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A Role for Membrane Lipid Polyunsaturation in Chloroplast Biogenesis at Low Temperature[1]

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ABSTRACT

Two different mutants of *Arabidopsis thaliana* deficient in chloroplast membrane lipid polyunsaturation were indistinguishable in appearance from the wild-type when grown at 22°C. By contrast, leaf tissues of the mutants that developed during growth at 5°C were chlorotic, whereas the wild type was not. This is the first direct evidence that chloroplast lipid polyunsaturation contributes to low-temperature fitness. Chloroplasts from mutant lines grown at 5°C were much smaller than those of the wild-type, and the thylakoid membrane content was reduced by up to 70%. However, there was no discernible effect of low temperature on chloroplasts that developed prior to exposure to low temperatures. These and related observations suggest that the high degree of chloroplast membrane lipid polyunsaturation is required for some aspect of chloroplast biogenesis.

The thylakoid membranes of chloroplasts have one of the highest contents of lipids containing polyunsaturated acyl groups known for any membrane. Polyunsaturated acyl groups, typically 16:3 (n-3) or 18:3 (n-3), account for 85 to 90% of the fatty acids in chloroplast lipids (13). The reasons for the high degree of lipid polyunsaturation of thylakoid membranes are not known. Because acyl lipids comprise only about 25 to 35% by weight of thylakoid membranes (13), it has been suggested that the high degree of polyunsaturation may be required to maintain the degree of fluidity required for the diffusion of lipophilic compounds such as plastoquinone and the protein complexes that catalyze photosynthetic electron transport (6). However, this proposal is not generally supported by the results of experiments in which 40% of the *cis* unsaturations in lipids of spinach chloroplasts were reduced by catalytic hydrogenation without significant loss of photosynthetic electron transport activity (14, 17).

An alternative proposal is that the high degree of unsaturation may confer a suitable geometry on the lipid molecules to facilitate intimate lateral contact between the membrane-spanning domains of integral membrane proteins and the lipid bilayer (5, 13, 15). To investigate the functional significance of chloroplast lipid composition, we have previously isolated and characterized a series of mutants of *Arabidopsis* that are defective in specific enzymic reactions involved in lipid metabolism (19). Four loci have been identified that encode gene products required for lipid-linked fatty acid desaturation of chloroplast lipids (*fad*A, *fad*B, *fad*C, *fad*D) and one locus has been identified that encodes a plastid glycerol-3-phosphate acyltransferase (*act1*). Each of these mutants exhibits significant changes in chloroplast fatty acid composition. However, the mutants have no visible phenotype and relatively minor effects were observed on growth rate, net photosynthetic CO_2 fixation, and photosynthetic electron transport, suggesting that a high degree of lipid unsaturation is not specifically required to support these processes. The most pronounced effects were that, under standard growth conditions (100–150 μmol m^{-2} s^{-1} PAR, 22°C), several of the mutant lines displayed changes in chloroplast organization, suggesting that polyunsaturated lipids are required for the development or maintenance of the characteristic ultrastructure of chloroplast membranes. However, because of lack of information about the factors that regulate chloroplast membrane ultrastructure, it has not been possible to propose a mechanistic explanation for the altered chloroplast morphology in the mutants.

In the studies reported here, we have compared the growth and development of the lipid mutants and wild-type *Arabidopsis* at low temperature. The results of these studies provide direct evidence that chloroplast membrane lipid polyunsaturation contributes to the low-temperature fitness of the organism.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The lines of *Arabidopsis thaliana* (L.) Henyh. described here were descended from the Columbia wild type. The mutant lines JB1, JB67, LK3, JB2, and JB25 carry defective alleles of the *fad*A (2), *fad*B (11, 12), *fad*C (4, 7), *fad*D (3), and *act1* (9) loci, respectively. The enzymic deficiencies in these mutants are listed in Table I. The *fad*B and *fad*C lines used here were backcrossed to the wild type a minimum of three times.

Plants were germinated at 22°C under continuous fluorescent illumination (100–150 μmol m^{-2} s^{-1}) on a potting mixture irrigated with mineral nutrients (7). After 7 d the seedlings were transferred to the growth conditions of a particular treatment as noted below. Plants grown at 5°C received continuous fluorescent illumination at 20 μmol m^{-2} s^{-1}.

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Table I. *Proposed or Demonstrated Enzymic Lesions in Mutants with Altered Chloroplast Lipid Composition*

Locus	Enzyme
act1	Glycerol-3-phosphate acyltransferase
*fad*A	*trans*-Δ3 desaturation of 16:0 on sn-2 of phosphatidylglycerol
*fad*B	*cis*-ω9 desaturation of 16:0 on sn-2 of galactolipids
*fad*C	*cis*-ω6 desaturation of 16:1 and 18:1 on sn-1 and sn-2 of all lipids
*fad*D	*cis*-ω3 desaturation of 16:2 and 18:2 on sn-1 and sn-2 of all lipids

Extraction and Analysis of Chl and Fatty Acids

For measurements of leaf fatty acid composition, plants were grown at 22°C for 21 d or germinated at 22°C for 7 d then grown at 5°C for 21 d. Fatty acid methyl esters were prepared and measured as described (2). Chl content was determined in 80% acetone.

Microscopy

Preparation and examination of leaf samples by EM were carried out as described (10).

RESULTS

Mutant Phenotype at Low Temperatures

Wild-type *Arabidopsis* plants are not injured by exposure to chilling temperature and continue to grow and develop during prolonged incubation at 5°C (Fig. 1). Although the growth rate is significantly reduced relative to that at higher temperature (7), the plants exhibit a normal morphology but have a significantly higher Chl content (Fig. 2). The *fad*A and *fad*D mutants were not visually distinguishable from the wild type at any growth temperature (results not presented) and were not examined further. The absence of an effect of the *fad*D mutation is expected because mutations at this locus do not have a significant effect on fatty acid composition below about 18°C (3). Although the *act1* mutant was slightly chlorotic, the effect was of similar magnitude at all temperatures (Fig. 2) so the effects of low temperature on the mutant were not examined further. By contrast, at 5°C the growth rate of the *fad*B and *fad*C mutants was significantly lower than wild type (Fig. 1), and the leaves at the center of the rosettes that developed during growth at low temperature were chlorotic (Fig. 3). The severity of the chlorosis varied with the growth temperature so that under standard growth conditions the mutants were visually indistinguishable from the wild type, but at 5°C rosettes of the mutants had only about half the Chl content of the wild type (Fig. 2).

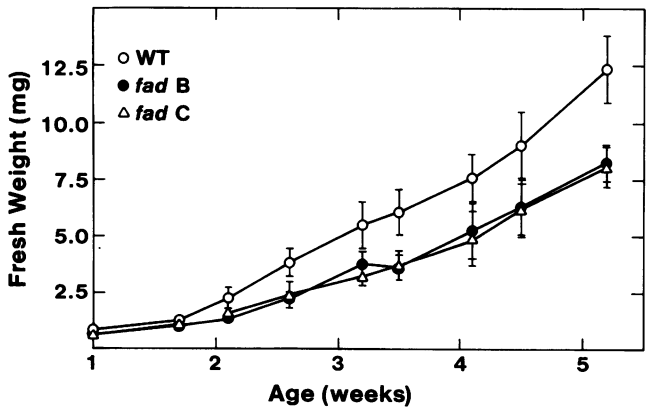

Figure 1. Growth rate of wild-type and mutant *Arabidopsis* at 5°C. Growth rate was measured by taking the fresh weight of the entire above-ground portions of plants at the indicated intervals. Values shown are the mean ± SD (n = 6).

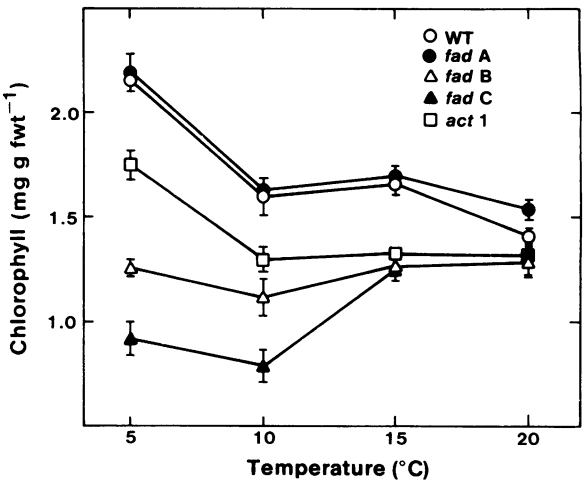

Figure 2. Chl content of wild-type and mutant *Arabidopsis* plants. Plants were germinated and grown for 7 d at 22°C then transferred to the temperatures shown for 14 d. Values shown are the mean ± SD (n = 6).

The effect of low temperature on Chl content of the *fad*B and *fad*C mutants was not restricted to the development of seedlings. When 16-d-old rosettes were transferred to 5°C for 6 weeks, the Chl content of the three youngest leaves from the wild-type, *fad*B, and *fad*C rosettes was 2.5 ± 0.3, 1.9 ± 0.1, and 1.6 ± 0.4 μg/mg fresh weight, respectively. The Chl *a*/*b* ratio of these leaves was 3.1 ± 0.2, 3.1 ± 0.1, 3.2 ± 0.1, respectively, indicating that there was a similar decrease in the amounts of both antenna and nonantenna Chl. By contrast, the Chl content of the three oldest leaves from the same plants was 2.5 ± 0.3, 2.6 ± 0.1, and 2.1 ± 0.1 μg/mg fresh weight, respectively.

Genetic Analysis

To exclude the possibility that the *fad*B and *fad*C mutant lines coincidentally contained an additional mutation at a locus other than the *fad* loci that was responsible for the chlorotic phenotype, cosegregation of the mutations responsible for the altered lipid composition of the mutant lines with the mutations that caused leaf chlorosis at low temperature was tested. Fifty F_2 plants from crosses between wild-type and the *fad*B and *fad*C mutants were individually scored for both the altered lipid composition and low temperature-induced

Figure 3. Phenotypes of wild-type and mutant *Arabidopsis*. Plants were germinated at 22°C. Plants at the cotyledon stage (7 d after germination) were either maintained at 22°C for 10 d (right panel) or transferred to 5°C for 3 weeks (left panel). Scale: 22°C, 0.66×; 4°C, 0.77×.

chlorosis. Eleven F_2 plants from the *fad*B × wild-type cross, and 13 plants from the *fad*C × wild-type cross, expressed both phenotypes. The remaining plants from these crosses did not display either phenotype. The cosegregation of the two phenotypes indicates that the loci controlling leaf chlorosis are tightly linked to the *fad*B and *fad*C loci and are, therefore, presumed to be identical.

Effect of Temperature on Fatty Acid Composition

It has previously been established that the *fad*B and *fad*C mutations affect chloroplast lipid fatty acid composition in plants grown at 22°C, but expression of the mutations at other temperatures was not examined (4, 11). Because the effects on fatty acid composition of mutations at the *fad*D locus are much less pronounced in plants grown at temperatures below about 18°C (3), it was considered essential to examine the effect of temperature on the fatty acid composition of the *fad*B and *fad*C mutants.

Comparison of the fatty acid composition of plants grown at 5 and 22°C showed that, in both mutants and wild type, growth at low temperature caused a significant increase in the proportion of 18:3 (Table II). There was also a significant decrease in the proportion of *trans*-16:1, as previously reported (8). Leaf lipids of the *fad*B mutant were completely deficient in *cis*-16:1 and 16:3 at both temperatures. Thus, the enzymic lesion in the *fad*B mutant (Table I) is not conditional on the temperature of growth. Similarly, at both temperatures the *fad*C mutant showed characteristic increases in the amount of 16:1 and 18:1 and corresponding decreases in the proportions of the more highly unsaturated 16- and 18-carbon fatty acids. Thus, *fad*C mutation is also not conditional on the growth temperature.

Chlorosis Affects Developing Leaves

To evaluate the effect of growth at low temperature on chloroplast ultrastructure, thin sections of chloroplasts were prepared from leaves of wild-type and mutant plants that had developed and expanded during growth at 5°C for 3 weeks. Following this treatment, the leaf Chl content of whole rosettes of wild-type, *fad*B, and *fad*C plants was 1.8 ± 0.1, 1.1

Table II. *Leaf Lipid Fatty Acid Composition of Wild-Type and Mutant Lines of Arabidopsis Grown at 22 and 5°C*

Values represent the mol % of total leaf fatty acids and are the mean ± SD (n = 6).

Acyl Group	Wild Type		*fad*B		*fad*C	
	22°C	5°C	22°C	5°C	22°C	5°C
16:0	12.4 ± 1.2	11.8 ± 1.2	20.8 ± 0.3	19.8 ± 0.9	10.9 ± 0.8	9.8 ± 0.1
16:1*cis*	1.1 ± 0.4	*t*[a]	*t*	*t*	10.0 ± 0.1	5.6 ± 0.7
16:1*trans*	2.0 ± 0.4	*t*	2.5 ± 0.2	*t*	2.5 ± 0.2	*t*
16:3	16.8 ± 1.2	14.4 ± 1.2	*t*	*t*	*t*	*t*
18:0	*t*	*t*	3.7 ± 0.4	1.6 ± 0.7	3.2 ± 1.2	2.5 ± 0.9
18:1	5.3 ± 0.6	3.6 ± 1.6	3.9 ± 0.8	2.7 ± 0.9	26.1 ± 0.6	25.2 ± 1.7
18:2	18.4 ± 0.7	17.8 ± 1.0	18.2 ± 0.9	16.6 ± 4.7	17.1 ± 0.5	20.7 ± 0.8
18:3	41.9 ± 0.5	50.4 ± 1.6	50.6 ± 1.8	58.5 ± 5.9	29.1 ± 0.6	35.3 ± 1.9

[a] *t* indicates less than 1% of total sample.

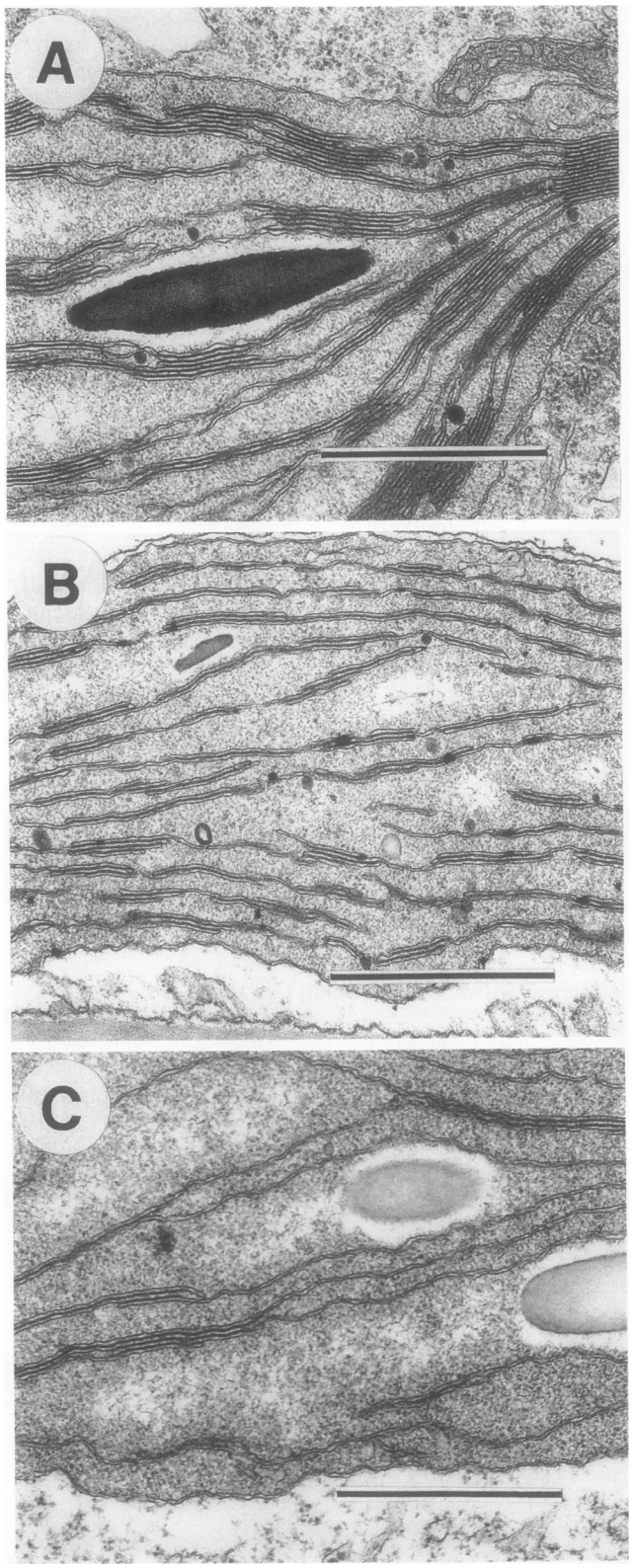

Figure 4. Transmission electron micrographs of chloroplasts from the youngest leaves of rosettes of wild-type and mutant *Arabidopsis*. Plants were germinated at 22°C and, after 7 d, were transferred to 5°C for 3 weeks. A, Wild type; B, *fad*B; C, *fad*C. Bar = 1 μm.

± 0.1, and 1.0 ± 0.1 μg/mg fresh weight, respectively. Electron micrographs of chloroplasts from these plants revealed several major differences in chloroplast ultrastructure between the wild-type and mutant lines (Fig. 4). Chloroplasts from the *fad*C mutant line contained only 30% as much thylakoid membrane as chloroplasts from wild-type plants (Table III). Similarly, the *fad*B mutant contained only 40% as much thylakoid membrane as the wild type. The average cross-sectional area of chloroplasts from the mutant lines were also greatly reduced, indicating a net decrease in chloroplast size under these conditions (Table III).

The reduced amount of chloroplast membranes present in the leaves of the mutants grown at low temperature could be due to either a defect in membrane biogenesis or membrane stability at low temperatures. To distinguish between these possibilities, leaf Chl content was measured in plants that were exposed to 5°C for 3 weeks at various stages of development. A defect in chloroplast membrane biogenesis would be expected to primarily affect developing leaves, whereas reduced stability would be expected to affect both developing and mature leaves.

Wild-type and mutant plants were grown at 22°C for 7 to 15 d then transferred to 5°C for 3 weeks. Under these conditions, the developmental stage at the time of transfer to low temperature ranged from cotyledon-stage seedlings lacking true leaves at 7 d to rosettes with six almost fully expanded leaves at 15 d (Fig. 5). Leaf chlorosis of the *fad*B and *fad*C mutants was most pronounced when very young seedlings were exposed to low temperatures. Leaves that were fully expanded at the time of transfer did not become chlorotic, suggesting that morphology of mature chloroplasts were unaffected by the low temperature treatment. Chloroplasts from 15-d-old rosettes that were transferred to 5°C for 3 weeks did not reveal the striking changes in chloroplast membrane

Table III. *Morphometric Analysis of Membranes in Thin Sections of Chloroplasts from Mutant Lines and Wild-Type Arabidopsis*

Plants at the cotyledon stage (7 d after germination) or rosette stage (21 d) were grown at 5°C for 3 weeks prior to fixation for microscopy. Micrographs of chloroplasts from 42-d-old plants were taken from the leaves that were fully expanded before transfer to growth at 5°C. Micrographs of chloroplasts from 28-d-old plants were taken from the leaves at the center of the rosettes that developed and expanded during the period of growth at 5°C. The conventions used to measure the various membranes were defined and illustrated in ref. 7. Values for amount of total membrane are means ± SD of measurements made on cross-sections of five representative chloroplasts from each treatment. Cross-sectional area was measured on 30 chloroplasts from each treatment.

Line	Age	Total Membrane[a]	Chloroplast Area
	d		μm^2
Wild type	28	2746 ± 132	15.56 ± 15.5
fadB	28	1126 ± 125	11.06 ± 18.7
fadC	28	826 ± 150	6.96 ± 13.4
Wild type	42	2516 ± 118	12.16 ± 14.4
fadB	42	2486 ± 125	13.46 ± 13.7
fadC	42	2456 ± 132	14.26 ± 13.7

[a] Amount per chloroplast cross-section in μm.

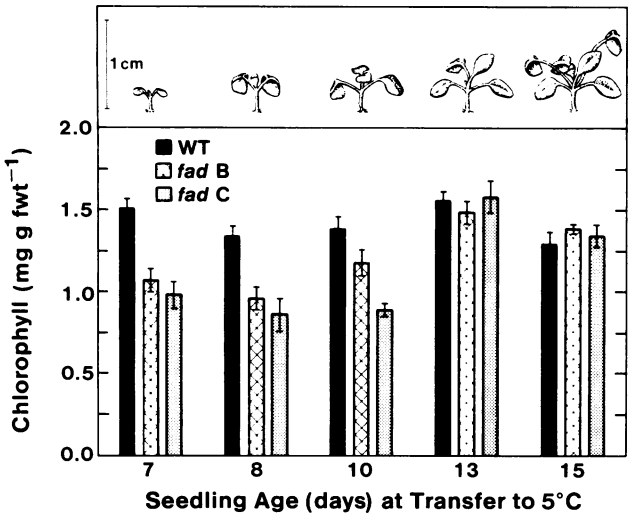

Figure 5. Dependence of plant age on the development of leaf chlorosis in mutants of *Arabidopsis*. Plants were germinated at 22°C and were transferred to 5°C after 7, 8, 10, 13, or 15 d. The approximate stage of development of the plants at these times is illustrated in the top panel. Three weeks after transfer to 5°C, the Chl and fresh weight of individual plants were determined. Values shown are the mean ± SD (n = 6).

content, size, or ultrastructure that were observed following growth of seedlings at 5°C (Table III). Thus, the cold-induced chlorotic phenotype of the mutant lines was dependent on the developmental stage of the leaves at the time of transfer to low temperature. These results indicate that the altered ultrastructure of *fad*B and *fad*C lines grown at low temperature was due to impaired chloroplast development rather than to reduced membrane stability.

DISCUSSION

Results presented here provide evidence that the high level of chloroplast lipid polyunsaturation contributes to the low temperature fitness of the organism. The only caveat to this conclusion is that, until the genes for the *fad* loci become available, it is not certain that the *fad*B and *fad*C mutations are in the structural genes for fatty acid desaturases. Each of these mutations could, in principle, be an alteration of a gene that regulates the activity of a specific desaturase and also regulates other proteins of relevance to low temperature fitness. However, because each of the *fad* mutations affects only one desaturation reaction, the simplest hypothesis is that the *fad* loci encode desaturases.

Whereas wild-type and mutant plants were similar in growth characteristics and appearance at temperatures above about 20°C, the developing leaves of the *fad*B and *fad*C lines became chlorotic during growth at temperatures below 5°C. Morphometric analysis of electron micrographs of chloroplasts from low temperature-grown wild-type, *fad*B, and *fad*C plants revealed dramatic reductions in chloroplast size, membrane content, and organization in developing leaves of the mutant lines. Because similar effects were not observed when fully expanded leaves were transferred to low temperature, the thylakoid membrane deficiency of the mutant lines appears to be due to impaired chloroplast development rather than to the reduced stability of the thylakoid membranes at low temperatures. Previous studies of the *fad*B and *fad*C mutants had revealed that the mutations caused altered chloroplast ultrastructure and slight reductions in leaf Chl content in plants growing at normal growth temperatures (7, 11, 12). It now seems likely that these effects were merely less severe manifestations of the same defect that causes the more dramatic inhibition of chloroplast development at low temperature. The implication is that a high level of lipid polyunsaturation has a fundamental role in chloroplast biogenesis at all temperatures but that the role is more pronounced at lower temperatures.

Studies on the thermal regulation of acyl chain composition have shown that as plants acclimate to warmer environments, there is a decrease in membrane lipid unsaturation (16). Thus, with respect to lipid composition, the *fad* mutants of *Arabidopsis* resemble, to some degree, high temperature-acclimated plants. In previous studies of the various mutants, it was observed that the chloroplast membranes of *fad*B, *fad*C, and *act1* mutants appeared more resistant than wild type to thermal inactivation of photosynthetic electron transport (7, 10, 12). Similarly, reduction in membrane unsaturation by catalytic hydrogenation also increased membrane stability by this criteria (15). In addition, the growth rates of the *fad*B and *act1* mutants were greater than wild type at temperatures near the upper limit for *Arabidopsis* (10, 12). These observations were interpreted as lending support to previous suggestions that chloroplast membrane lipid composition may be a component of the thermal acclimation response observed in many plant species (1). It is particularly noteworthy that several of the *fad* mutations have negative effects on plant fitness at low temperature, but appear to have positive effects at high temperature. This observation is consistent with the concept that chloroplast lipid polyunsaturation is normally regulated by the plant to provide an optimal acyl composition for a particular range of growth temperatures.

In considering possible mechanisms for the defect in chloroplast biogenesis, it is instructive to consider the nature of the changes in lipid composition in the mutants. The *fad*C mutant, which displays the most pronounced chlorosis at low temperature, is deficient in the conversion of 16:1 to 16:2 and 18:1 to 18:2 on the chloroplast glycerolipids monogalactosyldiacylglycerol, digalactosyldiacylglycerol, sulfolipid, and phosphatidylglycerol (4). Both the *sn*-1 and *sn*-2 positions of monogalactosyldiacylglycerol are affected. The net effect is that the mutant has only about 67% as many polyunsaturated chloroplast fatty acids as the wild type. By contrast, the *fad*B mutant is specifically deficient in the desaturation of 16:0 to 16:1 on the *sn*-2 position of monogalactosyldiacylglycerol (16), resulting in a less pronounced overall reduction in polyunsaturated chloroplast fatty acids. Thus, a positive correlation exists between the severity of chlorosis in the two mutants at low temperatures (Fig. 3) and the degree of reduction in polyunsaturated chloroplast lipid composition.

It seems unlikely that the defective chloroplast biogenesis in the *fad* mutants of *Arabidopsis* is due to an effect of temperature on a liquid-to-gel crystalline phase transition. Measurements of the fluidity of wild-type and *fad*B thylakoid

membranes by fluorescence polarization did not reveal any significant differences between the membranes in the range of 0 to 55°C (12). Similarly, differential scanning calorimetry of thylakoid lipids from wild-type, *fad*B, and *fad*C plants did not reveal significant differences between the lines over this temperature range (our unpublished results). Therefore, from these results, and from consideration of the relatively minor effects of polyunsaturation on the order parameter of pure lipid suspensions at physiologically relevant temperatures (18), we consider it unlikely that the mutant phenotype is due to alteration of chloroplast membrane fluidity.

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