω-functional group characteristic of cutin and suberin monomers. We reasoned that their incorporation into polyesters may require the coexpression of a specific isoform of fatty acid ω-oxidase normally expressed in roots, which would add hydroxyl and/or carboxyl groups to the terminal position of acyl chains. Members of the CYP86A family of cytochrome P450 monooxygenases are known to be fatty acyl oxidases involved in the synthesis of cutin (13) or essential for cuticle formation (27). Based on preferential expression in roots and seeds (28) and strong transcript coexpression under various conditions (29, 30), we identified *Arabidopsis* CYP86A1 (At5g58860) as an enzyme potentially involved with GPAT5 in lipid polyester synthesis. Also, the recent transcriptome analysis of cork phelloderm (16) identified homologs with close similarity to CYP86A1, rather than other members of this P450 subfamily, supporting the association of CYP86A1 with suberin biosynthesis. *In vitro* fatty acid ω-oxidase activity has also been detected for recombinant CYP86A1 expressed in yeast (31). The importance of CYP86A1 for accumulation of oxidized fatty acids in suberin was confirmed

by the chemical analysis of suberin in roots that showed a 50–75% reduction in several ω-oxidized monomers including 16- and 18-carbon dicarboxylic acids (SI Fig. 12).

The ectopic overexpression of CYP86A1 alone did not result in changes in cutin, nor in soluble lipids. But strikingly, the cooverexpression of CYP86A1 together with the acyltransferase GPAT5 resulted in an 80% increase in total lipid polyester content together with the appearance of very long-chain (C20-C22) fatty hydroxyacids and diacids that are typical of suberin (1) (Fig. 2*B*), as well as several-fold increases in C16:0 and C18:0 dicarboxylic acids. Peeling and analyzing the stem epidermal layer demonstrated that >90% of the new polyester monomers produced by ectopic coexpression of GPAT5 and CYP86A1 were present in the epidermis (SI Fig. 13).

Changes in Cutin Composition Affect Cuticle Structure and Function. In GPAT5/CYP86A1 double overexpressors, much of the 15- to 20-nm electron-dense outer layer seen by TEM in WT was replaced by a 50- to 70-nm diffuse multilayered structure (Fig. 3*C*, arrow), whereas a normal compact cuticle was observed in the GPAT5 or CYP86A1 single overexpressors (data not shown). These results thus showed that not only the amount and composition but also the organization of the cutin polymer within the cuticle has been modified by coexpression of GPAT5 and CYP86A1. The *GPAT5/CYP86A1* overexpressors however retained normally shaped stomata and the presence of cuticular ledges, as observed by confocal microscopy (data not shown). Thus, structural changes in the cutin polymer alone will not necessarily prevent formation of stomatal ledges. Their absence in *gpat4/gpat8* mutants is therefore likely due to cutin loads dropping below a certain threshold or to changes in critical structural features of the polymer.

Changes in the GPAT5/CYP86A1 double-overexpressor plants were not limited to compositional and structural differences in the cutin layer but also included altered resistance to water loss, a major function of the cuticle. Both initial (5–30 min) and steady state (>30 min) water-loss rates (21) were intermediate between WT and *gpat4/gpat8* double mutants, whereas load and composition of total cuticular waxes were similar to the *GPAT5* or *CYP86A1* single overexpressors (SI Fig. 14). By contrast, resistance to the fungal pathogen *A. brassicicola* was preserved (Fig. 5). Although *GPAT5/CYP86A1* plants were affected in the size and shape of pavement cells (SI Fig. 10) and had increased water loss, this was not detrimental to their vegetative growth (data not shown).

Discussion

GPATs Are Involved in Lipid Polyester Synthesis. In *Arabidopsis*, five of the eight GPATs (including GPAT4 and GPAT5) have been shown to possess a GPAT activity *in vitro* (17). *In vivo* acyl transfer to a glycerol-based acceptor by a GPAT has also been confirmed by ectopic expression of GPAT5 *in planta* (18). Here, using loss- and gain-of-function approaches, we identify GPAT4 as the first acyltransferase involved in cutin synthesis and show that it is functionally redundant with GPAT8. This finding confirms that several members of the *Arabidopsis* GPAT family of acyltransferases are essential for the synthesis of extracellular lipids. Our conclusions are in agreement with the recently published transcriptome analysis of the suberin-rich cork phelloderm, where several homologs of *Arabidopsis* GPATs are highly and specifically expressed (16), and with studies on the WIN1 transcription factor, where GPAT4 is one of the few genes induced coincident to increased cutin biosynthesis (32).

GPATs Are Partners of Fatty Acyl Oxidases. *Arabidopsis* genes have been identified for a long-chain acyl-CoA synthetase (LACS2) (11, 12), a P450 oxidase (CYP86A2, ATT1) (13), and two GPATs (this work), all of which when knocked out individually

(or as a pair in the case of the GPATs) give large reductions in cutin monomer loads and particularly in the C18:2 dicarboxylic acid. Although each of these genes encode a biosynthetic enzyme involved in cutin biosynthesis, the *in vivo* substrates may not be fully understood. In this regard, despite evidence of functional activity on free fatty acids of plant P450 fatty acid ω-oxidases from various sources when expressed in yeast or insect cells (26, 33, 34), the production of ω-hydroxy fatty acids or dicarboxylic acids *in planta* by overexpression of these enzymes (Fig 2*B*) has not previously been reported.

When GPAT4 or GPAT8 is overexpressed, a significant part of the increase in monomer load is the accumulation of C16 and C18 saturated dicarboxylic acids (Fig. 2*A*). These same products are formed when GPAT5 is coexpressed with CYP86A1 but not when either suberin-associated gene is expressed independently. It is clear that GPAT5 is a functioning enzyme in the absence of CYP86A1, in that it results in the accumulation of novel waxes (18), but, unlike GPAT4/8, it apparently cannot function with the resident epidermal P450s to direct increased acyl flux to cutin. Whatever the exact mechanisms of polyester biosynthesis, the two suberin-associated enzymes clearly function together to synthesize both the novel C20 and C22 monomers and maybe to increase endogenous C16- and C18-saturated monomers that were observed in the coexpression experiment (Fig. 2*B*).

Role of Cutin in Cuticle Functions. The loss of cutin monomers in *gpat4*/*gpat8* plants resulted in an increase in cuticle permeability in the presence of normal wax loads. This was consistent with previous observations on the *att1* and *lacs2* cutin mutants and thus confirmed that cutin is important for water barrier function. Because wax is embedded in cutin, the structural organization of the polyester may impact the ability of wax to form an effective barrier. In this regard, the *GPAT5*/*CYP86A1* expressors showed further that despite an 80% increase in cutin monomer content the water barrier function was impaired, suggesting that not only monomer load, but also structural organization is a determinant of the role of cutin in permeability to water.

The increase in susceptibility of *gpat4*/*gpat8* plants to infection by *A. brassicicola* infection demonstrates that normal cutin formation can be essential to prevent infection by a nonhost fungal pathogen and is consistent with the observations that cutinolytic enzymes produced by some fungi during the infection process enhance pathogenicity (1) . The normal resistance to fungi but alteration of water-barrier function that were observed in *GPAT5*/*CYP86A1* overexpressors show that targeted manipulation of acyltransferase and oxidase enzymes involved in lipid polyester metabolism is a useful complement to loss-of-function approaches to probe cutin functions and that water and pathogen barrier functions of the cuticle can be manipulated independently.

In addition to changes in pavement cells (Fig. 3*A* and SI Fig. 10), the range of stomatal alterations observed (Fig. 3 *D–I* and SI Fig. 11) in *gpat4*/*gpat8* might be reasons why the mutant showed a strong increase in water loss and susceptibility to infection by fungi. The loss of cuticular projections that surround stomatal pores has not been previously reported; this finding conclusively demonstrates that these projections depend on cutin for their structure. It also highlights the need of additional studies on the influence of cutinized walls (and possibly associated waxes) on the functions of stomata.

The fact that the suberin-associated enzymes GPAT5 and CYP86A1 are required to function together to produce typical suberin monomers in the transgenic cuticle and that both CYP86A2 and GPAT4/8 are required for endogenous cutin monomer synthesis highlights the existence of conserved features in the biosynthetic machineries of cutin and suberin. Further, the GPAT and ω-oxidase coexpression results indicate that these gene families may best be studied in unison and open a perspective on the production of bifunctional fatty acids in plants. Finally, beyond the value in dissecting fundamental biochemical processes of the epidermis, the synergy between GPATs and fatty acyl oxidases described here provides a tool for manipulating cutin quantity and composition that may provide useful variants in the barrier properties of the plant surface.

Materials and Methods

Plant Materials. *Arabidopsis thaliana* WT (Col-0) and transgenic plants were grown on a soil mixture (1:1:1 peat moss-enriched soil/vermiculite/perlite) in a growth-chamber at 21–22°C, 40–60% humidity, a 16/8-h light/dark cycle and a fluorescent light intensity of 80–100 μmol/m^2/s. To select transformants, surface-sterilized seeds were selected on agar with antibiotics, transferred to soil, and grown as described above.

Isolating Single and Double T-DNA Knockout Mutants. Seeds stocks of T-DNA insertional lines (35) for *GPAT4* (SALK_106893) and *GPAT8* (SALK_095122) were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University (Columbus, OH). Plants homozygous for *GPAT4* or *GPAT8* were screened by using gene-specific primers GP4-tF/GP4-tR or GP8-tF/GP8-tR together with T-DNA left-border primer LBa1 (SI Table 1). To generate plants homozygous for both *GPAT4* and *GPAT8* knockouts, parental *gpat4* and *gpat8* plants were crossed reciprocally. Several double-knockout *gpat4*/gpat8 plants were identified from both crossing events by PCR.

Creating Single and Double Overexpressors. The *35S::GPAT5* construct described in Li *et al.* (18) was used in this study. Genomic DNA sequences encoding the GPAT4, GPAT8, and CYP86A1 proteins were amplified by PCR using primers GP4-cF/GP4-cR, GP8-cF/GP8-cR, and CYP86A1-cF/CYP86A1-cR, respectively (SI Table 1). PCR products were initially cloned into pGEM-T easy vector, and then subcloned as a SmaI-SacI fragment for *GPAT4* and as a XbaI-SacI fragment for *CYP86A1* into binary vector pBI121 to replace the GUS gene. *GPAT8* was inserted as a SalI-EcoRI fragment into the binary vector pCambia1390 (CAMBIA, Canberra, Australia); to this vector, 35S promoter was cloned from pBI121 and inserted as a HindIII-PstI fragment. The constructs (*35S::GPAT5*, *35S::GPAT4*, *35S::GPAT8*, and *35S::CYP86A1*) were introduced into *Agrobacterium tumefaciens* strain C58C1 for *Arabidopsis* (Col-0) vacuum infiltration (36). To achieve cooverexpression of *GPAT5* and *CYP86A1*, cultures of *Agrobacterium* were mixed in a 1:1 ratio. Transgenic plants with both genes inserted were selected by PCR with primer pairs GPAT5-scR/35S-F and CYP86A1-scR/35S-F (SI Table 1).

Microscopy. For SEM to view epidermal surfaces, rosette leaves/stems were fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), dehydrated through a graded ethanol series and processed through a critical point dryer. Samples were mounted onto standard aluminum stubs for SEM, and then sputter coated with ≈30 nm of gold by using an SC-500 sputter coater (Emscope Laboratories, Ashford, U.K.). The images were taken with a 6400V scanning electron microscope (JEOL, Tokyo, Japan). For TEM, small stem/leaf pieces were vacuum infiltrated for 30 min with fixation solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer), followed by overnight fixation in the same solution at 4°C. Samples were postfixed in 1% buffered osmium tetroxide overnight at 4°C and dehydrated in an acetone series 30–100%. They were then infiltrated and polymerized in Poly/Bed resin for 2 days at 60°C. Resin blocks were sectioned by using a Power Tome-XL ultramicrotome (Boeckeler Instruments, Tucson, AZ). Silver–gold sections (≈70 nm thick) were mounted on copper grids and stained with 1% uranyl acetate and 1% lead citrate. Images were taken with a JEOL 100CX transmission electron microscope. For confocal microscopy, leaves were mounted into 5 μg/ml Nile

red solution (Eastman Kodak, Rochester, NY) and viewed immediately with a Zeiss Pascal (Zeiss Microimaging, Thornwood, NY) with a Plan-Apochromat 63×/1.4× oil DIC objective. Samples were excited with 488-nm laser, and emission was detected through a band-pass 560–615 nm filter.

Lipid Analyses. Fatty acid composition of *Arabidopsis* leaves and stems was analyzed after acidic transmethylation according to ref. 37. For polyester analysis, the NaOMe depolymerization and GC-MS analysis method (9) was performed on whole delipidated leaves and stem sections. Depolymerization conditions included the modifications described in ref. 38. Analysis of cuticular waxes was performed as described in ref. 18. Areas of rosette leaves were determined by ImageJ software (http://rsb.info.nih.gov/ij/) by using digital images of flattened leaves quickly blotted on filter paper after wax extraction. For stems, the surface area was estimated by multiplying by 3.14 the measured area of the two dimensional projection of the stems.

Fungal Pathogenicity Assays. *A. brassicicola* (strain MUCL20297) was grown on potato dextrose agar (Difco, St. Louis, MO) at 25°C for 10–14 days. Conidial spores of *A. brassicicola* were collected and resuspended in water and filtered through glass wool. The surface of rosette leaves of 30- to 35-day-old soil-grown plants was inoculated with 10 μl of 1.3×10^6/ml spores in water. Flats containing *A. brassicicola*-challenged plants were kept under a transparent plastic dome to maintain high humidity. Spore count assay of *A. brassicicola* challenged plants was performed 7 days after inoculation according to van Wees *et al.* (39) with slight modifications. Batches of lesions containing leaves were excised and placed in 6 ml of 0.1% Tween 20 in a test tube. After vigorous shaking, the suspension containing fungal spores was moved to a fresh test tube and centrifuged at $5,000 \times g$ for 15 min. Fungal spores in the pellet were resuspended in 200 μl of 0.1% Tween 20 and counted by using a hemocytometer. Spores formed *in planta* were dominant in number and were distinguished from spores used for inoculation based on their colorless appearance.

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