

A Mutation in the *LPAT1* Gene Suppresses the Sensitivity of *fab1* Plants to Low Temperature^{1[OA]}

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The *Arabidopsis* (*Arabidopsis thaliana*) *fatty acid biosynthesis1* (*fab1*) mutant grows as well as wild type at 22°C, but after transfer to 2°C *fab1* plants cannot maintain photosynthetic function and die after 5 to 7 weeks at 2°C. A *fab1* suppressor line, *S7*, was isolated in a screen that identified mutants that remained alive after 16 weeks at 2°C and were able to flower and produce seed after return to 22°C. Relative to wild type, *S7* plants had reduced levels of 16:3 fatty acid in leaf galactolipids, indicating reduced synthesis of chloroplast glycerolipids by the prokaryotic pathway of lipid metabolism. The suppressor mutation was identified, by map-based and candidate-gene approaches, as a hypomorphic allele of *lysophosphatidic acid acyltransferase1* (*lpat1*), *lpat1-3*. *LPAT1* encodes the enzyme that catalyzes the second reaction in the prokaryotic pathway. Several lines of evidence indicate that damage and death of *fab1* plants at 2°C may be a result of the increased proportion of phosphatidylglycerol (PG) in *fab1* that are high-melting-point molecular species (containing only 16:0, 18:0, and 16:1,Δ3-trans fatty acids). Consistent with this proposal, the *lpat1-3* mutation strongly affects the fatty acid composition of PG. The proportion of high-melting-point molecular species in PG is reduced from 48.2% in *fab1* to 10.7% in *fab1 lpat1-3* (*S7*), a value close to the 7.6% found in wild type.

There are two distinct pathways in higher plants for the synthesis of membrane glycerolipids. Both pathways are initiated by the synthesis of 16:0-acyl carrier protein (16:0-ACP) from acetyl-CoA by plastid acetyl-CoA carboxylase and enzymes of the fatty acid synthase. 16:0-ACP may be elongated by one additional cycle of the fatty acid synthase and then desaturated by the stromal 18:0-ACP desaturase (Lindqvist et al., 1996), so that 16:0-ACP and 18:1-ACP are the primary products of plastid fatty acid synthesis (Ohlrogge and Browse, 1995). The prokaryotic pathway located in the plastid envelope begins with the synthesis of 18:1-lysophosphatidic acid by acyl-ACP:glycerol-3-P acyltransferase. Then 16:0-ACP:lysophosphatidic acid acyltransferase (*LPAT*) completes the synthesis of phosphatidic acid (PA) that is a key intermediate in glycerolipid synthesis. The PA in the prokaryotic

pathway is used for the synthesis of phosphatidylglycerol (PG) and, in some plants, as a precursor via diacylglycerol for the synthesis of monogalactosyldiacylglycerol (MGD), digalactosyldiacylglycerol (DGD), and sulfoquinovosyldiacylglycerol (SQD), the four major glycerolipids of the photosynthetic thylakoid membranes. The alternative eukaryotic pathway begins with the hydrolysis of acyl-ACPs and the synthesis of 16:0-CoA and 18:1-CoA in the plastid envelope for export to the endoplasmic reticulum, where they are used for the synthesis of phosphatidylcholine (PC) and other phospholipids characteristic of the various extrachloroplast membranes of the cell. In addition, the diacylglycerol moiety of PC is returned to the chloroplast envelope—possibly in the form of PA (Benning, 2009)—where it enters the diacylglycerol pool and contributes to the synthesis of all the thylakoid glycerolipids, except PG (Browse et al., 1986; Benning, 2009). On both the prokaryotic and eukaryotic pathways, 16:0 and 18:1 are incorporated into the membrane glycerolipids before membrane-bound desaturases in the chloroplast and endoplasmic reticulum desaturate the acyl groups further to produce the polyunsaturated lipids typical of the different membrane systems of the cell. In many species of higher plants, PG is the only product of the prokaryotic pathway and the remaining chloroplast lipids are synthesized entirely by the eukaryotic pathway. However, in *Arabidopsis* (*Arabidopsis thaliana*) and some other species, the two pathways contribute about equally to the synthesis of MGD, DGD, and SQD (Browse et al., 1986; Wallis and Browse, 2010).

Investigations of *Arabidopsis* mutants deficient in enzymes of these two lipid synthesis pathways have provided considerable information about the relation-

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ship between membrane lipid composition and biological function. For example, a number of mutations that reduce the level of membrane unsaturation compromise plant growth at low temperatures (<10°C; Hugly and Somerville, 1992; Wu and Browse, 1995; Murakami et al., 2000; Routaboul et al., 2000). It has been proposed that damage occurring at these temperatures in chilling-sensitive plant species is caused by an L_{α} to L_{β} lipid phase (liquid crystalline to gel phase) transition in cellular membranes (Nishida and Murata, 1996), and, in particular, that molecular species of PG containing only 16:0, 18:0, and 16:1, Δ 3-trans (high-melting-point PG) confer chilling sensitivity on plants (Murata, 1983; Murata and Yamaya, 1984). PG purified from chilling-sensitive plants (containing 33%–71% high-melting-point molecular species) entered the gel phase at temperatures approximately 20°C higher than PG from chilling-resistant plants (6%–20% high-melting-point molecular species; Murata and Yamaya, 1984). A role for membrane unsaturation in maintaining plant growth and photosynthesis at low temperatures is also supported by experiments in which transgenic expression of fatty acid desaturases in chilling-sensitive plant species resulted in increased survival of plants at low temperatures (Kodama et al., 1994; Ishizaki-Nishizawa et al., 1996; Murakami et al., 2000).

One of the *Arabidopsis* mutants, *fatty acid biosynthesis1* (*fab1*), contains increased levels of the saturated fatty acid, 16:0, as a result of a mutation in the *KAS2* gene, which encodes the first step in elongation of 16:0 to 18:0 during fatty acid synthesis (Wu et al., 1994; Carlsson et al., 2002). Although *fab1* plants do not exhibit a classic chilling-sensitive phenotype (Wu and Browse, 1995), they are damaged and killed by long-term exposure to low temperatures. During the first 7 to 10 d after transfer from 22°C to 2°C, growth and photosynthetic characteristics of *fab1* plants were similar to wild type. However, between 10 and 28 d at 2°C the mutants suffered almost complete loss of photosynthetic function and destruction of chloroplasts, but not other organelles, within leaf cells, and then plants died after 4 to 6 weeks in the cold (Wu et al., 1997). This collapse of photosynthesis and breakdown of chloroplasts in *fab1* plants at 2°C suggests that further investigation of this mutant will lead to a better understanding of the relationship between thylakoid lipid structure and photosynthetic function.

We initiated a suppressor screen for mutations that rescue *fab1* from death at low temperatures (Barkan et al., 2006). One of the mutants isolated in this screen, *S7*, showed a reduced level of 16:3 in total leaf lipids, and this altered fatty acid composition cosegregated with the suppressive phenotype as a single, recessive mutation. The low 16:3 in *S7* plants indicated a possible block in synthesis of thylakoid lipids by the prokaryotic pathway (Kunst et al., 1988). Reductions in lipid synthesis by the prokaryotic pathway have previously been observed in the *gly1* mutant, which is deficient in glycerol-3-P dehydrogenase activity

(Miquel et al., 1998), and the *act1* mutant, which is deficient in activity of the chloroplast acyl-ACP:glycerol-3-P acyltransferase (Kunst et al., 1988). However, test crosses of *S7* with *gly1* and *act1* produced F_1 progeny with levels of leaf 16:3 similar to wild type, indicating that the lipid mutation in *S7* is not allelic to either of these previously characterized mutants (Barkan et al., 2006). Here we describe identification of the *S7* suppressor, by map-based cloning, as a new, hypomorphic allele of *LPAT1* that encodes acyl-ACP:LPAT, the second enzyme in the prokaryotic pathway. Our discovery provides new insight on the cause of the *fab1* low-temperature phenotype. In addition, because the *lpat1-1* and *lpat1-2* null alleles are lethal (Kim and Huang, 2004; Yu et al., 2004), our newly identified allele provides a useful tool to investigate this step in the biochemistry of thylakoid lipid synthesis.

RESULTS

Changes in Leaf Lipids of *S7* Plants

Because the overall fatty acid composition of leaf lipids from *S7* plants indicated a partial block of the prokaryotic pathway, we separated individual lipids from leaf extracts and analyzed their fatty acid compositions by gas chromatography (GC). The results of this analysis, shown in Table I, confirm that the *S7* suppressor line has a reduced flux through the prokaryotic pathway since 16:3, a fatty acid that is confined to the sn-2 position of lipids synthesized by this pathway (Browse et al., 1986), is present in much lower proportions on MGD and DGD of *S7* plants. The major chloroplast lipid, MGD, in *S7* contained 13.3% 16:3 compared to 33.3% in the parental *fab1* line, suggesting that MGD synthesis via the prokaryotic pathway is reduced by approximately 60% in *S7*. This deficiency appears to be compensated for by increased MGD synthesis by the eukaryotic pathway, since there was a very much smaller drop in total MGD as a percentage of leaf lipids (Table I).

Consistent with previous results (Wu et al., 1994; Barkan et al., 2006), all of the lipids in *fab1* leaves had increased 16:0 compared with wild type. Three of the major membrane lipids, MGD, DGD, and PC, from *S7* plants all showed a further increase in 16:0 relative to *fab1*. The only substantial decrease in 16:0 was seen in PG; for this lipid 16:0 was decreased from 48.2% in *fab1* to 34.5% in *S7*—close to the value for wild-type PG of 33.0%. This change in fatty acid composition of PG is significant because it likely reflects a decrease in high-melting-point species of PG (Roughan, 1985). We have previously proposed that high-melting-point PG contributes to the photosynthetic decline and death of *fab1* at low temperature (Wu et al., 1997; Barkan et al., 2006). To test whether *S7* plants contain substantially lower levels of high-melting-point PG than *fab1*, we submitted wild-type, *fab1*, and *S7* leaf lipids for lipidomics analysis by mass spectrometry (Esch et al., 2007). The results obtained (Table II) indicate that high-melting-point molecular species account for

Table I. Fatty acid compositions of leaf lipids from wild-type and mutant *Arabidopsis* grown at 22°C

Data are from one of three separate experiments that gave similar results. WT, Wild type; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

Lipid Class	Genotype	% of Total Polar Lipids	Fatty Acid Composition						
			16:0	16:1	16:3	18:0	18:1	18:2	18:3
MGD	WT	32.6	3.3	1.7	30.7	— ^a	1.4	3.4	57.2
	<i>fab1</i>	35.1	5.2	3.2	33.3	—	2.5	2.0	51.1
	S7	30.1	9.6	3.0	13.3	1.3	3.2	2.2	65.8
DGD	WT	14.2	16.8	—	2.9	2.7	1.1	4.1	71.8
	<i>fab1</i>	10.4	31.4	1.2	3.2	2.1	3.5	3.4	54.5
	S7	12.2	32.7	2.9	—	2.2	2.8	1.7	57.2
PG	WT	12.9	33.0	19.8	—	2.1	5.8	12.1	27.2
	<i>fab1</i>	14.8	48.2	18.4	—	1.5	5.1	7.5	18.4
	S7	11.5	34.5	21.3	—	1.4	5.2	11.0	25.3
SQD	WT	3.4	52.7	1.4	—	5.6	3.0	5.6	31.6
	<i>fab1</i>	3.5	58.3	2.0	2.9	4.2	1.8	5.8	24.4
	S7	3.6	55.6	3.2	—	8.2	2.4	3.0	24.9
PC	WT	18.7	26.6	—	—	3.2	6.7	32.7	30.1
	<i>fab1</i>	18.3	34.0	2.6	—	2.6	6.1	24.4	29.8
	S7	20.7	38.5	1.7	—	3.4	7.3	26.2	24.9
PE	WT	12.9	30.9	—	—	2.7	4.0	34.4	28.0
	<i>fab1</i>	12.9	34.2	1.1	—	1.8	5.6	26.4	29.8
	S7	14.7	38.1	—	—	2.3	6.3	29.5	23.7
PI	WT	5.3	52.3	—	—	5.3	2.5	19.3	20.6
	<i>fab1</i>	5.0	56.1	—	—	1.9	1.7	16.6	23.7
	S7	7.1	55.5	1.0	—	4.1	4.7	18.0	16.7

^a<1%.

7.6% of PG in wild type and 48.2% in *fab1*; these values agree with results obtained earlier using HPLC techniques (Wu and Browse, 1995). In S7, high-melting-point molecular species make up only 10.7% of PG. Thus the lipid mutation in S7 provides a molecular species composition in PG that is closer to that of wild type than to that of the *fab1* progenitor.

The S7 Line Contains a Mutation in *LPAT1*

To identify the suppressor locus in S7, we first crossed this line to Landsberg *erecta*, selfed the F₁

progeny, and grew F₂ plants. Based on the expected Mendelian segregation, 25% of the F₂ would be *fab1* homozygotes and 50% of these would be heterozygous for the recessive suppressor mutation. We identified six *fab1* plants by GC of leaf fatty acids, grew them to maturity, and collected seed from each plant separately. We chose one of the plants whose progeny segregated for the suppressor phenotype (approximately one-quarter surviving at 2°C) as the parent of an F₃ mapping population. A total of 800 F₃ seeds were planted and the resulting plants grown at 2°C for 6 weeks. DNA was prepared from leaf samples of

Table II. Molecular species composition of leaf PG from wild-type and mutant *Arabidopsis*

Analyses were conducted by collision-induced dissociation time-of-flight mass spectrometry as described under "Materials and Methods." Data are normalized from the means of five replicates with SEs < 10% of the means. WT, Wild type.

Fragment Mass	Molecular Species	Percentage of Total PG		
		WT	<i>fab1</i>	S7
760.5	18:3/16:1	40.3	21.6	37.4
762.5	18:3/16:0 + 18:2/16:1	30.0	18.5	29.2
764.5	18:2/16:0 + 18:1/16:1	13.5	7.3	14.8
766.5	18:1:16:0	8.1	4.3	7.8
—	Other unsaturated species	0.5	0.1	0.1
768.6	18:0/16:0	0.6	0.3	0.1
738.5	16:0/16:1	3.6	26.1	6.5
740.5	16:0/16:0	3.4	21.8	4.1
	Total high-melting-point species	7.6%	48.2%	10.7%

surviving plants. Bulk-segregant analysis (Lukowitz et al., 2000), using DNA pooled from 50 individuals, indicated Columbia DNA associated with marker NGA111 that is on the lower arm of chromosome1 close to the *fab1* locus. Columbia DNA was also associated with marker NGA1107 on chromosome 4, indicating that the suppressor mutation lay close to this marker. Analysis of DNA from individual plants indicated that the suppressor mutation was approximately 10 centimorgans from the marker. Because NGA1107 is close to the end of chromosome 4, this degree of linkage suggested a map window centered around 15,500 kb on the physical map. The 1,200 kb region around this position contains approximately 350 genes including 12 that have known or putative functions in lipid metabolism (Beisson et al., 2003). One of these, *LPAT1* (At4g30580), was a particularly appealing candidate since it encodes the second enzyme of the prokaryotic pathway. Null mutants at this locus, *lpat1* (=ats2), are embryo lethal (Kim and Huang, 2004; Yu et al., 2004), but the presence of 13% 16:3 in MGD from S7 leaves would be consistent with a hypomorphic *lpat1* allele.

We prepared RNA from wild-type, *fab1*, and S7 plants and amplified cDNAs of *LPAT1* from each. Sequencing showed that the wild-type and *fab1* cDNAs corresponded with the sequence in The Arabidopsis Information Resource database (www.arabidopsis.org), while the S7 allele contained a G to A change in the seventh exon of the gene. This change is predicted to encode an Ala-290Thr mutation in the LPAT1 protein (Fig. 1). The Ala-290 residue is located in one of the three domains (D1–D3 in Fig. 1) that are conserved in LPAT proteins from plants, animals, and microbes (Eberhardt et al., 1997), although Phe or Ser are found in place of Ala in some sequences. We designate this new mutant allele *lpat1-3*.

LPAT1 Expression Complements the Lipid and Suppressor Phenotypes of S7 Plants

To test whether the *lpat1-3* mutation is the basis of the altered lipid composition and the suppressor

allowing growth of S7 plants at 2°C, we transformed S7 plants with an *LPAT1* cDNA under control of the cauliflower mosaic virus 35S promoter. Eight independent T₁ transgenic plants had levels of 16:3 in total leaf lipids that were increased to those of *fab1* controls, indicating that the altered lipid composition of S7 plants is indeed the result of reduced LPAT1 activity. A line containing a single insert of the *35S:LPAT1* transgene was identified by a 3:1 segregation pattern in the T₂ generation, and a homozygous T₂ individual was identified by T₃ pedigree analysis. Homozygous S7 *35S:LPAT1* plants were grown together with wild-type, *fab1*, and S7 controls at 22°C for 25 d and then transferred to 2°C. After 36 d at 2°C wild-type and S7 plants were healthy while *fab1* and S7 *35S:LPAT1* plants showed symptoms of chlorosis and poor growth similar to those reported previously for *fab1* (Wu and Browse, 1995; Wu et al., 1997). Measurements of the potential quantum yield of PSII, F_v/F_m , showed that wild-type and S7 plants maintained F_v/F_m close to 0.7, while the values for *fab1* and S7 *35S:LPAT1* plants were less than 0.1 (Fig. 2A). After 78 d at 2°C, the older leaves on S7 plants were senescent but new green leaves had been produced (Fig. 2B), and the plants quickly recovered and set seed after being returned to 22°C (Fig. 2C). In contrast, *fab1* and S7 *35S:LPAT1* plants were completely dead as indicated by their failure to recover after transfer to 22°C (Fig. 2C). These experiments establish *lpat1-3* as the suppressor mutation in S7, and we now refer to this line as *fab1 lpat1-3*.

To investigate the biological consequences of the *lpat1-3* mutation independently of *fab1*, we crossed *fab1 lpat1-3* to wild type and isolated a homozygous *lpat1-3* mutant. Interestingly, the leaf fatty acid composition of this new line contained only 1.8% 16:3 (Table III), considerably less than the 5.6% 16:3 measured in *fab1 lpat1-3* (S7) leaves (Barkan et al., 2006). We interpret this difference as indicating that the reduced KASII activity caused by the *fab1* mutation (Wu et al., 1994) results in an increase in 16:0-ACP, the substrate for LPAT1, which, in turn, increases synthesis of PA and prokaryotic lipids by the mutant LPAT1 enzyme encoded by *lpat1-3*.

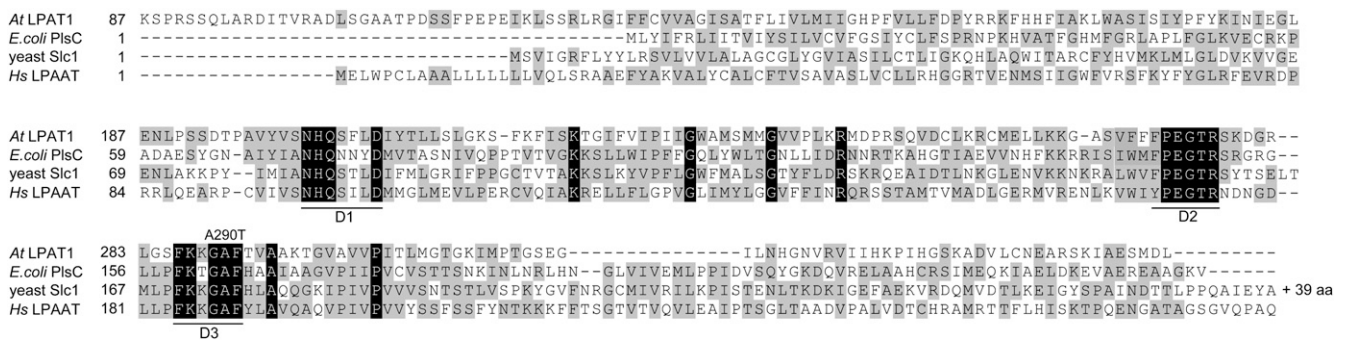


Figure 1. Sequence alignment of LPAT1 homologs. The Arabidopsis amino acid sequence was aligned with LPATs from *Escherichia coli* (PlsC), yeast (*Saccharomyces cerevisiae*; Slc1p), and human (*Hs* LPAAT; GenBank AAC51649). The three conserved domains (D1–D3) are shown and the site of the *lpat1-3* mutation, A290T, is indicated.

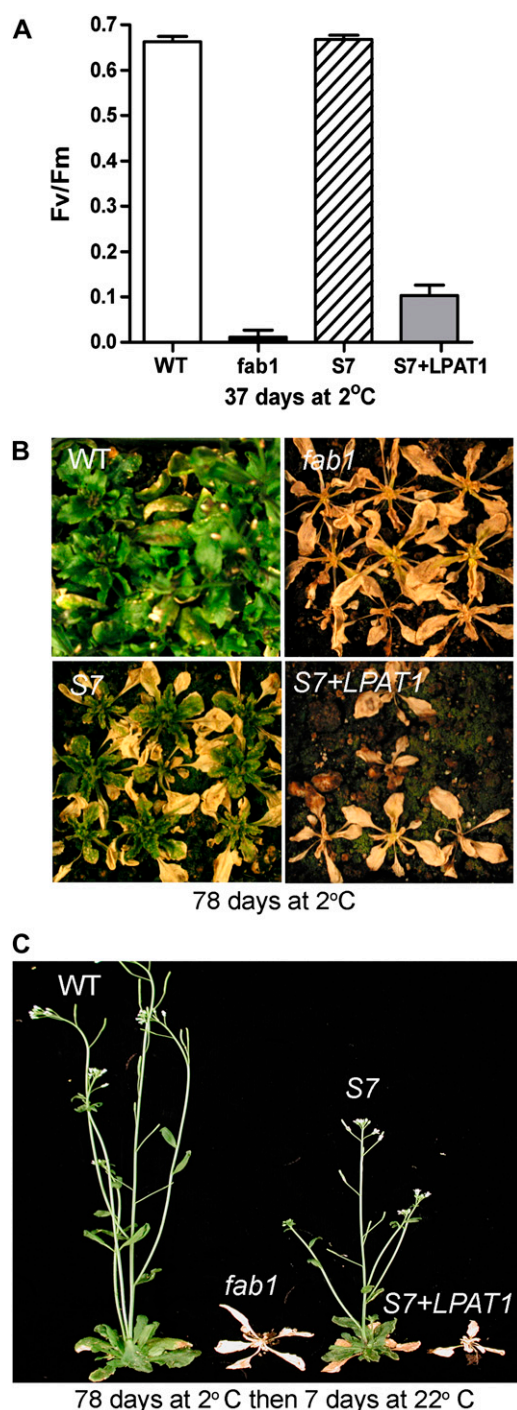


Figure 2. Expression of an *LPAT1* cDNA complements the S7 suppressor phenotype. **A**, After 37 d at 2°C, wild-type (WT) and S7 plants maintain high F_v/F_m , while S7 plants transformed with a *35S:LPAT1* transgene (S7 + *LPAT1*) and *fab1* plants have low F_v/F_m (data are mean \pm SE for 10 plants of each line). **B**, After 78 d at 2°C, only wild-type and S7 plants remain green. **C**, When returned to 22°C, wild-type and S7 plants flower and set seed, while *fab1* and S7 + *LPAT1* plants fail to recover.

Plants of the *fab1 lpat1-3* line are smaller than those of *fab1* and wild type (Barkan et al., 2006), and *lpat1-3* plants were also observed to be small and slow to develop. However, the *act1* mutants, which are also defective in prokaryotic lipid synthesis, are similar in growth and development to wild type (Kunst et al., 1988; Xu et al., 2006). We transformed *lpat1-3* with the *35S:LPAT1* transgene and complemented both the lipid and growth defects of the mutant (data not shown), thus demonstrating that *LPAT1* deficiency causes the mutant phenotype. Next, we crossed the *act1-1* and *lpat1-3* mutants and produced a double-mutant line. *act1-1*, *lpat1-3*, and *act1-1 lpat1-3* plants, along with wild-type controls, were grown side by side. The leaf fatty acid compositions of these plants (Table III) indicate that all three mutant lines have very low flux through the prokaryotic pathway, as judged by the very low levels of 16:3—a fatty acid that is only present in galactolipids synthesized by the prokaryotic pathway. The proportion of total fatty acids lost from 16:3 is distributed among the 18-carbon unsaturated fatty acids. The levels of 16:3 found in *act1-1 lpat1-3* and *act1* leaves suggest that prokaryotic flux in these mutants is less than one-tenth of that in wild type, while the *lpat1-3* mutant has a slightly higher flux (Table III). After 33 d of growth, wild-type and *act1-1* plants had well-developed bolts with flowers and filled siliques (Fig. 3). By contrast, the *lpat1-3* plants were mainly maturing rosettes that had only just initiated reproductive growth. Plants of the *act1-1 lpat1-3* line were noticeably smaller rosettes that had not yet bolted. The mutations in *lpat1-3* and *act1-1 lpat1-3* primarily result in a reduced growth rate. Mutant plants had a number of rosette leaves (10–12) similar to wild-type and *act1* plants at the time of floral initiation, indicating that developmental processes were substantially unaffected. The growth phenotype of the *act1-1 lpat1-3* double mutant is similar to that of *act1-1 (ats1-1)* plants in which *LPAT1* expression was reduced by RNA interference (Xu et al., 2006). Thus, the *lpat1-3* mutant causes reduced growth and development in wild-type and *act1-1* genetic backgrounds, as well as in the *fab1* background (Barkan et al., 2006).

DISCUSSION

The Arabidopsis *fab1* mutation causes a Leu-337Phe substitution in KASII (At1g74960), the 3-ketoacyl-ACP synthase responsible for elongation of 16:0-ACP during fatty acid synthesis (Carlsson et al., 2002). The amino acid substitution likely results in structural instability of the KASII protein due to the difficulty of accommodating the imidazole ring of the mutant Phe-337 residue. The result is a 40% reduction in KASII activity in extracts from *fab1* leaves relative to wild type and increased proportions of 16:0 in all the major leaf glycerolipids of *fab1* plants (Wu et al., 1994). Of particular interest is the change in fatty acid composition of PG, the major chloroplast phospholipid. The

Table III. Overall leaf fatty acid compositions of wild-type *Arabidopsis* and the *lpat1-3*, *act1*, and *act1 lpat1-3* mutants

Data are the means of five replicates with $SES < 10\%$ of the means. WT, Wild type.

Fatty Acid	Percentage of Total			
	WT	<i>lpat1-3</i>	<i>act1</i>	<i>act1 lpat1-3</i>
16:0	15.7	13.1	14.4	11.9
16:1, Δ 3-trans	2.2	1.8	2.2	1.5
16:3	13.3	1.8	1.1	0.8
18:0	0.9	1.5	1.5	1.5
18:1	2.5	9.9	7.2	15.4
18:2	12.4	17.1	17.5	19.6
18:3	51.4	54.4	55.3	48.8

PG from *fab1* leaves has a 50% increase in the proportion of 16:0 compared with PG from wild-type leaves (Table I; Wu et al., 1994, 1997). Although *fab1* plants contain 43% to 48% high-melting-point PG (Table II; Wu and Browse, 1995), this mutant does not show classic symptoms of chilling sensitivity, based on established tests that quickly lead to damage and death of chilling-sensitive plants (Wu and Browse, 1995). Instead, *fab1* plants show a decline in photosynthetic function (as indicated by the fluorescence parameter, F_v/F_m) starting 10 d after transfer to 2°C (Wu et al., 1997).

Ultrastructural studies revealed that the decline in photosynthetic capacity is accompanied by disruption of granal and stromal thylakoids, and the development of small vacuoles (0.5–1.0 μ m) in close association with the chloroplasts. This process continues with degradation and complete dismantling of chloroplasts in leaf mesophyll cells. Other organelles and cell structural features appear to be maintained during this period of chloroplast breakdown, so that affected leaf tissue is still alive but very chlorotic at the end of a 3-week cold treatment (Wu et al., 1997). *fab1* plants kept at 2°C for up to 5 weeks are able to recover and set seed after a return to 22°C (Wu et al., 1997). We originally suggested that this chloroplast degradation at low temperature was a form of autophagy; however, recent reports of vacuole formation and chloroplast degradation during natural or dark-induced senescence provide alternative models for the process that occurs following the collapse of photosynthesis in *fab1* plants at 2°C (Otegui et al., 2005; Ishida et al., 2008).

Based on these results, we have proposed that the levels of high-melting-point PG found in *fab1* plants (and chilling-sensitive species) disrupt long-term maintenance of the photosynthetic machinery at low temperatures. In this model, altered PG composition would be one of several traits that evolved in tropical and subtropical plants (perhaps because they confer a selective advantage) but are incompatible with growth in a cold climate (Wu and Browse, 1995). However, it is important to note that this characterization of the *fab1* mutant does not establish increased high-melting-

point PG as the cause of low-temperature damage. It could be argued that increased 16:0 in another membrane lipid (DGD, for example), or the general increase in the proportion of 16:0 in membrane lipids, is the key determinant of damage.

Our characterization of the *lpat1-3* mutation as a suppressor of the *fab1* low-temperature phenotype addresses these possible explanations. It therefore provides additional support for the proposal that high-melting-point PG is a barrier to growth and survival of *fab1* plants at low temperatures. Compared with *fab1*, the *fab1 lpat1-3* (S7) line contains a higher proportion of 16:0 in the total leaf lipids (25.5% versus 23.3%; Barkan et al., 2006). There are also increases in the level of 16:0 in all of the major leaf glycerolipids except PG and SQD (Table I). The decrease of 16:0 in SQD is relatively small (from 58.3% in *fab1* to 55.6% in *fab1 lpat1-3*) and is accompanied by a small increase in 18:0, the other saturated fatty acid. By contrast, 16:0 in PG decreases from 48.2% in *fab1* to 34.5% in *fab1 lpat1-3*. More importantly, the percentage of high-melting-point molecular species in leaf PG of *fab1 lpat1-3* is 10.7%, compared with 48.2% in *fab1* and 7.6% in wild type (Table II). Given the evidence linking PG molecular species composition with low-temperature damage in plants (Nishida and Murata, 1996), it is reasonable to conclude that the *lpat1-3* acts as a suppressor through its effect on reducing the proportion of high-melting-point PG. However, this is not the only alteration in thylakoid lipid composition that can suppress the *fab1* phenotype. Mutations at the *fad5* locus that reduce desaturation of 16:0 to 16:3 in MGD also result in *fab1 fad5* plants that survive at 2°C. We have proposed that the change in shape of MGD molecules caused by the *fad5* mutations may compensate for disruptive changes in the shape and packing

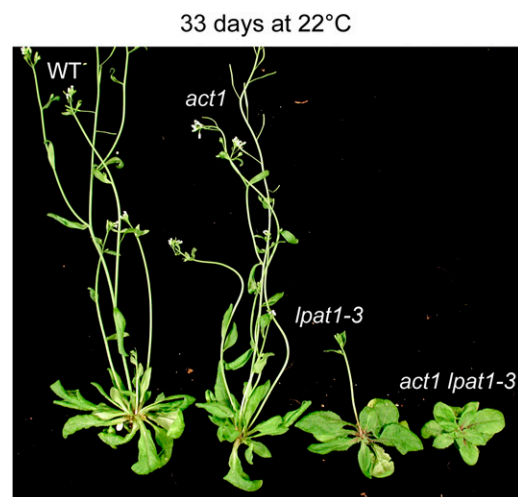


Figure 3. Growth and development of mutants deficient in chloroplast acyltransferases. Wild type (WT), *act1*, *lpat1-3*, and *act1 lpat1-3* were grown for 33 d at 22°C.

of PG molecules induced by the *fab1* mutation (Barkan et al., 2006).

Our identification of the *S7* suppressor as a hypomorphic *lpat1* allele is additionally useful because two, previously characterized, null *lpat1* alleles are not viable (Kim and Huang, 2004; Yu et al., 2004). Interestingly, these null alleles are embryo lethal, causing arrest of embryos between the globular and torpedo stages of development. Evidently, LPAT1 activity is not essential for growth and development of either the male or female gametophytes, nor for the earliest stages of embryo development. Furthermore, homozygous *lpat1* embryos were produced in the predicted Mendelian ratio (Kim and Huang, 2004; Yu et al., 2004), and this indicates that development and function of *lpat1-1* and *lpat1-2* sperm cells are sufficient for them to bring about fertilization as effectively as wild-type sperm cells.

LPAT1 is required for operation of the prokaryotic pathway that produces MGD, DGD, PG, and SQD for thylakoid biogenesis (Wallis and Browse, 2010), so it is reasonable to conclude that synthesis of one or more of these lipids is essential for embryo (and plant) development. In the *act1* mutant, which is blocked in acyl-ACP:glycerol-3-P acyltransferase—the step preceding LPAT1—accumulation of prokaryotic MDG + DGD + SQD is reduced by >98% (Kunst et al., 1988; Xu et al., 2006), making it unlikely that synthesis of any of these three lipids by the prokaryotic pathway is essential. Leaves of *act1* plants contain levels of MGD, DGD, and SQD that are similar to wild type due to increased flux through the eukaryotic pathway, and growth and development of the mutants are very similar to wild type (Fig. 3; Kunst et al., 1988, 1989). Chloroplast PG is not synthesized by the eukaryotic pathway, but the rate of PG synthesis by the prokaryotic pathway appears to be preferentially maintained in the *act1* mutant at 50% of the wild-type rate. There appears also to be reduced turnover of PG in *act1* so that PG is present in *act1* leaves in amounts that are approximately 70% of wild type (Kunst et al., 1988; Xu et al., 2006). These observations suggested the possibility that *lpat1* null mutants are embryo lethal because they are unable to synthesize chloroplast PG. However, a *pgp1* null mutant that is also deficient in chloroplast PG synthesis is not embryo lethal, but instead is able to germinate and grow on Suc media, although it is not autotrophic (Hagio et al., 2002). Characterization of this *pgp1* null mutant, and other evidence (Nussberger et al., 1993; Liu et al., 2004), indicate that PG is a component of the photosystem light-harvesting complexes and required for photosynthetic functions, but this does not provide an explanation for the embryo-lethal phenotype of *lpat1* null mutants.

The *lpat1-3* mutant is comparable to *act1* in the level of PG and of 16:3 in the leaf lipids (Table III; data not shown); however, *lpat1-3* plants grow more slowly than *act1* and wild type (Fig. 3). We considered the possibility that accumulation of lyso-PA, one of the substrates of the LPAT1 enzyme, might contribute to

the phenotypes of the hypomorphic and null *lpat* mutants. To test this possibility, we crossed the *lpat1-3* mutant to *act1-1*. Chloroplast lyso-PA synthesis is blocked by the *act1-1* mutation (Kunst et al., 1988), but this does not result in improved growth of *act1 lpat1-3* plants, instead these double mutants grow more slowly than *lpat1-3* plants (Fig. 3; Xu et al., 2006). It is unlikely that accumulation of 16:0-ACP, the other LPAT1 substrate, is related to the growth phenotype since the levels of this fatty acid synthase product are expected to be less in *lpat1-3* than in *fab1 lpat1-3* or *fab1* chloroplasts. Although these observations do not identify the mechanism through which reduced LPAT1 activity affects plant growth (and embryo viability), the *lpat1-3* allele that we have identified from our suppressor screen provides a new tool to investigate the biochemical and biology roles of the chloroplast LPAT1 enzyme.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 was used as the wild type in this study. Mutant plant lines *fab1*, *act1*, *S7*, and *lpat1-3* are in the Columbia-0 genetic background. Wild-type and mutant plants were grown at 22°C under 16 or 24 h of illumination, 120 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. For cold-resistant test, plants were grown at 2°C for up to 12 weeks.

Mapping the *S7* Suppressor Locus

To map the suppressor, an *S7* plant was crossed to the Landsberg *erecta* ecotype and the resulting F_1 plants were allowed to self. F_2 plants homozygous for the *fab1* mutation were selected and their progeny tested for segregation of the recessive suppressor phenotype. An F_2 plant that was homozygous *fab1* and heterozygous for the suppressor was used as the parent of an F_3 mapping population. Eight-hundred F_3 seeds were sown on soil and the resulting plants tested for survival at 2°C. DNA was prepared from leaves of individual surviving plants. Samples of 50 DNA preparations were pooled and used for bulk-segregant analysis. Subsequently, DNA from 170 individuals was tested using marker NGA1107 to identify an approximate position for the suppressor locus.

Cloning and Sequencing of LPAT1 Alleles

LPAT1 cDNAs were amplified from first-strand cDNA synthesized from total RNA of wild-type, *fab1*, and *S7* plants using reverse transcription (RT)-PCR. To amplify LPAT1 cDNAs from different mutant plant lines, gene-specific primers (5'-GTTTTTGCTCCAGATTCCGCC-3' for N terminus and 5'-AAGACAACCTTCTGAGATCAGC-3' for C terminus) were designed to sequences in 5'-untranslated and 3'-untranslated regions of the LPAT1 coding region. RNA samples from eight plants of each line were used to amplify RT-PCR products that were sequenced to identify the gene mutation.

Gene Cloning and Gene Construction

An LPAT1 cDNA containing full-length open reading frame was amplified using RT-PCR with gene-specific primers, 5'-CACCATGGATGTCGCTTCGTGCTCGGAGC-3' and 5'-TTAGAGATCCATTGATCTGCAAT-3' from first-strand cDNA from wild-type plants, and cloned into pENTR gateway entry vector using Topoisomerase ligation. The cDNAs were confirmed by DNA sequencing and inserted into Gateway plant expression vector, pB2GW7.0 (Karimi et al., 2002) under control of the cauliflower mosaic virus 35S promoter, by LR Clonase (Invitrogen) reaction.

S7 + *LPAT1* and *lpat1-3 + LPAT1* transgenic plants were generated by the floral-dip method (Clough and Bent, 1998) with use of *Agrobacterium tumefaciens* strain GV3101 containing the 35S:*LPAT1* construct. Plants resistant to BASTA were examined by PCR for genotype, and lipid composition using GC.

Extraction and Analysis of Lipids

The overall fatty acid compositions of leaf tissues were determined as previously described (Wu et al., 1994) with the exception that 1.5 mL water and 0.5 mL hexane were used to extract fatty acids into the organic phase. Samples (1 μ L) of the organic phase were analyzed by GC on a 30 m \times 0.53 mm Alltech Econo-cap column containing a 1.2 μ m EC-WAX phase (Alltech Associates, Inc.). The GC was programmed for an initial temperature of 160°C for 1 min, followed by an increase of 20°C/min to 190°C and a second increase of 4.5°C/min to the final temperature of 203°C.

The more detailed analyses of lipid and fatty acid composition were performed as described previously (Wu et al., 1994). Aliquots of the lipid extract were separated by one-dimensional thin-layer chromatography on (NH₄)₂SO₄-impregnated silica gel G (Wu et al., 1994) using acetone:benzene:water (30:10:2.7 v/v; Khan and Williams, 1997). To determine the fatty acid composition and the relative amounts of individual lipids, the silica gel from each spot was transferred to a screw-capped tube and fatty acid methyl esters were prepared and analyzed as described above. A known amount of 17:0 PC was added as internal standard prior to derivatization.

Quantification of PG molecular species was obtained by lipidomics analysis of lipid extracts performed at the Kansas State University Lipidomics Facility (Esch et al., 2007).

Measurements of Chlorophyll Fluorescence

Chlorophyll fluorescence from leaf tissues was measured using a PAM fluorometer (Walz). The ratio of variable fluorescence to maximal fluorescence (F_v/F_m), representing the potential quantum yield of PSII photochemistry, was measured in dark-adapted leaf tissues described by Barkan et al. (2006).

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