REVIEW ARTICLE

Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria

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INTRODUCTION

Cyanobacterial cells resemble chloroplasts of eukaryotic plants in terms of membrane structure and glycerolipid composition [1]. There are three types of membrane in the cyanobacterial cells, namely, the plasma membrane, the outer membrane and the thylakoid membranes. The thylakoid membranes are closed systems and are separated from the plasma membrane [1,2]. This architecture corresponds to that of the eukaryotic chloroplast, which has inner and outer envelope membranes and thylakoid membranes.

The major glycerolipid components of both plasma membranes and thylakoid membranes of cyanobacterial cells are monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulphoquinovosyl diacylglycerol (SQDG) and phosphatidylglycerol (PG) [2], as is true also for the inner envelope membranes and thylakoid membranes of eukaryotic chloroplasts [3,4]. MGDG contributes about half of the total glycerolipids, and the other three glycerolipids contribute the remaining half to different degrees, depending on the strains and growth conditions [5,6]. In addition to these four classes of glycerolipids, cyanobacterial cells contain a minor glycerolipid, monoglucosyl diacylglycerol, at a level of less than 1 % of the total glycerolipids [7]. At an early stage of glycerolipid biosynthesis, this lipid is synthesized from diacylglycerol and UDP-glucose [8] and is immediately converted into MGDG [7]. By contrast, in eukaryotic chloroplasts MGDG is synthesized directly from diacylglycerol and UDP-galactose [9].

Glycerolipids form bilayers and provide the necessary background for the functioning of membrane proteins [10]. 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 motion of these glycerolipids is affected by alterations in the extent of unsaturation of fatty acids [11–13]. 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, and the import and export of metabolites and proteins across the plasma membrane.

The unsaturation of fatty acids of glycerolipids in biological membranes can be altered by changing the growth temperature of the organism. Such temperature-induced changes in unsaturation of fatty acids are explained in terms of the regulation of membrane fluidity that is necessary for the proper functioning of biological membranes [14]. However, the contribution of the unsaturation of fatty acids to cold tolerance has not been obvious, since acclimatization to low temperature induces not only desaturation of fatty acids of membrane lipids but also a number of other metabolic modifications [15–17]. To determine whether the unsaturation of fatty acids contributes to the ability to tolerate low temperature, it is necessary to alter the extent of unsaturation of fatty acids of glycerolipids exclusively by manipulation of genes for fatty-acid desaturases, thereby minimizing effects on any other metabolic processes.

Desaturases introduce double bonds into fatty acids. There are three types of desaturase (Figure 1). Acyl-CoA desaturases introduce double bonds into fatty acids bound to coenzyme A; these enzymes are bound to the endoplasmic reticulum in animal, yeast and fungal cells [18]. Acyl-ACP desaturases introduce double bonds into fatty acids that are bound to ACP (acyl carrier protein); they are present in the stroma of plant plastids [19]. Acyl-lipid desaturases introduce double bonds into fatty acids that have been esterified to glycerolipids [20–22]; they are bound to the endoplasmic reticulum, the chloroplast membrane in plant cells [20], and the thylakoid membrane in cyanobacterial cells [21]. This last type of desaturase is the most efficient regulator of the extent of unsaturation of membrane lipids in response to

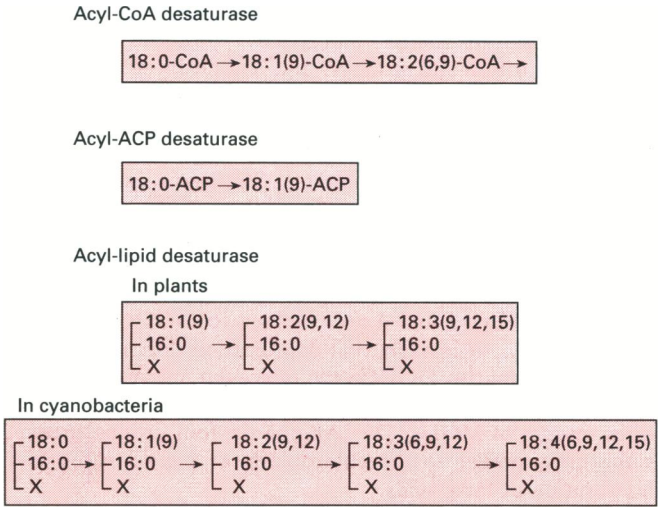

Figure 1 The three types of fatty-acid desaturase, with typical examples of desaturation reactions

X represents a polar head group.

Abbreviations used: ACP, acyl carrier protein; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulphoquinovosyl diacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; IPTG, isopropyl-1-thio-β-D-galactoside; Km^r, kanamycin-resistance gene; fatty acids are abbreviated as X:Y, where X represents the number of carbon atoms and Y represents the number of double bonds, or as X:Y (Z_1,Z_2,··) where, in addition, Z_1,Z_2,·· represent the positions of double bonds in the *cis* configuration from the C-terminus.

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changes in temperature. Recently, Jones et al. [23] suggested that the Δ12 desaturase of *Acanthamoeba castellanii*, a member of the animal kingdom, is of the acyl-lipid type.

The acyl-lipid desaturases can be further classified into subgroups according to their electron donors. One subgroup, present in the endoplasmic reticulum of plant cells, uses cytochrome b_5 as the electron donor [24,25]. The other, present in the chloroplasts of plant cells and in cyanobacterial cells, uses ferredoxin as the electron donor [21,26,27]. A unique characteristic of the acyl-lipid desaturases is that they recognize, by an unknown mechanism, exact positions within various carbon chains at which double bonds are to be specifically introduced.

In this review, we describe recent research on acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria, with reference also to higher plants. We include a discussion of the basic characteristics of fatty-acid desaturation, the characterization of acyl-lipid desaturases, the genetic manipulation of desaturases in relation to modification of cold tolerance, and the temperature-regulated expression of genes for desaturases.

FATTY-ACID DESATURATION IN CYANOBACTERIA AND HIGHER PLANTS

Cyanobacterial strains can be classified into four groups in terms of fatty-acid desaturation (Figure 2; [5]). Group 1 is characterized by the presence of only saturated and mono-unsaturated fatty acids, whereas groups 2, 3 and 4 contain polyunsaturated fatty acids. The latter fatty acids are unique in that the C_{18} and C_{16} fatty acids are esterified to the *sn*-1 and *sn*-2 positions of the glycerol moiety respectively. Strains in group 1 [e.g. *Synechococcus* sp. PCC 7942 (*Anacystis nidulans* R2) and *Mastigocladus laminosus*] only introduce a double bond at the Δ9 position of fatty acids, either at the *sn*-1 or the *sn*-2 position [5,28]. Strains in group 2 (e.g. *Synechococcus* sp. PCC 7002, *Anabaena variabilis*, *Plectonema boryanum* and *Nostoc muscorum*) can introduce double bonds at the Δ9, Δ12 and Δ15 (ω3) positions of C_{18} acids at the *sn*-1 position and at the Δ9 and Δ12 positions of C_{16} acids at the *sn*-2 position [28,29]. Strains in group 3 (e.g. *Synechocystis* sp. PCC 6714 and *Spirulina platensis*) can also introduce three double bonds, but these are at the Δ6, Δ9 and Δ12 positions of C_{18} acids at the *sn*-1 position [5]. Strains in group 4 (e.g. *Synechocystis* sp. PCC 6803 and *Tolypothrix tenuis*) can introduce double bonds at the Δ6, Δ9, Δ12 and Δ15 (ω3) positions of C_{18} acids at the *sn*-1 position [5,30]. However, desaturation at the *sn*-2 position in groups 1 and 2 and Δ6 desaturation at the *sn*-1 position in groups 3 and 4 are confined to MGDG, excluding SQDG and PG. It is likely that desaturation does not occur in fatty acids bound to DGDG; the molecular species of DGDG are synthesized by galactosylation of the corresponding molecular species of MGDG [29]. Among the four groups, group 2 is the most similar to the chloroplasts of plants in terms of desaturation of fatty acids.

The specificity of acyl-lipid desaturases to the position in the fatty-acyl chain at which the double bond is introduced was studied by feeding cells of the cyanobacterium *Synechocystis* sp. PCC 6803 with heptanoic acid, namely, a C_7 fatty acid [31]. This aliphatic acid was elongated to C_{17} and C_{19} fatty acids in the cells. The prevalent unsaturated fatty acids of membrane lipids synthesized in these cells were 17:4(6,9,12,14), 18:4(6,9,12,15) and 19:4(6,9,12,16). Thus, double bonds appeared at positions 6, 9 and 12 from the C-terminus, regardless of the chain length of the fatty acids. However, a fourth double bond was located at position 3, counted from the methyl terminus. From these physiological results, we designated the four desaturases present

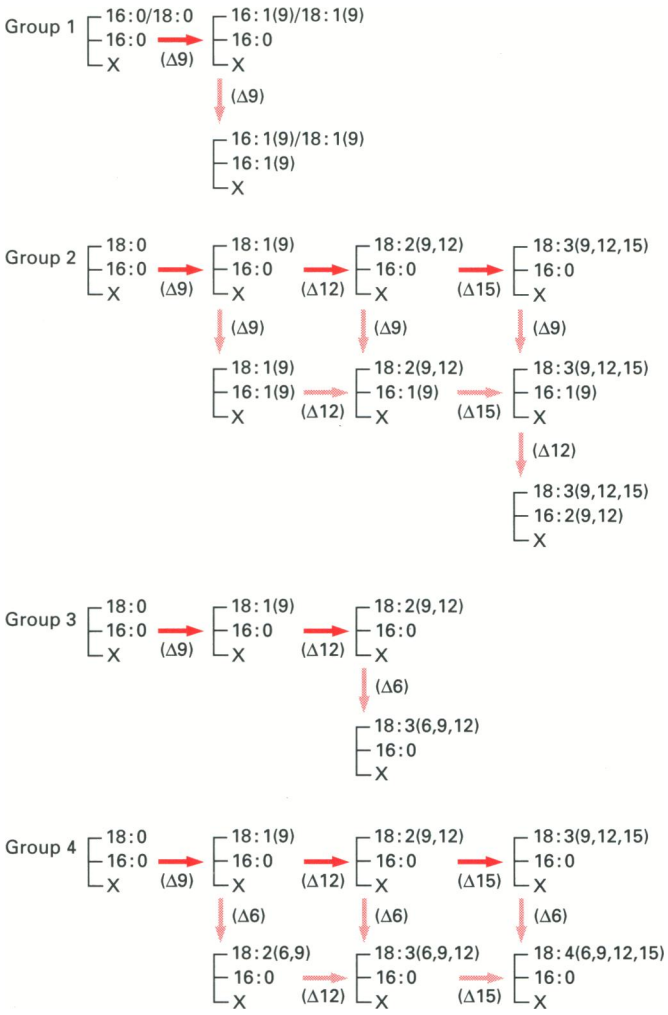

Figure 2 The acyl-lipid desaturation of fatty acids in the four groups of cyanobacterial strains

Red arrows, desaturation occurring in MGDG, SQDG and PG; pink arrows, desaturation occurring only in MGDG. Numbers in parentheses indicate the positions at which a double bond is introduced.

in *Synechocystis* sp. PCC 6803 as Δ6, Δ9, Δ12 and ω3 acyl-lipid desaturases [31].

In eukaryotic plant cells, the synthesis of fatty acids occurs exclusively in plastids [32,33]. The product of such synthesis is 16:0-ACP, some of which is elongated to 18:0-ACP and subsequently desaturated to 18:1(9)-ACP [20,33]. Although 18:0 is desaturated to 18:1(9) in the ACP-bound form in plastids by Δ9 acyl-ACP desaturase, all other desaturation reactions involve lipid-bound forms and acyl-lipid desaturases [20,34]. 18:1(9) bound to *sn*-1 and *sn*-2 positions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is desaturated to 18:2(9,12) and then to 18:3(9,12,15) in the endoplasmic reticulum by Δ12 and ω3 desaturases. The 18:1(9) bound to the *sn*-1 and *sn*-2 positions of glycolipids, MGDG and SQDG, and to the *sn*-1 position of PG, is desaturated to 18:2(9,12) and then to 18:3(9,12,15) in plastids by Δ12 and ω3 desaturases [35]. 16:0 bound to the *sn*-2 position of PG is desaturated to Δ^3-*trans*-hexadecenoic acid in plastids by an uncharacterized reaction. The 16:0 bound to the *sn*-2 position of MGDG is sequentially

desaturated to 16:1(7), 16:2(7,10) and 16:3(7,10,13) in plastids [35].

MOLECULAR CLONING OF ACYL-LIPID DESATURASES

Cyanobacterial desaturases

All known cyanobacterial desaturases are of the acyl-lipid and membrane-bound type. Since purification of these enzymes by conventional methods has proved difficult, we attempted the molecular cloning of the various desaturases. A mutant that was defective in 18:2(9,12), 18:3(6,9,12), 18:3(9,12,15) and 18:4(6,9,12,15) was initially isolated from *Synechocystis* sp. PCC 6803 after treatment of wild-type cells with ethyl methanesulphonate [30]. The mutant, designated Fad12, was defective in desaturation at the Δ12 position of C_{18} fatty acids at the *sn*-1 position of the glycerol moiety in all lipid classes. The growth rate at 22 °C of the mutant was much lower than that of the wild type, whereas mutant and wild-type cells grew at about the same rate at 34 °C.

A gene (*desA*) for the Δ12 desaturase was isolated [36] by screening of the genomic library of *Synechocystis* sp. PCC 6803 with the ability to complement the Fad12 mutation with respect to growth at low temperature and the desaturation at the Δ12 position of fatty acids after *in situ* transformation [37]. The *desA* gene contains an open-reading frame of 1053 bp that corresponds to 351 amino acid residues and encodes an acyl-lipid desaturase. The enzyme can introduce a second *cis* double bond at the Δ12 position of fatty acids bound to membrane glycerolipids. Similar *desA* genes were isolated by heterologous hybridization from *Synechococcus* sp. PCC 7002, *Synechocystis* sp. PCC 6714 and *Anabaena variabilis* using a probe derived from the *desA* gene of *Synechocystis* sp. PCC 6803 [38]. The amino acid sequence deduced from the nucleotide sequence of the *desA* gene of *Synechocystis* sp. PCC 6803 is similar to that of *Synechocystis* sp. PCC 6714. The extent of sequence similarity between the amino acid sequences of *Synechocystis* sp. PCC 6803 and *Synechocystis* sp. PCC 6714 is 96%. However, the extent of amino acid sequence conservation between the amino acid sequences of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 and that between the amino acid sequences of *Synechocystis* sp. PCC 6803 and *A. variabilis* are 57% and 59% respectively [38].

Comparison of the amino acid sequences reveals in cyanobacteria four conserved domains and indicates that certain residues, in particular histidine residues, are highly conserved in these desaturases. The 15 histidine residues in the four conserved domains are found in all the Δ12 desaturases. The cDNAs and genes for Δ12 acyl-lipid desaturases have also been isolated from higher plants [40,41]. Comparisons of amino acid sequences of cyanobacterial Δ12 desaturases with those of higher plant Δ12 desaturases reveals that histidine residues are highly conserved in these desaturases also.

The *desC* gene for the Δ9 acyl-lipid desaturase was found in the 5′-upstream region of the *desA* gene in the chromosome of *A. variabilis* [42]. The *desC* gene of *Synechocystis* sp. PCC 6803 was cloned by screening a genomic library with a probe derived from the *desC* gene of *A. variabilis*. The deduced amino acid sequences of the Δ9 acyl-lipid desaturases of *Synechocystis* sp. PCC 6803 and *A. variabilis* (318 and 272 amino acid residues) are similar to those of the Δ9 acyl-CoA desaturases from the rat [43], the mouse [44,45] and yeast [46] with amino acid sequence conservation of about 25%.

We recently cloned the *desB* gene for the ω3 acyl-lipid desaturase from *Synechocystis* sp. PCC 6803 by screening a genomic library with a probe derived from the *desA* gene of this strain [39]. The amino acid sequence of the ω3 desaturase, deduced from the *desB* gene [39], resembles those of the ω3 desaturases from higher plants [47–50] with amino acid sequence conservation of 45–50%.

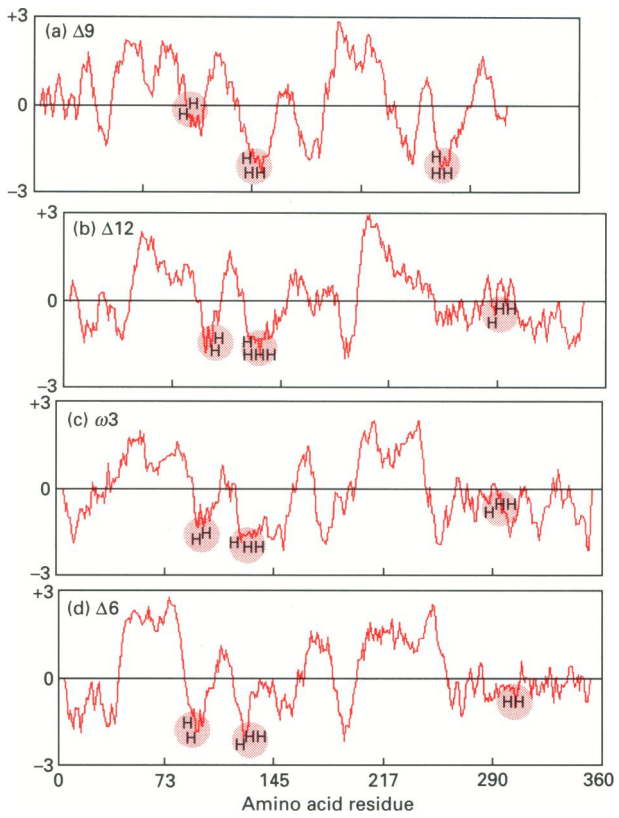

Figure 3 Hydropathy profiles and conserved histidine clusters of the acyl-lipid desaturases of *Synechocystis* sp. PCC 6803

The window size for the calculations of hydropathy indices was 12 amino acid residues. (**a**) Δ9 desaturase, (**b**) Δ12 desaturase, (**c**) ω3 desaturase (**d**) Δ6 desaturase. Histidine residues in conserved clusters are indicated by H and a shaded circle.

Reddy et al. [51] cloned the *desD* gene for Δ6 desaturase by the gain-of-function method using *Anabaena* sp. PCC 7120, which does not contain a Δ6 desaturase. Now genes for all the desaturases have been cloned from *Synechocystis* sp. PCC 6803. It is unlikely that a transit peptide is included in the product of translation of any of these genes.

When the deduced amino acid sequences of the four desaturases from *Synechocystis* sp. PCC 6803 are compared, the amino acid sequence conservation is highest (28%) between the Δ12 and ω3 desaturases. The amino acid sequence conservation between the other combinations of desaturases was less than 15%. The hydropathy profiles of the deduced amino acid sequences of the four desaturases (Figure 3) show two clusters of hydrophobic regions, which are putative membrane-spanning domains, in all the desaturases. There are some well-conserved regions of amino acid sequence, in particular three histidine-cluster motifs, one HXXXH and two HXXHH. However, in the Δ6 desaturase two HXXHH motifs are replaced by HXXXHH, and in the Δ9 desaturase the HXXXH motif is replaced by HXXXXH. Histidine residues are potential ligands of iron atoms, which are assumed to act at the catalytic centres of desaturases [53]. It seems likely that the histidine residues are located on the cytoplasmic surface of the membrane.

To characterize in further detail the Δ12 desaturase of *Synecho-*

Table 1 Molecular characteristics of the four acyl-lipid desaturases of *Synechocystis* sp. PCC 6803

	Desaturases			
	Δ6 (*desD*)	Δ9 (*desC*)	Δ12 (*desA*)	ω3 (*desB*)
Amino acid residues	359	319	351	359
Molecular mass	41.4 kDa	37.2 kDa	40.5 kDa	41.9 kDa
Specificity				
Position	*sn*-1	*sn*-1*	*sn*-1*	*sn*-1
Fatty acid	18:1(9) 18:2(9,12) 18:3(9,12,15)	18:0*	18:1(9)*	18:2(9,12) 18:3(6,9,12)
Head group				
MGDG	+	+	+	+
SQDG	−	+	+	+
PG	−	+*	+*	+
PE	?	+*	+*	?
CL	?	+*	?	?

* Confirmed in an overexpression system in *E. coli* [39,52]. ?, not determined. + and − represent occurrence and absence of desaturation in individual lipid classes respectively.

cystis sp. PCC 6803, the *desA* gene for Δ12 desaturase was overexpressed in *Escherichia coli* under the control of the promoter of the gene for RNA polymerase of bacteriophage T7 [52]. The coding region of the *desA* gene was introduced into the 3′-downstream region of the promoter in an expression vector, pET-3a, and the resultant vector with the chimeric gene construct (pET-desA) was used to transform *E. coli* strain BL21(DE3). Upon induction by isopropyl-1-thio-β-D-galactoside (IPTG), a large amount of the Δ12 desaturase with an apparent molecular mass of 36 kDa was produced. When *E. coli* cells transformed with pET-desA were supplied with 18:1(9) in the culture medium, 18:1(9) was incorporated into the glycerolipids during growth and was desaturated *in vivo*. The activity that desaturates 18:1(9) to 18:2(9,12) is referred to as the activity of Δ12 desaturase *in vivo*. By contrast, a homogenate of cells in which the *desA* gene had been overexpressed was active in desaturating 18:1(9) to 18:2(9,12) in the presence of reduced ferredoxin *in vitro* [52]. This activity is referred to as the activity of Δ12 desaturase *in vitro*.

The importance of the conserved histidine residues in the Δ12 desaturase was studied by site-directed mutagenesis (M.-H. Macherel, D. Macherel, H. Wada and N. Murata, unpublished work). Five conserved histidine residues, namely, His-90 (at position 90 of the Δ12 desaturase encoded by the *desA* gene of *Synechocystis* sp. PCC 6803), His-109, His-129, His-287 and His-290, were individually changed to arginine residues. Although none of the mutations affected the level of the expressed protein, the mutations of His-109, His-129, His-287 and His-290 totally eliminated the desaturase activity both *in vivo* and *in vitro*, whereas that of His-90 eliminated the activity *in vitro* but did not totally eliminate the activity *in vivo*. These results demonstrate that histidine residues His-109, His-129, His-287 and His-290 are essential for the Δ12 desaturase activity.

The *desC* genes of *Synechocystis* sp. PCC 6803 and *A. variabilis* were incorporated into pET-3a and overexpressed in *E. coli* under the control of the promoter of T7 RNA polymerase [42]. The cells were supplied with 18:0, which is present at very low levels in *E. coli* cells under normal conditions. The externally supplied 18:0 was esterified at both the *sn*-1 and *sn*-2 positions of the glycerol moiety of PG and PE. The overexpressed Δ9 acyl-lipid desaturases of *Synechocystis* sp. PCC 6803 and of *A. variabilis* were active in converting 18:0 into 18:1(9) at the *sn*-1 position but not at the *sn*-2 position. The enzymes did not desaturate 16:0 at the *sn*-1 or the *sn*-2 position. These observations indicate that the Δ9 acyl-lipid desaturases from *Synechocystis* sp. PCC 6803 and *A. variabilis* are specific to 18:0 and the *sn*-1 position but are non-specific with respect to the head group.

Table 1 summarizes the molecular characteristics of four acyl-lipid desaturases from *Synechocystis* sp. PCC 6803. Δ6, Δ12 and ω3 desaturases have about 360 amino acid residues each, whereas the Δ9 desaturase is shorter than the other three desaturases by about 40 amino acid residues. It is very likely that these desaturases are strictly specific to the *sn*-1 position. Their specificity to fatty acids is unique; the Δ9 desaturase is strictly specific to 18:0 and does not recognize 16:0 [42], and the Δ12 desaturase is strictly specific to 18:1(9), recognizing neither 16:1(9) nor 18:1(11) [52]. The ω3 desaturase acts on fatty acids that have double bonds at the Δ9 and Δ12 positions but not on those without a double bond at the Δ12 position [30,39]. It is likely that the Δ6 desaturase recognizes fatty acids that have a double bond at the Δ9 position [30,36,54]. The specificity to the polar head group varies: the Δ6 desaturase is specific to MGDG but the Δ9 and Δ12 desaturases do not discriminate among polar head groups, as far as we know [42,52]. The ω3 desaturase recognizes MGDG, SQDG and PG, but its recognition of PE and cardiolipin (CL) has not yet been examined. It is likely that none of the desaturases recognizes DGDG as a substrate [29].

Acyl-lipid desaturases of higher plants

Acyl-lipid desaturases of higher plants have been cloned from several plant sources. Heinz and his collaborators solubilized Δ12 acyl-lipid desaturase from the envelope membranes of chloroplasts and purified it by ion-exchange and ferredoxin-affinity chromatography [55]. Determination of the N-terminal sequence of the purified Δ12 desaturase allowed a cDNA clone for this desaturase to be isolated from spinach leaves [56]. The amino acid sequence deduced from the nucleotide sequence is homologous to that of the Δ12 desaturase encoded by the *desA* gene of *Synechocystis* sp. PCC 6803. The leader sequence of the Δ12 desaturase contains 65 amino acids and has features characteristic of chloroplast transit peptides, with a high level of serine and threonine residues.

Molecular-genetic approaches to the isolation of genes or cDNAs for acyl-lipid desaturases have been successful in the case of ω3 desaturases in the endoplasmic reticulum and plastids.

Somerville's group [47] isolated a YAC clone that contained the gene for the ω3 acyl-lipid desaturase in the endoplasmic reticulum of *Arabidopsis thaliana* by chromosome walking. The cDNA for the same enzyme in the endoplasmic reticulum of *Brassica napus* was then isolated by screening the cDNA library of *B. napus* with a probe derived from the YAC clone of *A. thaliana*. Upon introduction of the isolated cDNA, a mutation in *A. thaliana* associated with a defect in the desaturation of fatty acids at the ω3 position in the endoplasmic reticulum was complemented and a normal phenotype was restored.

Yamamoto et al. [57] isolated cDNAs of several auxin-regulated genes of mung bean and one of the cDNAs was identified as the cDNA for the ω3 desaturase in the endoplasmic reticulum [50]. Yadav et al. [49] isolated the gene for the ω3 desaturase in the endoplasmic reticulum of *A. thaliana* using mutant lines generated by T-DNA tagging [58]. They also isolated cDNAs for homologues of the ω3 desaturases in the endoplasmic reticulum and plastid of *A. thaliana*, soybean and rapeseed. Iba et al. [48] isolated a gene for the ω3 desaturase in the plastid of *A. thaliana*. In addition, the cDNA encoding the Δ12 desaturase in the endoplasmic reticulum was isolated from *A. thaliana* [41]. cDNAs for the Δ12 desaturase in plastids were also isolated from *A. thaliana*, *Glycine max* and *B. napus* [40]. When the cDNA of *A. thaliana* was used to transform the cyanobacterium *Synechococcus* sp. PCC 7942, the transformant acquired the ability to introduce a second double bond into the Δ12 position of fatty acids [40]. Although several genes and cDNAs for acyl-lipid desaturases have been isolated from higher plants, a gene or cDNA for the desaturase that introduces a double bond at the Δ3 position of 16:0 bound to the *sn*-2 position of PG in the *trans* configuration and for the enzyme that introduces a double bond at the Δ7 (ω9) position of 16:0 bound to the *sn*-2 position of MGDG in the *cis* configuration, have not yet been isolated.

Other desaturases

The Δ9 acyl-ACP desaturase is present in the stroma of plastids in higher plant cells. This enzyme was partially purified from maturing seeds of safflower and found to be a homodimer of 68 kDa [19]. It was purified to homogeneity from avocado mesocarp [59] and developing embryos of safflower seed [60]. cDNAs encoding stearoyl-ACP desaturases were isolated from castor bean [59,61], safflower seeds [60], cucumber [62], spinach [63], *B. rapa* [64] and *B. napus* [65]. cDNA encoding the 16:0-ACP desaturase that is involved in the biosynthesis of petroselinic acid was isolated from coriander [66,67]. This desaturase introduces a double bond at the Δ4 position of 16:0 bound to ACP [67]. Fox et al. [68] overexpressed cDNA for the stearoyl-ACP desaturase of castor bean in *E. coli* and found that the active homodimeric enzyme contained four iron atoms.

Δ9 Acyl-CoA desaturase (18:0-CoA desaturase) is present in the endoplasmic reticulum of animal and yeast cells. The enzyme purified from the microsomes of rat liver is a single polypeptide of 41.4 kDa, containing one atom of non-haem iron [43,53]. The level of the mRNA for the 18:0-CoA desaturase increases 50-fold upon feeding of fat-free foods after starvation [69]. cDNA for the enzyme in rat liver was isolated by differential hybridization with mRNAs extracted from liver cells before and after feeding of fat-free foods [69]. The cDNA included an open-reading frame of 1074 bp, coding for 358 amino acid residues [43]. Genes and cDNAs for stearoyl-CoA desaturases were also isolated from rat [70], mouse [44,45], bovine [71] and yeast [46]. Activities of Δ5 and Δ6 acyl-CoA desaturases have been identified in animal cells, but neither genes nor cDNAs have been isolated.

GENETIC MANIPULATION OF DESATURASES AND MODIFICATION OF COLD TOLERANCE

Disruption of desaturase genes

Insertional disruption [72] of *desA* and *desB* genes has enabled us to manipulate the number of double bonds in lipid molecules in the cyanobacterium *Synechocystis* sp. PCC 6803 [36,39,54]. Cells of the wild-type strain and the Fad6 mutant (defective in the ΔG desaturase) were transformed with genes that had been disrupted by insertion of a Km^r (kanamycin-resistance gene) cartridge. Figure 4(a) shows the changes in composition of molecular species of lipids upon mutation and transformation of cells of *Synechocystis* sp. PCC 6803. The results demonstrate that the extent of unsaturation of fatty acids in membrane lipids can be modified, step by step, by genetic manipulation of the *desA* gene.

The effects of the unsaturation of fatty acids in membrane lipids on physiological characteristics, such as growth and chilling tolerance, were studied using genetically manipulated strains of *Synechocystis* sp. PCC 6803 [54,73–75]. At 34 °C, there was no significant difference in growth rate during the exponential phase of growth between wild-type, mutant Fad6, and Fad6/*desA*::Km^r transformed cells. At 22 °C, the Fad6 cells grew at the same rate as the wild type. By contrast, the growth of Fad6/*desA*::Km^rcells was markedly slower than that of wild-type and Fad6 cells. These observations suggest that elimination of desaturation at the Δ12 position of fatty acids had a deleterious effect on the growth of *Synechocystis* sp. PCC 6803 at low temperatures.

The effect of fatty-acid unsaturation on chilling tolerance was also studied by comparing wild-type and Fad6/*desA*::Km^rcells

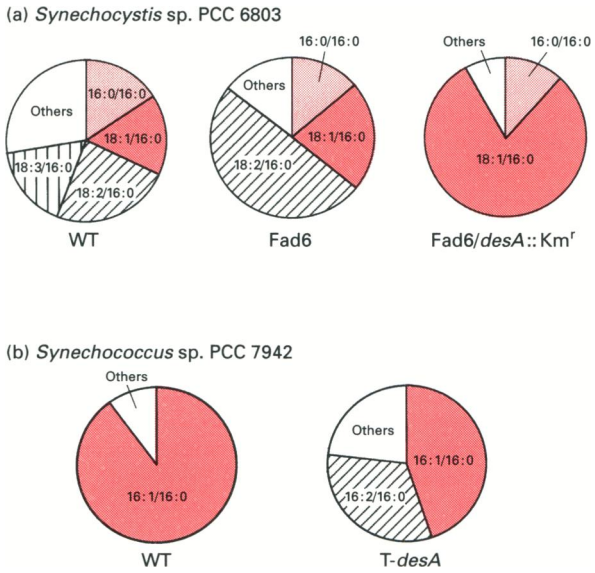

Figure 4 Changes in molecular species composition after manipulation of genes for desaturase in cyanobacteria

(**a**) Mutagen-induced and insertional mutations in genes for desaturases in *Synechocystis* sp. PCC 6803. Abbreviations: WT, wild type; Fad6, the mutant defective in the ΔG desaturase; Fad6/*desA*::Km^r, the double mutant in which the *desA* gene was disrupted by a Km^r cartridge in the Fad6 mutant. Cells were grown at 34 °C. Based on results of Wada et al. [54]. (**b**) Introduction of the *desA* gene from *Synechocystis* sp. PCC 6803 into *Synechococcus* sp. PCC 7942. WT, wild type; T-*desA*, the transformant produced by introduction of the *desA* gene on the shuttle vector pUC303. Cells were grown at 22 °C. Based on results of Wada et al. [80].

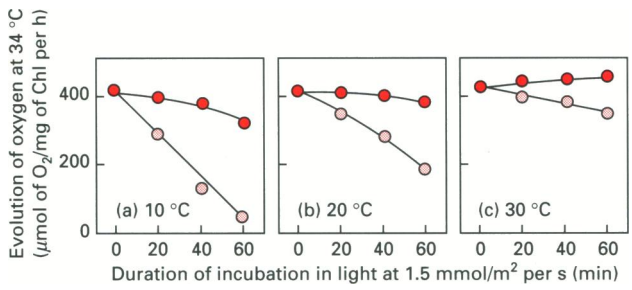

Figure 5 Photoinhibition of photosynthesis in wild-type and Fad6/*desA*::Km^r cells of *Synechocystis* sp. PCC 6803 grown at 34 °C

Pink symbols, wild type; red symbols, Fad6/*desA*::Km^r. Adapted from Gombos et al. [75]; Chl, chlorophyll.

that were grown at the same temperatures. When the cells were exposed to light at low temperature, photosynthesis was significantly inhibited (Figure 5; [54,74,75]). The extent of photoinhibition differed among strains: Fad6/*desA*::Km^r cells, which contain only monounsaturated and saturated lipids, were the most susceptible to the low-temperature photoinhibition of photosynthesis, whereas Fad6 and wild-type cells were indistinguishable in terms of sensitivity to photoinhibition. These results suggest that the second double bond at the Δ12 position in fatty acids is important for protection against low-temperature photoinhibition. Photoinhibition at room temperature, although much less significant than that at low temperature, was also affected by the unsaturation of fatty acids (Figure 5; [74,75]). By contrast, the photosynthetic transport of electrons, measured at various temperatures, was unaffected by changes in the extent of unsaturation of membrane lipids [74]. These results suggest that polyunsaturated fatty acids are important for growth and the ability to tolerate photoinhibition of photosynthesis at low temperatures.

Since the extent of photoinhibition of photosynthesis *in vivo* must depend on a balance between photoinduced inactivation and recovery from the photoinactivated state, the effects of the unsaturation of membrane lipids on individual processes were investigated [75]. In isolated thylakoid membranes and, also, in intact cells supplemented with chloramphenicol (an inhibitor of protein synthesis), the recovery process was blocked. Under these conditions, the unsaturation of membrane lipids did not affect the inactivation process. In a separate experiment in which the recovery process was isolated from the inactivation process, the unsaturation of membrane lipids increased the rate of recovery considerably. Therefore, the apparent increase in low-temperature photoinhibition of photosynthesis *in vivo* seems to be due to depressed recovery [75]. According to the current hypothesis of the mechanism of photoinhibition [76,77], photoinhibition is caused by light-induced damage to the D1 protein, which contributes to an essential part of the photochemical reaction centre II [78]. Subsequently, the inactivated D1 protein is degraded proteolytically leaving a photosystem II complex depleted of the D1 protein. The precursor to the D1 protein is synthesized *de novo*, incorporated into the D1 protein-depleted photosystem II complex and then processed to yield active D1 protein, with resultant recovery of an active photosystem II complex. Since the light-induced damage to the D1 protein is regarded as the inactivation process of photoinhibition, it is likely that the damage to the D1 protein is unaffected by the unsaturation of membrane lipids. Therefore, one of the subsequent processes can be assumed to be accelerated by the unsaturation of membrane lipids.

In contrast to the changes in cold tolerance that follow elimination of the unsaturation of membrane lipids, the heat tolerance of photosynthesis is barely affected or is slightly reduced by decreases in the unsaturation of membrane lipids [79,80]. Since the heat stability and the saturation of membrane lipids increase with increases in growth temperature, it was suggested previously that unsaturation of membrane lipids might lower heat tolerance [81,82]. Our results after manipulation of desaturase genes demonstrate clearly the absence of such a correlation. We found recently that changes in heat tolerance due to the growth temperature are caused by protein factors [83,84]. Photosynthesis and the photosynthetic transport of electrons from water to benzoquinone are unaffected by changes in unsaturation of membrane lipids [74,80]. These results suggest either that the diffusion of plastoquinone is unaffected by the unsaturation (and, ultimately, by the fluidity) of thylakoid membrane lipids or that the diffusion of plastquinone is not the rate-limiting step in electron transport [74,85].

Introduction of desaturase genes

Another cyanobacterium, *Synechococcus* sp. PCC 7942 (*Anacystis nidulans* strain R2), was transformed with the *desA* and *desB* genes to manipulate the unsaturation of membrane glycerolipids [36,38,39]. *Synechococcus* sp. PCC 7942 belongs to group 1 of the cyanobacteria, being completely incapable of desaturation at the Δ6, Δ12 and ω3 positions [5]. Figure 4(b) shows the changes in molecular species composition of total lipids upon transformation with the *desA* gene. It appears that the transformant with the *desA* gene acquired the ability to introduce a second double bond at the Δ12 position of fatty acids. In order to examine the effect of the introduction of this second double bond on chilling tolerance, wild-type and transformed cells of *Synechococcus* sp. PCC 7942 were compared [36,80]. When wild-type cells (and also cells that had been transformed with the vector plasmid alone) were exposed to low temperatures below 10 °C, photosynthetic activity decreased irreversibly. More than 50% of the activity was lost upon incubation at 5 °C for 60 min. By contrast, the transformant with the *desA* gene showed scarcely any change in photosynthetic activity during incubation at 5 °C for 60 min [36]. These observations demonstrate that chilling tolerance of *Synechococcus* sp. PCC 7942 was enhanced by transformation with the *desA* gene.

We compared the wild-type and transformed cells in terms of heat tolerance, photosynthesis and photosynthetic electron transport. No detectable changes were caused by the change in the unsaturation of membrane lipids. The results obtained upon an increase in the unsaturation of membrane lipids by transformation of *Synechococcus* sp. PCC 7942 are all consistent with those obtained by decreasing the unsaturation of membrane lipids by gene disruption in *Synechocystis* sp. PCC 6803.

In higher plants, we have successfully altered the extent of unsaturation of fatty acids in PG and cold tolerance by genetic engineering of glycerol-3-phosphate acyltransferase [86]. First, we isolated cDNAs for this enzyme from squash, a chilling-sensitive plant [87] and from *Arabidopsis*, a chilling-resistant plant [88]. We introduced the cDNAs into tobacco, which has intermediate chilling sensitivity [86]. Upon introduction of the cDNA for glycerol-3-phosphate acyltransferase from squash, the level of unsaturation of fatty acids in PG fell significantly and the chilling sensitivity of tobacco plants was markedly increased. By contrast, introduction of the cDNA from *Arabidopsis* increased both the unsaturation of fatty acids in PG and chilling tolerance.

Wolter et al. [89] confirmed the correlation between unsaturation of fatty acids in PG and chilling sensitivity by genetic engineering of *Arabidopsis* using glycerol-3-phosphate acyltransferase from *E. coli*, which is of chilling-sensitive type. The plant was changed from chilling-resistant to chilling-sensitive.

TEMPERATURE-REGULATED EXPRESSION OF DESATURASE GENES

In living organisms the fluidity of membranes is maintained by regulation of the levels of unsaturation of the fatty acids of membrane lipids [14,90]. Cyanobacterial cells respond to a decrease in temperature by the introduction of double bonds into fatty acids of membrane lipids [6,91–93]. Acyl-lipid desaturases are responsible for the desaturation and play important roles in cold acclimatization of cyanobacterial cells [6].

Several mechanisms have been proposed for the regulation of the unsaturation of fatty acids of membrane lipids [14,94]. We suggested that the crucial step in the regulation of unsaturation of fatty acids of membrane lipids is synthesis *de novo* of acyl-lipid desaturases in cyanobacterial cells as low-temperature-induced desaturation of fatty acids is inhibited by rifampicin and chloramphenicol but not by cerulenin, an inhibitor of the synthesis of fatty acids [93,95,96]. Other reports have suggested that the accelerated desaturation of fatty acids at low temperature results from the negative coefficient of desaturase activities, namely, the activity of desaturases is higher at lower temperatures [97,98]. An alternative hypothesis is that synthesis *de novo* of saturated lipids is depressed at low temperatures, whereas active desaturation of fatty acids continues with a resultant increase in the extent of unsaturation of membrane lipids at low temperatures.

The genes for the acyl-lipid desaturases of *Synechocystis* sp. PCC 6803 have been cloned, so it is possible to examine the effect of low temperature on their expression. The level of mRNA for the Δ12 desaturase from *Synechocystis* sp. PCC 6803 increases with a decrease in ambient temperature [96], suggesting that the low-temperature-induced desaturation of fatty acids of membrane lipids results from the low-temperature-induced up-regulation of expression of the genes for desaturases [96]. The lifetimes of mRNAs for the Δ12 desaturases at 22 °C and 34 °C were 20 min and 2 min respectively, suggesting that the level of the mRNA is regulated in response to temperature via changes in the rate of its degradation (D. Los and N. Murata, unpublished work). It is likely, moreover, that transcriptional regulation also operates to increase the level of the mRNA.

We have also obtained evidence that a decrease in the unsaturation of plasma membranes of *Synechocystis* sp. PCC 6803 by catalytic hydrogenation of the unsaturated fatty acids of the membrane lipids can mimic the effect of a decrease in temperature on the regulation of the level of *desA* mRNA [99]. The changes in the level of the mRNA are caused by saturation of a small portion of the lipids of the plasma membrane. The level of *desA* mRNA increases after a short lag time, reaching a level 10-fold higher than the original level within 1 h. This time course is similar to that observed after a temperature shift from 36 °C to 22 °C.

In summarizing the results of the low-temperature-induced and hydrogenation-induced regulation of expression of the desaturase gene, we propose a scheme for the acclimatization of the cyanobacterial cells to low temperature, as shown in Figure 6. Mechanisms for sensing a decrease in membrane fluidity and signal-transduction pathways are now being studied.

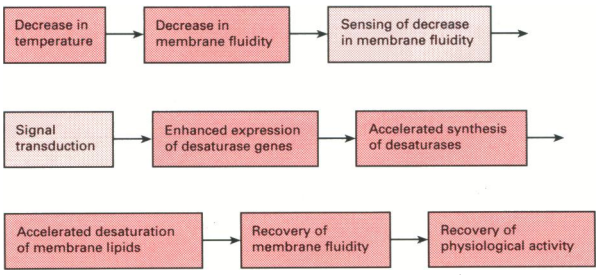

Figure 6 A scheme for the acclimatization of cyanobacterial cells to low temperature

The processes in dark pink boxes have been well documented, whereas evidence for the processes in pale pink boxes is not yet available.

CONCLUSIONS AND PERSPECTIVES

(a) Genes for acyl-lipid desaturases, which introduce double bonds into fatty acids at Δ6, Δ9, Δ12 and ω3 positions, have been cloned from cyanobacteria. By disrupting these genes in *Synechocystis* sp. PCC 6803 and by introducing them into *Synechococcus* sp. PCC 7942, we can manipulate sequentially the number of double bonds in membrane lipids.

(b) The unsaturation of membrane lipids is important in cold tolerance. It is now possible to alter the cold tolerance of cyanobacteria and plants genetically by manipulating the unsaturation of fatty acids of membrane lipids.

(c) Low temperature increases the desaturation of fatty acids of membrane lipids in cyanobacterial cells. The low-temperature-induced desaturation is caused by the up-regulation of the expression of genes for desaturase.

(d) To understand fully the biochemical and biophysical processes of desaturation of fatty acids, it is now important to study the structures of desaturases by X-ray crystallography and protein engineering.

(e) The methods described herein for the introduction and disruption of desaturase genes should be useful for future biophysical studies of membrane structure. The extent of unsaturation of membrane lipids can be manipulated with minimal perturbation of the protein composition of the membrane.

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