Insertional mutant analysis reveals that long-chain acyl-CoA synthetase 1 (LACS1), but not LACS8, functionally overlaps with LACS9 in Arabidopsis seed oil biosynthesis

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SUMMARY
Triacylglycerols (TAGs) are major storage materials that accumulate in developing seeds and serve as carbon and energy reserves for germination and growth of the seedling. One of the critical reactions in TAG biosynthesis is activation of fatty acyl chains to fatty acyl CoAs, catalyzed by long-chain acyl CoA synthetases (LACSs). Of the nine LACSs identified in Arabidopsis, only LACS9 is known to reside in the plastid, the site of de novo fatty acid synthesis, and is considered the major LACS isoform involved in plastidial fatty acid export for TAG formation. Because the lacs9 null mutant did not show any detectable phenotype, it was hypothesized that at least one additional LACS enzyme must be active in the plastid. Expression analyses to identify potential plastid-localized LACSs involved in TAG biosynthesis revealed that, in addition to LACS9, isoforms LACS1, LACS2, LACS4 and LACS8 are transcribed in the seed. LACS8 showed the highest expression level in the embryo and a high sequence similarity with LACS9, and was therefore characterized further and shown to be associated with the ER, not the plastid. Furthermore, disruption of LACS8 in the lacs8 mutant and lacs8 lacs9 double mutant, and over-expression of LACS8, did not affect the seed fatty acid content. In contrast, 11 and 12% decreases in fatty acid content were detected in lacs1 lacs9 and lacs1 lacs8 lacs9 seeds, respectively, indicating that LACS1 and LACS9 have overlapping functions in TAG biosynthesis. This result is surprising because, unlike LACS9, LACS1 is localized in the ER and has been shown to be involved in cuticular lipid synthesis.

Keywords: triacylglycerols, acyl CoA synthetase, LACS1, LACS8, LACS9, endoplasmic reticulum.

INTRODUCTION
Seed oils, which are comprised almost entirely of triacylglycerols (TAGs), are major storage materials that accumulate in developing embryos of higher plants. They serve as carbon and energy reserves used for germination and growth of the young seedling before photosynthesis is established. TAGs are also of great economic importance in human nutrition, as renewable feed stocks for a wide range of industrial applications, and for biofuel production (Cahoon et al., 2007; Dyer et al., 2008). Given the overall demand for TAGs, understanding factors that limit their accumulation in the seed is an essential first step in an effort to increase TAG content by genetic engineering.

Carbon for TAG biosynthesis in developing oilseeds, such as Arabidopsis thaliana, is derived from sucrose, which is unloaded from the phloem and metabolized to glycolytic intermediates including hexose phosphates, phosphoenolpyruvate and pyruvate (Hills, 2004). Phosphoenolpyruvate is the major substrate imported into the plastid, and is subsequently used for fatty acid synthesis (Baud et al., 2008). These fatty acids are activated to CoA thioesters before export from the plastid, and are used for stepwise acylation of glycerol-3-phosphate in the endoplasmic reticulum (ER) to generate TAGs (Kennedy, 1961; Stymne and Stobart, 1987).

Given that acyl-CoA thioesters are key metabolites required for TAG biosynthesis, we were interested in identifying the enzymes involved in TAG-related acyl-CoA formation, and assessing their contribution to TAG accumulation. Activation of free fatty acids to acyl-CoAs is catalyzed by long-chain acyl-CoA synthetase (LACS) enzymes by a two-step mechanism. First, a free fatty acid reacts with ATP to yield an acyl-AMP intermediate. In the second step, the enzyme bound acyl-AMP intermediate
reacts with CoA to generate acyl-CoA, with release of AMP (Groot et al., 1976). In Arabidopsis, a family of nine genes encodes LACS proteins, all of which are enzymatically active after heterologous expression in *Escherichia coli* (Shockey et al., 2002). Reverse genetic approaches allowed functional characterization of some of these enzymes, and established their roles in various aspects of lipid metabolism. For example, the LACS1 and LACS2 isozymes were reported to be preferentially involved in providing fatty acids for cuticular lipid synthesis (Schnurr et al., 2004; Bessire et al., 2007; Lü et al., 2009; Weng et al., 2010), whereas LACS6 and LACS7 functionally overlap in activating fatty acids for β-oxidation in the peroxisome (Fulda et al., 2002).

Another well-studied member of the LACS family is LACS9, which has been shown to reside in the plastid envelope (Schnurr et al., 2002). Because of its location in the plastid, the site of *de novo* fatty acid synthesis in plants, LACS9 is considered to be the major LACS isoform involved in the production of acyl-CoA for biosynthesis of membrane glycerolipids and storage TAGs. Consequently, LACS9 activity was expected to be essential for normal growth and development of the organism and seed filling. To verify this, a loss-of-function mutation in the LACS9 gene was identified and found to result in a 90% reduction of chloroplast LACS activity. Surprisingly, the *lacs9-1* mutant was indistinguishable from the wild-type in size and appearance, and produced normal-looking seed with wild-type amounts of fatty acids (Schnurr et al., 2002), suggesting that one or more additional LACS isoforms must be active in the plastid. Based on its high sequence similarity to LACS9, and preliminary results from *in vitro* chloroplast assays, LACS8 was predicted to be the most likely candidate for this role.

To test this hypothesis, and determine whether LACS8 and LACS9 jointly contribute acyl-CoAs for storage TAG synthesis, we investigated the subcellular localization of the LACS8 protein, the LACS8 transcript level in the seed, and the TAG content in the *lacs8* single mutant and *lacs8 lacs9* double mutant. Unexpectedly, we found that it is not LACS8 but LACS1, which is known to be the major isoform involved in cuticular lipid formation, that functionally overlaps with LACS9 in TAG biosynthesis.

**RESULTS**

**LACS8 resides in the ER**

Preliminary *in vitro* chloroplast import assays by Schnurr et al. (2002) suggested that LACS8 may co-localize in the chloroplast envelope with LACS9 and catalyze the activation of fatty acids to acyl-CoAs during their export from the plastid. In contrast, localization of Arabidopsis endomembrane system proteins by isotope tagging revealed that LACS8 is probably an ER-resident protein (Dunkley et al., 2006). To distinguish between these two possibilities, the yellow fluorescent protein (YFP) coding sequence was fused to the 5′ end of the LACS8 coding sequence and expressed under the control of the 35S promoter (35S::YFP-LACS8) in wild-type Arabidopsis. Leaves of several transgenic lines with various expression levels of 35S::YFP-LACS8 were analyzed by confocal microscopy. As shown in Figure 1, YFP fluorescence was found in a reticulate network, typical of the ER. The same reticulate network was also marked by hexyl rhodamine B, a dye that stains the ER in plants (Boevink et al., 1996).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** The YFP–LACS8 fusion protein is localized in the endoplasmic reticulum (ER).

(a–c) Confocal micrographs of leaf cells of transgenic plants expressing the 35S::YFP-LACS8 construct. Scale bar = 10 μm.

(a) ER network stained by hexyl rhodamine B.

(b) YFP–LACS8 fluorescence-labeled ER network.

(c) Overlay of images (a) and (b) indicating that the YFP–LACS8 fluorescence is coincident with that of hexyl rhodamine B.
Several LACS genes, including LACS8, are transcribed in the seed

Evidence that LACS8 was not localized to the plastid did not preclude the possibility that it might be involved in supplying acyl-CoAs for TAG biosynthesis. However, for involvement in TAG biosynthesis, it must be expressed in the embryo at the time of TAG accumulation. Data reported by Shockey et al. (2002) obtained by semi-quantitative RT-PCR showed low expression of LACS8 (At2g04350) in the developing seed. To more accurately assess the seed transcription levels of LACS8 during maximal oil deposition at 7 days post-anthesis (DPA), we used real-time PCR. Our results demonstrate that LACS8 is highly and predominantly expressed in the seed (Figure 2). LACS8 therefore appeared to be a good candidate for an enzyme involved in TAG biosynthesis. We also used real-time PCR to evaluate the transcription levels of additional LACS genes previously shown to be expressed in the developing seed (Shockey et al., 2002), and confirmed that besides LACS8, LACS1 (At2g47240), LACS2 (At1g49430), LACS4 (At4g23850) and LACS9 (At1g77850) were also expressed in seed tissues at seven DPA (Figure 2).

Additionally, these LACS genes were transcribed in other plant organs. For example, the highest LACS1 transcript levels were detected in the stem, consistent with LACS1 function in stem wax and cutin biosynthesis (Lü et al., 2009). LACS2 and LACS9 were predominantly transcribed in the floral tissues, and LACS4 was predominantly transcribed in the leaf.

LACS8 is expressed in various organs and in the embryo throughout development

To more precisely define the developmental pattern of LACS8 expression in the embryo and determine the organ specificity of LACS8 expression, a 1667 bp genomic fragment containing the 5’ UTR and part of the coding region of the LACS8 was fused upstream of the uidA reporter gene encoding β-glucuronidase (GUS), and the construct was introduced into wild-type plants. Consistent with the real-time PCR results, histochemical analysis of GUS activity in transgenic plants harboring the LACS8 promoter:uidA construct demonstrated LACS8 transcription in siliques, roots, stems, leaves and flowers, as well as embryos, throughout development (Figure 3). LACS8 transcription in Arabidopsis embryos was verified by in situ hybridization at seven and nine DPA (Figure 3h).

Seed fatty acid content of lacs8 and lacs9 mutants and the lacs8 lacs9 double mutant

To determine whether the LACS8 protein is involved in seed TAG biosynthesis, we obtained several lines with
T-DNA insertions in the LACS8 gene from the Arabidopsis SALK collection (Alonso et al., 2003). Two sets of PCR amplifications were performed for each line, and homozygous mutants with inserts in both homologous chromosomes were identified. The nature of gene disruptions and exact locations of the T-DNA in individual mutant lines were determined by sequencing after PCR amplification of the T-DNA/gene junction (Figure 4a). None of the plants homozygous for lacs8 alleles were distinguishable from wild-type plants in terms of appearance, growth rate, seed filling or germination. The effect of gene disruption on transcript accumulation in each homozygous mutant was examined by RT-PCR assays using total leaf RNA. As shown in Figure 4(b), virtually no LACS8 transcript was detected in the lacs8-1 mutant, so it was chosen for subsequent analyses.

To directly assess whether the LACS8 isoform is involved in seed TAG production, the fatty acid content of mature lacs8-1 seeds, which essentially corresponds to the TAG content in the seeds, was measured by gas chromatography. As shown in Figure 5, the total seed fatty acid content in lacs8-1 was 39.3 ± 0.8% of dry seed weight (mean ± SE, n = 3) and that in the wild-type was 40.0 ± 0.3%. Thus, our data indicate that LACS8 is not a major LACS isoform involved in supplying acyl-CoAs for TAG formation in the Arabidopsis seed. This conclusion is supported by measurements of the seed fatty acid content of additional independent lacs8 mutants (Figure 5).
Similar to our results with the lacs8 mutants, the lacs9-1 loss-of-function line also did not exhibit reduced seed fatty acid content (Schnurr et al., 2002). To verify this, we isolated additional alleles of LACS9 (Figure 4), and selected the lacs9-2, lacs9-3, lacs9-4 and lacs9-5 alleles for seed fatty acid analyses. The seed fatty acid content of all the lacs9 mutants was essentially indistinguishable from that of the wild-type (Figure 5), confirming the previous report (Schnurr et al., 2002) and demonstrating that, despite the greatly reduced LACS9 activity, transport of acyl groups from the plastid to the ER in the mutant is sufficient to support relatively normal levels of seed lipid production.

Even though loss-of-function mutations in either the LACS8 or LACS9 gene did not result in reduced seed fatty acid content, and LACS8 and LACS9 were localized in different cellular compartments, these proteins could have overlapping functions and potentially be involved in generating the ER acyl-CoA pool used for TAG biosynthesis. In this case, in addition to LACS9-mediated export of acyl-CoAs from the plastid, and in the absence of another plastid-localized LACS isoform, an alternative mechanism of fatty acid transport across the plastid envelope would have to exist. These fatty acids would then have to be transferred to the ER where they could be activated by LACS8, and fed into the acyl-CoA pool. To determine whether LACS8 and LACS9 have such overlapping functions in seed TAG production, and whether the absence of a fatty acid phenotype in the lacs8 mutant was due to compensatory activity of LACS9 and vice versa, we crossed lacs8-1 and lacs9-2 null alleles, and identified homozygous double mutants in the resulting F2 population. Seed fatty acid analyses of multiple lacs8-1 lacs9-2 individuals did not indicate a more pronounced reduction in fatty acid levels than those found in either parent (Figure 5). We therefore conclude that LACS8 and LACS9 do not have overlapping functions in the ER acyl-CoA pool formation for TAG biosynthesis.

Over-expression of the LACS8 gene in the seed does not affect fatty acid content

As the loss-of-function mutation in LACS8 did not result in a seed fatty acid phenotype, we decided to independently assess the potential contribution of LACS8 to seed storage TAG synthesis by over-expressing the LACS8 gene under the control of the seed-specific FAE1 promoter (Rossak et al., 2001) in wild-type plants. Seed-specific over-expression of LACS8 did not lead to increased fatty acid content or any alteration in the fatty acid composition of transgenic lines in the T2 generation (data not shown). In several lines, introduction of the FAE1promoter:LACS8 transgene actually resulted in decreased TAG accumulation, probably as a result of co-suppression, even though this has not been confirmed by expression analyses of transgenes in these lines. Re-evaluation of seed oil content in subsequent generations (T3 and T4; Figure 6) confirmed that the fatty acid content of the FAE1promoter:LACS8 transgenic lines was not significantly higher than that of the wild-type, indicating that LACS8 activity is not a limiting factor for TAG biosynthesis.

LACS1 is localized in the ER and functionally overlaps with LACS9 to supply acyl-CoAs for TAG biosynthesis

Real-time PCR analysis showed that, in addition to LACS8 and LACS9, the LACS1 gene is also highly expressed in oil-accumulating 7-day-old seeds (Figure 2). Subcellular localization of YFP-tagged LACS1 transiently expressed in Arabidopsis leaf protoplasts (data not shown), and in transgenic plants carrying the 35S:YFP-LACS1 or 3SS:LACS1-YFP transgenes, revealed that the LACS1 protein is uniformly localized in the ER (Figure 7). LACS1 has been shown to participate in stem and leaf wax and cutin biosynthesis (Lü et al., 2009; Weng et al., 2010), but we wished to determine whether it is also involved in provision of acyl-CoA moieties for seed TAG production. For this purpose, we obtained eight SALK T-DNA insertion lines (Alonso et al., 2003) from the Arabidopsis Biological Resource Center (ABRC), and analyzed them for disruption of the LACS1 gene using PCR.

Although T-DNA was detected in all the lacs1 mutant alleles (Figure 8), sequencing of the T-DNA/gene junctions revealed errors in the reported location of the T-DNA in two of the alleles, lacs1-3 and lacs1-4. In the lacs1-3 mutant (SALK_138782), T-DNA is inserted in the 9th exon rather than the 9th intron, whereas the lacs1-4 mutant (SALK_142182) has an insertion in the 13th intron rather than the 1st intron as indicated in TAIR (Figure 8a). The extent of gene disruption in these T-DNA mutants was examined by RT-PCR using total leaf RNA. Full-length LACS1 transcript was identified in the
Analyses of mature seeds of several double and triple mutant individuals by GC showed that the lacs1 single and lacs1 lacs8 double mutants had near wild-type fatty acid content (Figure 9). In contrast, the lacs1 lacs9 individuals showed an 11% reduction and the lacs1 lacs8 lacs9 triple mutants showed a 12% reduction in seed fatty acid content (Figure 9). These results indicate that, although there does not appear to be any functional redundancy between LACS1 and LACS8 in providing acyl-CoAs for TAG biosynthesis, the LACS1 and LACS9 isozymes apparently do have overlapping functions in this process.

Analyses of seed fatty acid compositions of the lacs1 lacs9 and lacs1 lacs8 lacs9 mutant lines revealed reduced amounts of C16:0, C18:0, C18:1 and C18:2 acyl groups (Table S2) and relatively normal amounts of the seed-specific very-long-chain fatty acyl groups (C26–C32). These data correlate well with the reported substrate preferences of LACS1 and LACS8 for the C16:0, C18:0, C18:1 and C18:2 fatty acids, and the low affinities of these LACS enzymes for C20:1, the most abundant very-long-chain fatty acid in Arabidopsis seeds (Shockey et al., 2002; Luk et al., 2009). We detected much greater than wild-type amounts of very-long-chain fatty acids in the stem cuticular wax of the lacs1 lacs9 double and lacs1 lacs8 lacs9 triple mutant lines, as well as the lacs1 mutant, as previously reported by Luk et al. (2009) (Table S1). These C26–C30 very-long-chain fatty acids are believed to be free fatty acids, which, in the absence of functional LACS1, which has high specificity for C26–C30 acyl groups (Luk et al., 2009), cannot be activated to fatty acyl-CoAs and used for cuticular wax biosynthesis.

**DISCUSSION**

The absence of a detectable phenotype in the lacs9-1 loss-of-function mutant, which is disrupted in the major plastidial LACS isoform, gave rise to the hypothesis that at least one additional LACS protein must reside in this cellular compartment. LACS8, which shares 67% amino acid identity and has similar substrate specificity to LACS9 (Shockey et al., 2002), was suggested as the most likely candidate (Schnurr et al., 2002). Our objective was to investigate whether LACS8 is indeed localized in the plastid and to determine whether it functionally overlaps with LACS9 to provide acyl-CoAs for TAG formation in the Arabidopsis seed.

To determine the subcellular localization of LACS8, double labeling experiments were performed using YFP-tagged LACS8 together with organelle-specific markers in transgenic plants. They revealed that YFP-LACS8 co-localizes with hexyl rhodamine B, a dye that stains the ER in plants (Boevink et al., 1996). Thus, unlike LACS9, which is localized to the plastid, LACS8 operates in the ER. Localization to the ER made it clear that LACS8 was not involved in acyl-CoA formation associated with fatty acid export from the plastid. However, despite being located in different cellular compartments, LACS8 and LACS9 could still have overlapping functions.
roles in generating the ER acyl-CoA pool required for seed TAG biosynthesis. In support of this possibility, LACS8 and LACS9 were among five LACS isoforms found to be expressed in Arabidopsis seed during maximal oil deposition at seven DPA.

To assess the contribution of LACS8 to seed TAG production, and re-evaluate the potential involvement of LACS9 in this process, we identified lacs8 insertional mutants and additional lacs9 alleles, and generated lacs8 lacs9 double mutants. As previously reported for the lacs9-1 allele (Schnurr et al., 2002), the seed fatty acid content of single lacs8 mutant lines was essentially unchanged in comparison to the wild-type, indicating that LACS8 does not play a major role in supplying activated acyl groups for seed TAG biosynthesis. Over-expression of LACS8 in the seed did not result in increased fatty acid accumulation, providing additional evidence that LACS8 activity is not a determining factor in this process. There was also no major reduction in the seed fatty acid content of the lacs8 lacs9 double mutant, an indication that either these two LACS enzymes do not have overlapping functions in TAG production, or that additional LACS family members contribute to the TAG-related acyl-CoA formation and compensate for the loss of LACS8 and LACS9 activity.

To distinguish between these two alternatives, we decided to investigate the effect of disruption in an additional LACS isoform on seed fatty acid content. Due to its relatively high expression levels in the seed, we chose LACS1 as the best candidate, even though this enzyme was recently shown to be involved in cuticular lipid biosynthesis (Lü et al., 2009). Expression of 3SS:YFP-LACS1 in transgenic plants resulted in a uniform distribution of YFP fluorescence in the ER, and demonstrated that, just like LACS8, LACS1 associates with ER membranes, the site of TAG biosynthesis. However, phenotypic analyses of lacs1 mutant seed and lacs1 lacs8 double mutant seed did not reveal any deficiencies in fatty acid accumulation. In contrast, the lacs1 lacs9 double mutant and the lacs1 lacs8 lacs9 triple mutant

Figure 7. The YFP-LACS1 protein is localized in the ER.
(a–d) Confocal micrograph of leaf epidermal cells of transgenic Arabidopsis plants expressing the 3SS:YFP-LACS1 construct. Scale bar = 10 μm.
(a, c) YFP-LACS1 fluorescence-labeled ER network.
(b) ER network stained by hexyl rhodamine B.
(d) Overlay of images (b) and (c), indicating that the YFP-LACS1 fluorescence is coincident with that of hexyl rhodamine B.

Figure 8. Mutant alleles of the LACS1 gene.
(a) The locations of T-DNA insertions in the LACS1 gene were mapped and are indicated by vertical arrows above the gene. Black boxes represent exons, gray boxes represent untranslated regions, and solid lines represent introns and intergenic regions. P1, P2 and P3 indicate the primers used for RT-PCR analyses.
(b) RT-PCR analyses of steady-state LACS1 transcript levels in mutant and wild-type leaves. RT-PCR was performed using total leaf RNA, and the expression level of GAPC was used as a control.
showed pronounced reductions in seed fatty acid content. Collectively, these results support the conclusion that there is no functional redundancy between LACS1 and LACS8 proteins, but the LACS1 and LACS9 isozymes both contribute acyl-CoAs for TAG biosynthesis. Thus, in addition to its significant role in cuticular lipid biosynthesis in the epidermis, LACS1 also participates in TAG biosynthesis in Arabidopsis seeds. The finding that loss-of-function mutations in LACS1 and LACS9 do not completely abolish TAG accumulation in the lacs1 lacs9 double mutant also implies that at least one additional LACS isozyme is still active in the seed. Based on our expression data, the best candidate is LACS4, and we have initiated a reverse genetic approach to investigate this possibility.

The failure to identify an additional plastid-localized LACS isoform that is functionally redundant with LACS9 raises the possibility that there may be an additional pathway for fatty acid export from the plastid in addition to the LACS-mediated pathway. Two pathways for fatty acid import into the peroxisome are well documented in yeast (Saccharomyces cerevisiae), in which the acyl-CoA synthetase Faa2p acts in parallel with the Pxa1p/Pxa2p ABC transporter (Hettema et al., 1996). Such pathways, involving LACS6/LACS7 proteins and the PxA1 ABC transporter, have also been suggested for Arabidopsis peroxisomes (Fulda et al., 2004). Even though there is currently no evidence for the existence of two parallel pathways for fatty acid export from the plastid, this type of organization would be beneficial, if not essential. It would allow greater export efficiency at times of accelerated membrane and/or TAG biosynthesis, and provide the cell with necessary acyl groups when the parallel export pathway fails.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

SALK T-DNA insertion lines, lacs8-1 (SALK_136060), lacs8-2 (SALK_105118), lacs8-3 (SALK_016704), lacs8-4 (SALK_047776), lacs9-2 (SALK_11135), lacs9-3 (SALK_053069), lacs9-4 (SALK_095484), lacs9-5 (SALK_113248), lacs9-6 (SALK_102518), lacs9-7 (SALK_040810), lacs9-8 (SALK_124615), lacs1-1 (SALK_127191), lacs1-2 (SALK_039128), lacs1-3 (SALK_138782), lacs1-4 (SALK_142182), lacs1-5 (SALK_142847), lacs1-6 (SALK_104847) and lacs1-7 (SALK_091414) (all Col-0 ecotype) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). Homozygous lines for each gene were identified by genotyping using primers provided by the SALK T-DNA primer design program (http://signal.salk.edu/tdnaprimers2.html). Double and triple mutants were obtained by crossing homozygous lines. Arabidopsis plants were transformed using the floral-dip method (Clough and Bent, 1998). Harvested seeds were sterilized and then germinated on AT medium (Somerville and Ogren, 1982) supplemented with agar (7 g L⁻¹) and appropriate antibiotics. Ten-day-old seedlings were transplanted to soil (Sunshine Mix 5, SunGro; http://www.sungro.com) and grown under continuous light (100–120 µE m⁻² sec⁻¹) of photosynthetically active radiation at 20°C until maturity.

**Plasmid constructs**

A 1667 bp long region that includes 1069 bp immediately upstream of the LACS8 translation start site (including all promoter regulatory sequences between the ATG and the gene upstream of LACS8) and 598 bp downstream of the LACS8 translation start site (including parts of the second and third exon and the second intron), hereafter referred to as the LACS8 promoter, was amplified from genomic DNA using gene-specific primers and high-fidelity Pfx polymerase (Invitrogen, http://www.invitrogen.com/). The forward primer 5’-GGATCCACATCCCTGGAAATTTACAACACCAAC-3’ and the reverse primer 5’-GAGGAGTGACGGCGCAGGTTAACAACCCTGTATTAACATC-3’ contained a SacII restriction enzyme cutting site (underlined) the reverse primer 5’-GCGGATCATCCATCCTGGAAAAATTACAAACACCAAC-3’ contained a BamHI cutting site (underlined). The reverse primer spans 21 bp of the second intron and 6 bp of the third exon, so that the resulting GUS fusion construct contains the whole ORF of the second exon starting with ATG and 6 bp of the third exon fused in-frame with the GUS coding sequence. Amplification was performed in a 50 µl reaction under the following conditions: initial denaturation at 94°C for 2 min and 30 sec, followed by 34 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 68°C for 1 min, then a final extension at 68°C for 5 min. The genomic fragment and the binary vector pH101.1 (Clontech, http://www.clontech.com/ CA, USA) were both digested with SalI and BamHI (Invitrogen). Purified vector and insert were ligated using T4 DNA ligase (Invitrogen) at room temperature overnight. The ligation product was transformed into chemically competent E. coli cells (DH5α) and selected based on resistance to kanamycin (50 mg L⁻¹). Insertion of the fragment was verified by colony PCR and test digestion. Sequencing confirmed that the PCR product corresponds to the region between −1069 and +598 bp relative to the LACS8 translational start point in the Arabidopsis database.

The coding sequence of the LACS8 gene used to generate the YFP–LACS8 fusion for determination of subcellular localization was obtained from leaf cDNA using forward primer 5’-attB1...
(5'-GGGGACAGGTGATCAAAAGCCAGCTTGCAGGAGGTAGATCTGAGTTGAGACCCATTGCAATAG-3') and reverse primer 5'-artB2 (5'-GGGGACACCTTGCAGGAGGTAGATCTGAGTTGAGACCCATTGCAATAG-3') were used in the PCR product was introduced into the pDONR207 entry vector (Invitrogen) using BP Clonase II enzyme mix (Invitrogen), and transformed into chemically competent E. coli cells (DH5α). Following selection on plates containing gentamycin (25 mg ml⁻¹), transfected colonies were re-precipitated. By adding DNase I (Invitrogen) to each sample, diethylpyrocarbonate (DEPC)-treated water and the sample was acetate (pH 5.2) was added after the RNA had been dissolved in the LACS2 coding sequence was amplified from leaf cDNA using a forward primer (5'-GCTCTAAGATGGAAGATCTGCTGAGTTGAGACCCATTGCAATAG-3') containing an XbaI cutting site (underlined) and a reverse primer (5'-GGGTAGATCTGAGTTGAGACCCATTGCAATAG-3') containing an SphiI cutting site (underlined). The LACS2 fragment was introduced into the pBluescript II KS(+) vector (Alding-Mees et al., 1992) before the FAE1 promoter. Then the whole FAE1 promoter:LACS2 cassette was excised using SphiI and Sph restriction enzymes (Invitrogen), producing a blunt end and a sticky end, respectively, and ligated into binary vector pCAMBIA 1380 (Deblaere et al., 1997) digested using SphiI and Smal (Invitrogen), which also created a blunt end and a sticky end, respectively.

All the binary vectors described above were introduced into Agrobacterium tumefaciens GV3101 (pMP90) cells (Koncz and Schell, 1986) and used for transformation of Arabidopsis inflorescences (Bechtold et al., 1993).

RNA isolation, RT-PCR and real-time PCR

Rosette leaves, whole stems, unopened flower buds, seven DPA seeds, 14-day-old seedlings and whole roots of wild-type Arabidopsis (Col-0 ecotype) and homozygous SALK T-DNA insertional lines, resulting in truncated mRNA. Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) was used to perform real-time PCR in an MJ Mini Opticon Personal Thermal Cycler (Bio-Rad Laboratories, http://www.bio-rad.com/) as specified by the manufacturer.

GUS histochemical assays and RNA in situ hybridization

Tissues from transgenic plants containing the LACS8promoter:uidA construct were removed and immersed in GUS staining buffer containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 100 mM NaHPO₄, 100 mM NaH₂PO₄, 0.2% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid (X-gluc) (Jefferson, 1987). Air was eliminated from the tissues by vacuum infiltration. Infiltrated tissues were incubated in the staining buffer for 4 h, or overnight. Stem and leaf tissues were cleared of chlorophyll by overnight incubation in a clearing solution (72% chloral hydrate, 11% glycerol, 17% water). Stained and cleared samples were examined visually either directly or under a dissecting microscope or compound light microscope.

In situ hybridization of Arabidopsis embryos (tissue fixation, sectioning, hybridization, signal detection and probe synthesis) was performed as described previously (Hooker et al., 2002; Hepworth et al., 2005). To synthesize the probes, DNA templates were amplified by PCR from the pDONR207-LACS8 construct containing LACS8cDNA using primers that add a T7 RNA polymerase binding site at the 5' end. For the sense probe, the primers used were 5'-CATATAACGACTCATAATGAGTGGAAGATCTGCTGAGTTGAGACCCATTGCAATAG-3' and 5'-GCTCTAGACCTTGCAGGAGGTAGATCTGAGTTGAGACCCATTGCAATAG-3'. For the antisense probe, the primers used were 5’-CATATAACGACTCATAATGAGTGGAAGATCTGCTGAGTTGAGACCCATTGCAATAG-3' and 5’-CATATAACGACTCATAATGAGTGGAAGATCTGCTGAGTTGAGACCCATTGCAATAG-3'.

Confocal microscopy

Leaves of Arabidopsis transformants containing the YFP-LACS8 transgene were immersed in hexyl rhodamine B solution (1.6 μM) for 10-30 min. YFP and hexyl rhodamine B fluorescence were examined using a Zeiss Pascal Excite laser scanning confocal microscope (http://www.zeiss.com/). A 488 nm excitation wavelength with the emission filter set at 500-530 nm were used for YFP.

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<th>Forward primers</th>
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<tr>
<td>LACS1(1)</td>
<td>5'-AAACACCAAGTCTTCTGATCTATAAACTA-3'</td>
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<tr>
<td>LACS1(2)</td>
<td>5'-ATACTCCTCGATAGATTTTCT-3'</td>
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<td>LACS1(3)</td>
<td>5'-CTGTTACCTTCTTCTGAGAA-3'</td>
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<td>LACS2</td>
<td>5'-TTGACCCGCGATTAGTGGTGTGTG-3'</td>
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<td>LACS4</td>
<td>5'-CGAGATCTTCTCTCAGCGGACCCCTTCT-3'</td>
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<tr>
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<td>LACS8(2)</td>
<td>5'-CTGTTACCTTCTGAGAGAAGATCTGCTGAGTTGAGACCCATTGCAATAG-3'</td>
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</tr>
<tr>
<td>GACP</td>
<td>5'-ATCTGAGAGAAGCTGCTGAC-3'</td>
</tr>
</tbody>
</table>
A 543 nm argon ion laser line and a 600 nm long-pass emission filter were used to excite hexyl rhodamine B. All confocal images obtained were processed using ImageJ (http://rsb.info.nih.gov/ij) and Adobe Photoshop 5.0 (http://www.adobe.com/).

**Seed fatty acid analysis**

Approximately 2.5 mg of dry seeds from each line were weighed and transferred into 1 × 10 cm glass tubes (pre-washed with chloroform and dried) with Teflon screw caps. Then 1 ml of freshly prepared 5% v/v concentrated sulfuric acid in methanol, 25 µl BHT solution (0.2% w/v butylated hydroxyl toluene in methanol) and 300 µl of toluene with internal standard (triheptadecanoin, 12.5 µg/300 µl) were added to each tube. All the tubes were then vortexed for 30 sec and heated at 90°C for 2 h. Then 1.5 ml 0.9% w/v NaCl were added to each sample after cooling on ice. Fatty acid methyl esters from each tube were extracted twice with 2 ml hexane, evaporated under N₂, dissolved in 50 µl hexane and transferred to glass vials. Fatty acid methyl esters were separated by gasliquid chromatography as described previously (Kunst et al., 1992).

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1:** Phenotypes of wild-type Arabidopsis and lacs1 mutant showing reduced cuticular wax load on the stem surface and reduced fertility.

**Table S1:** Cuticular wax composition of 25-day old stems of Arabidopsis Col-0 and lacs1-3, lacs1 lacs8, lacs1 lacs9 and lacs1 lacs8 lacs9 mutant lines.  

**Table S2:** Fatty acid composition of dry seeds of Arabidopsis Col-0, lacs1-3, lacs1 lacs9 and lacs1 lacs8 lacs9 mutant lines.  

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