the plant journal

SEB Society for Experimental Biology

The Plant Journal (2010) **64**, 1048–1058 doi: 10.1111/j.1365-313X.2010.04396.x

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

Lifang Zhao, Vesna Katavic, Fengling Li, George W. Haughn and Ljerka Kunst*

Department of Botany, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

Received 9 August 2010; revised 27 September 2010; accepted 5 October 2010; published online 15 November 2010.
*For correspondence (fax +1 604 822 6089; e-mail kunst@interchange.ubc.ca).

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).

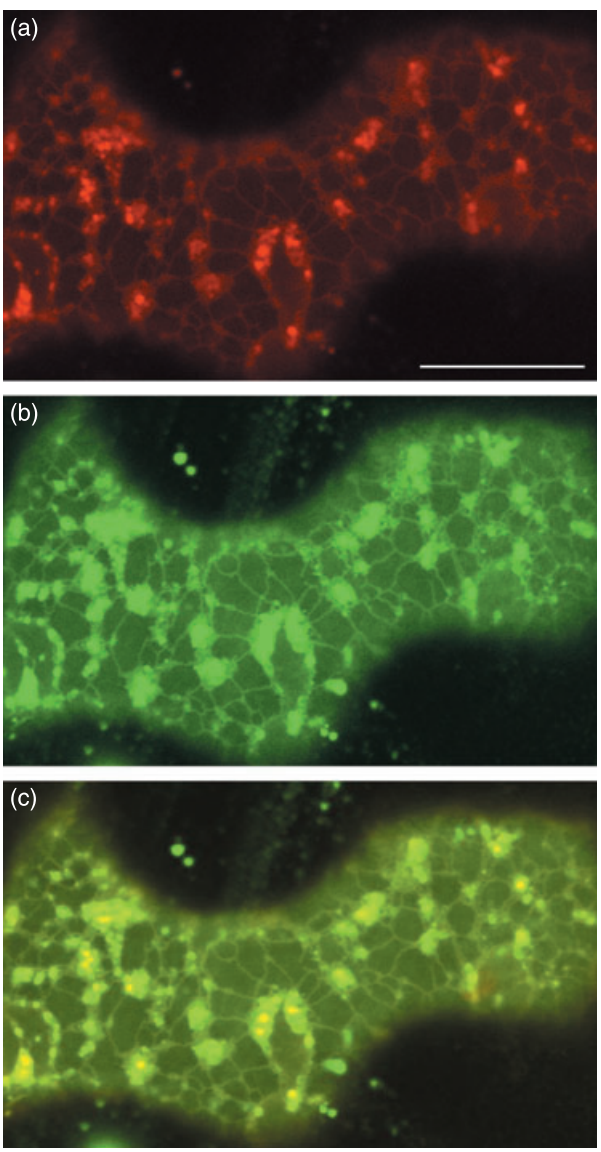

Figure 1. The YFP–LACS8 fusion protein is localized in the endoplasmic reticulum (ER).
(a–c) Confocal micrographs of leaf cells of transgenic plants expressing the *35S:YFP-LACS8* construct. Scale bar = 10 μm.
(a) ER network stained by hexyl rhodamine B.
(b) YFP–LACS8 fluorescence-labeled ER network.
(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 *LACS8promoter: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

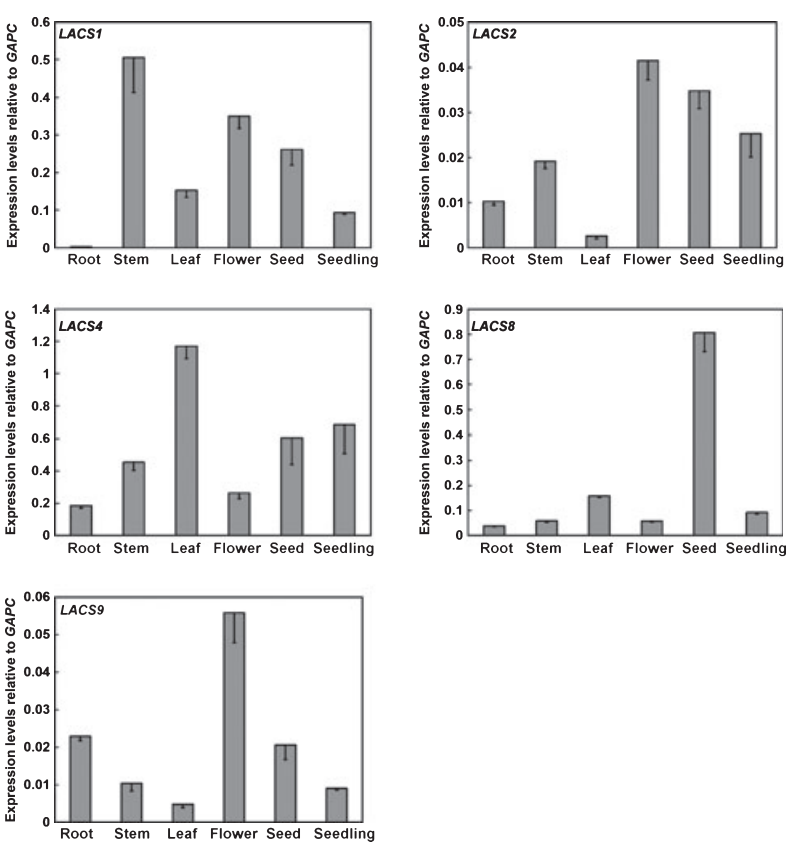

Figure 2. Comparison of organ-specific expression patterns of *LACS* genes.
Aliquots of total RNA from each organ were analyzed for expression of each *LACS* gene, as well as *GAPC* as a control, by quantitative real-time PCR using gene-specific primer pairs. *LACS* gene expression values were normalized to the *GAPC* expression values. Developing seeds were collected at 7 days post-anthesis (DPA). Roots and seedlings were 3 weeks old. Error bars represent SE (n = 3).

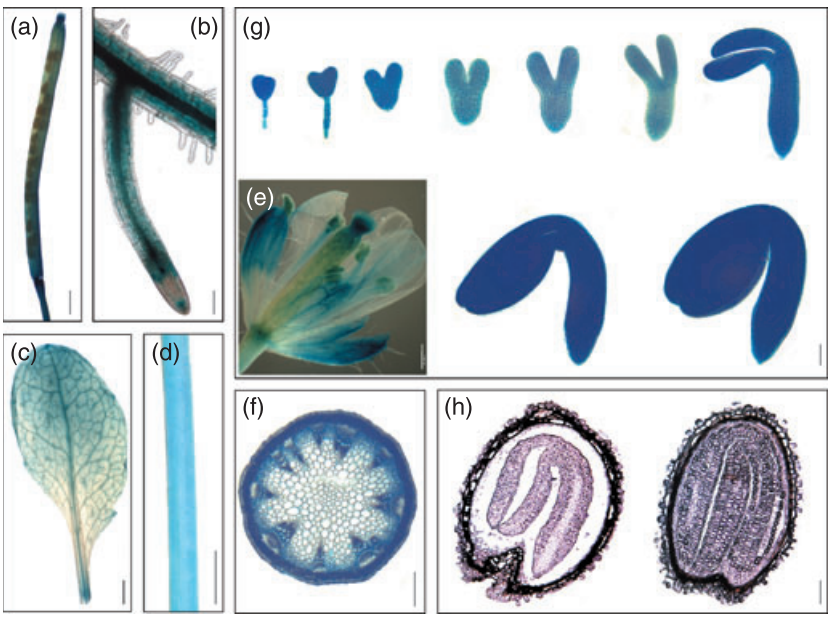

Figure 3. Organ-specific expression pattern of *LACS8* in Arabidopsis.
(a–g) Arabidopsis plants expressing the *uidA* reporter gene encoding β-glucuronidase (GUS) under the control of the *LACS8* promoter. Tissues were incubated in X-gluc assay buffer. GUS activity is indicated by a blue precipitate. (a) Silique. (b) Root. (c) Rosette leaf. (d) Top 3 cm of the stem. (e) Flower. (f) Stem cross-section. (g) Developing embryos from 3 to 9 DPA.
(h) Expression of *LACS8* in the developing seed as detected by *in situ* hybridization at seven and nine DPA.
Scale bars = 1 mm (a, c, d, e), 50 μm (b), 0.1 mm (f, g).

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).

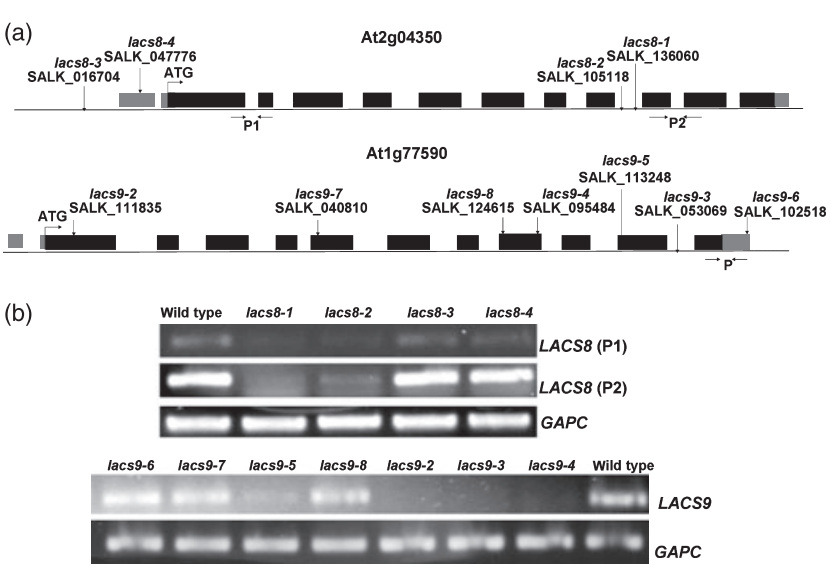

Figure 4. Mutant alleles of *LACS8* and *LACS9* genes.
(a) The locations of T-DNA insertions in *LACS8* and *LACS9* genes were mapped and are indicated by vertical arrows above the genes. Black boxes represent exons, gray boxes represent untranslated regions, and solid lines represent introns and intergenic regions. P1, P2 and P indicate the sets of primers used for RT-PCR.
(b) RT-PCR analyses of steady-state *LACS8* and *LACS9* 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. Two sets of primers were used to assess the transcript abundance of *LACS8* alleles: *LACS8* data were generated using a set of primers from the end of the 2nd exon to the start of the 3rd exon (P1) or a set of primers spanning the region from the 10th to the 11th exon (P2). *LACS9* data were generated using a single set of primers (P).

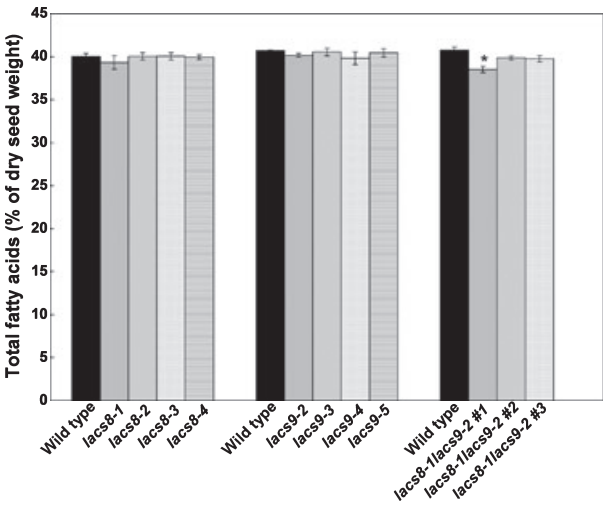

Figure 5. Comparisons of seed fatty acid content between single and double loss-of-function mutants and their corresponding wild-types.
Data are expressed as mean percentages ± SE (n = 3; replicate analyses performed on seed lots from each line, with 120–140 seeds analyzed per replicate). Student's t-test was applied to the data; the asterisk indicates that this value was statistically significantly different from wild-type at $P < 0.05$.

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 F_2 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 T_2 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 (T_3 and T_4; 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 *35S: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

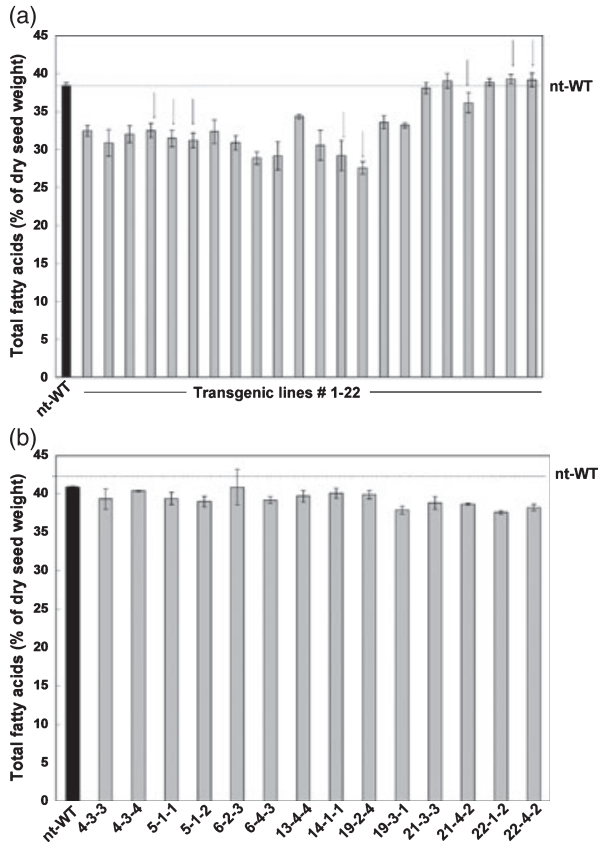

Figure 6. Fatty acid content of T_3 and T_4 seeds expressing the *FAE1p:LACS8* construct.
(a) Seed fatty acid content of independent T_3 lines. Error bars represent SD calculated from four batches of seeds from the same transgenic line. The black bar indicates the oil content of non-transformed wild-type (nt-WT) plants. Homozygous plants used for GC analysis in the T_4 generation were from the lines labeled with arrows.
(b) Fatty acid content of 14 homozygous T_4 plants. The first part of each line number indicates which transgenic T_3 line each T_4 line originated from. Error bars indicate SD (n = 3).

wild-type, and very low transcript levels were detected in *lacs1-6* and *lacs1-7* mutants, but not in *lacs1-1*, *lacs1-2*, *lacs1-3*, *lacs1-4* or *lacs1-5*, indicating that the latter are null alleles (Figure 8b). All these alleles displayed bright green glossy stems, an indication of reduced cuticular wax load on the stem surface (Figure S1). In addition, *lacs1-1*, *lacs1-2*, *lacs1-3* and *lacs1-4* also had small siliques, a sign of reduced fertility. Both these phenotypes are associated with loss-of-function mutations in genes involved in cuticular wax biosynthesis (Koornneef *et al.*, 1989). We used the *lacs1-3* mutant allele for crosses with the *lacs8-1*, *lacs 9-2* and *lacs8-1 lacs9-2* to generate double and triple mutants. The *lacs1 lacs8* and *lacs1 lacs9* double mutants and the *lacs1 lacs8 lacs9* triple mutant had reduced amounts of stem cuticular wax (Table S1) and low seed set phenotypes, similar to the *lacs1* single mutants, but were otherwise indistinguishable in appearance from the wild-type.

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 $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ acyl groups (Table S2) and relatively normal amounts of the seed-specific very-long-chain fatty acyl groups (C_{20}–C_{22}). These data correlate well with the reported substrate preferences of LACS1 and LACS9 for the $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ fatty acids, and the low affinities of these LACS enzymes for $C_{20:1}$, the most abundant very-long-chain fatty acid in Arabidopsis seeds (Shockey *et al.*, 2002; Lü *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 Lü *et al.* (2009) (Table S1). These C_{26}–C_{30} very-long-chain fatty acids are believed to be free fatty acids, which, in the absence of functional LACS1, which has high specificity for C_{26}–C_{30} acyl groups (Lü *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

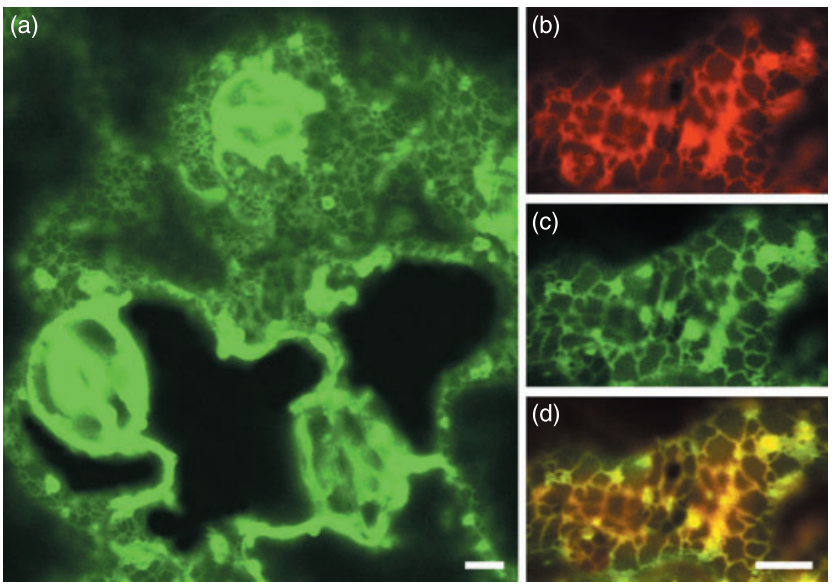

Figure 7. The YFP:LACS1 protein is localized in the ER.
(a–d) Confocal micrograph of leaf epidermal cells of transgenic Arabidopsis plants expressing the *35S:YFP-LACS1* construct. Scale bar = 10 μm.
(a, c) YFP–LACS1 fluorescence-labeled ER network.
(b) ER network stained by hexyl rhodamine B.
(d) Overlay of images (b) and (c), indicating that the YFP–LACS1 fluorescence is coincident with that of hexyl rhodamine B.

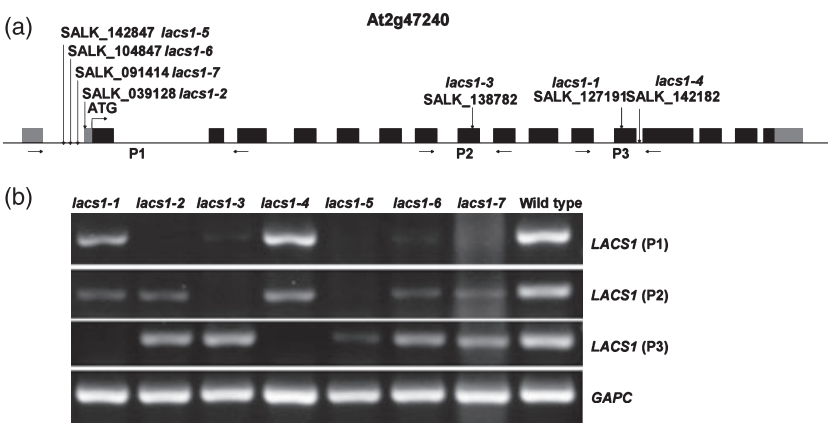

Figure 8. Mutant alleles of the *LACS1* gene.
(a) The locations of T-DNA insertions in the *LACS1* gene were mapped and are indicated by vertical arrows above the gene. Black boxes represent exons, gray boxes represent untranslated regions, and solid lines represent introns and intergenic regions. P1, 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.

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 *35S: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 production. 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

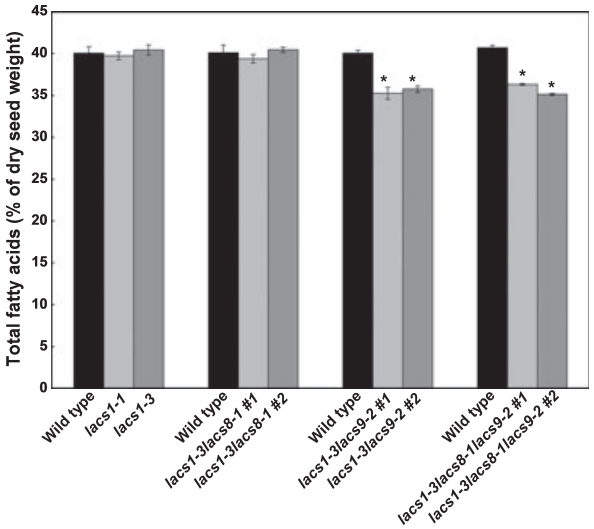

Figure 9. Comparisons of seed fatty acid content between single, double and triple mutants and their corresponding wild-types.
Data are expressed as mean percentages ± SE ($n = 3$ replicate analyses performed on seed lots from each line, with 120–140 seeds analyzed per replicate). Student's *t*-test was applied to the data; asterisks indicate values that were statistically significantly different from wild-type at $P < 0.05$.

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 insertional lines, *lacs8-1* (SALK_136060), *lacs8-2* (SALK_105118), *lacs8-3* (SALK_016704), *lacs8-4* (SALK_047776), *lacs9-2* (SALK_111835), *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/tdnaprimers.2.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′-GAGGTCGACCGCAAGGTAAACCGCTCTATTAAATC-3′) contained a *Sal*I restriction enzyme cutting site (underlined) the reverse primer (5′-GCGGATCCACATCCCTGGAAATTTACAACACCAAC-3′) contained a *Bam*HI 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 pBI101.1 (Clontech, http://www.clontech.com/ CA, USA) were both digested with *Sal*I and *Bam*HI (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′-*att*B1

(5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAGATTCTG GAGTGAATCCAATGGA-3′) and reverse primer 5′-*att*B2 (5′-GGG GACCACTTTGTACAAGAAAGCTGGGTCGGCATATAACTTGCTGAG TTCATCTTTGAA-3′), and high-fidelity *Pfx* polymerase (Invitrogen). 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}), sequencing of an individual clone confirmed that there were no errors in the *LACS8* coding sequence. The insert was then transferred to the binary vectors pEarleyGate 101 (C-YFP–HA) (Earley *et al.*, 2006) using LP Clonase™ II enzyme mix (Invitrogen) to obtain translational fusions between the *YFP* coding sequence and the *LACS8* coding sequence. Transformed colonies were selected on LB medium containing kanamycin (50 mg ml^{-1}).

To over-express *LACS8* in a seed-specific manner, the *LACS8* coding sequence was amplified from leaf cDNA using a forward primer (5′-GCTCTAGAATGGAAGATTCTGGAGTGAATCCAA-3′) containing an *Xba*I cutting site (underlined) and a reverse primer (5′-GCGTCGACTTAGGCATATAACTTGCTGAGTTCA-3′) containing an *Sal*I cutting site (underlined). The *LACS8* fragment was introduced into the pBluescript II KS(+) vector (Alting-Mees *et al.*, 1992) behind the *FAE1* promoter. Then the whole *FAE1* promoter:*LACS8* cassette was excised using *Sal*I and *Ssp*I restriction enzymes (Invitrogen), producing a blunt end and a sticky end, respectively, and ligated into binary vector pCAMBIA 1380 (Deblaere *et al.*, 1987) digested using *Sal*I and *Sma*I (Invitrogen), which also created a blunt end and a sticky end, respectively.

All the binary vectors described above were introduced into competent *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 mutants were collected and frozen immediately using liquid nitrogen. With the exception of developing green seeds, total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Because developing seeds usually contain a high level of polysaccharides, a method involving acid phenol/LiCl (Downing *et al.*, 1992) was used to isolate RNA from green seeds. To remove excess polysaccharides from the RNA sample, 3 M sodium acetate (pH 5.2) was added after the RNA had been dissolved in diethylpyrocarbonate (DEPC)-treated water and the sample was re-precipitated. By adding DNase I (Invitrogen) to each sample, possible residual DNA in the RNA sample was removed. For reverse transcription, 1–5 μg of total RNA, oligo(dT) and SuperScript II reverse transcriptase (Invitrogen) were mixed to synthesize first-strand cDNA as specified by the manufacturer. Amplification of a specific region overlapping two exons of each *LACS* gene or glyceraldehyde-3-phosphate dehydrogenase C (*GAPC*) for expression analysis by RT-PCR or real-time PCR was performed using the primers listed in Table 1.

Three pairs of primers amplifying various domains of *LACS1*, and two pairs of primers amplifying various domains of *LACS8* were used to demonstrate that transcription of these genes was impaired in the 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 Na_2HPO_4, 100 mM NaH_2PO_4, 0.2% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (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 perfomed 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 *LACS8* cDNA using primers that add a T7 RNA polymerase binding site at the 5′ end. For the sense probe, the primers used were 5′-CATAATACGACTCACTATAGGATGGAAGATTCTGGAGTGA-3′ and 5′-GCGTCGACTTAGGCATATAACTTGCTGAGTTCA-3′. For the antisense probe, the primers used were 5′-GCTCTAGAATGG AAGATTCTGGAGTGAATCCAA-3′ and 5′-CATAATACGACTCACTAT AGGTTAGGCATATAACTTGCT-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.

Table 1 Primers used for RT-PCR or real-time PCR

	Forward primers	Reverse primers
LACS1(1)	5′-TAAACCAGTCCTTGATCTATAAACTA-3′	5′-AATCTGCAAAACTTCTTCGTATACTT-3′
LACS1(2)	5′-ATACTCCTGGATCGATTTTCT-3′	5′-AGAGATACACATCATCATGTGT-3′
LACS1(3)	5′-CTGATTTCATTGCTTTCAGAAA-3′	5′-TCCTCCAAGTGTCTCCGTTA-3′
LACS2	5′-TTTAGCCGCGGATAATGTGTTGTTG-3′	5′-CGCTTCCTTATATGTGATCCACGTGT-3′
LACS4	5′-CGAGATCTTCTTACGCCGACCTTC-3′	5′-CAAATATTGTACACAGATGAGAGAAG-3′
LACS8(1)	5′-AGATTCCACCCTGATGGATGTCT-3′	5′-ATGCTCCTCGTGATGGAACAACAA-3′
LACS8(2)	5′-TTGATGATCGTGTTGCTATCT-3′	5′-CTCATTGAGTGAGTAAATCAAA-3′
LACS9	5′-GGAATTCTCTGAAGATCTCACCA-3′	5′-TCGTCCTAGTTCCCACACAA-3′
GAPC	5′-ACTCGAGAAAGCTGCTAC-3′	5′-ATTCGTTGTCGTACCATG-3′

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 N_2, dissolved in 50 μl hexane and transferred to glass vials. Fatty acid methyl esters were separated by gas/liquid chromatography as described previously (Kunst *et al.*, 1992).

ACKNOWLEDGEMENTS

We thank the Genomic Analysis Laboratory of the Salk Institute for providing sequence indexed Arabidopsis T-DNA insertion mutants, Keith Earley (Biology Department, Washington University, St Louis, MO) for the pEARLEYGATE101 vector, the Bio-Imaging Facility at the University of British Columbia for providing microscopy support, as well as Reinhard Jetter and Lin Shi (Department of Botany, University of British Columbia) for thoughtful discussion and comments on the manuscript. L.K. and G.W.H. gratefully acknowledge funding for this project from the Green Crop Strategic Research Network and the Strategic Network Grants Program of the Natural Sciences and Engineering Research Council of Canada (NSERC). F.L. was supported in part by a NSERC Strategic Project Grant.

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.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Alting-Mees, M.A., Sorge, J.A. and Short, J.M. (1992) pBluescriptII: multifunctional cloning and mapping vectors. *Methods Enzymol.* **216**, 483–495.

Baud, S., Dubreucq, B., Miquel, M., Rochat, C. and Lepiniec, L. (2008) Storage reserve accumulation in Arabidopsis: metabolic and developmental control of seed filling. In *The Arabidopsis Book* (Last, R.L., ed.). Rockville, MD: American Society of Plant Biologists. doi 10.1199/tab.0113, http://www.aspb.org/publications/arabidopsis/, pp. 1–24.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci. Paris Life Sci.* **316**, 1194–1199.

Bessire, M., Chassot, C., Jacquat, A.C., Humphry, M., Borel, S., Petétot, J.M., Métraux, J.P. and Nawrath, C. (2007) A permeable cuticle in Arabidopsis leads to a strong resistance to *Botrytis cinerea*. *EMBO J.* **26**, 2158–2168.

Boevink, P., Cruz, S., Hawes, C., Harris, N. and Oparka, K.J. (1996) Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells. *Plant J.* **10**, 935–941.

Cahoon, E.B., Shockey, J.M., Dietrich, C.R., Gidda, S.K., Mullen, R.T. and Dyer, J.M. (2007) Engineering oilseeds for sustainable production of industrial and nutritional feedstocks: solving bottlenecks in fatty acid flux. *Curr. Opin. Plant Biol.* **10**, 236–244.

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

Deblaere, R., Reynaerts, A., Hofte, H., Hernalsteens, J.P., Leemans, J. and Van Montagu, M. (1987) Vectors for cloning in plant cells. *Methods Enzymol.* **153**, 277–292.

Downing, W.L., Mauxion, F., Fauvarque, M.O., Reviron, M.P., de Vienne, D., Vartanian, N. and Giraudat, J. (1992) A *Brassica napus* transcript encoding a protein related to the Kunitz protease inhibitor family accumulates upon water stress in leaves, not in seeds. *Plant J.* **2**, 685–693.

Dunkley, T.P., Hester, S., Shadforth, I.P. *et al.* (2006) Mapping the Arabidopsis organelle proteome. *Proc. Natl. Acad. Sci. USA*, **103**, 6518–6523.

Dyer, J.M., Stymne, S., Green, A.G. and Carlsson, A.S. (2008) High-value oils from plants. *Plant J.* **54**, 640–655.

Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616–629.

Fulda, M., Shockey, J., Weber, M., Wolter, F.P. and Heinz, E. (2002) Two long-chain acyl-CoA synthetases from *Arabidopsis thaliana* involved in peroxisomal fatty acid β-oxidation. *Plant J.* **32**, 93–104.

Fulda, M., Schnurr, J., Abbadi, A., Heinz, E. and Browse, J. (2004) Peroxisomal acyl-CoA synthetase activity is essential for seedling development in *Arabidopsis thaliana*. *Plant Cell* **16**, 394–405.

Groot, P.H., Scholte, H.R. and Hulsmann, W.C. (1976) Fatty acid activation: specificity, localization, and function. *Adv. Lipid Res.* **14**, 75–126.

Hepworth, S.R., Zhang, Y., McKim, S., Li, X. and Haughn, G.W. (2005) BLADE-ON-PETIOLE-dependent signaling controls leaf and floral patterning in Arabidopsis. *Plant Cell*, **17**, 1434–1448.

Hettema, E.H., van Roermund, C.W., Distel, B., van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R.J. and Tabak, H.F. (1996) The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. *EMBO J.* **15**, 3813–3822.

Hills, M.J. (2004) Control of storage-product synthesis in seeds. *Curr. Opin. Plant Biol.* **7**, 302–308.

Hooker, T.S., Millar, A.A. and Kunst, L. (2002) Significance of the expression of the CER6 condensing enzyme for cuticular wax production in Arabidopsis. *Plant Physiol.* **129**, 1568–1580.

Jefferson, R.A. (1987) Assaying chimeric gene in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.

Kennedy, E.P. (1961) Biosynthesis of complex lipids. *Fed. Proc.* **20**, 934–940.

Koncz, C. and Schell, J. (1986) The promoter of T_L-DNA gene 5 controls the tissue specific expression of chimeric genes by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**, 383–396.

Koornneef, M., Hanhart, C.J. and Thiel, F. (1989) A genetic and phenotypic description of *eceriferum* (*cer*) mutants in *Arabidopsis thaliana*. *J. Hered.* **80**, 118–122.

Kunst, L., Taylor, D.C. and Underhill, E.W. (1992) Fatty acid elongation in developing seeds of *Arabidopsis thaliana*. *Plant Physiol. Biochem.* **30**, 425–434.

Lü, S., Song, T., Kosma, D.K., Parsons, E.P., Rowland, O. and Jenks, M.A. (2009) Arabidopsis *CER8* encodes LONG-CHAIN ACYL-COA SYNTHETASE 1 (LACS1) that has overlapping functions with LACS2 in plant wax and cutin synthesis. *Plant J.* **59**, 553–564.

Rossak, M., Smith, M. and Kunst, L. (2001) Expression of the *FAE1* gene and *FAE1* promoter activity in developing seeds of *Arabidopsis thaliana*. *Plant Mol. Biol.* **46**, 717–725.

Schnurr, J.A., Shockey, J.M., de Boer, G.-J. and Browse, J. (2002) Fatty acid export from the chloroplast. Molecular characterization of a major plastidial acyl-coenzyme A synthetase from Arabidopsis. *Plant Physiol.* **129**, 1700–1709.

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

Shockey, J.M., Fulda, M.S. and Browse, J.A. (2002) Arabidopsis contains nine long-chain acyl-coenzyme A synthetase genes that participate in fatty acid and glycerolipid metabolism. *Plant Physiol.* **129**, 1710–1722.

Somerville, C.R. and Ogren, W.L. (1982) Isolation of photorespiratory mutants of Arabidopsis. In *Methods in Chloroplast Molecular Biology* (Hallick, R.B. and Chua, N.H., eds). New York: Elsevier, pp. 129–139.

Stymne, S. and Stobart, A.K. (1987) Triacylglycerol biosynthesis. In *The Biochemistry of Plants: A Comprehensive Treatise, Volume 9* (Stumpf, P.K., ed.). Orlando, FL: Academic Press, pp. 175–214.

Weng, H., Molina, I., Shockey, J. and Browse, J. (2010) Organ fusion and defective cuticle function in a *lacs1 lacs2* double mutant of Arabidopsis. *Planta*, **231**, 1089–1100.