A Suppressor of *fab1* Challenges Hypotheses on the Role of Thylakoid Unsaturation in Photosynthetic Function[1]

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Leaf membrane lipids of the Arabidopsis (*Arabidopsis thaliana*) *fatty acid biosynthesis 1* (*fab1*) mutant contain a 35% to 40% increase in the predominant saturated fatty acid 16:0, relative to wild type. This increase in membrane saturation is associated with loss of photosynthetic function and death of mutant plants at low temperatures. We have initiated a suppressor screen for mutations that allow survival of *fab1* plants at 2°C. Five suppressor mutants identified in this screen all rescued the collapse of photosynthetic function observed in *fab1* plants. While *fab1* plants died after 5 to 7 weeks at 2°C, the suppressors remained viable after 16 weeks in the cold, as judged by their ability to resume growth following a return to 22°C and to subsequently produce viable seed. Three of the suppressors had changes in leaf fatty acid composition when compared to *fab1*, indicating that one mechanism of suppression may involve compensating changes in thylakoid lipid composition. Surprisingly, the suppressor phenotype in one line, S31, was associated with a further substantial increase in lipid saturation. The overall leaf fatty acid composition of S31 plants contained 31% 16:0 compared with 23% in *fab1* and 17% in wild type. Biochemical and genetic analysis showed that S31 plants contain a new allele of *fatty acid desaturation 5* (*fad5*), *fad5-2*, and are therefore partially deficient in activity of the chloroplast 16:0 Δ7 desaturase. A double mutant produced by crossing *fab1* to the original *fad5-1* allele also remained alive at 2°C, indicating that the *fad5-2* mutation is the suppressor in the S31 (*fab1 fad5-2*) line. Based on the biophysical characteristics of saturated and unsaturated fatty acids, the increased 16:0 in *fab1 fad5-2* plants would be expected to exacerbate, rather than ameliorate, low-temperature damage. We propose instead that a change in shape of the major thylakoid lipid, monogalactosyldiacylglycerol, mediated by the *fad5-2* mutation, may compensate for changes in lipid structure resulting from the original *fab1* mutation. Our identification of mutants that suppress the low-temperature phenotype of *fab1* provides new tools to understand the relationship between thylakoid lipid structure and photosynthetic function.

The chloroplast membranes that are host to the light-harvesting and electron-transport reactions of photosynthesis have a characteristic and unusual lipid composition. One remarkable feature is the high number of double bonds found in the lipid acyl chains. Typically, only about 10% of the fatty acids that compose the hydrophobic midportion of the thylakoid bilayer lack double bonds altogether, whereas more than 80% have two or more double bonds (Harwood, 1982). Because free-radical by-products of the photosynthetic light reactions can lead to oxidative degradation of polyunsaturated fatty acids, it has been inferred that these membrane components have some crucial roles in supporting photosynthesis. The characterization of an Arabidopsis (*Arabidopsis thaliana*) mutant largely deficient in polyunsaturated fatty acid synthesis, *fad2-2 fad6*, demonstrated that chloroplast function does indeed require polyunsaturated fatty acids, while most other membrane functions are adequately supported by lipids containing monounsaturated acyl groups (McConn and Browse, 1998). Mutants with smaller decreases in thylakoid unsaturation are substantially indistinguishable from wild type when grown at 22°C, but exhibit defects in biogenesis and maintenance of chloroplasts at temperatures below 5°C. These mutants include *fad5*, *fad6*, the double mutant *fad7 fad8*, and the triple mutant *fad3 fad7 fad8* (Hugly and Somerville, 1992; Murakami et al., 2000; Routaboul et al., 2000). A role for thylakoid unsaturation in maintaining photosynthetic function 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).

The Arabidopsis desaturation mutants *fad5*, *fad6*, *fad7 fad8*, and *fad3 fad7 fad8* all show chlorosis and reduced growth rates when grown at 4°C, but the plants are nevertheless able to complete their life cycle (Hugly and Somerville, 1992; Murakami et al., 2000;

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Routaboul et al., 2000). Another Arabidopsis mutant, *fatty acid biosynthesis 1* (*fab1*), exhibits a distinct low-temperature phenotype. This mutant contains increased levels of 16:0 due to a mutation in the *KAS2* gene, which encodes the condensing enzyme that catalyzes the first step in elongation of 16:0 to 18:0 during fatty acid synthesis (Wu et al., 1994; Carlsson et al., 2002). The *fab1* mutation leads to increased 16:0 in all the membrane lipids, although the increase in the major thylakoid lipid monogalactosyldiacylglycerol (MGD) is very modest, from 1.6% of total MGD acyl groups in wild type to 4.2% in the mutant (Wu et al., 1994). In the thylakoid phospholipid phosphatidylglycerol (PG), 16:0 is increased from 20% in wild type to 41% in *fab1* (Wu and Browse, 1995). This is significant because increased 16:0 in PG, and more specifically the sum of 16:0 + 18:0 + 16:1, Δ3 trans (sometimes referred to as high-melting-point fatty acids), has been correlated with chilling sensitivity through surveys of chilling-tolerant and chilling-sensitive plant species (Murata et al., 1982; Roughan, 1985). Typically, plants containing more than 60% high-melting-point fatty acids in PG have been shown to be chilling sensitive. The *fab1* mutant contains 69% high-melting-point fatty acids in PG: a higher percentage than is found in many chilling-sensitive plants. However, *fab1* plants were completely unaffected when compared with wild-type controls, by a range of chilling treatments that quickly killed chilling-sensitive plants such as cucumber (*Cucumis sativus*), mung bean (*Vigna radiata*), green bean (*Phaseolus vulgaris*), green pepper (*Capsicum annuum*), and castor bean (*Ricinus communis*; Wu and Browse, 1995). Instead, *fab1* plants are damaged only by long-term exposure to low temperature. During the first 7 to 10 d after transfer to 2°C, growth and photosynthetic characteristics of *fab1* plants remained indistinguishable from wild type, but between 10 and 28 d the mutants suffered almost complete loss of photosynthetic function and an associated destruction of chloroplasts (but not other organelles) within leaf cells (Wu et al., 1997). *fab1* plants die after 4 to 6 weeks at 2°C. We have previously speculated that elevated levels of high-melting-point fatty acids in PG may be the direct cause of the damage and death of *fab1* plants at 2°C, although it is not possible to rule out the involvement of other changes in thylakoid lipid composition (Wu and Browse, 1995; Wu et al., 1997).

The collapse of photosynthesis and breakdown of chloroplasts that occurs in *fab1* plants at 2°C is the most dramatic effect of temperature on photosynthesis among the Arabidopsis fatty acid mutants. Improved knowledge of this aspect of the *fab1* phenotype will likely lead to a better understanding of the relationship between thylakoid lipid structure and photosynthetic function. In this article, we describe the isolation of suppressor mutations that rescue *fab1* from death at low temperatures. Surprisingly, one of the suppressors is an allele of *fad5*; thus, a further increase in the proportion of saturated fatty acids in the photosynthetic membrane is associated with maintenance of photosynthetic function and viability of the plants at 2°C.

RESULTS

A Functional Screen for *fab1* Suppressors

Before beginning the suppressor screen, the *fab1* mutation was crossed into the *glabrous* (*gl1*) mutant background (Koornneef et al., 1982). The absence of trichomes on *fab1 gl1* plants provided a simple means to identify any pollen or seed contamination in the experiment (the *gl1* mutation was subsequently eliminated from each suppressor line during backcrossing to *fab1*). Approximately 50,000 *fab1 gl1* seeds were mutagenized with ethyl methanesulfonate and sown into 20 trays. M1 plants were grown to maturity and the resulting M2 seeds were collected, with each tray of plants providing a separate M2 pool. The suppressor screen was conducted on young seedlings grown in 15-cm petri dishes on agar medium containing mineral nutrients. Five-thousand sterilized M2 seeds were sown in each petri dish. After 12 d at 22°C, seedlings were well established with fully expanded green cotyledons. The plants were then transferred to 2°C. After 4 weeks at 2°C, most of the seedlings showed very little growth and were severely chlorotic (Fig. 1A). Against this background, putative suppressors were readily distinguished as much larger green plants with four to six true leaves (Fig. 1B). These plants were transferred to soil and grown to maturity at 22°C. A total of 110 putative mutants have been identified to date in this screen. For each of the putative mutants, 10 M3 plants were grown on soil at 22°C for 20 d and then transferred to 2°C for 6 weeks. Many

Figure 1. A functional screen for suppressors of the *fab1* low-temperature phenotype. Seedlings of M2 populations derived from the *fab1* mutant were grown for 12 d at 22°C and then transferred to 2°C. A, Representative view of plants after 4 weeks at 2°C. B, Putative suppressors were green and larger compared with other seedlings in the population.

of the mutants were dead at the end of this extended cold treatment, as were the *fab1* controls. However, plants of several mutant lines consistently survived for 6 weeks at 2°C. Based on the results of this secondary screen, we have so far identified 10 lines for further study. Here we report on five lines, S1, S7, S31, S101, and S106, for which an initial characterization has been completed.

Reciprocal crosses between mutant plants and *fab1* yielded F1 plants that were cold sensitive, like the *fab1* parent. Plants in the F2 populations segregated approximately 25% cold-tolerant individuals. These results indicate that each line contains a single recessive, nuclear mutation that confers suppression of the *fab1* cold-sensitive phenotype.

Four of the suppressors, S1, S31, S101, and S106, were indistinguishable from wild type and *fab1* in size and appearance when grown under standard culture conditions at 22°C. Plants of the S7 line were slightly reduced in size, but nevertheless were robust plants that produced abundant seed. The modestly smaller stature of S7 plants was still evident in plants that had been backcrossed to the *fab1* parental line through three generations. This result suggests that the suppressor mutation in the S7 line may have additional pleitropic effects on plant growth, although we cannot at present exclude the possibility that mutation(s) in other genes tightly linked to the suppressor locus are responsible for the altered phenotype.

Suppressor Mutations Rescue Photosynthetic Function of Cold-Treated *fab1*

To further investigate the suppressors, plants of the S1, S7, S31, S101, and S106 lines were grown together with *fab1* and wild-type controls at 22°C for 25 d, and then transferred to 2°C. After 3 weeks at 2°C, all the plants were similar in appearance. Between 3 and 4 weeks, *fab1* plants began to show some chlorosis relative to wild type, and all of the suppressors also became slightly chlorotic. In appearance, the suppressors were not readily distinguished from *fab1*, and wild-type plants were only slightly more green in color. These results are consistent with our previous observations that *fab1* leaves remain green at 2°C even after photosynthesis has collapsed and the chloroplasts show extensive damage (Wu et al., 1997).

Although *fab1* plants could not be easily distinguished from wild-type and suppressor plants by size or appearance, measurements of the potential quantum yield of photosynthesis, F_v/F_m, clearly established that the suppressors act by allowing photosynthetic function to be maintained. After 28 d at 2°C, F_v/F_m measured on *fab1* leaves was only 0.10, and after 38 d it was essentially 0 (Table I). By contrast, F_v/F_m in the suppressor lines was between 70% and 98% of the wild-type controls.

The Suppressors Remain Viable after Prolonged Cold Treatment

fab1 plants died after 5 to 7 weeks at 2°C as indicated by their failure to recover when transferred to warmer growth temperatures. Plants from all five suppressor lines remained alive at 2°C but they grew very little in size because the production and expansion of new leaves was substantially offset by senescence and death of mature leaves. Beyond 7 weeks at 2°C, all the suppressors were chlorotic and showed other symptoms of tissue damage. Figure 2 shows plants of the S1, S7, S31, and S106 lines after 14 weeks at 2°C. The *fab1* controls in this experiment are clearly dead. The wild-type controls, while healthy, are still small plants that have grown very slowly during 14 weeks at 2°C. The relative growth rate of wild-type Arabidopsis at 2°C is less than one-tenth of the rate at 22°C (Wu et al., 1997). When plants from these four suppressor lines were transferred to a higher growth temperature after 16 weeks at 2°C, they were all able to bolt, flower, and produce seed (Fig. 3).

In S31 Plants Suppression Is Associated with Increased 16:0

We next analyzed the overall fatty acid composition of leaf lipids in each of the suppressor lines. The compositions determined for plants of S101 and S106 were similar to that of the *fab1* mutants (data not shown), but the S1, S7, and S31 lines all showed substantial changes from the parental *fab1*. In both S1 and S7 plants, 16:3 is reduced to approximately

Table I. *Suppressors of the fab1 mutation preserve photosynthetic function at 2°C*

Plants of the suppressor lines were grown together with wild-type (WT) and *fab1* controls for 25 d at 22°C before transfer to 2°C. Values for the potential quantum yield of PSII, F_v/F_m, were measured after 28 d (experiment 1) or 38 d (experiment 2) at 2°C. Data are means ± SE.

Plant Line	WT	*fab1*	S1	S7	S31	S101	S106
Experiment 1							
F_v/F_m	0.646	0.102	0.525	0.544	0.490	–	–
SE	0.018	0.005	0.017	0.008	0.018	–	–
n	5	5	10	5	5	–	–
Experiment 2							
F_v/F_m	0.725	<0.02	–	0.707	0.662	0.540	0.495
SE	0.018	–	–	0.030	0.030	0.006	0.050
n	18	18	–	12	6	3	6

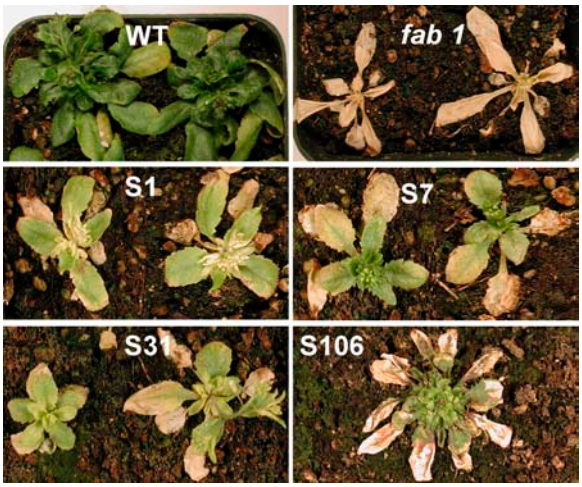

Figure 2. Survival of suppressor mutants after 14 weeks at 2°C. Plants were grown for 4 weeks at 22°C under a photoperiod with 16-h light and 8-h dark before transfer to 2°C with constant light.

one-third of the proportion found in *fab1*, and there are concomitant increases in the proportions of 18:2 and 18:3 fatty acids (Table II). Leaves of S31 plants also have reduced 16:3 but this is accompanied by a substantial increase in 16:0. These changes in leaf fatty acid composition suggest that each of these three suppressor lines has a mutation affecting the prokaryotic pathway of lipid synthesis: the chloroplast-resident pathway that is the only route to synthesis of 16:3 (Browse et al., 1986).

To find out if the S1, S7, and/or S31 lines are allelic, we made reciprocal crosses between them. Leaves of F_1 progeny from all six possible crosses had fatty acid compositions similar to *fab1* (>10% 16:3), indicating that S1, S7, and S31 contain mutations at distinct loci. Tests on plants from our backcrossing experiments indicated that the altered fatty acid composition in each of the lines cosegregated with the suppressor phenotype. The finding that three nonallelic suppressors all have reduced synthesis of the prokaryotic lipid 16:3 suggests that changes in chloroplast lipid metabolism may result in compensating changes in thylakoid lipid composition that alleviate the damage caused by the *fab1* lesion at 2°C. However, it is important to point out that cosegregation cannot definitively rule out the possibility in each case that the altered fatty acid composition is caused by a mutation that is distinct from, but closely linked to, the suppressor locus.

The overall fatty acid composition of S1 and S7 leaves is similar to that of the Arabidopsis *gly1* mutant, which is deficient in glycerol-3-P dehydrogenase activity (Miquel et al., 1998; Kachroo et al., 2004), and might also be expected of a leaky allele at the *act1* (= *ats1*) locus, which encodes the chloroplast acyl-acyl-carrier protein:glycerol-3-P acyltransferase (Kunst et al., 1988). To test these possibilities, we crossed S1 and S7 plants with the *gly1* and *act1* mutants. The F_1 progeny from all the crosses had leaf fatty acid compositions similar to wild type (>10% 16:3), indicating that S1 and S7 plants do not harbor mutations at either *gly1* or *act1*.

The Suppressor in S31 Is an Allele of *fad5*

The combination of increased 16:0 and decreased 16:3 found in leaf tissue of S31 plants suggested the possibility that this line contained a leaky mutation at the *fad5* locus. The *FAD5* gene encodes the chloroplast Δ7 desaturase that in Arabidopsis acts specifically on 16:0 esterified to the *sn-2* position of MGD (and possibly digalactosyldiacylglycerol [DGD]; Kunst et al., 1989). The product of the FAD5 enzyme is further desaturated to 16:3, which in wild-type plants accounts for 30% to 35% of the acyl groups of MGD, the major thylakoid lipid (Browse et al., 1986).

To discover more about lipid metabolism in the S31 line, we grew wild-type, *fab1*, and S31 plants side by side, separated individual lipids from leaf extracts, and analyzed their fatty acid compositions by gas chromatography (GC). In *fab1* plants, the fatty acid composition of MGD is similar to wild type, with 35% 16:3 and less than 5% 16:0 (Table III). This agrees with previous findings that MGD is the membrane lipid least affected by the accumulation of 16:0 in *fab1* leaves (Wu et al., 1994). By contrast, MGD from S31 leaves contains only 6% 16:3 and 24% 16:0. This change in fatty acid composition would be expected for a plant substantially deficient in FAD5 desaturase activity. The chloroplast lipid DGD, as well as extrachloroplast phospholipids, also contain increased proportions of 16:0 compared with *fab1*, and this is consistent with the lipid changes observed in the original *fad5* mutant (Kunst et al., 1989).

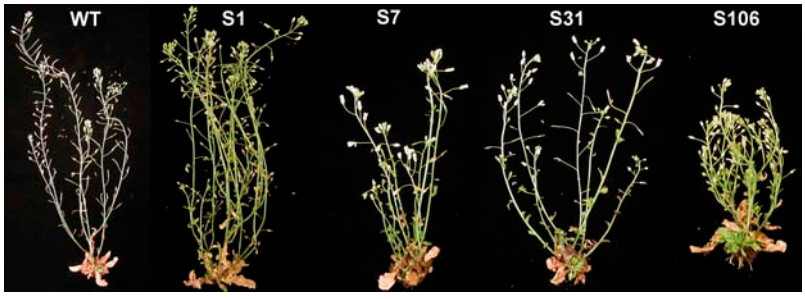

Figure 3. After prolonged cold treatment, suppressors recover after return to warm temperature. Plants were grown for 4 weeks at 22°C followed by 16 weeks at 2°C. They then were transferred to 19°C for 1 week before being photographed.

Table II. *Altered fatty acid compositions in three suppressors of fab1*

Plants of each suppressor line were grown together with *fab1* and wild-type (WT) plants at 22°C for 3 weeks. Data are means ± SE ($n = 4$).

Fatty Acid	WT	*fab1*	S1	S7	S31
16:0	17.1 ± 0.3	23.3 ± 0.3	25.6 ± 0.6	25.5 ± 0.5	30.5 ± 0.1
16:1 cis	0.5 ± 0.2	1.7 ± 0.2	1.2 ± 0.5	1.8 ± 0.5	1.4 ± 0.3
16:1 trans	2.1 ± 0.1	2.6 ± 0.3	2.4 ± 0.2	2.2 ± 0.2	2.7 ± 0.3
16:2	0.6 ± 0.1	1.0 ± 0.2	1.0 ± 0.3	0.9 ± 0.3	0.2 ± 0.2
16:3	12.2 ± 0.2	15.0 ± 0.2	4.0 ± 0.2	5.6 ± 0.3	3.7 ± 0.2
18:0	1.3 ± 0.3	0.7 ± 0.2	0.7 ± 0.3	1.0 ± 0.4	1.0 ± 0.3
18:1	3.1 ± 0.1	2.8 ± 0.5	3.6 ± 0.7	3.9 ± 0.8	3.2 ± 0.5
18:2	15.1 ± 0.1	10.9 ± 0.3	13.9 ± 0.8	14.6 ± 0.3	12.5 ± 0.4
18:3	48.6 ± 0.3	42.0 ± 0.5	47.6 ± 0.5	44.5 ± 1.1	44.8 ± 0.8

It is noteworthy that the fatty acid composition of PG from S31 plants is very similar to that of PG from *fab1* (Table III). The proportion of high-melting-point fatty acids in this lipid is 70% for S31, in this experiment, compared with 71% in *fab1* (and 58% in wild type).

To confirm that the S31 line contained a mutation at the *fad5* locus, we made a cross to the original *fad5* line characterized by Kunst et al. (1989), which we now designate *fad5-1*. The overall leaf fatty acid composition of leaves from F1 plants of this cross contained 3% 16:3 and 21% 16:0. The lack of complementation of the low 16:3 phenotype in this test cross demonstrates that S31 indeed contains an allele of *fad5* that we designate *fad5-2*.

We used PCR to amplify the *fad5-2* allele from genomic DNA prepared from S31 plants. Sequencing of the PCR products revealed a single base-pair change relative to the wild-type sequence (GenBank accession no. NM112455) that is a G to A substitution at position 1,638 of the GenBank sequence. This change is predicted to encode an Ala-286 Thr mutation in the sequence of the predicted FAD5 preprotein (Fig. 4). The *fad5-1* allele encodes a Trp-98 stop mutation and is thus likely to be a null allele (Heilmann et al., 2004).

We crossed *fab1* and *fad5-1* to produce a *fab1 fad5-1* double mutant. Analysis of the fatty acid compositions of individual leaf lipids of *fab1 fad5-1* plants provided data very similar to those shown for S31 in Table III. In particular, both MGD and DGD contained substantially higher proportions of 16:0 than *fab1* plants: 45.4% 16:0 in DGD and 30.1% 16:0 in MGD. The MGD from leaves of S31 plants contains 23.5% 16:0, but retains 6.5% 16:3 (Table III), and this suggests that the Ala-286 Thr mutant allele of FAD5 retains a low level of desaturase activity. The *fab1 fad5-1* double mutants (and the original *fad5-1* line) do not contain any 16:3 in MGD (Kunst et al., 1989; data not shown). We grew wild-type, *fab1*, S31, and *fab1 fad5-1* plants at 22°C for 3 weeks and then transferred them to 2°C. After 6 weeks at 2°C, we measured F_v/F_m in leaves of five plants of each line. The data from these measurements (average ± SE) were 0.723 ± 0.017, 0.092 ± 0.043, 0.613 ± 0.036, and 0.696 ± 0.044 for wild-type, *fab1*, S31, and *fab1 fad5-1*, respectively. Thus, suppression of the *fab1* phenotype mediated by the *fad5-1* allele is comparable to that observed in the S31 line (Table I). After 10 weeks at 2°C, leaf tissue on *fab1* plants was dead and brown but leaves of *fab1 fad5-1* double mutant plants were

Table III. *Fatty acid compositions of leaf lipids from wild-type (WT), fab1, and S31 plants*

Plants were grown together at 22°C for 3 weeks and leaf material harvested. Lipids were extracted, separated by thin-layer chromatography, and their fatty acids analyzed by GC, as described under "Materials and Methods." SQD, Sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

Fatty Acid	MGD[a]			DGD			SQD			PG			PC			PE			PI		
	WT	*fab1*	S31	WT	*fab1*	S31	WT	*fab1*	S31	WT	*fab1*	S31	WT	*fab1*	S31	WT	*fab1*	S31	WT	*fab1*	S31
16:0	1.7	4.2	23.5	15.0	27.8	46.5	45.6	56.0	57.8	34.9	49.6	47.8	23.2	32.0	34.0	32.8	36.8	39.4	49.5	51.8	59.6
16:1 c	1.5	2.4	1.2	–	1.3	–	–	1.5	–	–	–	–	–	2.1	1.8	–	1.2	0.6	–	–	–
16:1 t	–[a]	–	–	–	–	–	–	–	–	–	21.5	20.0	21.1	–	–	–	–	–	–	–	–
16:2	2.0	2.2	–	0.7	0.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16:3	32.0	35.1	6.5	2.9	3.5	0.8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18:0	–	–	0.8	1.3	1.4	2.3	–	2.8	3.1	1.6	1.4	1.3	2.5	2.4	2.2	2.5	1.8	2.1	4.1	3.6	3.5
18:1	1.5	1.1	1.9	1.8	2.3	0.9	4.0	2.1	3.7	6.2	4.1	3.7	8.7	5.2	6.5	4.3	5.4	6.7	3.0	1.8	2.3
18:2	3.2	2.4	2.1	4.1	3.8	2.1	10.2	7.3	4.8	8.6	5.3	5.9	34.7	26.3	27.1	35.3	27.6	28.7	20.8	18.1	17.1
18:3	57.7	52.0	63.8	73.3	59.1	46.1	36.1	29.0	30.6	27.0	18.6	19.8	30.6	31.5	28.2	24.2	26.6	22.6	22.3	23.8	16.9
Proportion of leaf polar lipids (%)	36.4	35.6	36.5	13.4	10.5	8.0	3.4	3.5	3.3	12.0	13.7	13.7	19.8	19.0	21.0	10.8	12.6	12.3	4.2	5.1	4.9

[a]<0.5%.

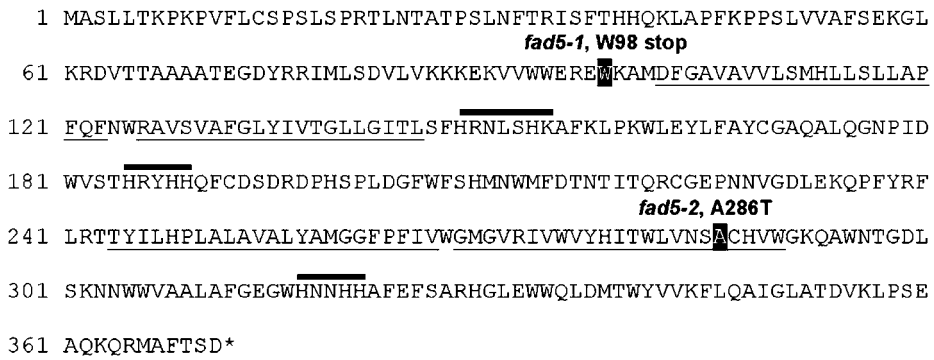

Figure 4. Peptide sequence of *fad5* alleles. The deduced amino acid sequence of FAD5 is shown. Heavy bars indicate the three His-rich sequences that coordinate the diioron-oxo group at the active site. Four predicted transmembrane domains are underlined. The mutations in *fad5-1* (W98 stop) and *fad5-2* (A286T) are shown.

similar in appearance to S31 (compare with Fig. 2). Plants were transferred back to 22°C and *fab1 fad5-1* and S31 plants (along with wild-type and *fad5* controls) had flowered and set seed 2 weeks later, while *fab1* plants had not recovered. These results strongly indicate that the *fad5-2* mutation is the basis of the S31 suppressor phenotype and we now refer to this line as *fab1 fad5-2*.

DISCUSSION

Considerable evidence indicates that membrane lipid composition is a key determinant of plant responses to temperature (Murata et al., 1992; Nishida and Murata, 1996; Browse and Xin, 2001; Iba, 2002). Improved knowledge of the responses of photosynthesis and other plant processes to both high and low temperatures is essential to understanding many phenomena, from the geographical ranges of plant species, to the response of ecosystems to global warming. In production agriculture, this knowledge is a prerequisite to modifying these processes for increased plant productivity in different environments.

Many plants undergo sharp reductions in growth rate and development at temperatures between 0°C and 12°C. These chilling-sensitive plants include many economically important crops such as cotton (*Gossypium hirsutum*), soybean (*Glycine max*), maize (*Zea mays*), and rice (*Oryza sativa*). Attempts to link the biochemical and physiological changes associated with chilling injury with a single trigger or site of damage have often focused on the possibility of an L_α to L_β lipid phase (liquid crystalline phase to gel phase) transition in cellular membranes (Lyons, 1973; Raison, 1973). One hypothesis, specific to chloroplast membranes, proposes that molecular species of chloroplast PG that contain high-melting-point fatty acids at both the *sn-1* and *sn-2* positions of the glycerol backbone confer chilling sensitivity on plants (Murata, 1983; Nishida and Murata, 1996). This hypothesis is supported by several lines of evidence, including the detection of L_α to L_β phase transitions in PG isolated from chilling-sensitive plants (Murata and Yamaya, 1984), a correlation across plant species between the proportion of high-melting-point fatty acids in PG and the degree of chilling sensitivity (Murata et al., 1982; Roughan, 1985), and evidence from transgenic plants with engineered alterations in the fatty acid composition of PG (Murata et al., 1992; Wolter et al., 1992).

Characterization of the *fab1* mutant demonstrated that high-melting-point PG can only be one component of plant-chilling sensitivity (Wu and Browse, 1995), but that its presence in thylakoid membranes is incompatible with maintenance of photosynthetic function and plant viability at low temperatures (Wu et al., 1997). The very complete collapse of photosynthesis and disruption of chloroplast structure that occurs in *fab1* plants at 2°C graphically demonstrates the critical role of membrane lipid composition in plant temperature responses. To explore this area of investigation further, we have carried out a screen for mutations that suppress the *fab1* low-temperature phenotype. In this article, we describe the screen and initial characterization of five mutants that were among the first suppressors isolated.

Two of the suppressor lines show no change in overall leaf fatty acid composition, relative to the parental *fab1* mutant. The suppressor mutations in these lines may have introduced a change in the amino acid sequence of a protein that allows the protein to better maintain its function in *fab1* thylakoids at 2°C. For example, PG is a component of the light-harvesting complex of PSII (LHCII) as determined by x-ray crystallography (Liu et al., 2004) and is required for oligomerization of the complexes (Nussberger et al., 1993). If the altered fatty acid composition of PG in the *fab1* mutant compromised assembly or maintenance of LHCII at low temperatures, then a mutation in one of the genes encoding LHCII (Andersson et al., 2003) might alleviate the defect, at least partially. Exploring such possibilities further will require us to identify the suppressors by map-based cloning techniques and this work is now under way.

Mutations may also produce a suppressor phenotype by causing a change in thylakoid fatty acid composition that complements the defect introduced by the *fab1* mutation. This appears to be the case for the mutations in the S1, S7, and S31 lines. For S31, the mutation has been defined as an allele of *fad5*. The overall

leaf fatty acid compositions of S1 and S7 plants suggest that they contain mutations affecting the prokaryotic pathway of chloroplast lipid synthesis. However, the mutations are not allelic to each other or to the *act1* and *gly1* mutations that affect enzymes of prokaryotic lipid synthesis (Kunst et al., 1988; Miquel et al., 1998; Kachroo et al., 2004). It is possible that map-based cloning of the suppressor loci in the S1 and S7 lines will identify new components involved in the biochemistry or regulation of chloroplast lipid synthesis.

The identification of *fad5-2* as the suppressor mutation in line S31 was a surprise to us. We believe that the collapse of photosynthesis and death of *fab1* plants at 2°C is caused by the increase in saturated 16:0 fatty acids and most probably by the presence of approximately 70% high-melting-point fatty acids in chloroplast PG (Wu et al., 1997). This hypothesis is consistent with the other evidence discussed above (Murata et al., 1982; Murata and Yamaya, 1984; Roughan, 1985; Murata et al., 1992; Ishizaki-Nishizawa et al., 1996). It is therefore unexpected that a further increase in chloroplast 16:0 should provide a suppressor phenotype, especially when *fad5* mutant plants are themselves compromised in growth and chloroplast biogenesis at low temperature (Hugly and Somerville, 1992).

The *fad5* mutations have complex effects on lipid metabolism. Although 16:0 accumulates in MGD, the increase is less than the decrease in 16:3 (Kunst et al., 1989; Table III). This is explained in part by decreased flux of fatty acids into the prokaryotic pathway, but there is also increased turnover of prokaryotic MGD, which results in transfer of 16:0 into DGD, phosphatidylcholine, and other lipids (Kunst et al., 1989). Synthesis of MGD via the eukaryotic pathway is increased, providing for a higher proportion of 18:3 in MGD and a level of MGD in leaf lipids that is similar to wild type (approximately 35%–38% of total leaf lipids). DGD is predominantly synthesized from eukaryotic MGD but the reaction favors molecular species containing 16:0 (Browse et al., 1986), and this partly explains the increased proportion of 16:0 in DGD of *fad5* leaves (Kunst et al., 1989). All these effects of the *fad5-2* mutation are reflected in the lipid analysis of S31 relative to *fab1* in Table III, and similar results were found for the *fab1 fad5-1* double mutant (data not shown).

It is noteworthy that the *fab1* mutation increases 16:0 in PG and DGD, but that MGD is largely unaffected, relative to wild type (Table III; Wu et al., 1994). In the *fad5-1* mutant, 16:0 is increased in MGD and DGD, while there is very little change in the fatty acid composition of PG, relative to wild type (Kunst et al., 1989). Consistent with these observations, introduction of the *fad5-2* allele into the *fab1* background did not substantially change the fatty acid composition of PG. In *fab1 fad5-2* (S31) plants, PG contains 70% high-melting-point fatty acids compared with 71% in PG from *fab1* plants (Table III). The data in Table III indicate that MGD in S31 thylakoids will contain greatly increased levels of 16:0/18:3 + 18:3/16:0 molecular species, nearly 50% of the total MGD molecules compared with only 8.4% in *fab1*. In DGD from S31 leaves, 16:0/18:3 + 18:3/16:0 will be >90% of the molecules, compared with 56% in *fab1*, but the change in MGD molecular species will have a larger effect on overall thylakoid lipid composition because MGD represents >36% of the total leaf glycerolipids versus 8% for DGD in S31.

We considered the possibility that the proportions of different lipids within the thylakoids might be a factor in suppressing the low-temperature phenotype. In both higher plants and algae, it has been observed that growth at low temperatures increases the ratio of DGD to MGD (Lynch and Thompson, 1982; Kuiper, 1985), although it is not clear whether this change reflects a mechanism of acclimation. In any case, *fab1 fad5* leaves contain proportions of DGD and MGD that are similar to those found in *fab1* leaves (Table III), and the DGD:MGD ratio calculated for the suppressor plants (0.22) is actually somewhat lower than that found in the *fab1* mutant (0.29).

The observation that the S1 and S7 lines as well as the *fab1 fad5-2* (S31) suppressor have reduced levels of 16:3 compared with *fab1* (and wild type) may make it tempting to suggest that the lower levels of 16:3 in MGD are somehow directly responsible for suppression. However, in S1 and S7, 16:3 MGD is largely replaced by 18:3 (data not shown), and our understanding of the biophysics of these two fatty acids does not provide any rationale for a substantial change in molecular or membrane properties. Furthermore, available evidence indicates that decreased 16:3 is correlated with lower leaf chlorophyll content (Heilmann et al., 2004) and reduced photosynthetic performance at low temperature (Routaboul et al., 2000). By contrast, the *fad5-2* mutation reduces overall unsaturation both in the individual chloroplast lipids (Table III) and in the overall leaf fatty acid profile (Table II). The decreased unsaturation is likely to reduce membrane fluidity and would be predicted to exacerbate rather than ameliorate the low-temperature phenotype of *fab1 fad5-2* plants.

However, the replacement of 16:3 in MGD of *fab1 fad5-2* plants by the saturated acyl group 16:0, is predicted to change the shape of the MGD molecule to a more narrow cone (Gounaris et al., 1983; Gruner et al., 1985). Thus, one possibility is that changes in the shape of MGD molecules compensate for disruptive changes in the shape of PG molecules induced by the *fab1* mutation, for example by altering the packing relationships between the thylakoid lipids and membrane proteins of the photosynthetic complexes (Gounaris and Barber, 1983; Simidjiev et al., 2000). Changes in the shape of lipid molecules is known to be important for a number of membrane functions including protein trafficking and membrane fusion (Rietveld et al., 1995; Chanturiya et al., 1997; Bruce, 1998). Certainly, the decreased unsaturation of lipids in *fab1 fad5-2* plants relative to *fab1* means that it is reasonable to consider molecular shape rather than membrane fluidity or phase changes as the basis of the suppressor phenotype.

Clearly, the relationship between thylakoid lipid structure, photosynthetic function, and plant temperature responses is very complex. Further analysis of the genes and mutants found in our suppressor screen will provide a means to develop and test new hypotheses about the mechanistic bases of this relationship.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used as the wild type in this study. Plants of the *fab1* mutant (Wu et al., 1994) were crossed with the *gl1* mutant (Koornneef et al., 1982). F3 seeds derived from F2 plants identified as being homozygous for *fab1* and *gl1* were collected. The F3 seeds were mutagenized with 0.3% (v/v) ethyl methanesulfonate for 12 h at room temperature, then rinsed in 10 changes of distilled water over 2 h and stored at 4°C. After 2 d of stratification, M1 seeds were sown on soil in plastic trays and the resulting plants grown to maturity at 22°C and continuous illumination of 120 μmol quanta m^{-2} s^{-1}. Seed from each tray of plants was harvested as a separate M2 pool.

Suppressor Screening

Approximately 5,000 seeds from each M2 pool were surface sterilized, stratified for 2 d at 4°C, and then spread evenly on a 15-cm petri dish containing 1× Gamborg's B-5 basal salts (Sigma Aldrich) in 1% agar. Petri dishes were sealed with porous tape and germinated at 22°C under a 16-h photoperiod of 80 μmol quanta m^{-2} s^{-1}. After 12 d, petri dishes were moved to the growth chamber at 2°C under constant illumination of 100 μmol quanta m^{-2} s^{-1}. After 4 weeks at 2°C, when the *fab1 gl1* control plants were chlorotic, survivors among the M2 seedlings were selected as putative suppressors of the *fab1* low-temperature phenotype. The selected surviving seedlings were transferred to soil and grown to maturity at 22°C.

For rescreening of the putative suppressor mutants, seeds were collected from each surviving M2 plant. Ten M3 plants of each suppressor line were grown in pots at 22°C for 20 d before being transferred to a growth chamber at 2°C. The M3 plants were scored for survival after 4 to 6 weeks at 2°C. From each suppressor line, several individual plants showing a surviving chilling phenotype were chosen, transferred to 22°C, and grown to maturity. Based on the second chilling screen, suppressor mutant lines originating from different M2 pools were chosen for further study. All the mutant lines discussed in this paper have been backcrossed to the original *fab1* line two to three times with the exception of S101 (one backcross).

Fatty Acid and Lipid Analysis

The overall fatty acid composition of leaves was 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 μL) of the organic phase were analyzed by GC on a 30 m × 0.53 mm Alltech Econo-cap column containing a 1.2 μm EC-WAX phase (Alltech Associates). 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 $(NH_4)_2SO_4$-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 phosphatidylcholine was added as internal standard prior to derivitization.

Measurements of Chlorophyll Fluorescence

Chlorophyll fluorescence from leaf tissue 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 tissue. Leaves on intact plants were dark adapted at 22°C for 30 min before each F_v/F_m measurement was made.

Cloning and Sequencing of *fad5-2*

A DNA fragment of At3g15850 was amplified from genomic DNA extracted from S31 plants with primers designed to the sequence of wild-type Arabidopsis (GenBank file 30698537). Restriction endonuclease sites suitable for cloning into conventional vectors were added to 5′ ends of the primers. The At3g15850 coding region was amplified using the primer combinations 5′-CGCGAATTCTCTTCCTTCTTTCTCTTAGCCAT-3′ (primer 1) and 5′-AATGGATCCAGTTGAGTATCTAGAATTGCCGT-3′ (primer 2). The amplification protocol included an initial 10-min denaturation step at 94°C, 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, and 3 min extension at 72°C, followed by 15 min extension at 72°C. To minimize PCR artifacts, Pfu polymerase was used. DNA fragments amplified from genomic DNA in three independent PCR reactions were cloned into pBlueScript (SK+) vectors (Stratagene) and were sequenced individually using universal and custom sequencing primers.

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