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Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress

(cyanobacteria/*desA* gene/fatty acid/desaturation/transformation)

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ABSTRACT **The contribution of the unsaturation of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress was studied in a transgenic cyanobacterium. *Anacystis nidulans* R2-SPc was transformed with the *desA* gene, which encodes the Δ^{12}-desaturase that desaturates the fatty acids of membrane lipids in *Synechocystis* PCC6803. The transformant acquired the ability to introduce a second double bond into palmitoleic and oleic acids. The transformation enhanced the tolerance of the photosynthetic machinery to chilling stress but it had no detectable effect on the ability to tolerate heat stress. The transformation itself did not have any effect on photosynthetic activity. These results imply that an increase in the unsaturation of membrane lipids enhances the tolerance of the photosynthetic machinery toward chilling stress but not toward heat stress and that such an increase does not affect photosynthesis within the range of physiological temperatures.**

Glycerolipids of thylakoid membranes form bilayers and provide the necessary background for the functioning of membrane proteins (1). There are four abundant glycerolipids in the thylakoid membranes of chloroplasts of higher plants and in the cells of cyanobacteria: monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG), and phosphatidylglycerol (PG). These glycerolipids are assumed to play important roles in the maintenance of photosynthetic electron-transport systems. SQDG is associated with ATP synthase (2), and MGDG is bound to the photosystem II (PSII) reaction-center complex (3).

The physical properties of glycerolipids depend on the degree of unsaturation of the fatty acids that are esterified to the glycerol backbone of the lipids, and consequently the molecular motions of these glycerolipids can be affected by alterations in the extent of unsaturation of fatty acids (4–6). We can postulate, therefore, that changes in the unsaturation of fatty acids should affect various functions of membrane-bound proteins, such as the photochemical and electron transport reactions in thylakoid membranes. It is of interest to determine, therefore, whether the unsaturation of fatty acids of glycerolipids in thylakoid membranes can modify the tolerance of the photosynthetic machinery to temperature stress.

The unsaturation of fatty acids of glycerolipids in thylakoid membranes can be altered by changing the growth temperature of the photosynthetic organism. Pearcy (7) and Raison *et al.* (8) reported that an increase in the growth temperature increased the degree of saturation of fatty acids and enhanced the stability of photosynthesis at high temperature. However, such studies failed to provide a direct correlation between the increase in the degree of saturation of fatty acids and the increase in high-temperature stability of the photosynthetic machinery, because not only the saturation of fatty acids but also other metabolic factors are affected by changes in growth temperature.

The contribution of the unsaturation of fatty acids to low-temperature tolerance is also unclear, since acclimation to low temperature induces not only desaturation of fatty acids but also a number of other metabolic modifications (9–11). To determine whether the unsaturation of fatty acids contributes to the ability to tolerate high and low temperatures, it is necessary to alter the unsaturation of fatty acids of glycerolipids by manipulation of the genes that encode the enzymes responsible for desaturation of fatty acids, without directly affecting any other metabolic processes.

We previously isolated mutants of the cyanobacterium *Synechocystis* PCC6803, Fad6 and Fad12, that were defective in the desaturation of fatty acids at the 6 and 12 positions, respectively (12). The Fad6 mutant lacked 6,9,12-octadecatrienoic acid [18:3(6,9,12)]. The Fad12 mutant lacked 9,12-octadecadienoic acid [18:2(9,12)] and 18:3(6,9,12) and contained a low level of 6,9-octadecadienoic acid [18:2(6,9)]. Although both mutants grew as rapidly as the wild type at 34°C, the growth rate of Fad12 at a low temperature such as 22°C was much lower than that of the wild type, whereas Fad6 grew at the same rate as the wild type. These results demonstrate that 18:2 but not 18:3 is important for growth at low temperature. The effect of unsaturation of fatty acids of membrane lipids on the stability of the photosynthetic machinery at high temperatures was also studied in the Fad12 mutant. Complete elimination of 18:3 and 18:2 from the membrane lipids by mutation of the Δ^{12}-desaturase did not affect the thermal stability of photosynthesis in the cells of *Synechocystis* PCC6803 (13). This observation suggests that the molecular species of lipids that are esterified with 18:3 are not determinative factors in the maintenance of the photosynthetic activity under heat-stress conditions.

In the case of higher plants, mutants of *Arabidopsis thaliana* have been isolated that are defective in the desaturation of fatty acids (14). In two mutants defective in the desaturation of hexadecanoic acid (16:0) and of 7-hexadecenoic acid [16:1(7)] and 9-octadecenoic acid [18:1(9)] the partial loss of polyunsaturated fatty acids increased the thermal tolerance of the photosynthetic machinery (15, 16) whereas in a mutant defective in the desaturation of 7,10-hexadecadienoic acid [16:2(7,10)] and 18:2, a decrease in levels of 7,10,13-hexadecatrienoic acid [16:3(7,10,13)] and 9,12,15-octadecatrienoic acid [18:3(9,12,15)] and an increase

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Abbreviations: DGDG, digalactosyl diacylglycerol; MGDG, monogalactosyl diacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyl diacylglycerol; PSII, photosystem II.

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in levels of 16:2(7,10) and 18:2(9,12) did not affect the thermal tolerance of the photosynthetic machinery (17).

After isolation of the gene for the Δ^{12}-desaturase, *desA*, from *Synechocystis* PCC6803 (18), we transformed the Fad6 mutant of *Synechocystis* PCC6803 with a *desA* gene that had been disrupted by insertion of a kanamycin-resistance gene cartridge (19). The resultant transformant contained only monounsaturated fatty acids and was very sensitive to low temperatures in the light (20). Our results revealed that the molecular species that contain polyunsaturated fatty acids are essential for protection against low-temperature photoinhibition.

In the present report, we describe changes in the lipid and fatty acid compositions of glycerolipids and in photosynthesis in *Anacystis nidulans* R2-SPc caused by transformation with the *desA* gene from *Synechocystis* PCC6803. Using the transformant, we addressed the question of whether the dienoic fatty acids play a determinative role in the tolerance of the photosynthetic machinery to temperature stress.

MATERIALS AND METHODS

Organisms and Culture Conditions. *A. nidulans* R2-SPc, which is a derivative of *A. nidulans* R2 (equivalent to *Synechococcus* PCC7942; ref. 21), was kindly provided by N. Sato of Tokyo Gakugei University. The wild-type and transformed cells of *A. nidulans* R2-SPc were grown photoautotrophically in BG-11 medium (22) supplemented with 20 mM Hepes/NaOH (pH 7.5) under illumination from incandescent lamps [0.07 $mE \cdot m^{-2} \cdot s^{-1}$; 1 E (einstein) = 1 mol of photons] with aeration with 1% CO_2 in air. The transformants were cultivated in the presence of chloramphenicol (7.5 μg/ml). Cultures at the exponential phase of growth were used for experiments.

Transformation. Transformation of *A. nidulans* R2-SPc with pUC303 (21) and pUC303/*desA* was carried out as described (18).

Membrane Isolation and Lipid Analysis. The cytoplasmic and thylakoid membranes of the wild type and the transformants were isolated by the method of Murata and Omata (23). Lipids were extracted from intact cells and from thylakoid and cytoplasmic membranes by the method of Bligh and Dyer (24). Lipids and fatty acids were analyzed as described (12).

Measurement of Photosynthetic Activity. Photosynthetic activity of cells suspended in BG-11 medium was measured with CO_2 as the electron acceptor by monitoring oxygen exchange with a Clark-type oxygen electrode. The activity of PSII of intact cells was measured with 1 mM 1,4-benzoquinone and 1 mM $K_3Fe(CN)_6$ as electron acceptors (25). Light (3.5 $mE \cdot m^{-2} \cdot s^{-1}$) was provided from an incandescent lamp after passage through a red optical filter (R-62; Hoya Glass, Tokyo). Chlorophyll concentrations were determined by the method of Arnon *et al.* (26).

RESULTS

Transformation with the *desA* Gene. The insert in pBluescript/1.5-kbp (18) that contained the *desA* gene of *Synechocystis* PCC6803 was subcloned into a shuttle vector that can operate between *Escherichia coli* and *A. nidulans* R2. The resultant plasmid, pUC303/*desA*, was used for transformation of *A. nidulans* R2-SPc. Ten chloramphenicol-resistant transformants were selected and the fatty acid compositions of their total lipids were analyzed. All the transformants had essentially the same changes in fatty acid composition, as compared with the wild type, and, therefore, two of them were arbitrarily selected and used for further analyses. Since there were no significant differences between the two transformants in their characteristics, the results for only one of the transformants, T-pUC303/*desA*, are described below. *A. nidulans* R2-SPc was also transformed with pUC303 in a similar manner to yield a control transformant, T-pUC303.

Alterations in Membrane Lipids Caused by the Transformation. The lipid compositions of wild-type and transformed cells of *A. nidulans* R2-SPc are compared in Table 1. This cyanobacterium contained four major glycerolipids—namely, MGDG, DGDG, SQDG and PG—as do other cyanobacteria (27, 28). In wild-type cells grown at 34°C, MGDG accounted for about 60% of the total glycerolipids, PG for about 20%, and DGDG and SQDG for about 10% each. Table 1 indicates that the lipid compositions of the transformants T-pUC303 and T-pUC303/*desA* were essentially the same as that of the wild type. The growth temperature had no significant effect on the lipid composition of either the wild type or the transformants.

The fatty acid compositions of individual lipid classes from wild-type and T-pUC303/*desA* cells grown at 22°C are presented in Table 2. In the wild-type cells, 16:0 and 9-hexadecenoic acid [16:1(9)] were major fatty acids in all lipid classes. These results are compatible with those obtained from another strain of *A. nidulans* (29). We found that 16:0 accounted for 40–50% of the total fatty acid in MGDG, DGDG, and PG, and for about 60% in SQDG. In T-pUC303 cells, the fatty acid compositions of individual lipid classes were found to be essentially the same as those obtained from wild-type cells (data not shown), suggesting that transformation with pUC303 did not affect the fatty acid compositions of the various lipid classes. In T-pUC303/*desA* cells, levels of 9,12-hexadecadienoic acid [16:2(9,12)] increased at the expense of 16:1(9) in all lipid classes, indicating that 16:1(9) was desaturated to 16:2(9,12). These findings suggest that T-pUC303/*desA* cells had acquired the ability to introduce a double bond into fatty acids at the 12 position. The 18:2(9,12) acid also became detectable in all lipid classes from pUC303/*desA* cells, but at a very low level, about 1% of the total fatty acids.

The growth temperature had a significant effect on the fatty acid composition of the wild type and the transformants. In wild-type and T-pUC303 cells that had been grown at 34°C, 16:0, 16:1(9), 18:0, 18:1(9) and 11-octadecenoic acid [18:1(11)] were major fatty acids in all lipid classes (data not shown). As in the case of lipids from cells grown at 22°C, in the cells grown at 34°C the 16:0 species accounted for about 50% of the fatty acids in MGDG, DGDG, and PG, and about 60% in SQDG. In T-pUC303/*desA* cells, 16:2(9,12) and 18:2(9,12) became detectable in all lipid classes at respective levels of about 5% of the total fatty acids. These findings suggest that, at 34°C, T-pUC303/*desA* cells can desaturate 16:1(9) and 18:1(9) to 16:2(9,12) and 18:2(9,12), respectively, in all lipid classes.

The fatty acid compositions of lipid classes from cytoplasmic membranes isolated from wild-type and T-pUC303/*desA* cells grown at 22°C and 34°C were also analyzed (data not shown). The levels of two saturated fatty acids, 16:0 and

Table 1. Compositions of lipid classes in wild-type, T-pUC303, and T-pUC303/*desA* cells of *A. nidulans* R2-SPc grown at 34°C and 22°C

Strain	Growth temperature, °C	Lipid, mol %			
		MGDG	DGDG	SQDG	PG
Wild type	34	60	13	7	20
	22	69	7	7	17
T-pUC303	34	56	12	11	21
	22	66	9	7	18
T-pUC303/*desA*	34	59	13	6	22
	22	66	10	5	19

Values are means from two independent experiments. Deviation of values was within 2.5%.

Table 2. Composition of fatty acids from wild-type and T-pUC303/*desA* cells of *A. nidulans* R2-SPc grown at 22°C

Lipid class	Fatty acid, mol %										
	14:0	14:1(9)	14:2(9,12)	16:0	16:1(9)	16:2(9,12)	18:0	18:1(9)	18:1(11)	18:2(9,12)	18:2*
Total lipids											
Wild type	1	4	0	46	45	0	1	1	2	0	0
T-pUC303/*desA*	2	1	1	52	19	22	1	t	t	1	1
MGDG											
Wild type	1	4	0	44	45	0	1	2	3	0	0
T-pUC303/*desA*	2	1	2	49	16	26	1	t	1	1	1
DGDG											
Wild type	2	3	0	47	41	0	2	2	3	0	0
T-pUC303/*desA*	3	1	1	55	21	14	1	1	1	1	1
SQDG											
Wild type	1	2	0	60	33	0	1	2	1	0	0
T-pUC303/*desA*	1	1	t	66	19	7	2	1	1	1	t
PG											
Wild type	1	1	0	50	39	0	2	3	4	0	0
T-pUC303/*desA*	1	1	t	59	19	13	3	1	1	1	1

t, Trace (<0.5%). Numbers in parentheses represent the positions of the cis double bonds, which are counted from the carboxyl-carbon. Values are means from two independent experiments.
*Positions of double bonds differed from those of 18:2(9,12) but were not identified.

octadecanoic acid (18:0), in all lipid classes from cytoplasmic membranes were higher than those from intact cells (data not shown). This result is consistent with results obtained with another strain of *A. nidulans* (30). Nevertheless, the nature of the changes in the desaturation of fatty acids by the transformation—namely, the emergence of 16:2(9,12) and 18:2(9,12)—was essentially the same as that in the lipids from the intact cells. The fatty acid compositions of lipid classes from thylakoid membranes isolated from both strains, grown at 22°C and at 34°C, were also analyzed. They were the same as those of lipid classes from the intact cells in the case of both strains (data not shown). This result is reasonable, since about 90% of the total lipids of cyanobacterial cells are located in the thylakoid membranes (31).

Effect of Transformation on Photosynthesis. The photosynthetic activities of wild-type and T-pUC303/*desA* cells grown at 22°C and 34°C are compared in Table 3. Despite differences in the extent of unsaturation of fatty acids between the two strains (Table 2), we failed to find any significant differences between wild-type and T-pUC303/*desA* cells in terms of photosynthetic activity, assayed as photosynthesis with CO_2 or the transport of electrons from H_2O to 1,4-benzoquinone, when cells were grown at the same temperature. The photosynthetic activities of the intact cells grown at 22°C were higher than those of cells grown at 34°C in both strains. This result is in good agreement with that obtained by Ono and Murata (32). A comparison of wild-type and T-pUC303/*desA* cells allowed us to characterize the effect of the introduction of the dienoic fatty acids on the thermal properties of the photosynthetic machinery.

Table 3. Photosynthetic and PSII activities in wild-type and T-pUC303/*desA* cells of *A. nidulans* R2-SPc grown at 22°C and 34°C

Strain	Growth temperature, °C	O_2 evolution, μmol per mg of chlorophyll per hr	
		CO_2	1,4-benzoquinone
Wild type	22	410 ± 20	720 ± 40
T-pUC303/*desA*	22	390 ± 20	700 ± 40
Wild type	34	220 ± 10	840 ± 40
T-pUC303/*desA*	34	240 ± 10	790 ± 40

Activities were measured at 34°C with CO_2 or 1,4-benzoquinone as electron acceptor. Values are means from three independent experiments.

The effects of the dienoic fatty acids on chilling tolerance are shown in Fig. 1, in which wild-type and T-pUC303/*desA* cells are compared. When the wild-type cells grown at 34°C were exposed to a temperature below 10°C for 1 hr, the oxygen-evolving activity of PSII—i.e., the transport of electrons from H_2O to 1,4-benzoquinone—was irreversibly decreased. More than 50% of the original activity of wild-type

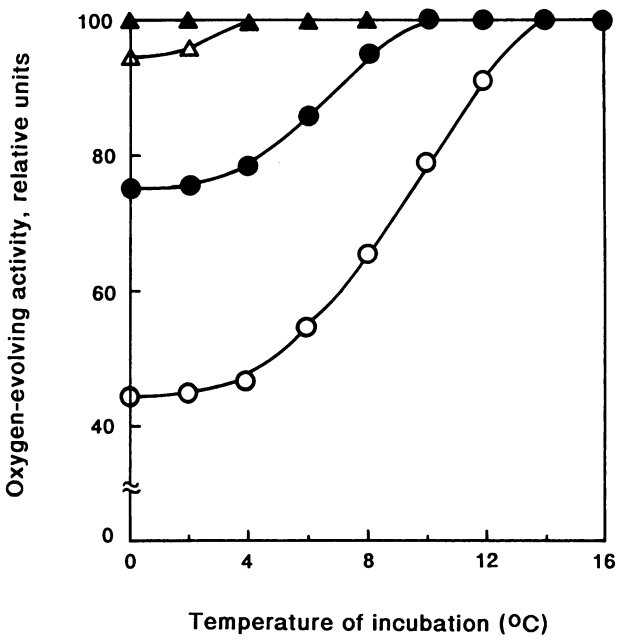

FIG. 1. Effects of low temperature on the oxygen-evolving activity of PSII of wild-type and T-pUC303/*desA* cells of *A. nidulans* R2-SPc. Cells grown at either 22°C or 34°C were incubated at designated temperatures for 60 min and were further incubated at the growth temperature for 10 min. Then PSII activity was measured by monitoring the evolution of oxygen at the growth temperature with 1,4-benzoquinone added as the acceptor of electrons. PSII activity is expressed as a percentage of that of untreated cells. The oxygen-evolving activities taken as 100% were 720 ± 30 and 630 ± 30 μmol of O_2 per mg of chlorophyll per hr for wild-type cells grown at 22°C and 34°C, respectively, and 750 ± 30 and 680 ± 30 μmol of O_2 per mg of chlorophyll per hr for T-pUC303/*desA* cells grown at 22°C and 34°C, respectively. △, Wild-type cells grown at 22°C; ▲, T-pUC303/*desA* cells grown at 22°C; ○, wild-type cells grown at 34°C; ●, T-pUC303/*desA* cells grown at 34°C. Values are from two independent experiments.

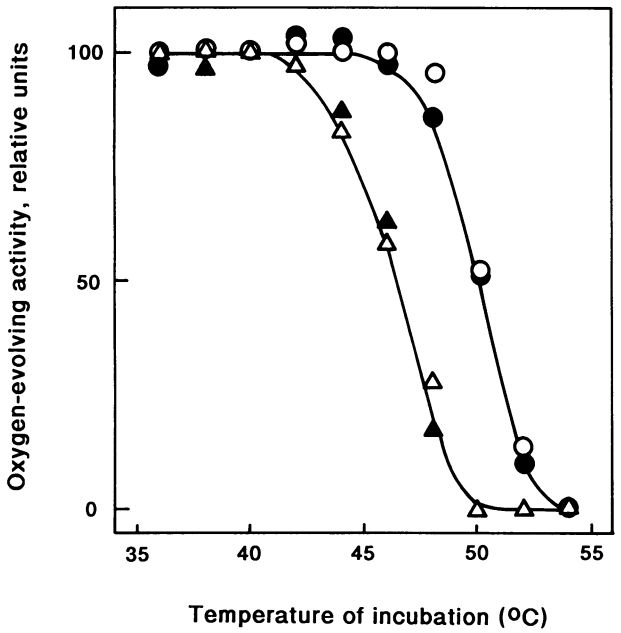

FIG. 2. Profiles of high-temperature inactivation of evolution of oxygen in wild-type and T-pUC303/*desA* cells of *A. nidulans* R2-SPc. Cells grown at 22°C or 34°C were incubated at designated temperatures in darkness for 20 min. The oxygen-evolving activity was measured at the growth temperature with 1,4-benzoquinone added as the acceptor of electrons. △, Wild-type cells grown at 22°C; ▲, T-pUC303/*desA* cells grown at 22°C; ○, wild-type cells grown at 34°C; ●, T-pUC303/*desA* cells grown at 34°C. Values are from three independent experiments.

cells was lost during incubation at 0°C for 1 hr. By contrast, the pUC303/*desA* cells did not lose any of the activity until they were chilled to 8°C, and the cells retained 75% of their original activity after incubation at 0°C for 1 hr. The oxygen-evolving activity of the cells grown at 22°C was much more resistant to low-temperature treatment.

Effect of Transformation on Thermal Tolerance of the Photosynthetic Machinery. The effects of incubation at high temperature on the oxygen-evolving activity of PSII—i.e., the transport of electrons from H_2O to 1,4-benzoquinone—of wild-type and T-pUC303/*desA* cells grown at 22°C and 34°C were compared (Fig. 2). The heat-induced inactivation of photosynthesis with CO_2 was also compared (data not shown). The inactivation profiles of photosynthesis and of the oxygen-evolving activity of PSII were very similar. However, the growth temperature markedly affected the thermal tolerance of these activities. The temperatures for 50% inactivation of the oxygen-evolving activity of wild-type cells grown at 22°C and 34°C were 46–47°C and 50–51°C, respectively. The inactivation profile of the oxygen-evolving activity of T-pUC303/*desA* cells was apparently indistinguishable from that of the wild-type cells (Fig. 2). The temperatures for 50% inactivation of the oxygen-evolving capability of T-pUC303/*desA* cells grown at 22°C and 34°C were 46–47°C and 50–51°C, respectively, and were identical to those of wild-type cells grown at 22°C and 34°C.

DISCUSSION

Our aim was to characterize the effects of molecular species of lipids that had been esterified with dienoic fatty acids on the temperature tolerance of PSII, which is the most thermosensitive of all the components of the photosynthetic machinery (33, 34). For this purpose we used wild-type and transformant cells of *A. nidulans* R2-SPc.

The wild-type cells of *A. nidulans* R2-SPc contained 16:1/16:0 as the most abundant molecular species, whereas molecular species that contained dienoic fatty acids were completely absent both from cells grown at 34°C and from cells grown at 22°C (Table 4). By contrast, T-pUC303/*desA* cells grown at 22°C synthesized 16:2/16:0 and 18:2/16:0 at levels that corresponded to about 40% and 2% of total lipids, respectively. At 34°C, 16:2/16:0 and 18:2/16:0 each accounted for about 10% of the total lipids.

Irreversible damage to the photosynthetic machinery caused by chilling temperatures is regarded as a consequence of the phase transition of cytoplasmic membranes from a liquid-crystalline state to a gel state (35). Transformation of *A. nidulans* R2-SPc with the *desA* gene resulted in the emergence of molecular species of lipids that were esterified with 16:2 and 18:2 at the *sn*-1 position of glycerolipids in the cytoplasmic membrane. This alteration in the unsaturation of lipids enhanced the tolerance of this cyanobacterium to chilling temperatures. Since the phase-transition temperature depends on the extent of unsaturation of membrane lipids (4), we suggest that the enhancement of chilling tolerance by transformation with the *desA* gene can be attributed to a decrease in the phase-transition temperature of the cytoplasmic membranes as a result of the emergence of dienoic glycerolipids (18).

The direct effects of high-temperature inactivation of the oxygen-evolving complex of PSII are triggered by the release of Mn^{2+} ions from the complex (36). Our results support the hypothesis that the oxygen-evolving complex is the primary site of heat damage. Although the evolution of oxygen by PSII in both wild-type and T-pUC303 cells grown at 22°C was inactivated at significantly lower temperature than that in the corresponding cells grown at 34°C, the profile of the high-temperature inactivation of oxygen evolution was not affected by the changes in the unsaturation of the lipids of the thylakoid membranes.

In higher plants and in cyanobacteria the threshold temperature for heat-induced reductions in photosynthetic activity can be modulated by changing the growth temperature (37–40). The adaptation of photosynthesis to high tempera-

Table 4. Estimated composition of major molecular species (*sn*-1/*sn*-2 fatty acyl groups) of glycerolipids in wild-type and T-pUC303/*desA* cells of *A. nidulans* R2-SPc grown at 22°C and 34°C

Strain	Growth temperature, °C	Molecular species, mol % 16:1/16:0	18:1(9)/16:0	16:2(9,12)/16:0	18:2(9,12)/16:0
Wild type	22	90	2	0	0
T-pUC303/*desA*	22	38	t	44	2
Wild type	34	72	12	0	0
T-pUC303/*desA*	34	58	4	10	12

Estimations were made by calculation on the basis of the composition of fatty acids of total lipids from cells grown at 22°C (Table 2) and at 34°C (18), as well as on the assumption that, in the glycerolipids of *A. nidulans* R2-SPc, the *sn*-2 position of the glycerol backbone is esterified exclusively by 16:0 (29).

ture is associated with changes in the thylakoid membranes (40). However, our experimental results indicate that the molecular species of lipids that contained 16:2 and 18:2 did not affect the ability of *A. nidulans* R2-SPc to tolerate high temperature, suggesting that the adaptation to high temperatures is associated with some factors other than the extent of unsaturation of glycerolipids.

Our results demonstrate that polyunsaturated fatty acids in the glycerolipids of thylakoid membranes are important for the ability to tolerate low temperature but not high temperature. If we are to understand the importance of the dienoic molecular species of glycerolipids in low-temperature tolerance, detailed studies of the effects of the unsaturated lipid molecules on membrane structure and on photosynthetic activities are required.

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