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Fatty Acid Desaturation during Chilling Acclimation Is One of the Factors Involved in Conferring Low-Temperature Tolerance to Young Tobacco Leaves[1]

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The *FAD7* gene, a gene for a chloroplast ω-3 fatty acid desaturase, is responsible for the trienoic fatty acid (TA) formation in leaf tissues. The TA content of the leaf tissue of the 25°C-grown transgenic tobacco (*Nicotiana tabacum* cv SR1) plants, in which the *FAD7* gene from *Arabidopsis thaliana* was overexpressed, increased uniformly by about 10%. Fatty acid unsaturation in all major leaf polar lipid species increased in the 25°C-grown *FAD7* transformants but was approximately the same between the control plants and the *FAD7* transformants when grown at 15°C. Therefore, the overexpression of the exogenous *FAD7* gene leads to the same consequence in the tobacco plants as the low-temperature-induced TA production that may be catalyzed by an endogenous, temperature-regulated chloroplast ω-3 fatty acid desaturase. In the 25°C-grown control plants, the chilling treatment caused symptoms of leaf chlorosis and suppression of leaf growth. The 25°C-grown *FAD7* transgenic plants conferred alleviation of these chilling-induced symptoms. A reduction of the chilling injury similar to that of the *FAD7* transformants was also observed in the 15°C-preincubated control plants. These results indicate that the increased TA production during chilling acclimation is one of the prerequisites for the normal leaf development at low, nonfreezing temperatures.

Many plants of temperate origin develop higher degrees of tolerance to low temperatures when exposed to low, nonfreezing temperatures. Two major classes of processes are involved in this adaptive development of acclimation to low temperatures (Guy, 1990). One of the processes of acclimation to low temperatures is the metabolic adaptation to low, nonfreezing temperatures and this is termed chilling acclimation. Chilling acclimation can be achieved in some plants by exposure to a moderately low temperature such as 14°C for several days (Cabané et al., 1993; Prasad et al., 1994). The second process of acclimation to low temperatures for some plants is the induction of freezing tolerance (Steponkus, 1984). Treatment of plants at a few degrees above the freezing point leads to an increase in their ability to tolerate temperatures below 0°C, and this adaptive phenomenon is termed freezing acclimation.

A general increase in level of polyunsaturated fatty acids is observed in most plants during growth at low temperatures (Graham and Patterson, 1982). Fluidity of membranes has been considered to play an important role in survival at low temperatures (Lyons et al., 1964; Lyons and Asmundson, 1965). Although many attempts to find a causal relationship between unsaturation of fatty acids and chilling or freezing acclimation have been made in higher plants, the results are controversial (Steponkus, 1978). Therefore, the precise role of increased production of polyunsaturated fatty acids during acclimation to low temperatures remains unclear.

On the other hand, the function of the unsaturated fatty acids in chilling tolerance can be demonstrated using the *fad* mutants of *Arabidopsis thaliana*, which are defective in desaturation of membrane lipids (Browse and Somerville, 1991; Somerville and Browse, 1991). The *fad* mutants, *fad2* (Lemieux et al., 1990), *fad5* (Kunst et al., 1989), and *fad6* (Browse et al., 1989), have reduced amounts of polyunsaturated fatty acids and are more sensitive to chilling (Hugly and Somerville, 1992; Miquel et al., 1993). The *fad7* mutant of *A. thaliana* has been characterized as being deficient in the activity of chloroplast ω-3 fatty acid desaturase, which is responsible for the production of TAs (16:3 and 18:3) in the prokaryotic pathway of lipid synthesis (Browse et al., 1986a). The *fad7* phenotype is strongly expressed in leaf tissues, and the *FAD7* desaturase is the main enzyme for the production of TAs in leaf tissues. A gene (*FAD7*) for chloroplast ω-3 fatty acid desaturase was isolated from *A. thaliana* by chromosome walking with yeast artificial chromosomes and was shown to complement the *fad7* mutation (Iba et al., 1993). The *FAD7* gene has also been isolated from several other species by heterologous probing with the *Arabidopsis* or *Brassica* genes (Yadav et al., 1993; van de Loo and Somerville, 1994). Transgenic tobacco plants that contain increased TA levels and correspondingly decreased

[1] The convention for nomenclature of gene symbols, genotypes, etc., recommended at the Third International *Arabidopsis* Meeting (East Lansing, MI, 1987) is adopted in this paper (Koornneef and Stam, 1992). This research was supported by Grants-in Aid for Scientific Research (06259214 and 06804050 to K.I.) from the Ministry of Education, Science and Culture, Japan. This research was also supported in part by Sapporo Bioscience Foundation.

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Abbreviations: 16:2, hexadecadienoic acid; 16:3, hexadecatrienoic acid; 18:2, linoleic acid; 18:3, linolenic acid; DA, dienoic fatty acid; DGD, digalactosyldiacylglycerol; EV, empty vector; MGD, monogalactosyldiacylglycerol; MS, Murashige-Skoog; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; RGR, relative growth rate; SL, sulfolipid; TA, trienoic fatty acid. ω-3 refers to the position of the double bond from the methyl end of a fatty acid.

levels of their precursors, 16:2 and 18:2, were engineered by the introduction of the *FAD7* cDNA, and in such resultant plants, chilling injury was reduced (Kodama et al., 1994). These results suggest the importance of polyunsaturated fatty acids in chilling tolerance of higher plants. We report here the findings of detailed analysis of high-TA transgenic tobacco plants with an emphasis on the relationship between the chilling tolerance and increased TA formation during acclimation to low temperatures.

MATERIALS AND METHODS

Transformation of Tobacco Plants

Primary transformed tobacco (*Nicotiana tabacum* cv SR1) plants (R_0) introduced with the *FAD7* gene or with an EV were generated as previously described (Kodama et al., 1994). The R_1 seeds obtained from the self-pollination of the R_0 transformants were aseptically germinated in continuous light (2000 lux) at 25°C on MS medium (Murashige and Skoog, 1962) supplemented with 100 mg/L kanamycin. The kanamycin-resistant R_1 seedlings were subjected to further analysis of lipids and the chilling tolerance.

Segregation of the Transgene in R_1 Seedlings

The R_1 seeds of the *FAD7* transformant (SRT-5) were aseptically germinated on MS medium without antibiotics. The fatty acid composition of each third leaf of the R_1 seedlings was determined. For investigation of the inheritance of the neomycin phosphotransferase II gene, the second leaves were cut from the R_1 seedlings, transferred to MS medium supplemented with 100 mg/L kanamycin and 1 mg/L 6-BA, and then cultured in continuous light (2000 lux) at 25°C. After 2 weeks of culture, the leaves were transferred onto the same medium and cultured for another 1 week. Abundant adventitious buds appeared from the leaves containing the neomycin phosphotransferase II gene. In the wild-type tobacco plants, regeneration from the detached second leaves was completely inhibited in the presence of kanamycin, whereas all of the second leaves were able to regenerate in the absence of kanamycin.

Lipid and Fatty Acid Analysis

Leaf samples were frozen in liquid N_2 and lipids were extracted according to the method of Miquel and Browse (1992). Individual lipids were purified either by a one-dimensional TLC method on $(NH_4)_2SO_4$-impregnated plates (Khan and Williams, 1977) or by two-dimensional TLC (Roughan et al., 1978). Fatty acid methyl esters of individual lipids or of leaf samples were prepared as described by Lemieux et al. (1990), and fatty acid compositions were determined by GC (GC-14B; Shimadzu, Kyoto, Japan) as previously described (Kodama et al., 1994).

Determination of Levels of Transcript of the *FAD7* Gene

Total RNA was purified from leaf tissue as described by Ausubel et al. (1991). Eight micrograms of total RNA were denatured and fractionated as described previously (Kodama et al., 1991). RNAs were blotted onto nylon membranes. The blots were probed simultaneously with the ^{32}P-labeled 3′ noncoding sequence of the *FAD7* cDNA clone (Iba et al., 1993) and the ^{32}P-labeled rice tRNA-Gly(GCC) gene (Reddy and Padayatty, 1988). The labeled DNA fragments were visualized by autoradiography and each band was quantified with a densitometer (Dual-Wavelength Flying-Spot Scanner, CS-9000; Shimadzu).

Plant Growth Conditions and Low-Temperature Treatment

Except for the experiments in which the chilling tolerance and T-DNA segregation were investigated, the kanamycin-resistant R_1 seedlings of the *FAD7* transformants or wild-type plants were transferred to soil and cultured in continuous light (2000 lux) at 25°C unless the growth temperature was indicated otherwise.

The pre-exposure of seedlings to 15°C was performed as follows. The seeds of the EV and the SRT-1 plants were germinated aseptically in Petri dishes at 25°C in continuous light (2000 lux) on MS agar medium with 100 mg/L kanamycin. Six days after imbibition, the seedlings were transferred to 15°C and cultured for 10 d under continuous light (2000 lux). During the 10 d of incubation at 15°C, very small (approximately 2 mm in length) first and second leaves appeared. The seedlings without the pre-exposure to 15°C were germinated and grown on MS agar medium supplemented with 100 mg/L kanamycin at 25°C in continuous light (2000 lux). The seedlings were grown in Petri dishes until very small first and second leaves (2 mm in length) appeared. These seedlings pre-exposed or not to 15°C were transferred to 1°C, kept for 7 d in continuous light (2000 lux) as described previously (Kodama et al., 1994), and then returned to the normal growth conditions at 25°C in continuous light (2000 lux).

Areas of the second leaves of seedlings were measured by approximation to an ellipse. Rates of leaf growth are compared in terms of RGR, which is defined as follows:

$$RGR = (\ln A_t - \ln A_0)/t$$

where A_0 and A_t are the initial and the final leaf areas, respectively, and t (in d) is the duration of incubation.

RESULTS

Overexpression of the *FAD7* Gene and Fatty Acid Composition

The *FAD7* gene was introduced into tobacco plants under the transcriptional control of the cauliflower mosaic virus 35S promoter by *Agrobacterium*-mediated transformation. Three transgenic lines (designated SRT-1, SRT-5, and SRT-6) had higher TA proportions than either the wild-type tobacco or a control transformant with an EV (Kodama et al., 1994). The phenotype of the *FAD7* transformants with increased TA levels is referred to as "high-TA phenotype" for convenience. To correlate the high-TA phenotype with the functional T-DNA insertion, the fatty acid compositions of null segregants from the *FAD7* trans-

formed seed line (SRT-5) were investigated (Table I). The high-TA phenotype was consistently observed in the kanamycin-resistant seedlings, and it indicated that the altered profile of fatty acids was due to the functional insertion of T-DNA in the SRT-5 line. The TA content of the kanamycin-sensitive seedlings (about 70% of total fatty acids) was higher than those previously reported in the wild-type and EV plants (about 60% of total fatty acids) (Kodama et al., 1994). This discrepancy could be due to the difference of culture conditions. In this study, to investigate the inheritance of the neomycin phosphotransferase II gene, leaf fatty acid compositions of the seedlings cultured in Petri dishes were determined. However, in our previous study (Kodama et al., 1994) and in the following studies except for that reported in Table V, the fatty acid compositions of the wild-type and EV plants were determined in the seedlings that had been transferred on soil and cultured for a few weeks.

Because three *FAD7* transformants (SRT-1, SRT-5, and SRT-6) reached the same TA level (Kodama et al., 1994), we examined whether there was a limit to the increase in the relative content of TA in the *FAD7* transgenic tobacco plants. Twenty-nine independent *FAD7* transformants were generated in total, and the fatty acid composition of whole leaves of each kanamycin-resistant R_1 seedling was determined. Of the 29 transformants, 25 lines showed the high-TA phenotype. Figure 1 shows the TA level plotted as a percentage of total fatty acids in these 25 lines. The mean value ± SD of the TA level was 70.6 ± 1.9 in these 25 lines, whereas it was 61.2 ± 0.5 in the wild-type plants and 58.8 ± 1.1 in the EV plants. The steady-state level of the *FAD7* transcript was investigated in leaves of nine independent transformants. The ratio of the amount of the *FAD7* mRNA normalized to the amount of tRNA-Gly varied from 0.19 to 0.52, whereas the TA content in these nine transformants increased uniformly (Fig. 2). These observations imply that there is a limit to the increase in the TA level in the *FAD7* transgenic tobacco plants.

Browse et al. (1993) reported that in *A. thaliana* the 18:3 content of PC was relatively low in young leaves and that the proportion of 18:3 in this lipid species increased with plant age. Thus, we examined the changes of the fatty acid composition during leaf expansion. The fifth leaves with different lengths were sampled from the EV plants and from the *FAD7* transgenic plants, SRT-1 (Fig. 3). The fifth leaves are suitable for this study because, unlike the third leaves, all fifth leaves can grow up to about 5 cm in length. The TA levels were very low in the small leaves but increased during leaf expansion and reached about 60% in 3-cm-long leaves from the EV plants. A similar pattern of the increase of TA contents parallel with leaf expansion was also observed in the SRT-1 plants, although their TA levels were higher by about 10% than those in the leaves of comparable sizes in the EV plants. These results indicate that the high-TA phenotype is also observed in young leaves of the *FAD7* transformants but that the TA contents of the *FAD7* transgenic line increase with leaf age in a way similar to the control plants.

Table I. *Fatty acid compositions of whole leaves of kanamycin-sensitive and kanamycin-resistant seedlings of the SRT-5 line*

The fatty acid composition was determined in the 1.5-cm-long third leaves. The values represent mol% ± SD from five plants. Molar ratios of TA to DA are also shown.

Fatty Acid	SRT-5 Seedlings	
	Kanamycin sensitive	Kanamycin resistant
16:0	11.5 ± 0.6	9.9 ± 0.2
16:1	2.4 ± 0.2	2.6 ± 0.1
16:2	1.4 ± 0.2	0.6 ± 0.1
16:3	13.5 ± 2.1	16.1 ± 0.6
18:0	0.9 ± 0.1	0.6 ± 0.1
18:1	1.2 ± 0.2	1.3 ± 0.1
18:2	11.9 ± 0.7	9.2 ± 0.2
18:3	57.1 ± 2.3	59.8 ± 0.6
TA	70.7 ± 0.3	75.8 ± 0.1
TA/DA	5.4 ± 0.3	7.7 ± 0.1

Effects of Temperature on Fatty Acid Composition

An increase of the TA content can be observed during growth of many plants at low temperatures (Graham and Patterson, 1982). We examined changes in the TA content upon temperature shift from 25°C to various temperatures in the wild-type tobacco plants. The amount of TAs, especially 16:3, increased when exposed to 15 or 20°C, suggesting that a low-temperature-induced production of TAs occurs indeed in tobacco plants (Fig. 4).

To compare the degree of unsaturation of fatty acids of the control (EV plants) with that of the *FAD7* transformants (SRT-1 plants) under low-temperature conditions, the seedlings with very small first and second leaves were grown at 15, 20, 25, and 30°C. Because the wild-type tobacco plants exhibited extremely reduced growth at 10°C, experiments at the temperatures below 15°C were omitted. After development of the third leaf to a length of 2 cm, the third leaf of each plant was harvested and its fatty acid composition was determined (Fig. 5). The TA proportion in leaves of the SRT-1 plants remained approximately constant (about 70% of total fatty acids) under all the temperatures investigated. When grown at 25 and 30°C, differences in the TA level due to the overexpression of the *FAD7* gene could be clearly observed between the EV and the SRT-1 plants. The TA level in the EV plants increased and reached a plateau of about 70% of total fatty acids at the temperatures below 20°C. Therefore, at a relatively low temperature of 15°C there was no significant difference in fatty acid compositions of total lipids between the control plants and the *FAD7* transformants. In the 20°C-grown EV plants the TA contents ranged widely (60.4–71.6% of total fatty acids). On the contrary, the 15°C-grown EV plants exhibited almost the same TA levels (68.0–70.5% of total fatty acids). The variation of the TA contents at 20°C may have been caused by local temperature variations in the growth chamber. Therefore, the 15°C-grown plants, instead of the 20°C-grown plants, were used for the comparisons of fatty acids and of the chilling tolerance with the 25°C-grown plants in the following experiments.

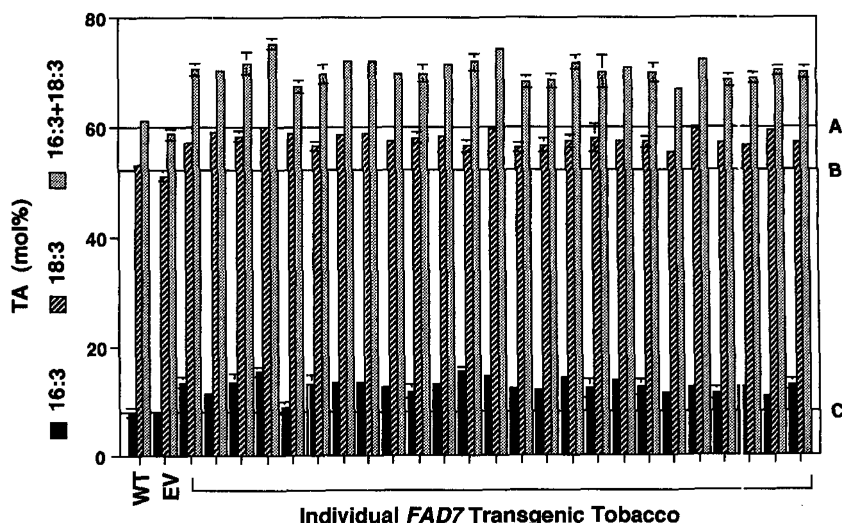

Figure 1. TA contents of the *FAD7* transgenic plants. Contents of TA in molar percentage of total fatty acids in 1-cm-long third leaves of kanamycin-resistant R_1 seedlings of the 25 independent *FAD7* transformants. Control transformants with an EV and the wild-type plants (WT) are included for comparison. The seedlings were transferred to soil and cultured in continuous light (2000 lux) at 25°C. Horizontal lines A, B, and C show the levels of 18:3 plus 16:3, 18:3, and 16:3 of the control plants, respectively. Vertical lines indicate SD (n = 4 or 5).

The *fad7* mutant had reduced levels of TA at growth temperatures above 26°C but had the wild-type fatty acid composition at temperatures below about 20°C (Browse et al., 1986a). This observation suggests that an isozyme of the *FAD7* desaturase is induced at temperatures between 20 and 26°C and compensates for the deficiency. Recently, a locus (*FAD8*) encoding such temperature-regulated chloroplast ω-3 fatty acid desaturase was identified from *A. thaliana* (McConn et al., 1994) and the corresponding cDNA clone was isolated (Gibson et al., 1994). Therefore, our results indicate that an overexpression of the *FAD7* gene in tobacco plants leads to the same compositions of fatty acids as found in whole leaves exposed to the temperatures in which endogenous *FAD8*-type desaturases are induced.

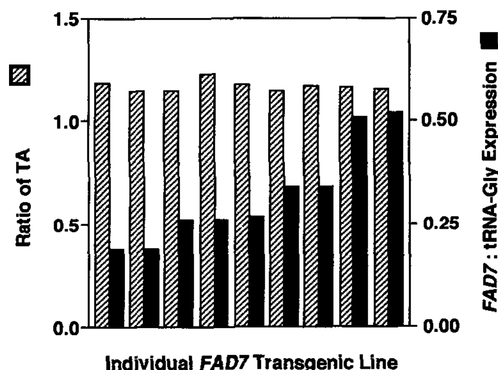

Figure 2. Transcript levels of the *FAD7* gene and the TA ratios in the transgenic lines. The ratio of the relative TA content (in mol%) in each of nine individual *FAD7* transformants to that of the EV plants. Fatty acids in 1-cm-long third leaves were analyzed. Total RNA was isolated from mature, 7- to 9-cm-long leaves of each of the transformants and was subjected to gel blot analysis (8 μg per lane). The amount of the *FAD7* mRNA in each sample was normalized to the amount of tRNA-Gly in the same sample by densitometry of autoradiograms exposed at less than saturation. Values are the means of samples taken during two independent experiments.

Fatty Acid Compositions of Individual Lipid Species

Analysis of the fatty acid compositions of individual lipids extracted from the leaf tissue of the EV and SRT-1 plants grown at 25°C revealed that all the major lipids were affected by the overexpression of the *FAD7* gene (Table II). The most notable effect of unsaturation in the *FAD7* transformants was observed in MGD and DGD. The TA content in MGD and DGD increased by about 6.4 and 12.5% in the *FAD7* transformants, respectively. The fatty acid compositions of other lipids, PC, PE, PI, SL, and PG, showed increases in TA by about 3 to 5%.

In 16:3 plants, such as tobacco, MGD has 16:3 exclusively at position *sn*-2 of glycerol. Chloroplast PG, which constitutes about 85% of the total PG in leaf tissues of *A. thaliana* (Browse et al., 1986b), has 18:3 only at position *sn*-1 of glycerol (Browse et al., 1989). Since both 16:3 of MGD and 18:3 of PG increased in the transgenic SRT-1 line, the effects of the overexpression of the *FAD7* gene appear in fatty acids at both positions of the glycerol backbone. Our results are in agreement with the features of the *FAD7* de-

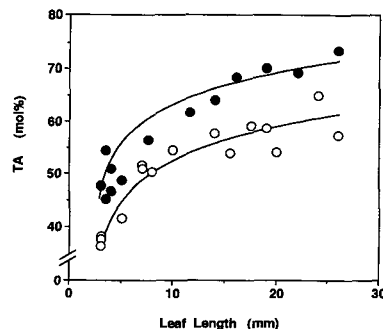

Figure 3. TA contents in the fifth leaves with different lengths from the EV (○) and SRT-1 (●) plants. The kanamycin-resistant R_1 seedlings were grown as described in the legend for Figure 1. The fifth leaves of differing lengths were harvested and their TA contents of whole leaves were determined.

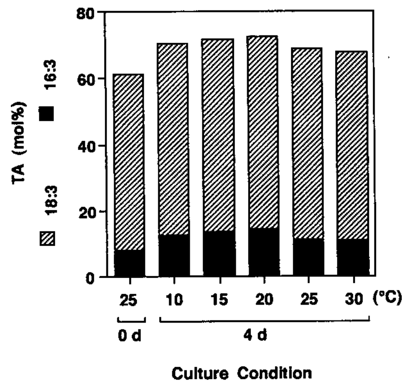

Figure 4. The proportion of 16:3 and 18:3 in leaves of the wild-type tobacco grown under different temperature conditions. Seedlings were grown in continuous light (2000 lux) at 25°C on soil. After the third leaves developed to the length of 1 cm, seedlings were transferred from 25°C to various temperatures as indicated in continuous light (2000 lux). At the time of the transfer (0 d) and after the transfer (4 d) the fatty acid compositions of the third leaves were determined. The values are the means obtained in three independent experiments.

saturase as deduced from the observation that the effect of the *fad7* mutation shows no specificity in length (16- or 18-carbon), glycerol backbone (*sn*-1 or *sn*-2), or lipid head group (Browse et al., 1986a).

As shown in Figure 5, the levels of TAs in leaves were similar in the EV and the SRT-1 plants when grown below 20°C. These consequences are shown more clearly by an analysis of individual lipids extracted from the leaf tissue of the EV and SRT-1 plants grown at 15°C (Table III). There were no major differences in the TA contents of individual lipids between the EV and the SRT-1 plants. Furthermore, the TA levels of individual lipids shown in Table III are comparable with those of the SRT-1 plants grown at 25°C as shown in Table II. These results agree well with the data in Figure 5 in indicating that the overexpression of the *FAD7* gene at 25°C brings about the same fatty acid composition of major lipids as those seen when grown at temperatures below 20°C. Therefore, the data in Table III show again that the overexpression of the *FAD7* gene at normal culture temperatures exhibits a profile of fatty acid composition similar to the temperature-regulated expression of the gene for endogenous *FAD8*-type chloroplast ω-3 desaturase in the wild-type plants.

Chilling Tolerance in Plants Pre-Exposed to Temperatures below 20°C

When exposed to 1°C for 7 d and then cultured at 25°C, the chilling-induced symptoms observed in the wild-type tobacco plants, namely leaf chlorosis and suppression of leaf growth, were significantly alleviated in the *FAD7* transgenic plants (Kodama et al., 1994). Because the wild-type tobacco plants grown at 15°C showed the same TA proportion as did the *FAD7* transformants (Fig. 5), we analyzed the chilling tolerance of the control plants that had been grown at 15°C prior to the chilling treatment.

The susceptibility to the chilling injury in tobacco leaf tissues is greatly affected by leaf age. The chilling injury appeared only in young leaves and no visible symptoms were observed in fully expanded leaves (Kodama et al., 1994). Therefore, it is important that leaves exposed to the chilling conditions should be of the same length. The outline of the experiment is shown in Figure 6. Seedlings both with and without pre-exposure to 15°C, all of which had very small first and second leaves of the same length (about 2 mm), were treated at 1°C for 7 d and then cultured at 25°C for 5 d. The RGRs were calculated from leaf growth during 5 d of culture at 25°C (Fig. 7). The RGRs of the 25°C-grown EV plants following the chilling treatment at 1°C were reduced to about 70% of those of the EV plants that did not undergo the chilling treatment. The suppression of leaf growth seen in the EV plants was alleviated by the introduction of the *FAD7* gene, as previously reported (Kodama et al., 1994). However, the RGRs of the EV plants that had been exposed to 15°C for 10 d and then to 1°C for 7 d were more than 90% of those of the EV plants that did not undergo the chilling treatment at 1°C, indicating that the 15°C-preincubated EV plants did not show the inhibition of leaf growth by the chilling treatment.

After the chilling treatment at 1°C for 7 d, symptoms of leaf chlorosis were obvious in the 25°C-grown EV and wild-type plants. However, the number of seedlings with leaf chlorosis was small in the 25°C-grown *FAD7* transgenic plants and was nearly zero in the control plants pre-exposed to 15°C for 10 d (Table IV).

The leaf and cotyledon tissues of the 25°C-grown EV and SRT-1 plants did not show significant changes in the fatty acid compositions during the chilling treatment at 1°C for 7 d (Table V). During the pre-exposure of seedlings to 15°C for 10 d, the TA level in these tissues increased by about 10% in the EV plants. It was approximately the same as that of the SRT-1 plants (Table V). Thus, the exposure to 15°C for 10 d was sufficient to modify the fatty acid composition.

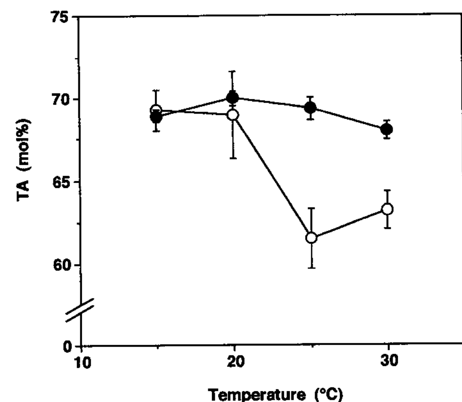

Figure 5. Effects of temperature on TA in the third leaves of the EV (○) and SRT-1 (●) plants. The kanamycin-resistant R_1 seedlings with very small first and second leaves grown on MS medium were transferred to soil and cultured in continuous light (2000 lux) at various temperatures as indicated. The fatty acid composition was determined in the 2-cm-long third leaves. Vertical lines indicate SD (n = 4 or 5).

Table II. *Fatty acid compositions of polar lipid species in leaves from the EV and SRT-1 plants grown at 25°C*

Each value is the mean of two independent experiments (in mol%). Dashes indicate that an acyl group was not detected.

Fatty Acid	MGD		DGD		SL		PG		PC		PE		PI	
	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1
16:0	2.8	2.5	16.6	15.0	45.2	38.7	24.7	27.6	20.0	20.3	28.3	26.7	41.8	42.5
16:1	–	–	2.4	–	5.9	3.6	34.6	31.2	–	–	1.0	–	2.3	1.0
16:2	2.8	–	–	–	1.2	1.5	–	–	–	–	–	–	–	–
16:3	18.8	21.0	2.6	1.9	1.2	1.6	–	–	–	–	–	–	–	–
18:0	–	–	2.5	2.1	7.4	11.0	2.0	2.6	4.7	7.1	4.7	5.8	5.7	3.7
18:1	–	–	4.6	1.5	7.5	5.5	5.7	5.5	4.3	3.6	2.0	1.6	2.9	2.3
18:2	6.4	2.7	8.2	3.7	19.3	20.9	11.6	8.3	51.9	45.4	49.6	47.2	25.0	23.1
18:3	69.6	73.8	63.2	76.4	12.2	17.2	21.3	24.7	19.1	23.6	14.3	18.7	22.3	27.4

Although the TA contents were found to be the same between the 15°C-preincubated EV and the SRT-1 plants, the contents were somewhat higher (about 4%) than that in the 25°C-grown SRT-1 plants. During the chilling treatment at 1°C the TA contents of the 15°C-preincubated EV and the SRT-1 plants decreased by about 4%. The levels of TAs were similar to that of the 25°C-grown SRT-1 plants, and the decreases in TAs were associated with corresponding increases in DA.

DISCUSSION

Involvement of Fatty Acid Desaturation in Chilling Acclimation

The use of the *FAD7* transformants allowed us to investigate the effects of fatty acid desaturation on chilling tolerance without regard to other biochemical changes associated with chilling acclimation. The low-temperature-induced chlorosis and suppression of leaf growth observed in the wild-type tobacco grown at 25°C were significantly alleviated in the wild-type tobacco grown at 15°C (Fig. 7; Table IV), indicating that in tobacco plants the chilling acclimation can be achieved by exposure to 15°C. The data in Figure 4 show that the largest effect in unsaturation was observed in plants exposed to temperatures below 20°C. Therefore, the chilling acclimation at 15°C involves increased TA production. Although the process of acclimation is considered to include complex and various biochemical events, our results show that the increase in TAs during chilling acclimation is one of the important factors in chilling tolerance. At least the increased TA formation during the chilling acclimation contributes to the normal leaf development following the chilling treatment. The following evidence supports this conclusion: (a) When cultured at 25°C, *FAD7* transgenic tobaccos are characterized by an increased TA level as compared with the wild-type tobacco plants (Kodama et al., 1994). However, almost the same profile of the fatty acid composition of the *FAD7* transformants was obtained in the 15°C-grown control plants (Fig. 5; Tables II and III). (b) The enhanced chilling tolerance shown in the *FAD7* transformants (Kodama et al., 1994) can be observed in those control tobacco plants that were pre-exposed to 15°C for 10 d (Fig. 7; Table IV).

In *A. thaliana* the effects of the *fad7* mutation are observed only at temperatures above about 26°C (Browse et al., 1986a). Because the *FAD7* transcripts are not detectable in the mutant at either normal or low temperatures (Iba et al., 1993), the existence of a temperature-regulated chloroplast ω-3 fatty acid desaturase has been expected. Another mutation has been identified at a new locus that may correspond to another chloroplast ω-3 fatty acid desaturase (McConn et al., 1994). Moreover, a gene for a temperature-regulated chloroplast ω-3 fatty acid desaturase, which has been designated as the *FAD8* gene, was isolated from *A. thaliana* (Gibson et al., 1994). Thus, at temperatures below 26°C the genes for the *FAD8*-type desaturases are expressed and DAs are unsaturated actively to produce TAs.

Several genes that are believed to contribute to chilling tolerance are expressed at temperatures ranging from 10 to 20°C. Soybean is acclimated to a lower temperature of 8°C by a 5-d exposure to 14/8°C (day/night) cycles. The synthesis of a heat-shock protein 70-related protein is stimu-

Table III. *Fatty acid compositions of polar lipid species in leaves from the EV and SRT-1 plants grown at 15°C*

Each value is the mean of two independent experiments (in mol%). Dashes indicate that an acyl group was not detected.

Fatty Acid	MGD		DGD		SL		PG		PC		PE		PI	
	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1	EV	SRT-1
16:0	1.9	2.4	13.3	15.9	34.0	31.4	34.4	36.6	20.7	18.6	26.3	24.2	36.2	37.6
16:1	–	–	–	–	1.8	2.1	27.8	30.2	–	–	1.5	–	–	1.0
16:2	1.2	–	–	–	–	–	–	–	–	–	–	–	–	–
16:3	18.6	21.7	1.4	1.7	–	–	–	–	–	–	–	–	–	–
18:0	–	–	2.1	1.6	9.3	6.1	1.6	1.7	4.8	4.1	3.5	3.1	3.9	3.9
18:1	–	–	1.5	2.0	5.3	5.7	2.6	2.7	3.5	3.9	1.7	1.8	2.2	2.6
18:2	4.6	2.2	4.1	2.8	30.0	33.1	10.1	5.6	47.7	50.6	47.8	52.1	26.0	26.0
18:3	73.7	73.7	77.6	75.9	19.5	21.7	23.5	23.3	23.2	22.7	19.2	18.8	31.7	28.9

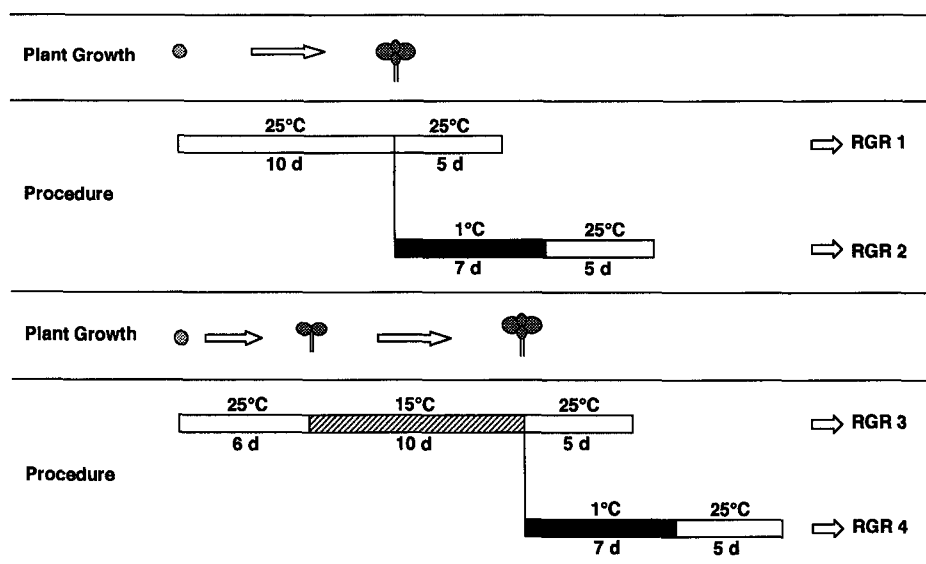

Figure 6. Schematic description of pre-exposure at 15°C and low-temperature treatment at 1°C. After the low-temperature treatment (1°C for 7 d) growth rate was determined by measuring the size of the second leaf at 25°C for 5 d. The sample size was 10 in all cases. Growth rates are compared in Figure 7 as the ratio of RGR2 to RGR1 in seedlings without the pre-exposure to 15°C and as the ratio of RGR4 to RGR3 in seedlings with the pre-exposure to 15°C. The approximate stages of development of the plants are illustrated in the "plant growth" panel.

lated during this acclimation period (Cabané et al., 1993). In the chilling-sensitive maize, a gene (*cat3*) that encodes the mitochondrial catalase 3 isozyme has been found to be expressed more abundantly during acclimation treatment at 14°C for 3 d (Prasad et al., 1994). Genes for *cat3*, *FAD8*, and heat-shock protein 70-related proteins may be induced during chilling acclimation at moderately low temperatures (10–20°C) and may act in concert to increase chilling tolerance.

It should be noted that the growth conditions prior to chilling treatment caused significant changes in the fatty acid composition during chilling treatment. When 15°C-preincubated plants were treated at 1°C for 7 d, the TA contents decreased and the DA contents correspondingly increased during the chilling treatment (Table V). These changes in the fatty acid composition occurred under the conditions in which the *FAD8*-type chloroplast ω-3 fatty

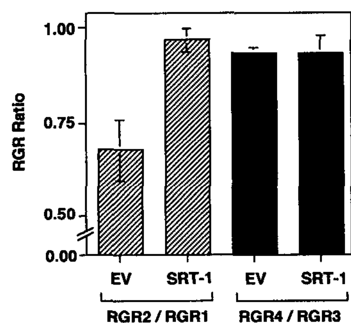

Figure 7. Growth rates of the EV and the SRT-1 plants after the chilling treatment. RGR ratios were determined as described in the legend for Figure 6. Data taken in three independent replicates are expressed as means ± SD.

acid desaturase was induced by low temperatures or the *FAD7* gene was overexpressed under the control of the cauliflower mosaic virus 35S promoter. However, the TA levels remained constant during the chilling treatment when 25°C-grown plants were incubated at 1°C for 7 d (Table V; Kodama et al., 1994). Therefore, in studies concerning lipid changes under temperature stress, it is important to clarify whether the additional *FAD8*-type desaturase is induced under the "normal" growth conditions.

Fatty Acid Desaturation as a Factor in Chilling Tolerance

Increased polyunsaturation during acclimation is associated with reduced chilling sensitivity. However, the molecular mechanism by which the increased TA levels confer enhanced tolerance to chilling temperatures remains unsolved.

Several mutants of *A. thaliana* that are deficient in chloroplast membrane lipid polyunsaturation exhibit changes in the ultrastructure of their chloroplast membranes (Somerville and Browse, 1991). The mutants, *fad5* and *fad6*, are deficient in chloroplast ω-9 desaturase (Kunst et al., 1989) and chloroplast ω-6 desaturase (Browse et al., 1989), respectively. Leaf tissues of the *fad5* and *fad6* mutants grown at 5°C were chlorotic and chloroplasts of these chlorotic leaf tissues were much smaller than those of the wild-type plants (Hugly and Somerville, 1992). Although the *fad6* mutation causes altered chloroplast ultrastructures in plants grown at normal growth temperatures (Hugly et al., 1989), more dramatic inhibition of chloroplast development at low temperatures was observed in the *fad5* and *fad6* mutants (Hugly and Somerville, 1992). Furthermore the cross-sectional area of chloroplasts in the *fad7* mutant

Table IV. *Leaf chlorosis in tobacco plants*

Each tobacco plant was cultured under the growth conditions indicated, exposed to 1°C for 7 d, and then transferred to 25°C. Visual assessment was carried out 3 d after the transfer to 25°C, when the symptoms of leaf chlorosis became obvious.

Growth Condition	Plant Line	Plants with Leaf Chlorosis	Total No. of Plants
Experiment 1			
25°C, 10 d	EV	5	10
	SRT-1	1	10
25°C, 6 d, and 15°C, 10 d	EV	0	10
	SRT-1	0	10
Experiment 2			
25°C, 10 d	Wild type	9	10
	EV	5	10
	SRT-1	0	10
	SRT-6	1	10
25°C, 6 d, and 15°C, 10 d	Wild type	0	10
	EV	0	10
	SRT-1	0	10
	SRT-6	0	10

grown at 27°C was only about 50% of the corresponding area in the wild-type plants (McCourt et al., 1987).

The reduced chloroplast size was accompanied by a corresponding increase in the chloroplast number (McCourt et al., 1987). These results imply that a high level of lipid polyunsaturation plays a fundamental role in chloroplast biogenesis at all temperatures and that the role is manifested more strongly at lower temperatures (Hugly and Somerville, 1992). The fatty acid compositions were indistinguishable between the wild-type and the *FAD7* transgenic tobacco plants when grown at 15°C (Fig. 5). Thus, the effects of the overexpression of the *FAD7* gene on chloroplast development may be much less pronounced at low temperatures. The appearance of the chilling-injury symptoms in tobacco plants is dependent on the developmental stage of the leaves at the time of transfer to low temperatures. These low-temperature-induced damages appear on very young leaves, whereas there is no discernible effect of low temperature on tobacco leaves that are fully developed prior to exposure to low temperatures (Kodama et al., 1994). The observed results with tobacco plants are consistent with observations for the *fad5* and *fad6* mutants (Hugly and Somerville, 1992). In tobacco plants, most plastids are in the proplastid phase or in early developmental phase of chloroplasts in the young, 2-cm-long leaves and they enter into the later stage of differentiation or maturation in leaves with a length ranging from 6 to 8 cm (Ehara and Misawa, 1975). Because the TA content in young leaves was remarkably low (Fig. 3), the plastidic membranes in young leaves can be considered to be composed of lipids with relatively low TA levels. It seems likely that these plastids in the early phase of development are more susceptible to chilling treatment and liable to suffer low-temperature-induced deleterious effects. In chilling-acclimated plants, the fatty acids of the membrane lipids of proplastids are polyunsaturated by the temperature-regulated *FAD8*-type chloroplast ω-3 fatty acid desaturase. This fatty acid polyunsaturation may assure the normal chloroplast biogenesis and leaf development under both chilling and normal growth conditions following chilling treatment and may contribute to the increase in chilling tolerance.

Regulation Involved in the Expression of the *FAD7* Gene

The high-TA phenotype observed in the *FAD7* transformants was clearly dependent on the existence of the T-DNA (Table I). However, the increased TA level was not apparently correlated with the level of the *FAD7* transcript (Fig. 2). All of the *FAD7* transformants had similarly increased TA levels (Fig. 1). In spite of the fact that the *FAD7* transcripts can be detected in the wild-type *A. thaliana*

Table V. *Levels of TA and DA in leaf and cotyledon tissues before and after chilling treatment at 1°C for 7 d under continuous illumination (2000 lux)*

Each tobacco plant was cultured under the growth conditions indicated and then fatty acids were analyzed before and after a chilling treatment at 1°C for 7 d. The values represent mean mol% ± SD from three to five seedlings.

Growth Condition	Plant Line	DA		TA	
		1°C, 0 d	1°C, 7 d	1°C, 0 d	1°C, 7 d
25°C, 10 d	EV	25.4 ± 1.7	26.4 ± 0.9	52.5 ± 1.8	52.1 ± 2.1
	SRT-1	18.4 ± 0.8	19.6 ± 0.7	58.2 ± 1.9	58.8 ± 0.7
25°C, 6 d, and 15°C, 10 d	EV	20.0 ± 1.2	24.8 ± 0.8	62.0 ± 1.4	57.9 ± 1.5
	SRT-1	18.5 ± 0.6	24.4 ± 0.5	62.5 ± 1.3	57.6 ± 0.6

grown at temperatures of 12 and 30°C (Iba et al., 1993), any additional chloroplast ω-3 desaturase, because of the expression of the *FAD8*-type gene at low temperatures or the overexpression of a foreign gene for chloroplast ω-3 desaturase, can cause a similar increase in TA content (Fig. 5). In fact, seedlings of the *fad7* mutant that were transformed with the *FAD7* cDNA showed complemented compositions of fatty acids, but none had significantly higher TA levels than those of the wild-type plants (Iba et al., 1993). These observations imply that the activity of the *FAD7* gene product is not controlled solely by the level of transcription. It remains to be clarified what mechanisms are involved in the posttranscriptional regulation of the chloroplast ω-3 fatty acid desaturase. The elucidation of the mechanism will contribute to the understanding of regulatory mechanisms determining the level of polyunsaturated fatty acids.

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