Glucosylglycerol, a Compatible Solute, Sustains Cell Division under Salt Stress in *Synechocystis* sp. PCC 6803

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To

My family

My teachers:

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Sadok BOUZID *(Faculty of Mathematical, Physical and Natural Sciences, TUNIS)*

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My beloved countries:

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Abbreviations

BG-11	Culture medium for *Synechocystis* cells
GG	glucosylglycerol
GB	glycine betaine
Synechocystis	*Synechocystis* sp. PCC 6803
PCC	Pasteur Culture Collection
Δ*ggpS*	mutant deficient in glucosylglycerol phosphate synthase
OD_{730}	optical density at 730 nm
Cell count	number of cells per milliliter of culture
Cell size	diameter of cell measured by flow cytometer
PCR	polymerase chain reaction

Abstract

In response to an upward shift in salt concentration of the growth medium, the cyanobacterium *Synechocystis* sp. PCC 6803 accumulates compatible solutes glucosylglycerol (GG) and transiently traces of sucrose. While the molecular mechanism for GG synthesis including regulation of the expression of *ggpS* gene, which encodes glucosylglycerol phosphate synthase, the key enzyme for GG biosynthesis has been intensively investigated, the role of GG in protection against salt stress remains poorly understood.

To study of the role of GG in the tolerance to salt stress, we generated Δ*ggpS* mutant cells of *Synechocystis*, which are deficient in GG biosynthesis. We found that salt stress due to 450 mM NaCl inhibited cell division and significantly increased cell size in Δ*ggpS* mutant cells, whereas this concentration of the salt did not affect wild-type cells. Inhibition of cell division and increase in cell size were observed in wild-type cells at much more higher concentrations of NaCl, such as 800 mM. Electron microscopic analysis revealed that, in Δ*ggpS* cells, septation and separation of daughter cells were incomplete and the aborted division was recognized by the presence of a structure that resembled a division ring. On the other hand, osmotic stress due to 900 mM sorbitol, which has approximately the same osmotic effect as 450 mM NaCl, totally arrested the growth of wild-type and Δ*ggpS* cells. While 450 mM NaCl significantly increased the size of Δ*ggpS* cells, 900 mM sorbitol slightly reduced the size (about 10~20%) of both wild-type and Δ*ggpS* cells, suggesting that the inhibition of cell division and the increase in cell size were due to the ionic effect but not the osmotic effect of NaCl.

Synechocystis cells are also able to uptake GG and trehalose by an ABC-type transport system encoded by *ggtA* gene and *ggtBCD* gene cluster. Sucrose is also uptaken by the same uptake system but with lower affinity than GG and trehalose. Exogenous supplemention of GG to the culture medium protected Δ*ggpS* cells against salt stress and reversed the adverse effects of NaCl on cell division and cell size. These observations suggested that GG is important for salt tolerance and thus for the proper division of *Synechocystis* cells under salt stress conditions. All other compatible solutes added failed to rescue the salt-sensitive phenotype of Δ*ggpS* cells. Thus, it appears that various compatible solutes are qualitatively different and might play different roles in the protection of cells against salt stress.

Chapter 1 summarises the response of organisms to abiotic stress and gives an overview of recently discovered stress-sensing mechanisms and signal transduction pathways. The use of genetic engeneering of compatible solutes to enhance stress tolerance of plants is also discussed.

Chapter 2 describes concisely the experimental procedures and various techniques used along this work.

Chapter 3 describes in details a comparative analysis of dramatic effects of NaCl stress on cell division, size and ultrastructure of both wild-type and Δ*ggpS* cells. Special emphasis is given to the specific inhibition of cell division by NaCl stress.

Chapter 4 is a description of the protective effects of exogenously supplemented GG and shows how GG was able to rescue the morphological aberrations and to stimulate cell division in the Δ*ggpS* cells under salt stress. In this chapter we also show that compatible solutes other than GG failed to protect Δ*ggpS* cells from the inhibition due to NaCl.

In Chapter 5 we show that salt stress and osmotic stress represent two different types of stress and that they act differently to impair the growth and the cell division of *Synechocystis* cells.

Finally, Chapter 6 represents general discussion and conclusion of all the chapters cited above. At the end of this chapter we also propose a hypothetical model by which NaCl and GG might affect the cell cycle in *Synechocystis* cells.

Chapter 1

General introduction

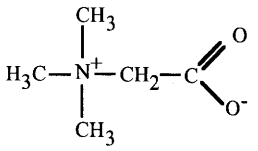
1.1. Definition of compatible solutes

Compatible solutes are low-molecular-weight highly soluble compounds that are nontoxic for cellular activities even at high concentrations. On the basis of their chemical structures, compatible solutes can be divided in the following four groups (i) carbohydrates (*e.g.,* trehalose), (ii) heterosides (*e.g.,* glucosylglycerol), (iii) amino acids (*e.g.,* proline) and their derivatives (*e.g.,* glycinebetaine and ectoine), and (iv) polyols (*e.g.,* glycerol and mannitol) (Galinski, 1993; Fig. 1).

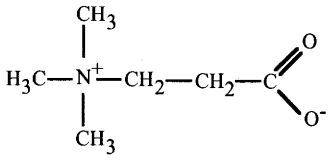
The accumulation of compatible solutes is, in most cases, a stress-inducible process and is a universal process during cellular acclimation to environments with low water potentials. The importance of this process for successful acclimation to high-salinity conditions has been demonstrated by two kinds of experiment. The first is when mutations affect genes for enzymes which synthesize compatible solutes lead, in all cases, to salt-sensitive phenotype with significant reduction in both salt tolerance and osmotic tolerance. Therefore, these genes are essential for survival under low water potentials. The second is the phenotypic complementation of the salt-sensitive phenotype by feeding the cells with compatible solutes in the medium.

1.2. Response of bacteria to salt stress

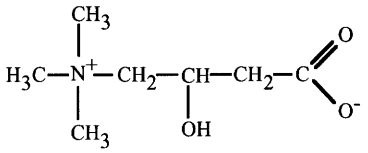
To acclimate to the continuously changing environments, cells regulate the expression of several genes which are specific to each kind of stress. For example, genes which are induced after cold shock are different from those which are induced by salt stress with few exceptions. The cytoplasmic membrane of bacteria is permeable to water but



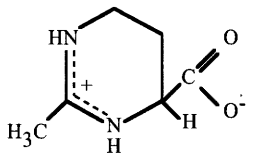
Glycine betaine



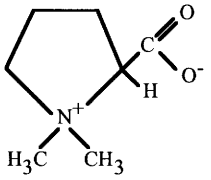
β -Alanine betaine



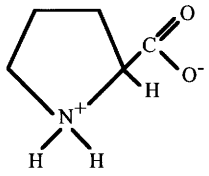
Carnitine



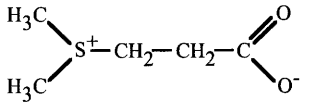
Ectoine



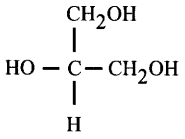
Proline betaine



Proline



Dimethylsulfoniopropinate (DMSP)



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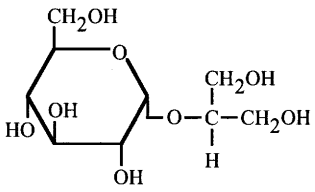
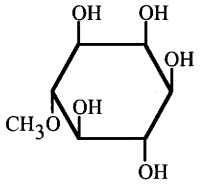
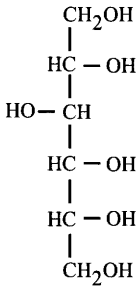


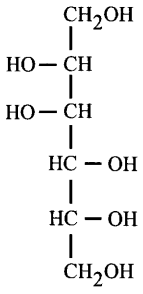
Glucosylglycerol



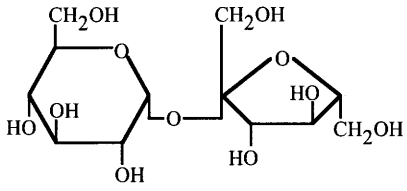
D-Ononitol



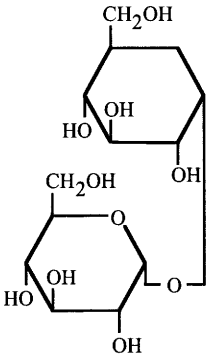
Sorbitol



Mannitol



Sucrose



Trehalose

Figure 1. Chemical structures of various compatible solutes that occur in higher plants, marine algae, bacteria and yeasts.

forms an effective barrier for most solutes present in the medium and metabolites present in the cytoplasm. Hyperosmotic stress causes a rapid efflux of water and loss of turgor; ultimately, the cells may plasmolyse (Kock, 1984), *i.e.,* the cytoplasmic membrane may retract from the cell wall. The degree of shrinkage and plasmolysis is different between Gram-negative and Gram-positive becteria. Cells respond to an upward shift in the osmotic pressure of the medium by expressing specific sets of genes including those involved in the biosynthesis or uptake of compatible solutes. Such solutes include K^+, amino acids (*e.g.,* glutamate and proline), amino acid derivatives (peptides and *N*-acetylated amino acids), quaternary amines (*e.g.,* glycine betaine and carnitine), sugars (*e.g.,* sucrose and trehalose) and tetrahydropyrimidines (ectoines) (Csonka, 1989; Galinski and Truper, 1994). It is worth to mention that the accumulation of compatible solutes in response to salt stress and osmotic stress is a common response to almost all living organisms from bacteria to higher plants.

Response of cells to salt stress and osmotic stress has been widely investigated; in bacteria, such as *Escherichia coli* and *Bacillus subtilis*, or yeasts, such as *Saccharomyces cerevisiae* which represent good models for prokaryotes or eukaryotes, respectively. For example, the initial response of bacteria, such as *Escherichia coli*, to an osmotic upshock involves the uptake of potassium via Kdp and TrkG or TrkH, the main transport systems for K^+ (Bakker, 1992; Schlosser *et al.*, 1995). To maintain electroneutrality, the accumulation of K^+ is accompanied by increases in the glutamate pool by *de novo* synthesis (Caylay *et al.*, 1991; McLaggan *et al.*, 1994).

The uptake of glycine betaine (hereafter GB, for short) and proline in enteric bacteria follows the uptake of potassium, provided these compatible solutes are present in the medium (Kempf and Bremer, 1998; Record et al., 1998). The uptake of these

compatible solutes is affected by various transport systems such as ProP and the ABC-type transport system ProU of *Escherichia coli* (Table I). These transporters were investigated in details and it seems that they are activated by different signals. For example upon salt upshock, the ProP protein is activated within seconds but its activation requires the presence of K^+ in the medium (Koo *et al.*, 1991). On the other hand, the activation of ProU is slower than that of ProP and takes several minutes after salt shock (Faatz et al., 1988). It has been also suggested that the activation of ProU also depends on the presence of substrate GB since the transport activity increases up to 3 min after salt upshock in the absence but not in the presence of GB (Faatz *et al.*, 1988).

However, the *bet* gene cluster is induced after salt shock in *Escherichia coli* cells when compatible solutes are not provided in the medium. The *bet* gene cluster is composed of four genes, namely *betABIT* (*i.e., betA* for choline dehydrogenase; *betB* for betaine aldehyde dehydrogenase; *betI* for putative regulatory protein of *betT*; *betT* for choline transport system). Thus, it appears that the enteric bacterium *Escherichia coli* has adopted two different strategies to survive under high-salinity conditions. The first strategy is the uptake of compatible solutes from the medium *via* the secondary transporter ProP or the ABC-type transporter ProU. The second is the biosynthesis of GB by BetAB proteins. The uptake of choline, which is the substrate for GB biosynthesis, is efficiently regulated by the BetIT system (Lamark et al., 1996; Rkenes et al., 1996).

When yeast cells are exposed to a hyperosmotic shock, cytoplasmic water is effluxed. Then several mechanisms are initiated to counteract the dehydration of cells and to protect the cellular structures. A MAP-kinase pathway has been involved in the transduction of the signal that is generated by an increase in osmolarity (reviewed in Toone and Jones, 1998). Among the yeast responses to an increase in external osmolarity,

Table I. Properties of compatible solute transport systems. Taken and modified from Kempf and Bremer (1998).

Organism	Gene	Type of transporter	Components	Substrates
Escherichia coli	ProP	Secondary transporter	ProP	GB, PB, Pro, Ect, PIP, DMP, DMG, DMSP, HB, BB
Escherichia coli	ProU	ABC transporter(1)	ProV, ProW, ProX	GB, PB, Pro, Car, DMP, HB, BB, Cho
Escherichia coli	BetT	Secondary transporter	BetT	Cho
Corynebacterium glutamicum	BetP	Secondary transporter	BetP	GB
Bacillus subtilis	OpuA	ABC transporter	OpuAA, OpuAB, OpuAC	GB, PB, DMSA, DMSP, Cho
Bacillus subtilis	OpuB	ABC transporter	OpuBA, OpuBB, OpuBC, OpuBD	Cho
Bacillus subtilis	OpuC	ABC transporter	OpuCA, OpuCB, OpuCC, OpuCD	GB, PB, Car, DMSA, DMSP, Cho, BB, CB, Ect, Choline-O-sulfate
Bacillus subtilis	OpuD	Secondary transporter	OpuD	GB, DMSA, DMSP, Pro
Bacillus subtilis	OpuE	Secondary transporter	OpuE	Pro
Erwinia chrysanthemi	OusA	Secondary transporter	OusA	GB, Pro, Ect, PIP

(1) ABC transporter, ATP-binding cassette transporter.

Abbreviations: BB, γ-butyrobetaine; Car, carnitine; CB, crotonobetaine; Cho, choline; GB, glycinebetaine; DMG, dimethylglycine; DMSA, dimethylsulfonioacetate; DMSP, dimethylsulfoniopropinate; Ect, ectoine; HB, homobetaine; PB, proline betaine; PIP, L-pipecolate.

accumulation of glycerol deserves special attention (reviewed in Hohmann, 1997). Glycerol accumulation is important for cell survival, since all mutants lacking glycerol accumulation are highly sensitive to high osmolarity.

It is worth to mention that, up to date, many research groups used a confusing terminology such as *osmoprotectants* instead of compatible solutes and *osmotic stress* when the stressing agent is NaCl. However, it has been already demonstrated that the accumulation of compatible solutes occurs not only in response to an increase in the osmolarity of the medium but also in response to high temperature, drought, etc. Thus, it became evident that compatible solutes are not exclusively involved in osmoregulation but also they have other functions. Moreover, the terminology used for osmotic stress is confusing because it does not descriminate between salt stress (ionic and osmotic effect), and a pure osmotic stress. Kanesaki et al. (2002) have demonstrated by using DNA microarrays that *Synechocystis* cells recognize salt stress and osmotic stress as different stimuli. Both types of stress induced different sets of genes after exposure of cells for 30 minutes to either 500 mM NaCl or 500 mM sorbitol. Detailed analysis of the differential effects of salt stress and osmotic stress on photosynthetic activities of *Synechococcus* sp. PCC 7942 cells demonstrated that both types of stress act differently to impair photosynthetic machineries (Allakhverdiev et al., 2000a, b).

When NaCl is the stressing agent, salt ions (Na^+ and Cl^-) penetrate inside the cells and disturb the metabolic functions by interacting directly with various macromolecules (such as proteins, DNA, mRNAs). However, osmotic stress is a pure physical process which increases the osmotic pressure in the medium and mainly induces an efflux of water, thus leading to dehydration of the cytosol. In the case of osmotic stress there is no direct interaction between the stressing agent and the macromolecules because

the stressing agent is not uptaken by the cell. This terminology is very important and will be carefully considered in this paper. Stress due to NaCl or other salts will be collectively called "salt stress". The term "osmotic stress" will be used when the stressing agent is sorbitol.

Many compatible solutes, such as GB, proved to be effective stabilizers of enzymes, providing protection not only against high salt but also against high temperature, cold, high light, freeze-thawing and drying (Yancey *et al.*, 1982, Deshnium et al., 1995, 1997; Hayashi et al., 1997; Alia et al., 1998; Sakamoto et al., 1998). Solutes that are non-charged or zwitterionic are generally more favourable to protein stability than ionic solutes. In most eubacterial species GB is the preferred compatible solute and generally provides the highest level of protection, which may reflect among others its favourable interaction with macromolecules.

1.3. Cell signaling in response to several types of abiotic stress

The cellular and molecular responses of organisms to environmental stress have been studied intensively (Thomashow, 1999; Hasegawa et al., 2000; Suzuki et al., 2001; Mikami et al., 2002). Understanding the mechanisms by which cells perceive environmental signals and transmit the signals to cellular machinery to activate acclimative responses is of fundamental importance to applied biology of breeding and transgenic strategies to improve stress tolerance in plants.

1.3.1. Stress signal transduction pathways

A signal transduction pathway starts with signal perception which is followed by the generation of the second messenger. This response often initiates a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors that control the expression of specific sets of stress-regulated genes. The products of these genes may participate in the generation of regulatory molecules like the plant hormones, such as abscisic acid, or might contribute to synthesize compatible solutes, such as GB, glucosylglycerol. One of the most well studied transduction pathways is that of yeast cells, namely the high-osmolarity glycerol mitogen-activated protein kinase pathway (HOG pathway for short) (For review, see Hohmann, 1997, 2002; O'Rourke et al., 2002).

The signal transduction requires the proper spatial and temporal coordination of all signaling molecules. Thus, there are certain molecules that participate in the modification, delivery, or assembly of signaling components, but do not directly relay the signal. They are also critical for the accurate transmission of stress signals.

1.3.2. Multiplicity of abiotic stresses and the need for multiple sensors

Abiotic stresses, such as low temperature, drought, and high salinity are very complex stimuli, each of which may provide cells with quite different signal. For example, low temperature may immediately result in mechanical constraints and changes the fluidity of membranes. High salinity includes both an ionic (chemical) and an osmotic (physical) component. The multiplicity of information embedded in abiotic stress signals underlies

one aspect of the complexity of stress signaling.

On the basis of this multiplicity, it is unlikely that there is only one sensor that perceives the stress conditions and controls all subsequent signals. Rather, a single sensor might only regulate branches of the signaling cascade that are initiated by one aspect of the stress conditions. For example, low temperature is known to change membrane fluidity (Murata and Los, 1997). A sensor which detects this change would initiate a signaling cascade responsive to membrane fluidity but would not necessarily control the signaling which is initiated by an intracellular protein whose conformation/activity is directly altered by low temperature. Thus, there may be multiple primary sensors that perceive the initial stress signal.

In our group we are studying these signaling pathways in response to several kinds of abiotic stress such as salt stress (due to NaCl, KCl, LiCl and Na_2SO_4), osmotic stress (due to sorbitol and mannitol), cold stress, high-temperature stress, oxidative stress (due to H_2O_2 and methyl viologen), and high-light stress. The whole genome sequence of the cyanobacterium *Synechocystis* sp. PCC 6803 was already determined (Kaneko et al., 1996). In the genome of this organism, 43 histidine kinases have been identified. A histidine kinase, Hik33, appears to be a sensor which perceives decreases in temperature and regulates the expression of certain cold-inducible genes in *Synechocystis* (Suzuki et al., 2001). Recent results indicated that the cold sensor Hik33 also acts as a sensor of osmotic stress in *Synechocystis* but that the mechanisms of its involvement in the sensing of cold stress and osmotic stress are not identical (Mikami et al., 2002). We also identified a membrane-bound histidine kinase as a possible sensor and a response regulator of Mn^{2+} ions that together regulate the expression of the *mntCAB* operon for an ABC-type translocater of Mn^{2+} ions (Yamaguchi et al., 2002). Another putative high-temperature

stress sensor, salt-stress sensor and osmotic-stress sensors were also found (Data not published yet). So it appears that histidine kinases in *Synechocystis* are involved in sensing various kinds of abiotic stress.

1.3.3. Potential sensors for abiotic stress signals

During the last few years the whole genome sequences of several organisms were accomplished. After great advances of genomics and proteomics the attention in the research field was shifted to look for the whole network that is working to let organisms sense, respond and acclimate to particular stress condition. Given the multiplicity of stress signals, the presence of many stress sensors is expected. Although sensors of drought or salinity stress have not been yet found, a membrane-bound sensor for low-temperature perception could be a two-component histidine kinase. Evidence suggests that the histidine kinase Hik33 in *Synechocystis* (Suzuki et al., 2001) and the histidine kinase DesK in *Bacillus subtilis* (Aguilar et al., 2001) are thermosensors that regulate the expression of genes for desaturases in response to temperature downshifts. In the genome of *Arabidopsis thaliana,* several putative two-component histidine kinases have been identified (Urao et al., 2000), although no evidence has been reported for any of these histidine kinases as thermosensors.

Another well-documented case of an osmoregulated system that senses a specific molecule is the membrane-bound sensor kinase KdpD. Consistent with the suggestion of ionic strength as a major stimulus, the autophosphorylation activity of KdpD increases up to at least 300 mM NaCl. In the wild-type system, this activity is specifically opposed by K^+ ions, whereas NaCl and KCl stimulate autophosphorylation activity of KdpD to the

same extent in a mutant defective in K^+ sensing (Jung et al., 1997).

1.4. Genetic engineering of compatible solutes in higher plants

Since plants are sessile organisms, their geographical distribution is very much influenced by the climate. It is always hard to find plants in the areas of extreme environments (*i.e.,* very hot and dry such as deserts or very cold such as the Tarctic and Antarctic areas). However, in such areas with extreme climates you might still find a large number of species of microorganisms. For example, thermophilic becteria in hot springs or cyanobacterial strains in the Antarctic continent. With the increasing population of our planet and with the increasing surface of deserts (such as Sahara Desert) due to desertification there is a great need to genitically engeneer crop plants of great nutritional value (such as rice, wheat and barley). Such genetic tools (*i.e.,* genes of interest) are mostly found in microorganisms which are adapted to extreme environments. These genes are, in part, those which are related to the biosynthesis of compatible solutes.

In plants, compatible solutes are usually accumulated in response to stress and they are largely confined to the cytoplasm (including organelles) and are almost absent in the vacuole, which generally occupies about 90% of the cell volume. For example, the halophyte *Atriplex gmelini* was found to have 320 mM GB in the cytoplasm, but only 0.24 mM in the vacuole (Matoh et al., 1987). Isolated chloroplasts contained high concentrations of GB, in particular when isolated from salt-stressed plants. Certain plants, such as spinach, accumulate significant amounts of GB in response to high salinity, cold and drought. GB occurs in diverse marine algae and at least 10 flowering plant families, including Chenopodiaceae, Amaranthaceae, Gramineae, Compositae, and Malvaceae

(Blunden and Gordon, 1986; Rhodes and Hanson, 1993). The physicochemical basis for this striking protective effect is not yet fully understood.

Plants represent a good tool to study the effectiveness of genes from bacterial origins. In fact, many genes that encode enzymes for biosynthesis of compatible solutes have been introduced to plants which are "nonaccumulators" of compatible solutes tempting to enhance their stress tolerance. Several pioneer groups have taken the first step toward this goal by expressing choline-oxidizing enzymes from bacteria (Hayashi et al., 1997; Alia et al., 1998, 1999; Sakamoto et al., 1998) or spinach CMO (Nuccio et al., 1998) in tobacco and other plants that do not contain GB (Table II). The transgenic plants produced a low level of GB and, in most cases, showed significant enhancement of tolerance to various stresses (for review, see McNeil et al., 1999, Sakamoto and Murata, 2000, 2001, 2002; Chen and Murata, 2002). However, the GB levels obtained to date in transgenic plants (typically about 0.1-1 μmol g^{-1} fresh weight) are only a small percentage of those in spinach, sugar beet, and other plants that are natural accumulators of GB. The main constraint on GB production in transgenic plants appears to be the endogenous choline supply, because providing choline exogenously leads to a massive increase in GB synthesis (Nuccio et al., 1998). Therefore, it will be necessary to up-regulate the *de novo* synthesis of choline in order to increase the activity of GB synthesis in nonaccumulators that express foreign genes for choline-oxidizing enzymes (Nuccio et al., 1998).

We will summarize and discuss below the contributions brought by genetic engeneering of compatible solutes in higher plants in terms of stress tolerance and also propose several hypotheses for the protective roles of compatible solutes.

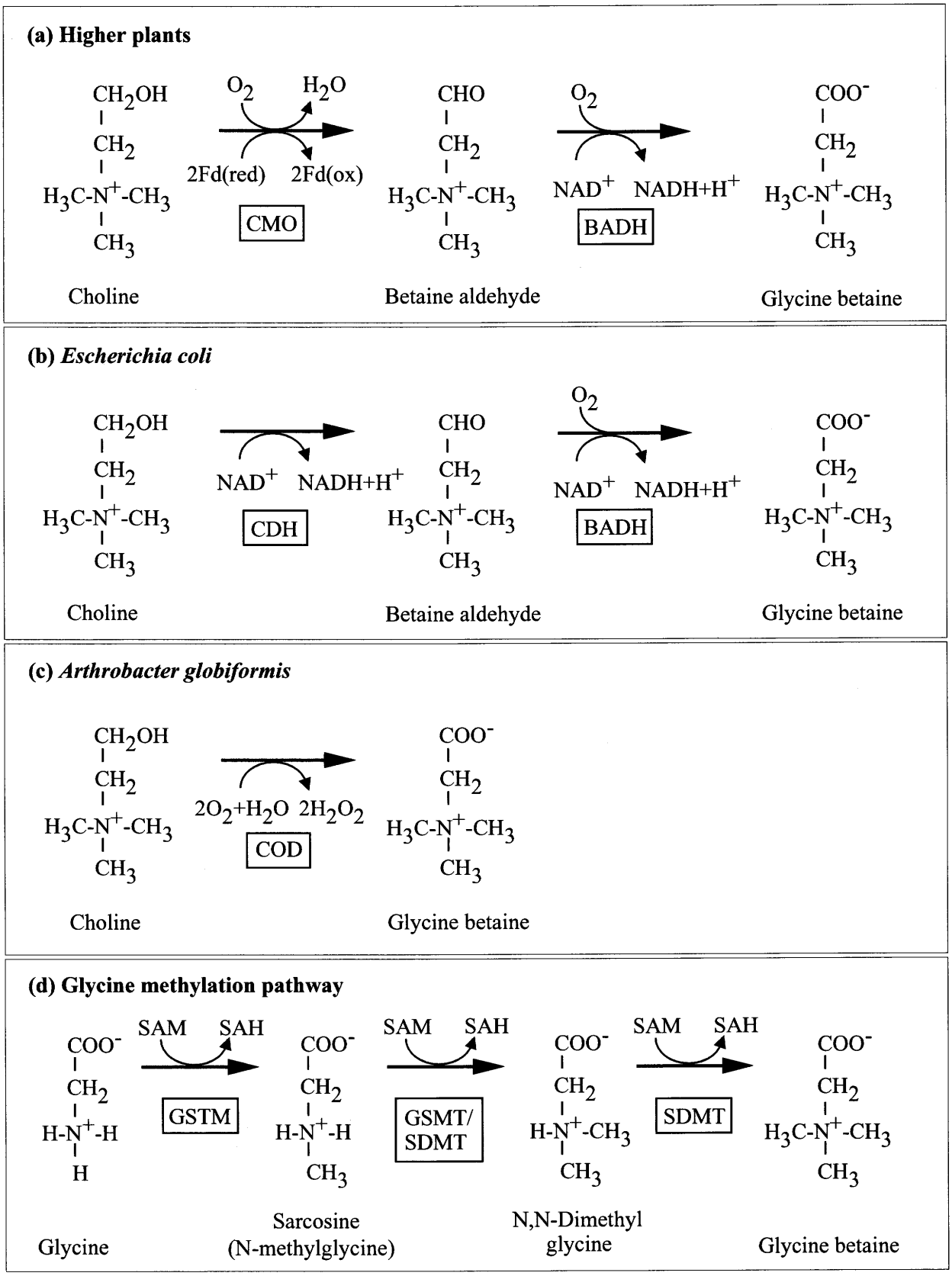
Table II. Transgenic plants engineered to synthesize glycine betaine and the enhanced tolerance to particular forms of abiotic stress. This table summarizes almost all the transgenic plants that have been engeneered to synthesize glycinebetaine. It also indicates for each transgenic line the enhanced tolerance to abiotic stress. Taken and modified from Chen and Murata (2002).

Species	Gene	Maximal accumulation	Enhanced tolerance	Reference
Arabidopsis thaliana	*codA*	1.2 μmol g^{-1} fw	Chilling, salt	Hayashi et al (1997)
	codA	1.2 μmol g^{-1} fw	Chilling	Alia et al (1998)
	codA	1.2 μmol g^{-1} fw	Heat	Alia et al (1998)
	codA	1.2 μmol g^{-1} fw	Strong light	Alia et al (1999)
	codA	1.2 μmol g^{-1} fw	Salt	Hayashi et al (1998)
	codA	1.2 μmol g^{-1} fw	Freezing	Sakamoto et al (2000)
	cox	19 μmol g^{-1} dw	Freezing, salt	Huang et al (2000)
Brassica napus	*cox*	13 μmol g^{-1} dw	Drought, salt	Huang et al (2000)
Brassica juncea	*codA*	0.82 μmol g^{-1} fw	Salt	Prasad et al (2000)
Diospyros kaki	*codA*	0.3 μmol g^{-1} fw	Salt	Gao et al (2000)
Nicotiana tabacum	*cox*	13 μmol g^{-1} dw	Salt	Huang et al (2000)
	betA/betB	0.035 μmol g^{-1} fw	Chilling, salt	Holmstrom et al (2000)
Oryza sativa, japonica	*codA*	5.3 μmol g^{-1} fw	Chilling, salt	Sakamoto et al (1998)
	betA (modified)	5.0 μmol g^{-1} fw	Drought, salt	Takabe te al (1998)
Oryza sativa, indica	*codA*	2.12 μmol g^{-1} dw	Salt	Mohanty et al (2002)

1.4.1. Expression of choline oxidase (COD) from *Arthrobacter globiformis* in *Arabidopsis thaliana*

The *codA* gene for choline oxidase (COD) was first cloned from *Arthrobacter globiformis* (for review, see Deshnium et al., 1995). This gene was introduced to the cyanobacterium *Synechococcus* sp. PCC 7942. The transgenic cells of *Synechococcus*, namely PAMCOD cells, became more tolerant than control cells (PAM) to salt stress (Deshnium et al., 1995), and cold stress (Deshnium et al., 1997). These results encouraged the researchers to use the *codA* gene to transform *Arabidopsis thaliana*, a model organism of non-accumulators of GB, to enhance its stress tolerance. The major advantage of using COD, as distinct from CMO/BADH (for choline monooxygenase and betaine aldehyde dehydrogenase, respectively) in spinach, or CDH/BADH (for choline dehydrogenase and betaine aldehyde dehydrogenase, respectively) in *Escherichia coli*, as a tool for engineering the synthesis of GB is that the introduction of only a single gene (*codA*) for this enzyme is sufficient for the conversion of choline to GB in transgenic plants (Fig. 2). *Arabidopsis thaliana* was transformed with a modified *codA* gene that encoded COD with a signal for targeting to chloroplasts (Hayashi *et al.*, 1997). The transformation resulted in the accumulation of GB in leaves at 1.0 μmol g^{-1} FW. Based on the assumption that GB is localized exclusively in the chloroplasts, its concentration was estimated to be about 50 mM (Hayashi *et al.*, 1997). Seeds of the transgenic plant also accumulated GB at 12-18 μmol g^{-1} DW (Hayashi *et al.*, 1998). These experiments provided the first example of genetically engineered plants with the ability to synthesize GB at appreciable levels.

Transgenic strains were more tolerant to salt stress than wild-type strains during germination of seeds (up to 300 mM NaCl) and during the early and late stages of



Chen and Murata (2002). *Current Opinion in Plant Biology*. 5: 250-257

Figure 2. Choline-dehydrogenation/oxidation and glycine methylation pathways. Pathways of choline dehydrogenation/oxidation in higher plants (a); in the enteric baterium *Escherichia coli* (b); and in the soil bacterium *Arthrobacter globiformis* (c). The pathway of glycine methylation in *Actinopolyspora halophila* and *Ectothiorhodospira halochloris* is shown in (d).

development of plants (up to 200 mM NaCl; Hayashi *et al.*, 1997, 1998). In addition, transgenic *Arabidopsis* exhibited significantly enhanced tolerance to both low- and high-temperature stresses (Hayashi *et al.*, 1997; Alia *et al.*, 1998, 1999). Seeds of transgenic plants remained able to germinate after the exposure to extreme temperatures, such as 0°C and 50°C, during imbibition. Germination and the subsequent growth of seedlings of transgenic plants were more rapid than those of wild-type *Arabidopsis* at extreme temperatures (Alia *et al.*, 1998, 1999). Transgenic plants were also visibly less damaged than wild-type plants after exposure to low temperature in the light (Hayashi *et al.*, 1997). The degree of tolerance was correlated, in every case, with the level of GB in transgenic *Arabidopsis*. Enhanced tolerance was also evident at the cellular level. The activity of PSII was maintained at a higher level in transgenic plants than in wild-type plants under several kinds of abiotic stress (Hayashi *et al.*, 1997; Alia *et al.*, 1999). Enhanced tolerance of the photosynthetic machinery was the result of accelerated recovery of the PSII complex from photo-induced inactivation (Alia *et al.*, 1999).

The possible side effects of the introduction of the gene for COD were examined since the enzyme produces H_2O_2 as a by-product of catalysis (Alia *et al.*, 1999 and Fig. 2c). Leaves of transgenic *Arabidopsis* that expressed COD had elevated levels of hydrogen peroxide (Alia *et al.*, 1999). Moreover, the activities of scavenging enzymes, namely ascorbate peroxidase, and to a lesser extent, catalase, were significantly higher in transgenic plants than in wild-type plants (Alia *et al.*, 1999). These observations suggest that the hydrogen peroxide generated by choline oxidase might have stimulated the expression of scavenging enzymes with the resultant maintenance of intracellular levels of hydrogen peroxide within a certain limited range.

In summary, the accumulation of GB in transgenic *Arabidopsis* as a result of expression of the *codA* gene for COD from *Arthrobacter globiformis* enhanced tolerance to salt, cold, heat, and high-intensity light stress. Enhancement of tolerance was not confined to specific stages of development but was recognized throughout the course of various developmental stages that included the imbibition and germination of seeds, as well as the early and later stages of vegetative growth.

1.4.2. Expression of choline oxidase (COD) from *Arthrobacter globiformis* in *Oryza sativa* (rice)

Transgenic rice plants were generated similarly to the transgenic *Arabidopsis* plants described above with the *codA* gene for COD which was targeted either to the chloroplast or to the cytosol. In the former case, GB accumulated at about 1 μmol g^{-1} FW in leaves; in the latter plants, it accumulated at about 5 μmol g^{-1} FW (Sakamoto *et al.*, 1998). Transgenic rice plants with COD targeted either to chloroplasts or to the cytosol exhibited enhanced tolerance to salt- or cold-induced photoinhibition. Moreover, the photosynthetic machinery was more efficiently protected when COD was targeted to the chloroplasts than to the cytosol of transgenic *Oryza sativa, japonica* (Sakamoto *et al.*, 1998) and *Oryza sativa, indica* (Mohanty et al., 2002). This observation indicates that the subcellular site of GB synthesis might be important in efforts to improve the stress tolerance of plants.

1.4.3. Transgenic plants engineered to produce compatible solutes other than glycine betaine

In addition to plants that have been engineered to synthesize GB, transgenic plants that accumulate other compatible solutes have also been produced. The resultant transgenic plants and their phenotypes are summarized in table III (for review, see Nuccio et al., 1999; Chen and Murata, 2002 and references therein).

1.5. Mechanisms of protection by compatible solutes

With the exception of the transgenic plants that accumulated proline and D-Ononitol, most of the transgenic plants that we have discussed contained only low levels of the compatible solute of interest, and these concentrations were insufficient to be of osmotic importance. Nevertheless, moderate to high levels of stress tolerance were reported for these transgenics. In addition, to our knowledge none of the transgenic plants gained tolerance to osmotic stress. Therefore, protective mechanisms other than direct osmoprotection were probably responsible for the increased tolerance of stress. Possible roles for GB in stress tolerance include stabilization by GB of complex proteins and membranes *in vivo*, protection by GB of the transcriptional and translational machinery, and intervention by GB as a molecular chaperone in the refolding of enzymes (Sakamoto and Murata, 2001). In addition, GB might reduce the peroxidation of membrane lipids (Chen et al., 2000) and might protect electron transport via complex II in mitochondria (Hamilton and Heckathorn, 2001).

Table III. Transgenic plants engineered to produce compatible solutes other than glycine betaine for enhanced tolerance to stress. From Chen and Murata (2002).

Compatible solute	Gene	Host plant	Max accumulation	Enhanced tolerance	Reference(s)
Fructan	*SacB*	Tobacco	0.35 mg g^{-1} fw	Drought	Pilon-Smits et al (1995)
	SacB	Sugar beet	0.5%dw	Drought	Pilon-Smits et al (1999)
Mannitol	*mt1D*	*Arabidopsis*	10 μmol g^{-1} fw	Salt	Thomas et al (1995)
	mt1D	Tobacco	6 μmol g^{-1} fw	Salt	Tarkzinski et al (1992, 1993)
	mt1D	Tobacco	3.8 mg g^{-1} dw	Salt	Karakas et al (1997)
	mt1D	Tobacco	7 μmol g^{-1} fw	Oxidative stress	Shen et al (1997)
D-Ononitol	*imt1*	Tobacco	35 μmol g^{-1} fw	Drought, salt	Sheveleva et al (1997)
Proline	*Anti-ProDH*	*Arabidopsis*	600 μmol g^{-1} fw	Freezing, salt	Nanjo et al (1999)
	P5CF127A	Tobacco	4 mg g^{-1} fw	Salt	Hong et al (2000)
Sorbitol	*S6PDH*	Persimmon	61.5 μmol g^{-1} fw	Salt	Gao et al (2001)
Trehalose	*TPS1*	Tobacco	32 mg g^{-1} dw	Drought	Holmstrom et al (1996)
	TPS1	Potato	?*	Drought	Yeo et al (2000)
	TPS1	Tobacco	?*	Drought	Zhao et al (2000)
	otsA, otsB	Tobacco	90 μg g^{-1} fw	Drought	Pilon-Smits et al (1998)

*No data were given.

Abbreviations: Anti-*ProDH*, antisense form of cDNA for proline dehydrogenase from *Arabidopsis;*

(Table III continued)

imt1, gene for myo-inositol *O*-methyltransferase from ice plant; *mt1D,* gene for mannitol-1-phosphate dehydrogenase from *E. coli; otsA and otsB*, genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively, from *E. coli*; *P5CS*, cDNA for D1-pyrroline-5-carboxylate synthetase from *Vigna aconitifolia*; *P5CF127A*, gene for a mutated form of D1-pyrroline-5-carboxylate synthetase from *V. aconitifolia*; *SacB,* gene for levansucrase from *B. subtilis; S6PDH*, gene for sorbitol-6-phosphate dehydrogenase from apple; *TPS1*, gene for trehalose-6-phosphate synthase from yeast.

The role of reactive oxygen species (ROS) in inducing damage to plants under stress conditions and the role of compatible solutes in the protection of plants against stress have been discussed by many authors. It was suggested that ROS, which are synthesized by plants experiencing various stress conditions would directly damage cellular components, such as membrane lipids and the photosystem II complex (Smirnoff, 1993). It was also suggetsed that ROS would be scavenged by compatible solutes, resulting in the protection of plants against stress conditions. Furthermore, some compatible solutes, such as mannitol, proline and sorbitol, but not GB, have been shown to be effective scavengers of ROS *in vitro* (Smirnoff and Cumbes, 1989). Transgenic plants that synthesize compatible solutes, with the exception of GB, exhibit enhanced tolerance to oxidative stress (Shen et al., 1997; Hong et al., 2000). However, these suggestions have not been evidenced *in vivo*.

There is, however, a plausible scheme that might better explain the results described above. The fates of cellular components under stress conditions depend on the balance between rate of damage and rate of repair. When the rate of repair of a given component is more rapid than the rate of damage, no damage becomes apparent. This situation is probably normal in plants under non-stress conditions. As the level of stress increases, the balance tends gradually toward damage. When the rate of repair is slower than the rate of damage, the latter becomes apparent. This is probably the situation in plants under stress. Such a relationship between damage and repair has been clearly demonstrated in the photoinhibition of photosystem II (PSII) complexes (Nishiyama et al., 2001). Nishiyama and collaborators provided evidence that increased intracellular concentrations of ROS inhibit repair of damaged PSII. Therefore, various kinds of stress increase the levels of ROS which might inhibits repair processes, which are linked (for the

most part) to protein synthesis. In cyanobacterial cells, a model system for studies of chloroplasts, H_2O_2 inhibits protein synthesis, which is essential for the repair of the photodamaged PS II (Nishiyama et al., 2001). It is possible that compatible solutes protect the protein-synthesizing machinery against various kinds of stress thereby maintaining conditions under which repair processes occur more rapidly than damaging processes.

1.6. Compatible solutes in cyanobacteria

Cyanobacteria represent an ancient group of eubacteria in which oxygen-evolving photosynthesis is believed to have arisen. These photoautotrophic bacteria are found in almost all habitats, including waters that contain salt at very low to saturating concentrations. The salt-induced accumulation of compatible solutes in these organisms has been investigated with more than 100 different strains (Mackay et al., 1984; Reed et al., 1984, 1986; Reed and Stewart, 1985). This led to classification of cyanobacteria in three main groups on the basis of their salt tolerance characterstics and dominant compatible solutes:(i) Strains that tolerate the lowest levels of salt accumulate the disaccharides sucrose and trehalose, such as *Synechococcus* sp. PCC 7942 and *Synechococcus* sp. PCC 6307. (ii) Moderately halotolerant strains synthesize the heterozide GG, such as *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002. (iii) Halophilic strains synthesizing the quaternary ammonium compounds GB and glutamate betaine such as *Galothrix* sp. PCC 7426 and *Synechococcus* sp. PCC 7418 (Mackay et al., 1984 and Table IV).

Table IV. Major compatible solutes of cyanobacteria. Taken and modified from Mackay et al (1984).

Taxonomic assignement *	Strain		Compatible solutes accumulated	Strain origin	Maximum tolerance (g NaCl l^{-1})
	ACMM	PCC			
Anabaena (IV)	N115	7122	Sucrose	Pond water	14.3
Synechococcus (I)	N158	6307	Sucrose	Lake water	23.8
Calothrix (IV)	N103	7601	Sucrose, trehalose	Unknown	24.7
Synechocystis (I)	N104	6701	Sucrose	Fresh water	28.5
Synechococcus (I)	N102	6301	Sucrose	Fresh water	33.8
Synechocystis (I)	N110	6308	Sucrose	Fresh water	33.8
Synechocystis		6803	Glucosylglycerol, sucrose	Fresh water	70.1
Synechococcus (I)	N111	7202	Glucosylglycerol	Alkaline pond	70.4
Synechocystis (I)	N167	6906	Glucosylglycerol	Hypersaline lake	70.4
Synechocystis (I)	N106	6714	Glucosylglycerol	Fresh water	80.2
Synechococcus (I)	N166	7002	Glucosylglycerol	Marine mud	100.1
Myxosarcina (II)	N108	7312	Glucosylglycerol	Supralittoral	104.8
Gloeocapsa (I)	N107		Trehalose, glycine betaine	Salt lake	130.4
Spirulina (III)	439		Glycine betaine	Salt lake	≥ 150.7
Synechococcus (I)	N161		glycine betaine	Salt works	≥ 157.6
Synechococcus (I)	N163		glycine betaine	Salt lake	≥ 157.6
Synechococcus (I) Φ	351		glycine betaine Σ	Hypersaline pond	210-240

* Cyanobacteria have been traditionally assigned to the algae. Because cultures are not recognized

(Table IV continued)

as valid type materials under the Botanical Code, Rippka *et al.* (1979) redefined certain cyanobacterial genera so that simple, clear-cut generic assignments could be made for cultures. Differences in structure and development of genera allow recognition of five sub-groups or sections of cyanobacteria. These sections do not precisely correspond to ordinal groups of phycologists, but do represent the broadest taxonomic sub-groups recognized for cyanobacterial cultures. We have used the classification scheme of Rippka *et al.* (1979) and show generic and sectional (in parentheses) assignments of strains. ψ Culture collections from which strains were acquired are as follows: ACMM, Australian Collection of Marine Microorganisms, Sir George Fisher Center for Tropical Marine Studies, James Cook University, Queensland 4811, Australia; PCC, Pasteur Culture Collection, Institute Pasteur, 28 Rue du Docteur Roux, Paris 75015, France. <> Strains are arranged in order of increasing salinity tolerance, measured as *maximum* concentration of NaCl ($g\ l^{-1}$) permitting growth. Φ The solute contents of these were tested at one salinity only. ❦ Strains tested using media solidified with 7 g Difco Bacto agar l^{-1}, grow at 38-41°C and 18-24 $\mu E\ m^{-2}s^{-1}$. Strains grew at the lower salinity shown, but failed to grow at the higher salinity. Σ Low concentrtion of glucosylglycerol present (see Mackay et al., 1984)

During the evolution of the modern biosphere, photosynthetic processes in cyanobacteria have a central role by elevating the oxygen level in the Earth's atmosphere about 3.5 billion years ago (Pfenning, 1978; Nisbet and Sleep, 2001). Compared with other eubacteria, a unique feature of cyanobacteria, the largest group of oxygenic photosynthetic prokaryotic organisms, is the presence of a differentiated membrane system. Similar to other Gram-negative bacteria, cyanobacteria have an envelope layer consisting of an outer membrane, a peptidoglycan layer, and a plasma membrane (Stanier and Cohen-Bazire, 1977; Gantt, 1994). In addition, these organisms have a distinct intracellular membrane system, the thylakoids, which are the sites for both oxygenic photosynthesis and respiration (Stanier and Cohen-Bazire, 1977; Gantt, 1994). Cyanobacteria are the progenitors of chloroplasts in green plants (Gray, 1989).

1.7. Aim of the present research

The accumulation of compatible solutes, such as betaines, proline and sugar alcohols, is a widespread response that may protect plants against environmental stress. Although a number of hypothesis have been suggested to explain the role of compatible solutes, it is not yet fully understood how these compounds are involved in the stress tolerance of whole plants. Some plants have been genetically engineered to express enzymes that catalyze the synthesis of various compatible solutes. Some interventions have increased the tolerance of some crop plants to abiotic stress. Furthermore, analysis of such transgenic plants has begun to clarify the roles of compatible solutes in stress tolerance.

The reasons for using *Synechocystis* sp. PCC 6803 (Fig. 3) are: (i) Its entire genome (some 3.6 million base pairs) has been sequenced; (ii) It is spontaneously and

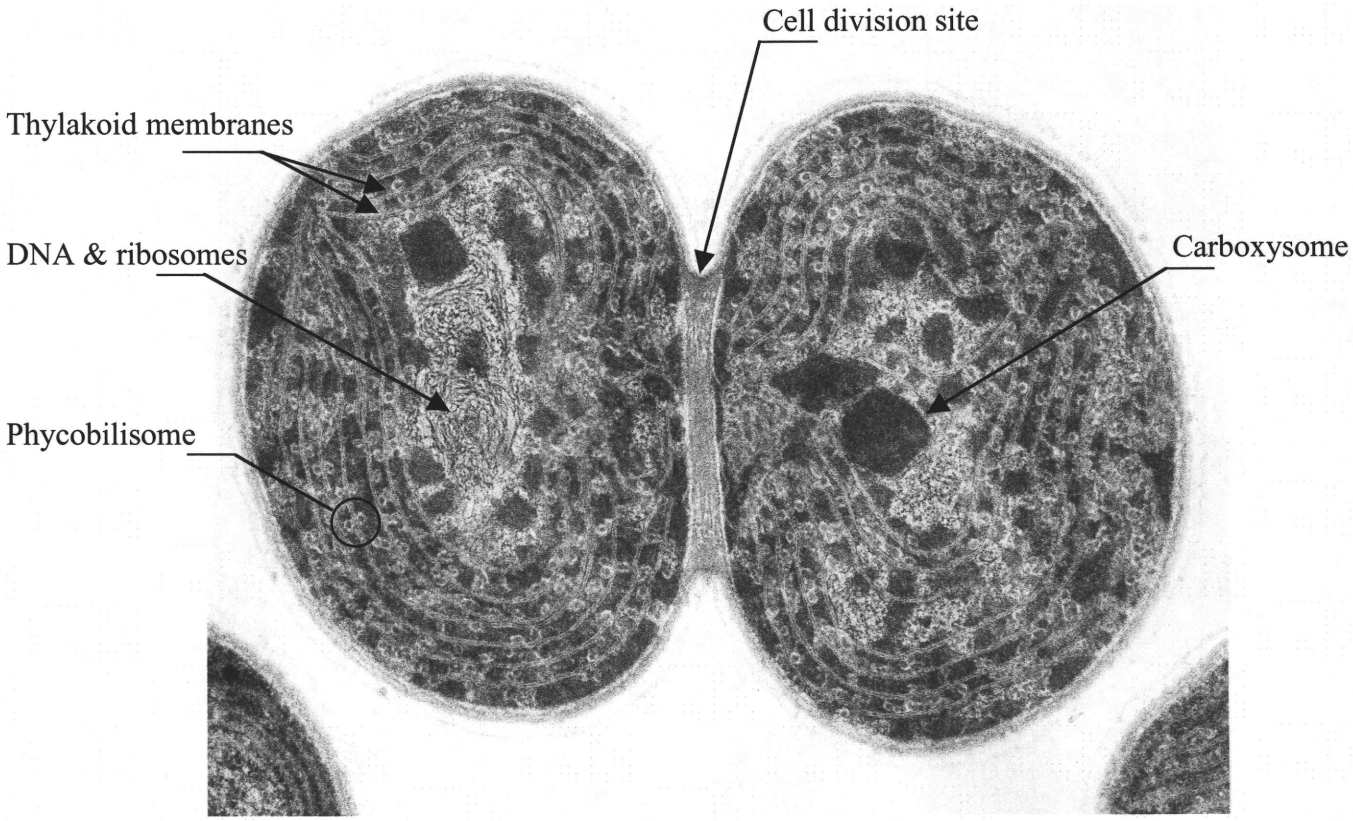


Figure 3. Electronmicrograph of the cyanobacterium *Synechocystis* sp. PCC 6803 during cell division. Cells were grown in BG-11 medium at 34°C, bubbled with air containing 1% CO_2 and under constant illumination at 70 μE m^{-2} s^{-1} (Courtesy of Dr. Toku Kanaseki).

easily transformable (*i.e.*, it takes up DNA by itself), and it can integrate DNA into its genome by homologous recombination; (iii) Its photosynthetic system is essentially identical to that of plants so that it is considered as agood model organism for higher plants evolving oxygenic photosynthesis. The cyanobacterium *Synechocystis* sp. PCC 6803 accumulates GG and sucrose as compatible solutes under salt stress. While the molecular mechanisms for GG synthesis including regulation of the *ggpS* gene, which encodes glucosylglycerol phosphate synthase, has been intensively investigated, the role of GG in protection against salt stress remains poorly understood. In our study we aimed to identify the protective mechanism by which GG molecules protect *Synechocystis* against salt stress. Thus *Synechocystis* is the model organism of choice for our purpose.

Chapter 2

Materials and methods

2.1. Growth conditions and salt stress

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was kindly provided by Dr. J. G. K. Williams (Du Pont de Nemours & Co., Inc., Wilmington, DE, USA). The mutant deficient in GG biosynthesis of this strain (*i.e.*, Δ*ggpS*) was produced in our laboratory as previously (Marin et al., 1998). The cells of wild type and Δ*ggpS* mutant were cultured at 34°C in BG-11 medium (Stanier et al., 1971) which had been buffered with 20 mM Hepes-NaOH (pH 7.6). This buffered BG-11 medium contained 20 mM Na^+ ions. Cell cultures were bubbled with air containing 1% CO_2 and under constant illumination at 70 μE m^{-2} s^{-1} from incandescent lamps (Ono and Murata, 1981).

Salt stress and osmotic stress were applied by adding an appropriate volume of a 5 M stock solutions of NaCl or sorbitol to cultures to give the desired final concentration. Compatible solutes were added exogenously to the cultures to a final concentrtion of 1 mM. Growth of cells was monitored by measuring changes in optical density at 730 nm using a spectrophotometer (model 200-20; HITACHI, Tokyo, Japan) after suitable dilution of aliquots from cell cultures.

2.2. Optical and electron microscopy

Optical microscopy was performed with a microscope (Axioskop FL; Carl Zeiss, Gottingen, Germany) that was equipped with a high-definition image-capture camera (model HC-1000; FUJIX, Tokyo, Japan).

For electron microscopy, cells were pelleted by centrifugation at 3,000x*g* for 5 min

and then immediately fixed for 1 h with 2% glutaraldehyde in 100 mM sodium phosphate (pH 7.2). After rinsing overnight in sodium phosphate buffer, samples were post-fixed in 1% osmium tetroxide for 1 h prior to dehydration by passage through a graded ethanol series (50% to 100%). Then samples were infiltrated with and embedded in resin (Araldite CY-212; OUKEN, Tokyo, Japan). Thin sections were mounted on copper grids, stained with uranyl acetate and examined under an electron microscope (model 1200EX; JEOL, Tokyo, Japan).

2.3. Flow-cytometric analysis

For flow-cytometric analysis, aliquots of culture (1 ml) were withdrawn at 24-h intervals. Samples were analyzed with a flow cytometer (EPICS XL; Beckman Coulter, Inc., Miami, FL, USA). As size standards, we used 2-, 5- and 10-μm polystyrene latex beads (Coulter Corporation, Miami, FL, USA) and excitation at 488 nm was provided by an argon-ion laser. For the determination of cell count of cultures, count fluorospheres (Coulter Corporation) were mixed with samples (1:1, v/v) and the system was programmed to stop the cell count at 30,000. All data were collected and analyzed with the cytometer software (EPICS XL System II, version 3.0; Beckman Coulter, Inc.).

2.4. Quantitation of glucosylglycerol and sucrose

Aliquots (4 ml) were withdrawn from cultures (OD_{730}= 0.6) and cells were collected by centrifugation at 3,000x*g* for 10 min at 4°C. Absolute ethanol (1 ml) was added to each pellet and tubes were shaken vigorously for extraction of sugars. Ethanol was then

evaporated on a centrifugal concentrator (model CC-101; TOMY, Tokyo, Japan). Dried pellets were suspended in distilled water. Sugars, amino acids and organic acids were separated and the sugar fraction was analyzed by gas chromatography, as described previously by Adams et al. (1999). Minor modifications and additions to the protocol were made to improve the separation of various sugars.

2.5. Quantitation of total proteins, DNA and chlorophyll

For quantitation of proteins, 1 ml of cell suspension was supplemented with 0.1 g of trichloroacetic acid and then the precipitate was collected by centrifugation at 15,000x*g* for 10 min at 4°C. The pellet was suspended in 1 N NaOH. The suspension was boiled for 30 min, cooled, and then centrifuged at 15,000x*g* for 5 min. The protein in the supernatant was quantitated as described by Lowry et al. (1951) with bovine serum albumin as the standard.

Concentrations of DNA were estimated as described by Labarca and Paigen (1980) with λ-HindIII DNA fragments as the standard (Takara Shuzo, Kyoto, Japan).

For the quantitation of chlorophyll, cells in 1 ml of culture were collected by centrifugation at 15,000x*g* for 10 min at 4°C. Pigments were extracted by suspending cells in 1 ml of a mixture of methanol and H_2O (9:1, v/v). After removal of the precipitate by centrifugation at 15,000x*g* for 5 min, chlorophyll in the supernatant was quantitated in terms of absorbance at 665 nm (Talling and Driver, 1961; Porra, 1991).

2.6. Purification of glucosylglycerol from *Synechocystis* cells

Cultures of *Synechocystis* cells that had been acclimated to 4% NaCl (5~10 liters) were harvested by centrifugation at 8,000x*g* for 10 min. The supernatant was discarded and pellets were suspended in ethanol (10 ml per 100 ml culture). The suspension was incubated at 65°C for 3 h in a water bath under continuous shaking and then centrifuged at 8000x*g* for 10 min at room temperature. The supernatant was collected. The pellets were suspended in a mixture mixture of ethanol and H_2O (8:2, v/v) and the suspension was centrifuged under the same conditions as above. The supernatant was collected and combined with the above mentioned supernatant.

The combined supernatant was evaporated on a centrifugal concentrator. The sirup obtained was dissolved with H_2O. After centrifugation at 10,000x*g* for 1 h at room temperature, the supernatant was collected and frozen at -30°C over night to precipitate impurities. After centrifugation at 10,000x*g* for 1 h at room temperature, the supernatant was collected, mixed with the same volume of ethylacetate, and shaked intensively. A separation funnel was used to collect the water phase at the bottom. The remaining ethylacetate was evaporated until odorlessness by a rotary evaporator. Anions and cations were removed by the use of an anionic exchange column (Dowex 50Wx8, 200-400 mesh, H^+-form) and a cationic exchange column (Dowex 1x2, 200-400 mesh, OH^--form), respectively. Finally, GG was concentrated by a rotary evaporator and its concentration was determined by gas chromatography (Karsten et al., 1993).

Chapter 3

Arrest of cell division under salt stress

3.1. Summary

To study the protective mechanism of GG under salt stress conditions, we produced a deletion mutant deficient in GG biosynthesis, namely Δ*ggpS,* which was very sensitive to salt stress and had a maximal tolerance to NaCl about 300 mM (Marin et al., 1998; Karandashova et al., 2002). To investigate the role of GG with greater details, we incubated wild-type and Δ*ggpS* cells with various concentrations of NaCl. We found that 450 mM NaCl was a critical concentration that totally arrested cell division, significantly increased size and subsequently induced cell lysis in Δ*ggpS* mutant cells. The same concentration of NaCl did not have significant effects on wild-type cells in respect to cell division and cell size. A comparable phenotype, *i.e.,* arrest of cell division and increase in cell size, could be observed in wild-type cells only at very high NaCl concentrations such as 800 mM. Moreover, the arrest of cell division in Δ*ggpS* cells was recognized by structures that resembled a division ring at the equator of mother cells. These observations suggested that NaCl inhibited specifically the cell division machinery in *Synechocystis* cells, which resulted in significant enlargement of cells and subsequently induced cell lysis.

3.2. Segragation of *ggpS* gene

We examined levels of segregation of the wild-type gene in Δ*ggpS* mutant by PCR using synthetic primers specifically designed for *ggpS* gene (Marin et al., 1998). In all cases, when genomic DNA of Δ*ggpS* mutant cells was used as template, the PCR analyses showed fragments that were larger than the fragments obtained with wild-type genomic DNA (Fig. 4). The difference in size exactly corresponded to the increase expected by insertion of *aph*II gene cassette (~1.2 kb). Furtheremore, the wild-type fragment was completely absent. Thus, the *ggpS* gene was completely segregated in Δ*ggpS* mutant cells (Fig. 4)

3.3. Growth of wild-type and mutant cells under salt-stress conditions

As it was previously mentioned, our aim is to study in details the mechanism by which GG protects *Synechocystis* cells against salt stress. For this, we first examined the effects of several different concentrations of NaCl on the growth of wild-type and Δ*ggpS* cells (200- 800 mM). When the concentration of NaCl was lower than 300 mM, Δ*ggpS* cells were able to grow similarly to wild-type cells. However, the growth of Δ*ggpS* cells in the presence of 450 mM NaCl was markedly retarded (Fig. 5) and, at ≥ 500 mM NaCl, Δ*ggpS* cells did not grow at all. By contrast, the growth of wild-type cells was not significantly affected at 450 mM NaCl (Fig. 5), but was seriously retarded at ≥ 800 mM NaCl.

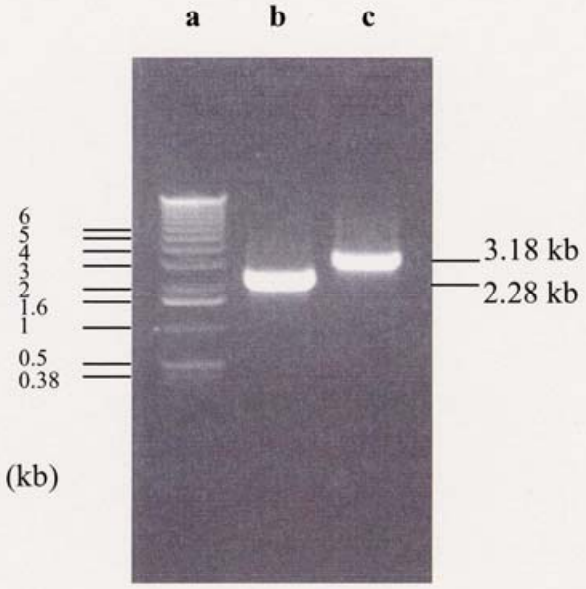


Figure 4. Analysis by PCR of genomic DNA from wild-type and Δ*ggpS* mutant cells. Lane a, 1 kb ladder fragments (molecular size marker); Lane b, the product of PCR obtained with *ggpS* gene primers and DNA from wild-type cells of *Synechocystis* sp. PCC 6803 as template; lane c, the product of PCR obtained with *ggpS* gene primers and DNA from Δ*ggpS* mutant cells as template.

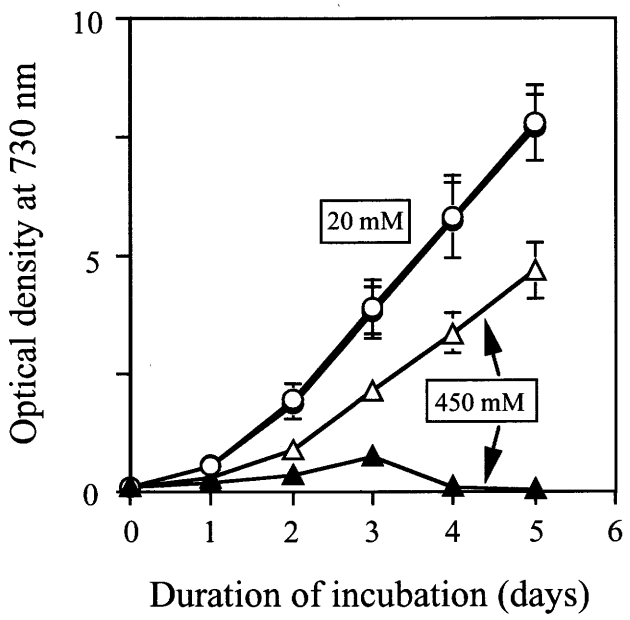


Figure 5. Effects of 450 mM NaCl on the growth of wild-type and Δ*ggpS* cells. Cells that had been grown under normal conditions in BG-11 medium that contained 20 mM Na^+ ions were inoculated, at an optical density of 0.1 at 730 nm, in BG-11 medium (abbreviated as 20 mM NaCl) or in BG-11 medium that had been supplemented with NaCl to a final concentration of 450 mM (abbreviated as 450 mM NaCl). Growth was monitored in terms of optical density at 730 nm. Graphs show growth of wild-type (open circles) and Δ*ggpS* mutant (closed circles) cells in 20 mM NaCl and growth of wild-type (open triangles) and Δ*ggpS* (closed triangles) cells in the presence of 450 mM NaCl. Data and error bars were calculated from the results of at least five independent experiments.

3.4. Effects of salt stress on the size of cells

From the growth curves we observed that at 3 or 4 days of salt stress (for concentrations of NaCl between 300 mM and 450 mM), the apparent optical density of cultures was suddenly decreased. To investigate the reasons for this unexpected phenomenon, we examined the condition and shape of wild-type and Δ*ggpS* cells during incubation with 450 mM NaCl by optical microscopy (Fig. 6) and we detected no significant changes in the size of wild-type cells during incubation with 450 mM NaCl (Figs. 6A and 6B). On the other hand, the diameter of Δ*ggpS* cells almost doubled during incubation for three days in medium supplemented with 450 mM NaCl (Figs. 6D and 6E). Furthermore, after three days all of the Δ*ggpS* cells seemed to be in the process of dividing. These observations suggested that the increase in cell size might have been caused by inhibition of cell division. From these observations we could conclude that 450 mM NaCl induced a significant increase in cell size of Δ*ggpS* cells. Increase in cell size was maximal after three days of stress. During the fourth day, the decrease in optical density was probably due to cell lysis.

Thus, it became evident that, 450 mM NaCl is a critical concentration that induced a significant increase in cell size of Δ*ggpS* cells. Under the same conditions, wild-type cells were almost unaffected by NaCl.

The growth of *Synechocystis* cells is usually monitored by changes in the optical density of the culture. However, since we observed a significant increase in cell size after incubation with 450 mM NaCl. Therefore, we decided to estimate the growth by measuring the cell count (*i.e.,* cell density of culture) by flow cytometry.

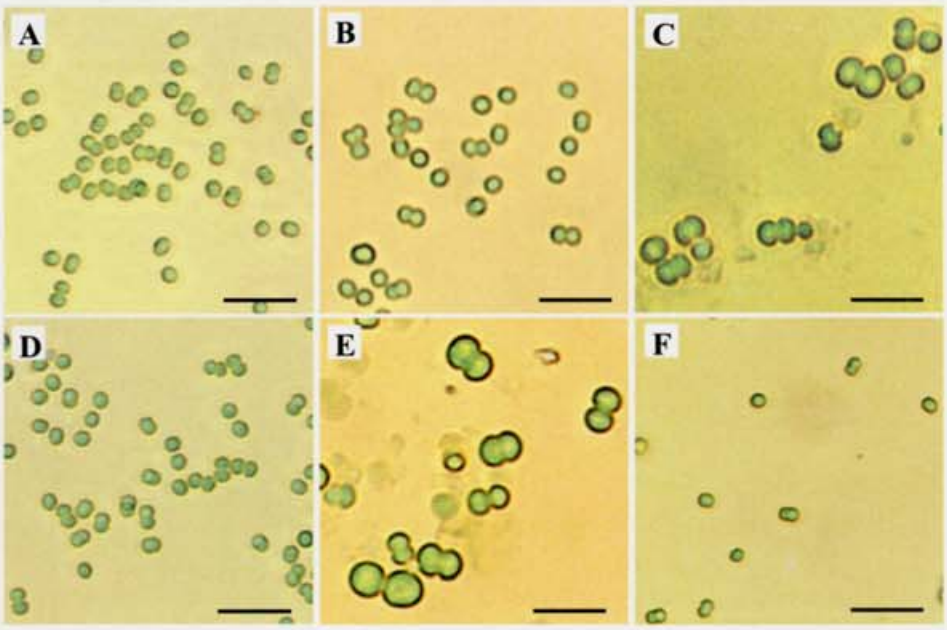


Figure 6. Effects of NaCl on the size of *Synechocystis* cells. Wild-type and Δ*ggpS* cells that had been grown in 20 mM NaCl were cultured in the presence of 20 mM, 450 mM or 800 mM NaCl for three days. The other experimental conditions were the same as those described in the legend to Figure 1. Light micrographs show (A) wild-type cells in 20 mM NaCl; (B) wild-type cells in 450 mM NaCl; (C) wild-type cells in 800 mM NaCl; (D) Δ*ggpS* cells in 20 mM NaCl; (E) Δ*ggpS* cells in 450 mM NaCl; (F) Δ*ggpS* cells in 800 mM NaCl; The scale bars represent 10 μm.

We analyzed the cell size and cell count by flow cytometry for the Δ*ggpS* cells during incubation with 200, 300, 400, 450 and 500 mM NaCl (Fig. 7). As shown in Figure 7, the cell size and the cell count of Δ*ggpS* cells were dependent on the concentration of NaCl. Increase in cell size was significant for concentrations of NaCl varying between 300 and 450 mM. However, at 200 mM NaCl, Δ*ggpS* cells could grow quite normally and after incubation for 5 days we could observe a slight increase in cell size. On the other hand, at 500 mM NaCl, the size of Δ*ggpS* cells did not change significantly. We subsequently determined the cell count of the culture of Δ*ggpS* cells in the NaCl concentration range between 200 and 500 mM. The results are summarized in Table V.

As the first conclusion, we could understand that the increase in cell size of Δ*ggpS* cells was mainly due to inhibition of cell division. Cell division in Δ*ggpS* cells was either partially arrested at concentration of NaCl between (300~ 400 mM) or totally arrested at concentrations of NaCl ≥ 450 mM (Table V). The second conclusion is that the occurance of inhibition of cell division and cell lysis was observed at different NaCl concentrations (Table VI). It is important to mention that wild- type cells were not significantly affected by incubation for several days with NaCl concentration up to 500 mM.

We attempted to check the effect of higher concentrations of salt on the growth and cell size of wild-type cells. As expected, a significant increase in the size of wild-type cells was observed with NaCl at concentrations ≥ 800 mM (Fig. 6C). Salt stress due to 800 mM NaCl did not change the size of Δ*ggpS* cells (Fig. 6F).

Finally, it is important to mention that all the phenotypical aberrations observed in Δ*ggpS* cells are due to the mutation of *ggpS* gene. The importance of this gene for *Synechocystis* to survive high concentrations of NaCl became more evident.

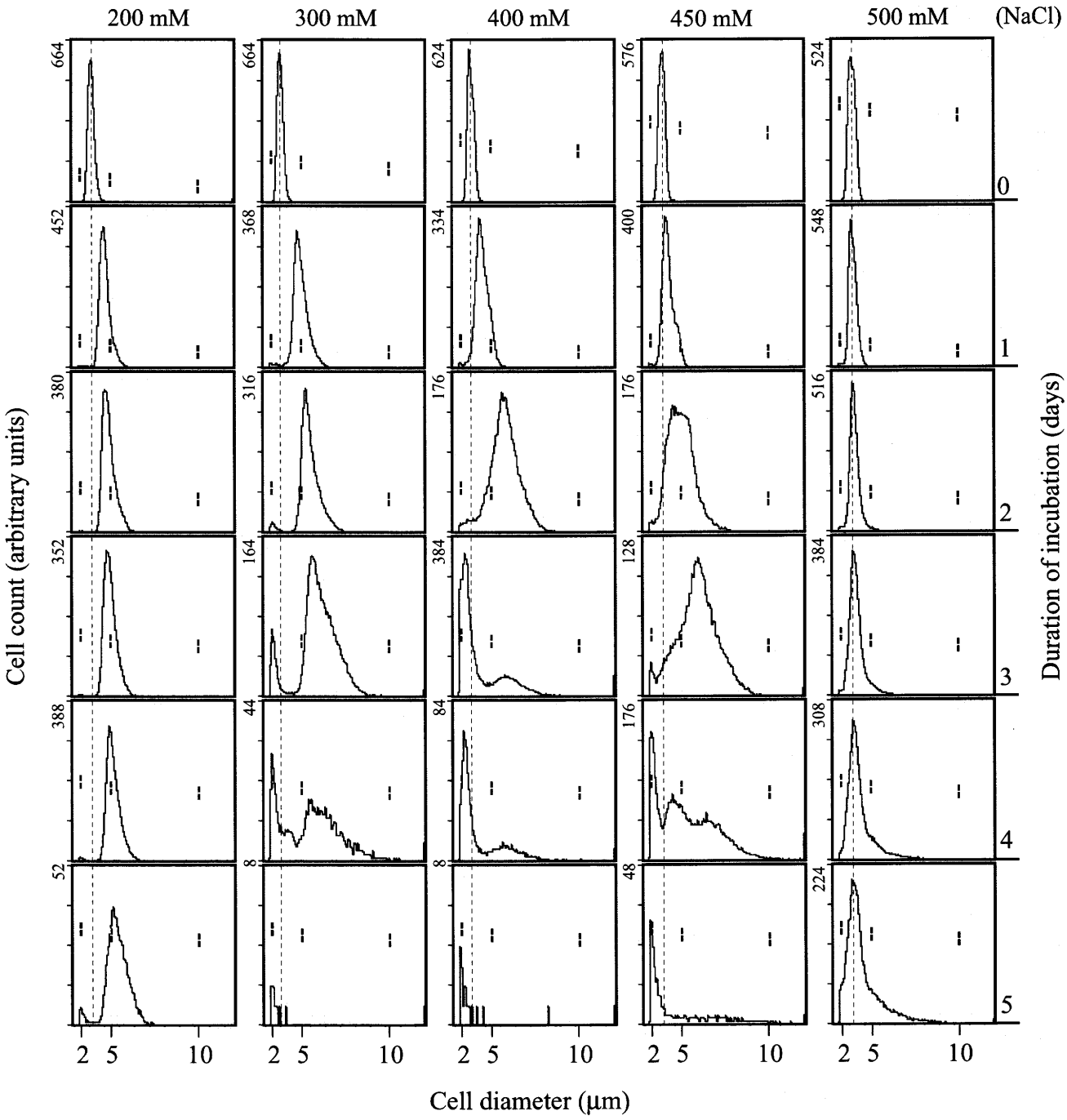


Figure 7. Effects of different concentrations of NaCl on cell size of Δ*ggpS* cells. Δ*ggpS* cells that had been grown with 20 mM NaCl were cultured in the presence of 200, 300, 400, 450 or 500 mM NaCl. Aliquots of cells were withdrawn and the distribution of cell sizes was analyzed by flow cytometry after appropriate dilutions. Arbitrary scales are used to provide a better view of the distribution of cell sizes. Numbers in the top-left part of plots represent the cell count of each plot but do not have a quantitative meaning for the cell density of cultures during the 5-day time course. Vertical dashed lines represent the initial sizes of Δ*ggpS* cells before salt stress. The three pairs of vertical dashes represent the positions of size markers with diameters of 2, 5 and 10 μm.

Table V. The variation of cell count of Δ*ggpS* cells during incubation in BG-11 medium that contained various concentrations of NaCl. The cell count was measured by flow cytometry. The results and standard deviations represent the average of four independent experiments.

	NaCl concentration (mM)				
Days of incubation	**200**	**300**	**400**	**450**	**500**
	Cell count (x10^7 cell ml^{-1})				
0	0.81±0.08	0.80±0.04	0.80±0.05	0.80±0.06	0.80±0.03
1	1.92±0.22	1.27±0.13	1.02±0.03	0.79±0.03	0.80±0.01
2	7.38±0.91	4.25±0.50	1.34±0.03	0.82±0.02	0.80±0.02
3	11.97±1.60	0.85±0.03	0.05±0.01	0.92±0.05	0.82±0.03
4	9.76±1.03	0.06±0.02	0.025±0.004	0.055±0.008	0.75±0.02

Table VI. The effects of various concentrations of NaCl on cell division, cell size, occurance of cell lysis and time necessay for occurance of cell lysis. These data represent the average from four independent experiments. (++) very significant; (+) significant; (-) negative.

	NaCl concentration (mM)				
	200	**300**	**400**	**450**	**500**
Cell division	++	++	+	-	-
Increase in cell size	+	++	++	++	+
Cell lysis	+	++	++	++	+
Time for cell lysis (days)	≥ 4	≥ 2	≥ 2	4	≥ 5

Therefore, to investigate the protection of Δ*ggpS* cells by GG, we used 450 mM NaCl as the salt stress in our subsequent experiments. This concentration of NaCl was selected among others because it totally arrested cell division and increased significantly cell size. The effects of incubation for 5 days of wild-type and Δ*ggpS* cells with 450 mM NaCl are presented in Figure 8 and Table VII. It is noticeable that the increase in cell size of Δ*ggpS* cells represented an increase in the average cell diameter plus the formation of cell duplets.

3.5. Effects of salt stress on the ultrastructure of *Synechocystis* cells

In order to clarify the effects of salt stress in greater details, we examined the ultrastructure of cells by transmission electron microscopy. The size and the ultrastructure of wild-type cells were not significantly affected by salt stress (Figs. 9A and 9B). However, after incubation for three days in the presence of 450 mM NaCl, Δ*ggpS* cells were much larger than wild-type cells and a structure that resembled a division ring was visible at the equator of cells (Figs. 9C and 9D). Moreover, the presence of division ring-like structures, as shown in Figure 9D suggested that NaCl specifically inhibited the cell-division machinery in Δ*ggpS* cells. These results confirmed the previous observations with optical microscopy and flow cytometry.

We next examined Δ*ggpS* cells that had been incubated with 450 mM NaCl for four days. Figures 10A and 10B show that Δ*ggpS* cells were unable to complete cell division and lysed, leaving a division ring that adhered closely to the cell envelope. Figure 10C shows a cell that appears to have burst during preparation for electron microscopy. Figure 10D shows a triplet with an unusual division pattern, demonstrating again the dramatic effects of NaCl on the cell-division machinery.

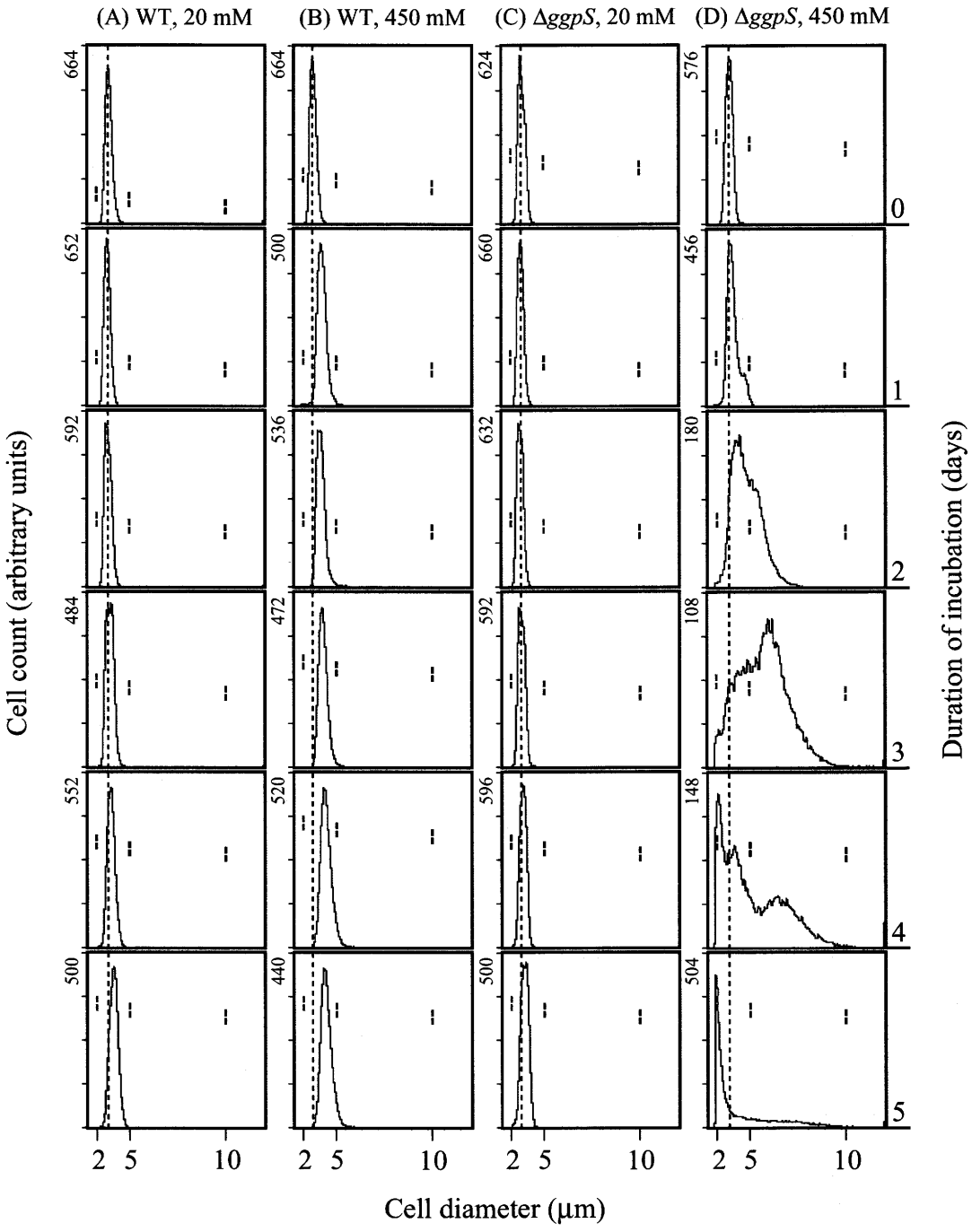


Figure 8. Effects of 450 mM NaCl on the distribution of the sizes of *Synechocystis* cells as determined by flow cytometry. Wild-type and Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated in the presence of 20 mM or 450 mM NaCl. Aliquots of both types of cell were withdrawn and the distribution of cell sizes was analyzed by flow cytometry after appropriate dilutions. (A) Wild-type cells in 20 mM NaCl; (B) wild-type cells in 450 mM NaCl; (C) Δ*ggpS* cells in 20 mM NaCl; (D) Δ*ggpS* cells in 450 mM NaCl. Arbitrary scales are used to provide a better view of the distribution of cell sizes. Numbers in the top-left part of plots represent the cell count of each plot but do not have a quantitative meaning for the cell density of cultures during the time course for 5 days. Vertical dashed lines represent the initial sizes of wild-type and Δ*ggpS* cells before salt stress. The three pairs of vertical dashes represent the positions of size markers with diameters of 2, 5 and 10 μm.

Table VII. Effect of incubation with 450 mM NaCl on the cell count of wild-type and Δ*ggpS* cells. Wild-type and Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated with 20 mM or 450 mM NaCl. Cell count of cultures was estimated by flow cytometry. Means and standard deviations were calculated from the results of three independent experiments.

	Cell count ($x10^7$ cells ml^{-1})			
	Wild type		Δ*ggpS*	
Day of incubation	**20 mM NaCl**	**450 mM NaCl**	**20 mM NaCl**	**450 mM NaCl**
0	0.80±0.10	0.80±0.07	0.80±0.10	0.80±0.03
1	3.70±0.39	1.49±0.15	3.76±0.12	0.79±0.03
2	13.22±1.03	4.62±0.20	13.44±1.31	0.82±0.02
3	22.12±1.66	10.42±1.10	22.48±2.11	0.92±0.03
4	30.14±2.12	15.22±1.70	29.53±2.54	0.055±0.008
5	38.32±2.56	20.56±2.91	37.92±2.75	0.016±0.009

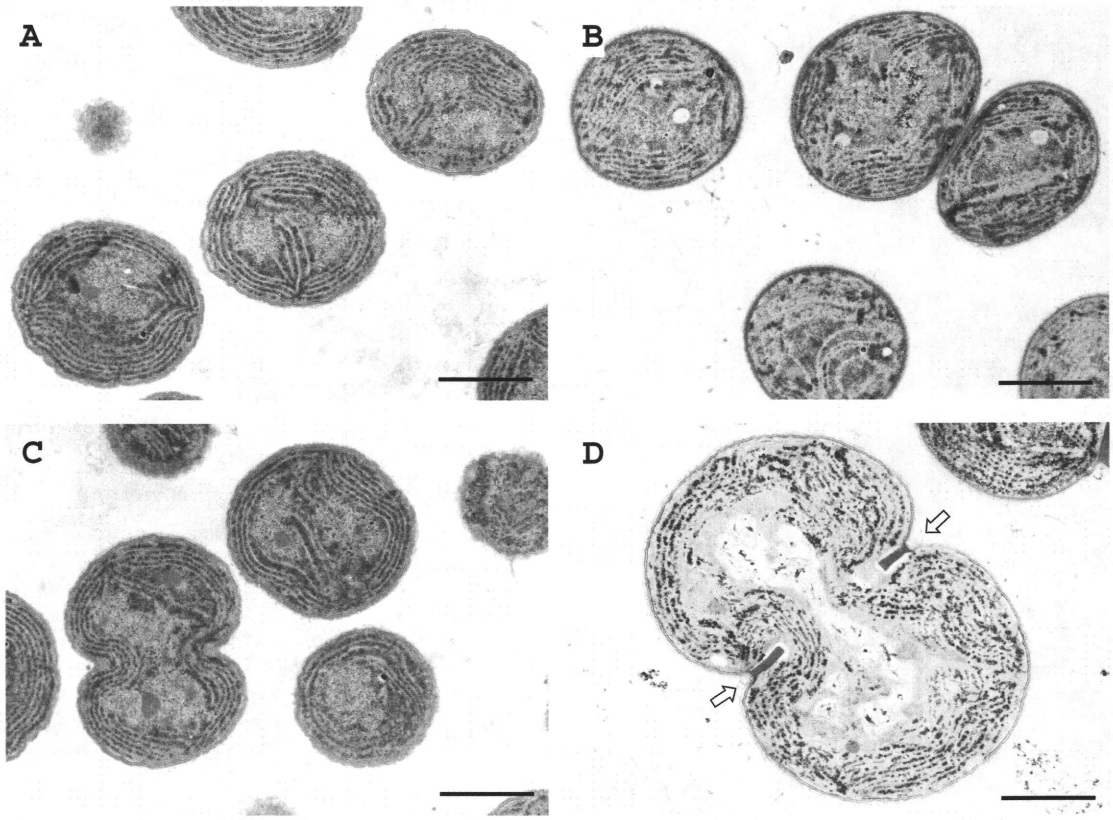


Figure 9. Effects of incubation with 450 mM NaCl for three days on the ultrastructure of wild-type and Δ*ggpS* cells of *Synechocystis*. Wild-type and Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated in the presence of 20 mM or 450 mM NaCl. (A) Wild-type cells in 20 mM NaCl for three days; (B) wild-type cells in 450 mM NaCl for three days; (C) Δ*ggpS* cells in 20 mM NaCl for three days;(D) Δ*ggpS* cells in 450 mM NaCl for three days. Arrows in (D) indicate a division ring-like structure at the cell's equator. Each scale bar represents 1 μm.

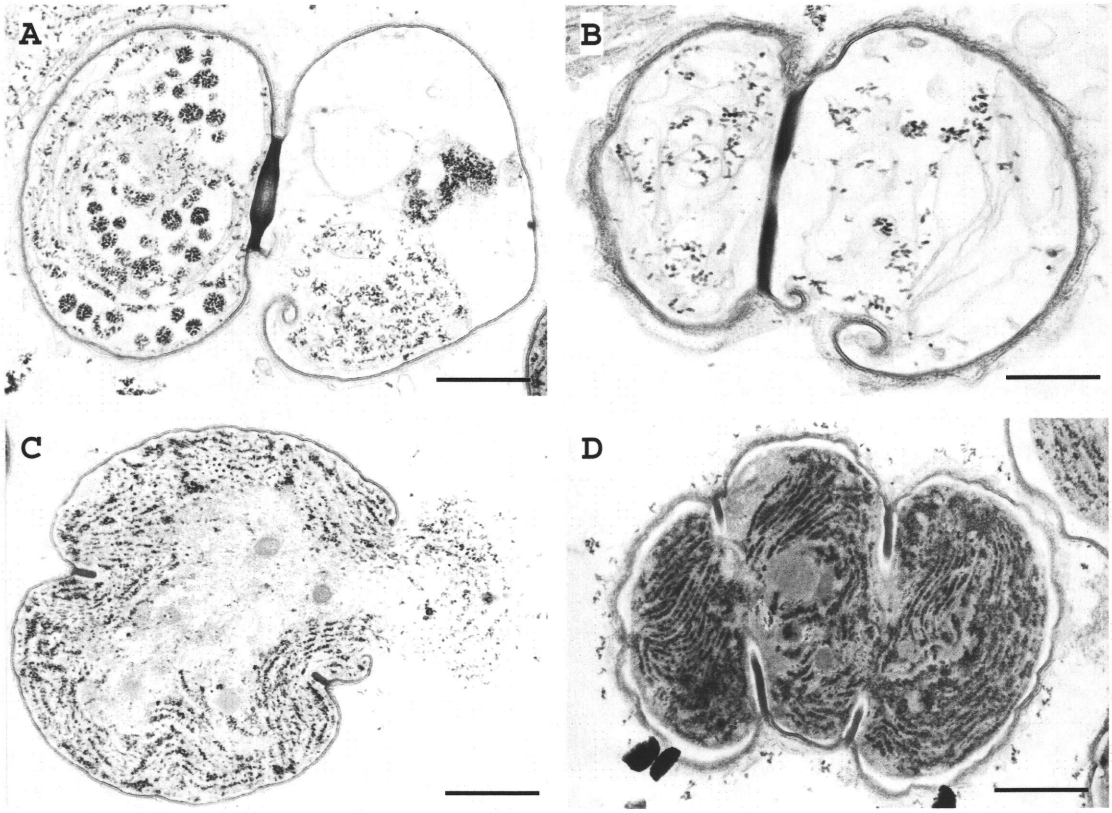


Figure 10. Effects of incubation with 450 mM NaCl for four days on the ultrastructure of Δ*ggpS* cells of *Synechocystis*. Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated in the presence 450 mM NaCl. A, B, C and D represent various shapes of cells. Each scale bar represents 1 μm.

Thus, it became evident that the sudden decrease in the optical density or in the cell count previously described during incubation of Δ*ggpS* cells with 450 mM NaCl (Fig. 5) is due to cell lysis.

3.6. Effects of salt stress on levels of proteins, DNA and chlorophyll in Δ*ggpS* cells

In *Escherichia coli*, cell cycle is investigated in details and could be subdivided into three major events, namely cell elongation, cell septation and cell separation (Vollmer and Holtje, 2001). Cell elongation is a preparative step for cell division and is mainly characterized by the duplication of DNA and by an active biosynthesis of proteins and other compounds necessary for cell division. Cell septation is marked by the formation of division ring that will split the mother cell to give to two identical daughter cells. The two daughter cells will be separated during cell separation which indicates the end of cell cycle. From the results found, it seems that salt stress inhibited cell septation. To determine the effects of salt stress on cell elongation, we measured the levels of proteins, DNA and chlorophyll in Δ*ggpS* cells during incubation with 450 mM NaCl. The results in Table VIII show that, in 20 mM NaCl, levels of these macromolecules remained almost constant during incubation for three days. However, when Δ*ggpS* cells were incubated in 450 mM NaCl, levels of proteins, DNA and chlorophyll per cell increased approximately 7-, 4- and 4-fold, respectively (Table VIII).

Under salt-stress conditions, Δ*ggpS* cells almost doubled in diameter, an increase that is equivalent to an approximately 8-fold increase in cell volume. Taken together, the results suggest that the concentration of proteins in each cell remained almost constant, whereas concentrations of DNA and chlorophyll were reduced to about 50% under salt stress

Table VIII. Effects of 450 mM NaCl on levels of proteins, DNA and chlorophyll in Δ*ggpS* cells.

Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated with 20 mM or 450 mM NaCl for three days. Aliquots of cultures were withdrawn at designated times and levels of proteins, DNA and chlorophyll were determined. Means and standard deviations were calculated from the results of three independent experiments.

	Proteins ($\mu g/10^7$ cells)		**DNA ($\mu g/10^7$ cells)**		**Chlorophyll ($\mu g/10^7$ cells)**	
	NaCl		**NaCl**		**NaCl**	
Day of incubation	20 mM	450 mM	20 mM	450 mM	20 mM	450 mM
0	0.98±0.10 (1.00)*	0.98±0.10 (1.00)	1.21±0.30 (1.00)	1.33±0.19 (1.00)	0.48±0.02 (1.00)	0.48±0.02 (1.00)
1	1.25±0.18 (1.27)	1.42±0.08 (1.44)	1.36±0.52 (1.12)	1.61±0.40 (1.21)	0.44±0.03 (0.91)	0.47±0.08 (0.97)
2	1.73±0.08 (1.76)	1.83±0.13 (1.86)	1.15±0.50 (0.95)	3.08±0.22 (2.31)	0.44±0.04 (0.91)	0.79±0.05 (1.64)
3	1.34±0.14 (1.36)	6.96±1.31 **(7.1)**	1.32±0.47 (1.09)	5.71±0.50 **(4.29)**	0.55±0.05 (1.14)	2.16±0.02 **(4.5)**

*Numbers in parentheses indicate increases relative to day 0.

(Table VIII). It is noteworthy that the amount of DNA in each cell increased only four-fold during incubation with 450 mM NaCl for three days, whereas the level of proteins increased in parallel with the increase in cell volume. Thus, although NaCl totally arrested the division of Δ*ggpS* cells, the biosynthesis of proteins, DNA and chlorophyll was not totally inhibited.

This result suggested that even during incubation with 450 mM NaCl cell elongation was successfully performed by Δ*ggpS* cells. Thus, the inhibition of cell division probably occurs in the cellular processes following cell elongation such as cell septation. Finally, the molecular mechanism by which NaCl inhibits cell sepatation remains to be elucidated.

3.7. Accumulation of glucosylglycerol and sucrose under salt stress

Synechocystis cells accumulate GG and transiently traces of sucrose in response to salt stress (Hagemann and Marin, 1999; Marin et al., 1998; Reed and Stewart, 1985). We examined the effects of the mutation in Δ*ggpS* cells on levels of GG and sucrose in wild-type and Δ*ggpS* cells during incubation in 450 mM NaCl (Fig. 11). Figure 11A shows that the concentration of GG reached a maximum in wild-type cells 8 h after the onset of salt stress and remained constant thereafter. However, sucrose accumulated transiently and at only a low level and had almost disappeared within 10 h. By contrast, Δ*ggpS* cells were unable to synthesize GG but synthesized and accumulated a much higher level of sucrose than wild-type cells (Fig. 11B).

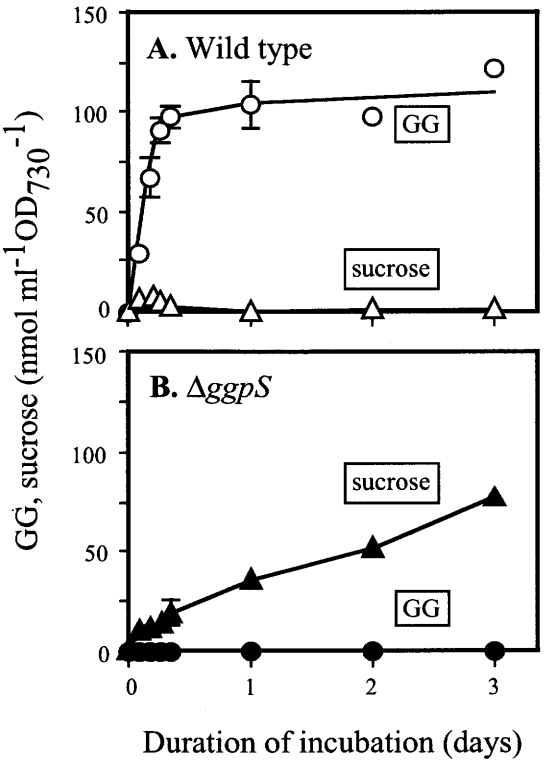


Figure 11. Effects of 450 mM NaCl on levels of GG and sucrose in wild-type and *ΔggpS* cells. Wild-type and Δ*ggpS* cells that had been grown in the presence of 20 mM NaCl were transferred to medium that contained 450 mM NaCl at zero time. (A) Levels of GG (open circles) and sucrose (open triangles) in wild-type cells. (B) Levels of GG (closed circles) and sucrose (closed triangles) in Δ*ggpS* cells.

Chapter 4

Glucosylglycerol sustains cell division and reverses the adverse effect of salt stress

4.1. Summary

As it was clearly shown in Chapter 3, salt stress due to 450 mM NaCl was able to totally arrest cell division in Δ*ggpS* mutant cells, but not in wild-type cells. Electron microscopic analysis revealed that, in Δ*ggpS* cells, cytokinesis was incomplete and aborted division could be recognized by the presence of a structure that resembled a division ring. Although Δ*ggpS* cells are unable to synthesize GG in response to salt stress, they are capable in taking up GG from the medium via an ABC-type transport system, which is encoded by the *ggtA* and *ggtBCD* gene cluster (Mikkat et al., 1996, 1997; Mikkat and Hagemann, 2000; Hagemann et al., 1997). Thus, we examined the effect of exogenously supplemented GG on both cell size and cell count of Δ*ggpS* cells. In addition to this, we have also examined the effects of compatible solutes other than GG, such as trehalose, sorbitol and sucrose. It is noteworthy that trehalose is uptaken by the ABC-type transporter with almost the same affinity as GG. Sucrose is also uptaken by the same transporter but with lower affinity compared to GG and trehalose (Mikkat et al., 1997). Finally, sorbitol is not uptaken by *Synechocystis* cells.

4.2. Effects of exogenous glucosylglycerol on cell division of Δ*ggpS* cells

Although Δ*ggpS* cells are unable to synthesize GG in response to salt stress, they are capable in taking up GG from the medium via an ABC-type transport system, which is encoded by the *ggtA* and *ggtBCD* gene cluster (Mikkat et al., 1996, 1997; Mikkat and Hagemann, 2000; Hagemann et al., 1997). Thus, we examined the effect of exogenously supplemented GG on both cell size and cell count of Δ*ggpS* cells.

Flow-cytometric analysis revealed that exogenously supplied GG at 1 mM reversed the effects of 450 mM NaCl on cell size (Fig. 12) and cell count (Table IX). Figure 12D shows the distribution of Δ*ggpS* cells which were incubated with 450 mM NaCl for 5 days. When GG was added to the medium 24 h and 48 h after the onset of salt stress, cell size decreased significantly over the course of the next few days (Figs. 12A and 12B). GG also increased cell count for as long as five days (Table IX), and the results suggested that the uptake of exogenous GG accelerated cell division and prevented cell lysis. However, when added at three days after the onset of salt stress, GG was only partially effective in preventing cell lysis (Fig. 12C and Table IX). Nonetheless, even in this case, cell lysis was somewhat retarded and Δ*ggpS* cells began to divide again quiet fastly two days after the addition of GG (Table IX). Finally, we found that the presence of GG during incubation of wild-type cells with 450 mM NaCl had no effect at all.

4.3. Effects of exogenous glucosylglycerol on ultrastructure of Δ*ggpS* cells

We also examined the effects of exogenous GG on Δ*ggpS* cells by electron microscopy. When GG was added simultaneously with exposure of cells to salt stress

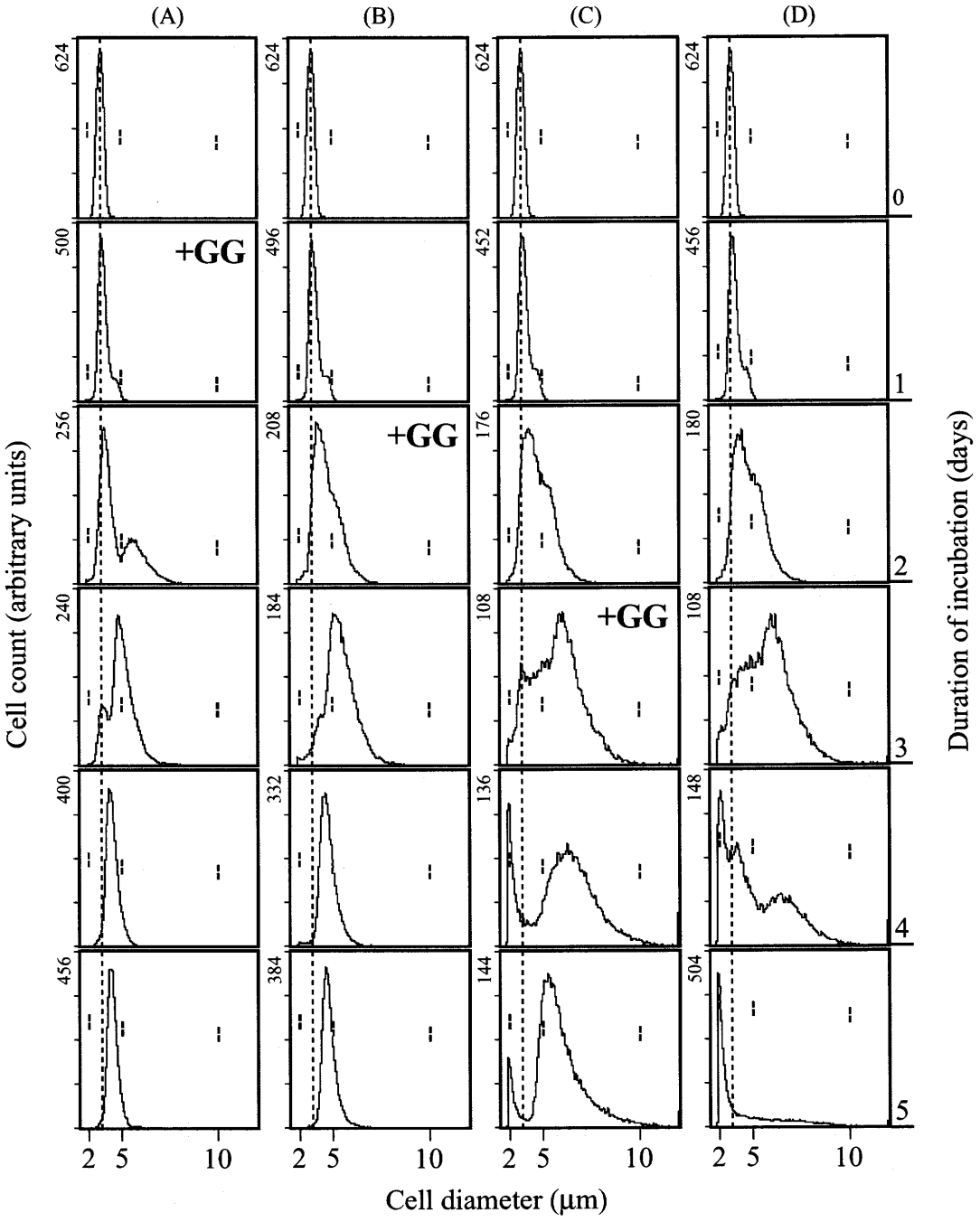


Figure 12. Effects of exogenous GG on the distribution of sizes of Δ*ggpS* mutant cells. Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated with 450 mM NaCl in the presence of exogenous GG, which was added to independent cultures one day (A), two days (B) or three days (C) after the onset of salt stress, or in the absence of GG (D). The sizes of cells were analyzed by flow cytometry. Numbers in the top-left part of plots represent the cell count of each plot but do not have a quantitative meaning for the cell density of cultures during the 5-day time course. Vertical dashed lines represent initial sizes of Δ*ggpS* cells before the onset of salt stress. The three pairs of vertical dashes represent the positions of size markers of 2, 5 and 10 μm in diameter.

Table IX. Effects of exogenous GG on the density of Δ*ggpS* cells under salt stress. Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated with 20 mM or 450 mM NaCl in the absence or presence of 1 mM GG that was added to the medium 1, 2 or 3 days after the start of salt stress. Means and standard deviations were calculated from the results of three independent experiments.

Cell count of Δ*ggpS* (x10^7 cells ml^{-1})					
	20 mM NaCl	**450 mM NaCl**			
	No GG added	**1 mM GG added after**			**No GG added**
Day of incubation		**1 day**	**2 day**	**3 day**	
0	0.80±0.10	0.81±0.08	0.80±0.08	0.79±0.05	0.80±0.03
1	3.76±0.12	0.87±0.04	0.82±0.05	0.78±0.04	0.79±0.03
2	13.44±1.31	1.10±0.11	0.90±0.11	0.83±0.03	0.82±0.02
3	22.48±2.11	3.03±0.01	1.82±0.01	1.00±0.02	0.92±0.03
4	29.53±2.54	7.85±0.33	6.11±0.13	0.17±0.30	0.055±0.008
5	37.92±2.75	11.0±0.1	9.17±0.14	0.62±0.05	0.016±0.009

(450 mM), the size and ultrastructure of Δ*ggpS* cells were unaffected (Figs. 13A and 13B). These results suggested that the uptake of exogenous GG counteracted the effects of salt stress. Figure 13C shows that incubation with 450 mM NaCl for 48 h caused a significant increase in the size of Δ*ggpS* cells as compared to Δ*ggpS* cells that had been grown in the presence of 20 mM NaCl (see Fig. 13A). After addition of GG to Δ*ggpS* cells that had been incubated with 450 mM NaCl for two days, cell size decreased significantly (Fig. 13D), suggesting that cell division resumed. These observations were in good agreement with the effect of exogenous GG on cell size, as determined by flow cytometry (Fig. 12 and Table IX). Finally, upon addition of GG to a culture of Δ*ggpS* cells that had been incubated with 450 mM NaCl for three days, we observed very large tetrads (Figs. 13E and 13F). Although most cells lysed subsequently, cell lysis was retarded by the addition of GG and tetrads might have represented an intermediate stage before the complete accoplishment of cell division.

4.4. Effects of supplemntation of compatible solutes other than glucosylglycerol on growth of Δ*ggpS* cells

We next examined the protective effects of compatible solutes other than GG. For this we selected three kinds of compatible solutes: sucrose, trehalose and sorbitol. Δ*ggpS* cells that have been incubated for 48 hours in the presence of 450 mM NaCl were supplemented with 1 mM (final concentration) of the compatible solutes mentioned above. The effects of these compounds on cell count and cell size was analyzed by flow cytometry (Figs. 14 and 15). As expected, pre-incubation for 48 hours with 450 mM NaCl significantly increased the cell size of Δ*ggpS* cells. Further incubation further increased the cell size and

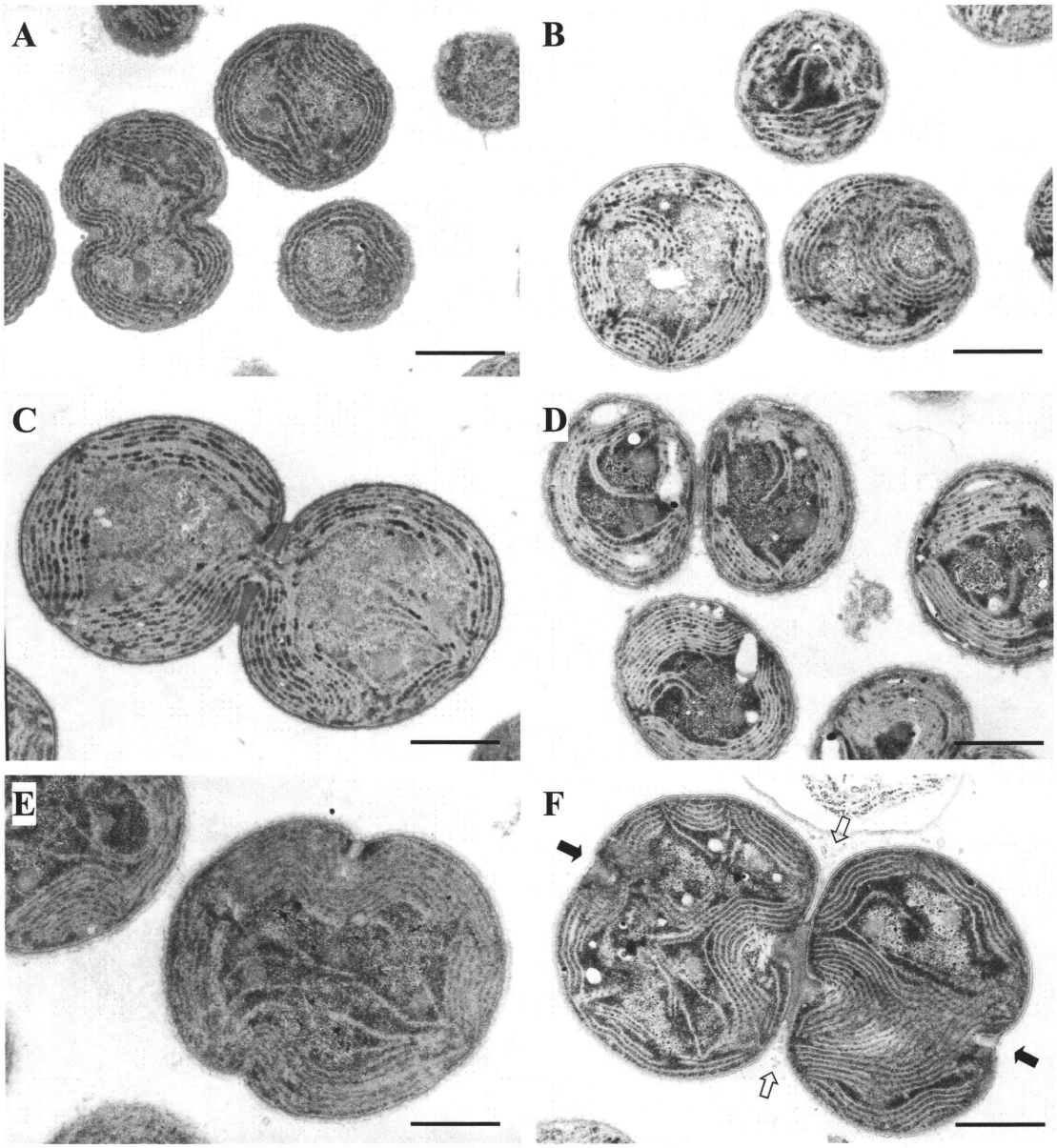


Figure 13. Effects of exogenous GG on the ultrastructure of Δ*ggpS* cells cultured with 450 mM NaCl. Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated for three days with 20 mM NaCl (A) or with 450 mM NaCl plus 1 mM GG (B). Δ*ggpS* cells that had been incubated for two days with 450 mM NaCl (C) were incubated for two further days in the presence of 1 mM GG (D). Δ*ggpS* cells that had been incubated for three days with 450 mM NaCl (E) were incubated for a further two days in the presence of 1 mM GG (F). Open arrows indicate the first septation site and closed arrows indicate the starting positions of the second septation site. Each scale bar represents 1 μm.

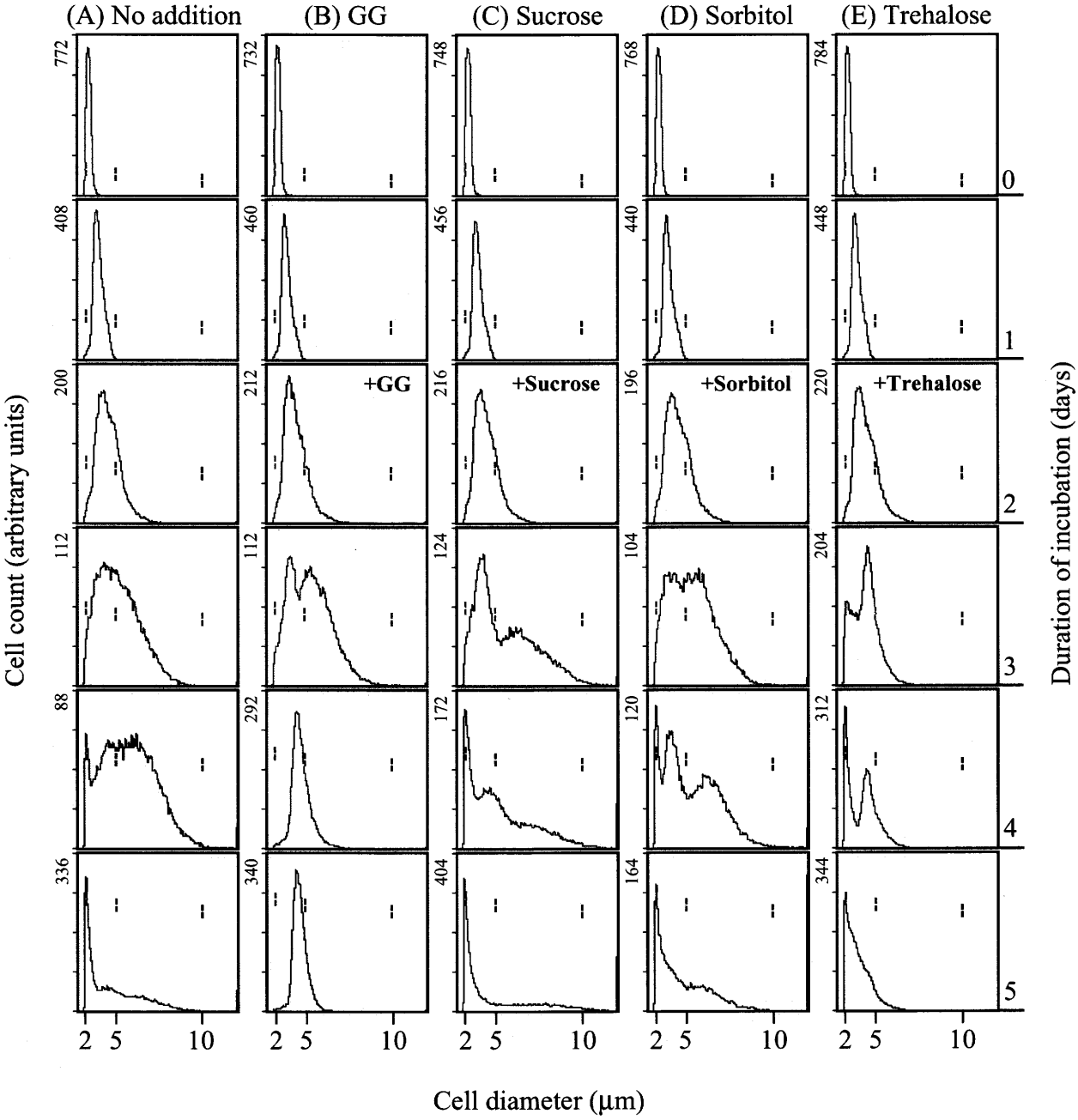


Figure 14. Effects of various compatible solutes on the size of *Synechocystis* cells under salt stress. Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated with 450 mM NaCl for 5 days (A). Δ*ggpS* cells that had been incubated for two days with 450 mM NaCl were incubated for three further days in the presence of 1 mM GG (B); 1 mM sucrose (C); 1 mM sorbitol (D); or 1 mM trehalose (E).

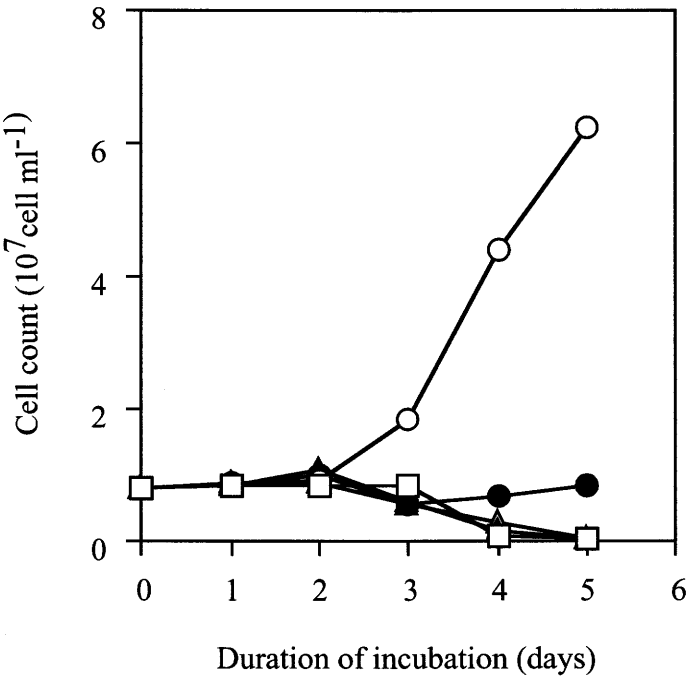


Figure 15. Effects of various compatible solutes on cell division of Δ*ggpS* cells under NaCl stress. Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated with 450 mM NaCl for 5 days (▲). Δ*ggpS* cells that had been cultured for two days with 450 mM NaCl were cultured for three further days in the presence of 1 mM GG (○); 1mM sucrose (□); 1mM sorbitol (△); or 1 mM trehalose (●)

induced cell lysis at the fourth day of the time course (Fig 14, panel A). Panel B shows the effects of exogenous GG and this result represent a positive control for the other kinds of compatible solutes. Sucrose had apparently the weakest effect in reducing the size of Δ*ggpS* cells that started to lyse at the fourth day of stress (Panel C). As expected, sorbitol also failed to protect Δ*ggpS* cells against salt stress because it is not uptaken by *Synechocystis* cells (Panel D). Surprizingly, trehalose revealed a slight protective effect (Panel E). The stress-induced increase in cell size and consequently cell lysis were somehow retarded by trehalose.

The determination of cell count clarified the effect of each compatible solute on cell division. In fact, as shown in Figure 15, when GG was added to culture of Δ*ggpS* cells which had been incubated with 450 mM NaCl for two days, the cell count doubled after only one day from the addition of GG. This indicated that cell division could resume after the uptake of exogenous GG. However, none of the other compatible solutes, namely sucrose, sorbitol and trehalose, could reinitiate cell division under salt stress conditions (Fig. 15). Although trehalose is efficiently uptaken and accumulated almost the same levels as GG (Mikkat et al., 1997), increase in cell size of Δ*ggpS* cells occurred slowly in the presence of trehalose but the latter failed to let cell division restart. Thus, except GG, other compatible solutes failed to protect Δ*ggpS* cells and to stimulate cell division under salt-stress conditions.

Chapter 5

Different effects of salt stress and osmotic stress

5.1. Summary

Ionic stress due to NaCl disturbs ion homeostasis but NaCl also has an osmotic effect (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998). In our laboratory, the effects of salt and osmotic stress on photosynthetic machinery (Allakhverdiev et al., 2000a,b) and gene expression (Kanesaki et al., 2002) were investigated in details. Results revealed that both types of stress act differently to impair photosynthetic activity and are recognized as two different stimuli by *Synechocystis* cells. Thus, it became of great importance to examine whether the phenotypical aberrations (*i.e.,* inhibition of cells division, increase in cell size and subsequent cell lysis) in Δ*ggpS* cells are caused by the ionic effects or by the osmotic effects of NaCl stress. For this two different concentrations of sorbitol were used, namely 450 mM and 900 mM. 450 mM sorbitol was used because it inhibits the growth of wild-type cells in the same manner as 450 mM NaCl. 900 mM was used because it has approximately the same osmotic effect as 450 mM NaCl. Results indicated that osmotic stress due to 900 mM sorbitol totally and similarly arrested cell growth and cell division in both wild-type and Δ*ggpS* cells. In addition, osmotic stress slightly decreased cell size (10~20%), and did not induce cell lysis in both wild-type and Δ*ggpS* cells. 450 mM sorbitol did not arrest the growth and cell division in both type of cells. Morever, no increase in cell size was found. These results suggested that the phenotypic aberrations observed in Δ*ggpS* are due to the ionic effects of NaCl stress and that GG does not provide protection against osmotic stress.

5.2. Effect of osmotic stress on cell count of wild-type and Δ*ggpS* cells

Osmotic stress due to 900 mM sorbitol, which has approximately the same osmotic effect as 450 mM NaCl, completely and similarly arrested the growth of both wild-type and Δ*ggpS* mutant cells which was measured by optical density at 730 nm. In addition, the cell density of cultures of wild-type and Δ*ggpS* cells, as determined by flow cytometry, did not increase in response to 900 mM sorbitol, suggesting that cell division was totally arrested (Table X). In addition, 450 mM sorbitol reduced partially and similarly the growth of wild-type and Δ*ggpS* cells (Fig. 16).

5.3. Effect of osmotic stress on cell size of wild-type and Δ*ggpS* cells

While 450 mM NaCl caused a clear increase in the size of Δ*ggpS* cells, we observed a slight decrease (10~20%) in the size of wild-type and Δ*ggpS* cells due to incubation with 900 mM sorbitol, when we examined cells by flow cytometry (Fig 17). Similarly, 450 mM sorbitol had no significant effect on cell size in both wild-type and Δ*ggpS* cells (Fig. 17). From these results we conclude that the effect of NaCl on cell division and cell size was probably due to the ionic effect but not to the osmotic effect exerted by NaCl.

In addition, osmotic stress due to sorbitol did not induce cell lysis. Since osmotic stress affected similarly the growth of wild-type and Δ*ggpS* cells, it is likely that GG has no effect in protecting cells against osmotic stress. The growth of wild-type cells was totally arrested by osmotic stress due to 900 mM sorbitol, suggesting that NaCl stress induces mainly ionic stress but not osmotic stress.

Table X. Effect of incubation with 900 mM sorbitol on the cell count of wild-type and Δ*ggpS* cells. Wild-type and Δ*ggpS* cells that had been grown in BG-11 medium were incubated in BG-11 medium with or without addition of 900 mM sorbitol. Cell count of was estimated by flow cytometry. Means and standard deviations were calculated from the results of three independent experiments.

	Cell count ($x10^7$ cells ml^{-1})			
	Wild type		Δ*ggpS*	
Day of incubation	control	900 mM sorbitol	control	900 mM sorbitol
0	0.80±0.10	0.82±0.07	0.80±0.10	0.79±0.06
1	3.70±0.39	0.83±0.03	3.76±0.12	0.79±0.05
2	13.22±1.03	0.81±0.08	13.44±1.31	0.78±0.07
3	22.12±1.66	0.86±0.10	22.48±2.11	0.83±0.11
4	30.14±2.12	0.87±0.07	29.53±2.54	0.88±0.10
5	38.32±2.56	0.91±0.11	37.92±2.75	0.90±0.15

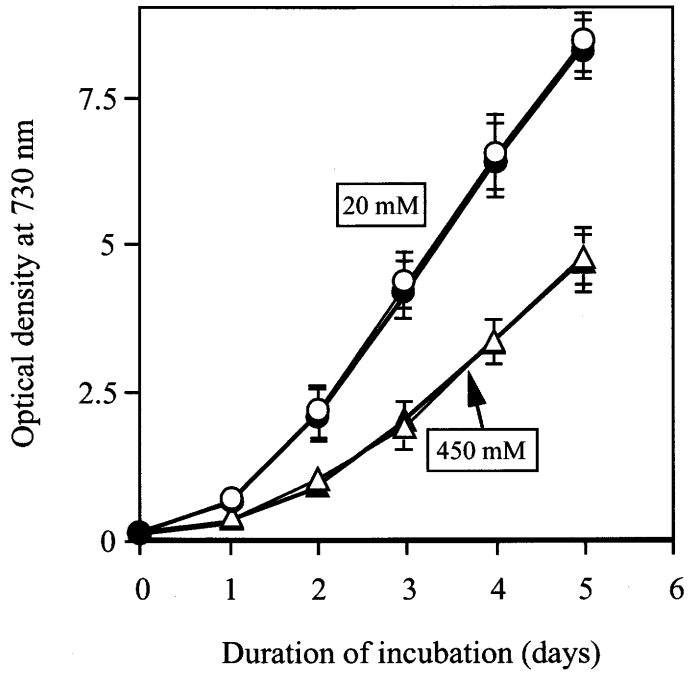


Figure 16. Effects of 450 mM sorbitolon the growth of wild-type and Δ*ggpS* cells. Cells that had been grown under normal conditions in BG-11 medium that contained 20 mM Na^+ ions were inoculated, at an optical density of 0.1 at 730 nm, in BG-11 medium (abbreviated as 20 mM NaCl) or in BG-11 medium that had been supplemented with sorbitol to a final concentration of 450 mM (abbreviated as 450 mM sorbitol). Growth was monitored in terms of optical density at 730 nm. Graphs show growth of wild-type (open circles) and Δ*ggpS* mutant (closed circles) cells in 20 mM NaCl and growth of wild-type (open triangles) and Δ*ggpS* (closed triangles) cells in the presence of 450 mM sorbitol. Data and error bars were calculated from the results of three independent experiments.

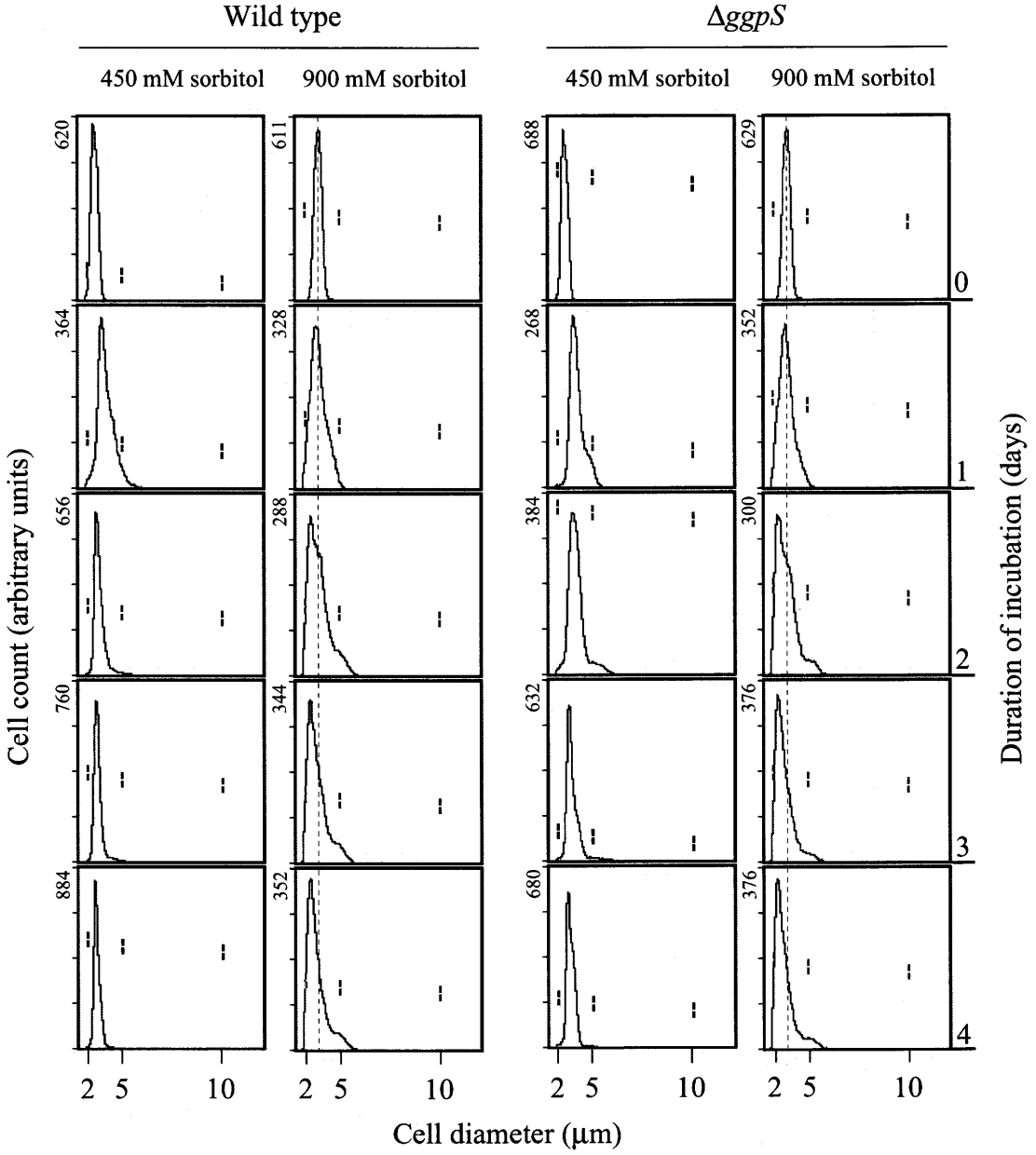


Figure 17. Effects of osmotic stress on cell size of *Synechocystis* cells. Wild-type and Δ*ggpS* cells that had been grown in 20 mM NaCl were incubated in medium that contained either 450 mM or 900 mM sorbitol. Aliquots of both types of cell were withdrawn and the distribution of cell sizes was analyzed by flow cytometry after appropriate dilutions. Arbitrary scales are used to provide a better view of the distribution of cell sizes. Numbers in the top-left part of plots represent the cell count of each plot but do not have a quantitative meaning for the cell density of cultures during the 4-day time course. Vertical dashed lines represent the initial sizes of wild-type and Δ*ggpS* cells before salt and osmotic stress. The three pairs of vertical dashes represent the positions of size markers with diameters of 2, 5 and 10 μm.

Chapter 6

General discussion and conclusions

6.1. Inhibition of cell division and induction of cell lysis by salt stress

In this study we found that Δ*ggpS* mutant cells, which were unable to synthesize GG, were sensitive to salt stress due to 450 mM NaCl. At this concentration, NaCl arrested cell division but did not inhibit cell growth. As a result, cell size increased significantly during salt stress for three days, then cells started to lyse. Osmotic stress due to 900 mM sorbitol also prevented cell division, inhibited cell growth but did not induce cell lysis. Thus, salt stress and osmotic stress had different effects on the proliferation of Δ*ggpS* cells of *Synechocystis*. Indeed, DNA microarray analysis in a previous study indicated that salt stress and osmotic stress are recognized by *Synechocystis* cells as different stimuli and induce the expression of different sets of genes (Kanesaki et al., 2002). Furtheremore, a similar effect of salt stress on cell size was observed previously in the halotolerant eubacterium *Staphylococcus aureus* (Vijaranakul et al., 1995).

To date, little attention has been paid to the morphological changes that occur in *Synechocystis* cells under stress conditions. After careful examination of cellular ultrastructure, we identified structure which had not been reported previously. We named it "*division ring-like structures*", the formation of which was specifically induced by NaCl stress (Fig. 9).

After incubation for three days in the presence of 450 mM NaCl, Δ*ggpS* cells were so fragile that we sometimes observed cells lysing during examination by optical microscopy. Although we have no direct experimental evidence for a specific mechanism of cell lysis, it is possible that NaCl might somehow change the structure of peptidoglycans by decreasing the degree of cross-linking, as previously suggested for the cell lysis in *Staphylococcus*

aureus (Vijaranakul et al., 1995). However, since Δ*ggpS* cells dramatically increased in size after three days of salt stress, (Figs. 6D and 6E), it is likely that the lysis of Δ*ggpS* cells might be attributable in part to weakened cell walls (*i.e.,* cellular envelopes including peptidoglycan layers) and the swelling of Δ*ggpS* cells that was induced by the active biosynthesis of macromolecules such as proteins, DNA and chlorophyll (Table VIII).

Bacterial cells usually divide by generating a central septum across the middle of the mother cell (for reviews, see Bramhill, 1997; Rothfield et al., 1999; Vollmer and Holtje, 2001). Recent studies indicate that the biosynthesis and the assembly of a fairly complicated protein complex is required at the site of division for orchestration of the division into daughter cells. Several of our electron micrographs revealed the formation of incomplete septa (Fig. 9), suggesting that NaCl might act to arrest the formation of the septum and the separation of daughter cells. It is also likely that the high concentration (450 mM) of NaCl inhibited the correct assembly of such a protein complex, thereby inhibiting cell division.

The results of flow cytometry demonstrated clearly that Δ*ggpS* cells under salt stress were highly heterogeneous with respect to size, ranging from 2 μm to 10 μm in diameter (Fig. 8D). Since we used non-synchronized cultures of Δ*ggpS* cells, this heterogeneity might have been due to differential effects of NaCl on cells at various stages of the cell cycle. It will be of interest to identify the stage in the cell cycle at which stress exerts its effects.

6.2. Glucosylglycerol counteracts the effects of salt stress

Ionic homeostasis within the cell is disturbed by salt stress and such stress may be

ultimately toxic. Upon exposure to salt stress, *Synechocystis* cells synthesize GG, which reaches a maximal level within 8 h (Marin et al., 1998, 2002 and Fig. 11). This rapid response to NaCl stress, in combination with the exclusion of Na^+ ions by Na^+/H^+ antiporters (Reed et al., 1985; Inaba et al., 2001; Elanskaya et al., 2002), protects wild-type cells from the toxic effects of excess Na^+ ions. Thus, wild-type cells are able to proliferate in the presence of high concentrations of NaCl (Reed and Stewart, 1985). However, the Δ*ggpS* null mutant lacked this acclimative response to NaCl stress. The negative effects of NaCl on several metabolic pathways, including photosynthesis (Allakhverdiev et al., 2002; Marin et al., 1998), are much more serious in Δ*ggpS* cells than in wild-type cells.

The structural aberrations and morphological abnormalities induced by NaCl stress (Figs. 6 and 9) were efficiently reversed by the addition of GG to the growth medium (Fig. 13). In Δ*ggpS* cells, GG had an "*anti-NaCl effect*", returning cell size close to normal and stimulating both cell growth and cell division (Figs. 12 and 13 and Table IX). Moreover, addition of GG to cultures of Δ*ggpS* cells that had been stressed for three days by exposure to 450 mM NaCl induced the formation of tetrads (Fig. 13F), suggesting that GG might have stimulated specifically the formation of the cell septum. These findings suggest that GG might play an essential role in protecting the cell-division machinery under salt stress conditions. The mechanism(s) whereby GG protects cell division machinery remain(s) to be elucidated.

The timing of inclusion of GG in the growth medium after the onset of salt stress was also critical in the protection of Δ*ggpS* cells from salt stress (Figs. 12 and 13), suggesting that GG is able to rescue *Synechocystis* cells until a certain extent of NaCl-induced damage has occurred. However, when most cell functions are seriously damaged after incubation with NaCl for long time, GG can no longer efficiently reverse the damage caused by NaCl.

6.3. Different effects of osmotic stress and salt stress

Ionic stress due to NaCl disturbs ion homeostasis but NaCl also has an osmotic effect (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998). Therefore, we attempted to examine the effects of osmotic stress on the growth, division and size of *Synechocystis* cells. Osmotic stress due to 900 mM sorbitol, which has approximately the same osmotic potential as 450 mM NaCl, arrested the growth of both wild-type and Δ*ggpS* mutant cells in the same manner. In addition, the cell density of cultures of wild-type and Δ*ggpS* cells, as determined by flow cytometry, did not increase in response to 900 mM sorbitol, suggesting that cell division was inhibited (Table X). However, while 450 mM NaCl caused a clear increase in the size of Δ*ggpS* cells, we observed a slight decrease (about 10~20%) in the size of wild-type and Δ*ggpS* cells when they were incubated with 900 mM sorbitol (Fig. 16). The inhibitory effect due to 900 mM sorbitol on the growth of cells was much stronger than that of 450 mM NaCl, suggesting that NaCl stress is mainly due to the ionic but not the osmotic effect.

These results suggested that osmotic stress and salt stress act differently to impair the growth of *Synechocystis* cells. It became also evident that salt stress due to NaCl and osmotic stress due to sorbitol represent two different stimili, thus, confirming the previous finding that we previously reported in the genome-wide gene expression (Kanesaki et al., 2002) and the inhibitory effect on photosystem II complexes (Allakhverdiev et al., 2000a, b). In fact DNA microarray analysis indicated that salt stress and osmotic stress regulate the expression of different sets of genes (Kanesaki et al., 2002), and salt stress and osmotic stress had different effects on photosynthetic activity of *Synechocystis* cells (Allakhverdiev et al., 2000a, b).

6.4. Qualitative differences between compatible solutes

During the last decade, compatible solutes have been the subject of intensive investigations (Chen and Murata, 2002). Most researchers used bacterial genes coding for biosythesis of compatible solutes to transform higher plants seeking for enhancement of their stress tolerance. On the other hand, it is interesting to understand how compatible solutes rescue cells from damage induced by stress conditions. Up to date, GB represents one of the most studied compatible solutes. However, provided that we think that all compatible solutes differ qualitatively, we found it very interesting to study other compatible solutes, such as GG, which did not receive as much attention as GB. This work represents a study in detail of the role of GG in the protection of cell division machiney in *Synechocystis*.

The outcome of this study suggested that the effects of GB on cell division in *Staphylococcus aureus* that was peviously described by Vijaranakul et al. (1995, 1997) is similar to that of GG in *Synechocystis* cells, as described herein. Thus, GG and GB might play similar roles in the protection of cell division. However, such does not seem to be the case for all compatible solutes. Thus, despite the high concentration of sucrose accumulated in Δ*ggpS* cells under salt stress, protection from salt stress was insufficient (Fig. 11). Moreover, trehalose which is accumulated as efficiently as GG by *Synechocystis* failed to protect Δ*ggpS* cells under salt stress conditions. These observations demonstrate that a qualitative difference should exist between various kinds of compatible solutes. Thus, sucrose, trehalose and GG should play different roles in protection against salt stress. Moreover, this observation suggests that compatible solutes are not accumulated simply for the osmotic pressure adjustment as some research groups still believe. If such hypoythesis

was correct, then the high level of sucrose accumulated in Δ*ggpS* should be enough to protect Δ*ggpS* cells from NaCl. Since sucrose was not protective, we suggest that sucrose and GG are qualitatively different and that the protection by compatible solutes is not accomplished by a simple adjustment of the osmotic potential between the extracellular and intracellular media. Thus, it seems that compatible solutes, such as GB and GG, actively interact with molecules or protein complexes and protect cellular functions of high importance such as translation machinery, photosynthetic complexes, and cell division machinery.

6.5. Hypothetical model for the effect of NaCl and GG on cell cycle of *Synechocystis*

The cell cycle and mechanism of cell division in *Escherichia coli* is well understood (For review, see Vollmer and Holtje, 2001) (Fig. 18). However, effects of various abiotic stresses such as salt stress on cell cycle has not been reported. The results found in this study suggested that salt stress inhibited specifically cell-division machinery which resulted in significant increase in cell size and subsequently induced cell lysis. Δ*ggpS* cells accumulated high levels of proteins, DNA and chlorophyll suggesting that the first step of cell division occurred normally, but cells were not able to separate. The uptake of GG from the medium restored cell division and cell size and protected Δ*ggpS* cells from lysis, suggesting that NaCl may act to inhibit cell septation and that GG probably protect this critical step of cell division in *Synechocystis* cells. These results are summarized in a hypothetical model shown in Figure 19.

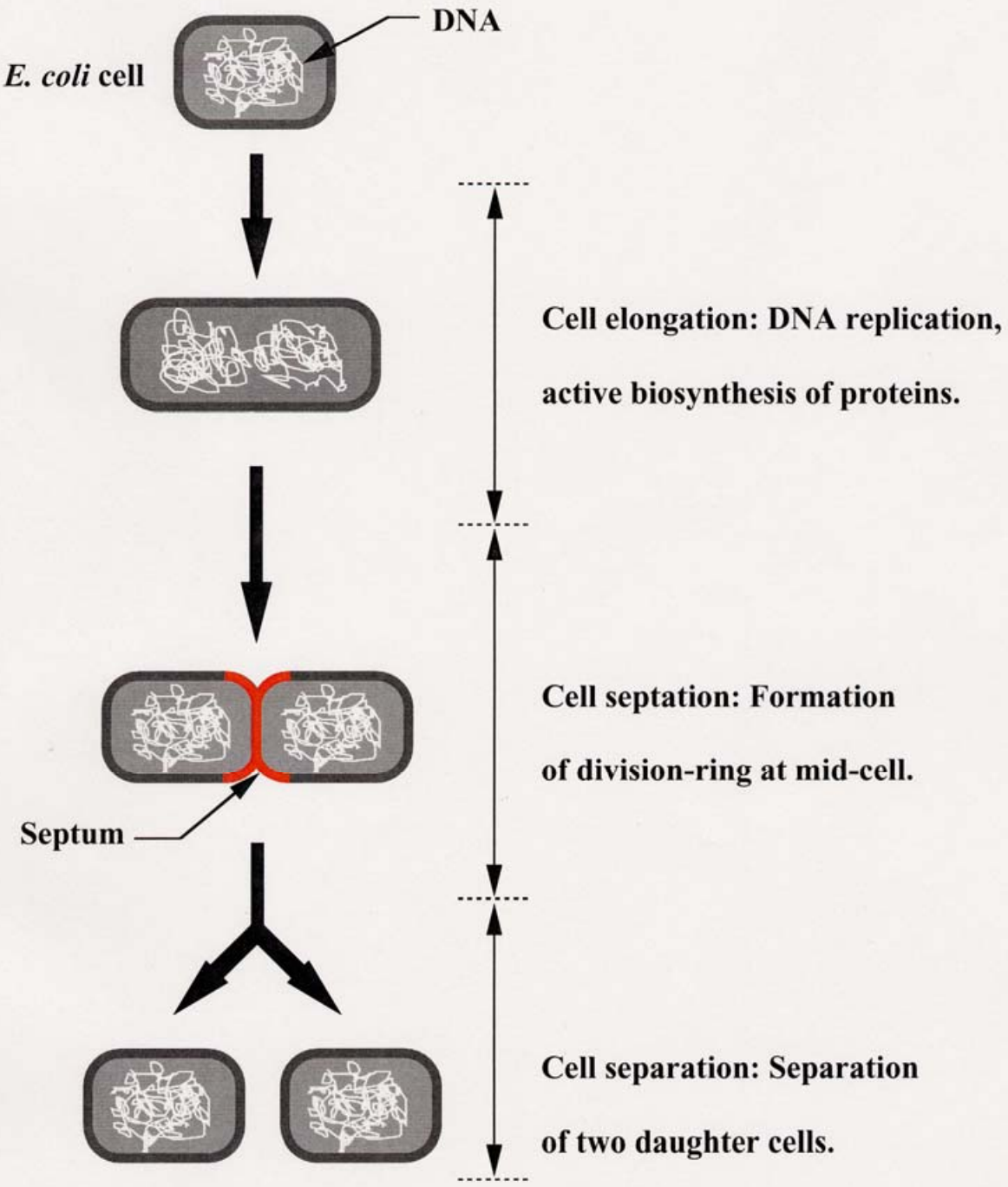


Figure 18. Model for cell cycle of *Escherichia coli* cells. Taken and modified from Vollmer and Holtje (2001).

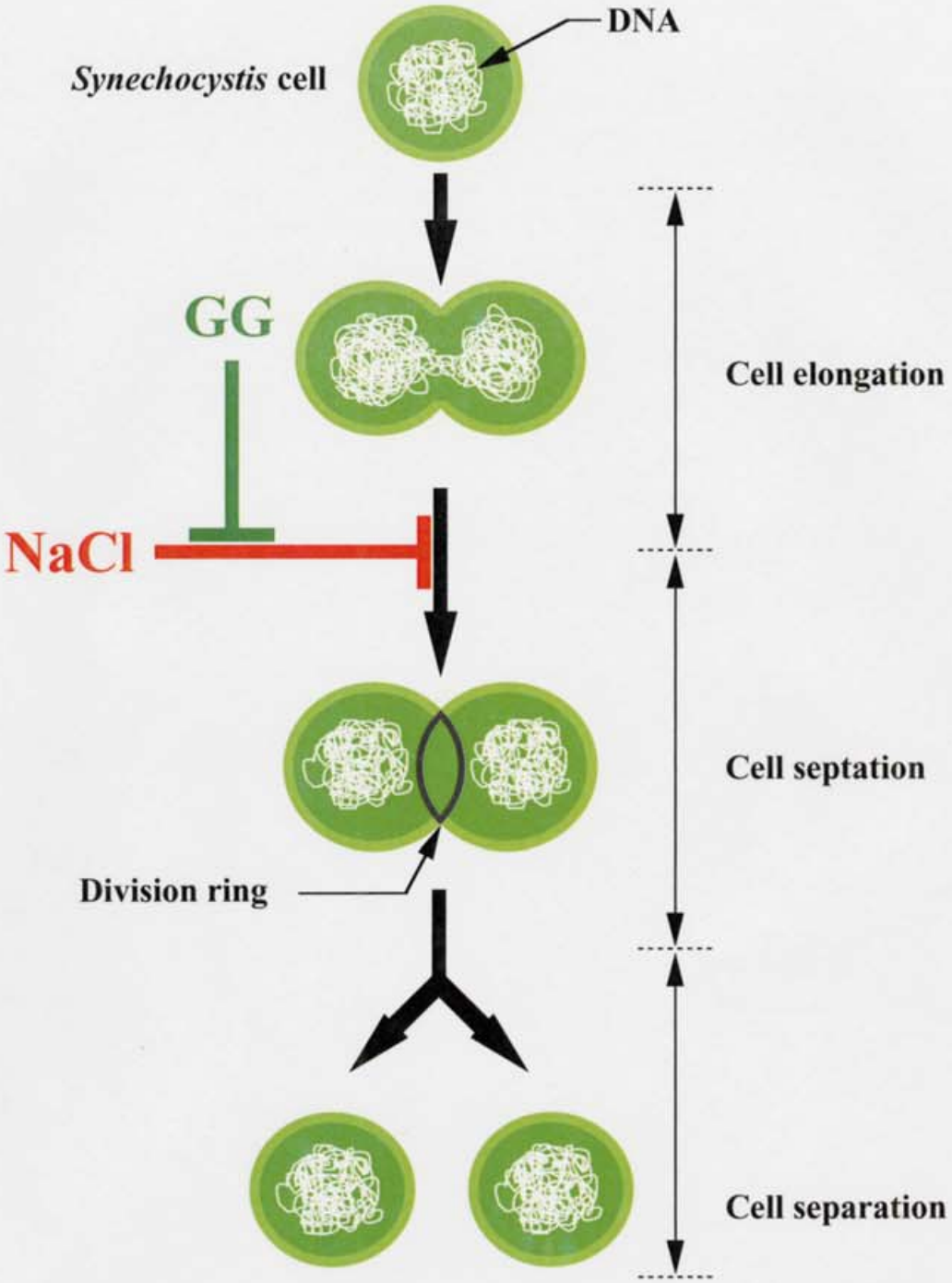


Figure 19. Hypothetical model for the effect of NaCl and glucosylglycerol on cell cycle of *Synechocystis* cells under salt stress conditions.

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Original papers

Ferjani A, Mustardy L, Sulpice R, Marin K, Suzuki I, Hagemann M, Murata N (2003) Glucosylglycerol, a Compatible Solute, Sustains Cell Division under Salt Stress. *Plant Physiol.* **131**:1628-1637.

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Glucosylglycerol, a Compatible Solute, Sustains Cell Division under Salt Stress[1]

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The cyanobacterium *Synechocystis* sp. PCC 6803 accumulates the compatible solute glucosylglycerol (GG) and sucrose under salt stress. Although the molecular mechanisms for GG synthesis including regulation of the GG-phosphate synthase (*ggpS*) gene, which encodes GgpS, has been intensively investigated, the role of GG in protection against salt stress remains poorly understood. In our study of the role of GG in the tolerance to salt stress, we found that salt stress due to 450 mM NaCl inhibited cell division and significantly increased cell size in Δ*ggpS* mutant cells, whereas the inhibition of cell division and increase in cell size were observed in wild-type cells at high concentrations of NaCl, such as 800 mM. Electron microscopy revealed that, in Δ*ggpS* cells, separation of daughter cells was incomplete, and aborted division could be recognized by the presence of a structure that resembled a division ring. The addition of GG to the culture medium protected Δ*ggpS* cells against salt stress and reversed the adverse effects of NaCl on cell division and cell size. These observations suggest that GG is important for salt tolerance and thus for the proper division of cells under salt stress conditions.

When a bacterium, such as *Escherichia coli*, is exposed to a sudden increase in the external concentration of NaCl, three major events occur. The first event is the rapid influx of Na^+ and Cl^- ions into the cytoplasm (Koch, 1984; Csonka, 1989); the second is the removal of Na^+ ions via the actions of Na^+/H^+ antiporters and the exchange of Na^+ for K^+ ions (Goldberg et al., 1987; Pinner et al., 1992; Ivey et al., 1993); and the third is the accumulation of compatible solutes, such as trehalose and Gly betaine, as a result of synthesis de novo or uptake (Vijaranakul et al., 1995; Kempf and Bremer, 1998; Record et al., 1998). These responses enable cells to exclude toxic cations and to acclimate to high concentrations of salt in the growth medium.

Cyanobacteria are prokaryotic microorganisms that perform oxygenic photosynthesis (Pfenning, 1978). Upon an upward shift in the concentration of NaCl in the medium, cells of *Synechocystis* sp. PCC 6803 accumulate glucosylglycerol (GG) as a major compatible solute and transiently accumulate traces of Suc, which enable cells to tolerate as much as 1.2 M NaCl (Reed and Stewart, 1985). *Synechocystis* sp. PCC 6803 cells synthesize GG-phosphate from ADP-Glc and glycerol 3-phosphate in a reaction catalyzed by GG-phosphate synthase (GgpS), and they dephosphorylate the intermediate GG-phosphate to yield GG in a reaction catalyzed by GG-phosphate phosphatase (Hagemann and Erdmann, 1994). GgpS and GG-phosphate phosphatase are encoded by the *ggpS* and *stpA* genes, respectively (Hagemann et al., 1997b; Marin et al., 1998). The *ggpS* gene has been cloned, and Δ*ggpS* mutant cells, which are defective in the *ggpS* gene, have been generated (Marin et al., 1998; Hagemann et al., 2001). Δ*ggpS* cells are sensitive to salt stress, and their tolerance threshold is below 0.3 M NaCl in liquid medium (Marin et al., 1998) and 0.425 M on agar plates (Karandashova et al., 2002). Wild-type cells can also take up GG from the medium via an ABC-type transport system, which is encoded by the *ggtA* gene and the *ggtBCD* gene cluster (Mikkat et al., 1996, 1997; Hagemann et al., 1997a; Mikkat and Hagemann, 2000). In an earlier study by DNA microarray analysis, we demonstrated that an increase in the concentration of NaCl to 0.5 M alters the expression of 375 genes, which include the *ggpS* gene (Kanesaki et al., 2002). The level of *ggpS* mRNA increases more than 10-fold during incubation in 0.5 M NaCl for 30 min.

In this study, we found that Δ*ggpS* cells were unable to divide when subjected to 450 mM NaCl stress.

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Furthermore, these cells almost doubled in diameter. Addition of GG to the growth medium reversed the effects of NaCl on both cell size and cell division. Our results suggest that salt stress inhibits cell division and that GG is crucial for successful cell division by *Synechocystis* sp. PCC 6803 under salt stress.

RESULTS

Growth of Wild-Type and Δ*ggpS* Cells under Salt Stress Conditions

We examined the effects of several different concentrations of NaCl (200–800 mM) in our investigations of the tolerance of wild-type and Δ*ggpS* mutant cells to salt stress. When the concentration of NaCl was lower than 300 mM, Δ*ggpS* cells were able to grow similarly to wild-type cells (data not shown). However, the growth of Δ*ggpS* cells in the presence of 450 mM NaCl was markedly retarded (Fig. 1), and at 500 mM NaCl, Δ*ggpS* cells did not grow at all (data not shown). By contrast, the growth of wild-type cells was not significantly affected at 450 mM NaCl (Fig. 1), but was seriously retarded at ≥800 mM NaCl (data not shown).

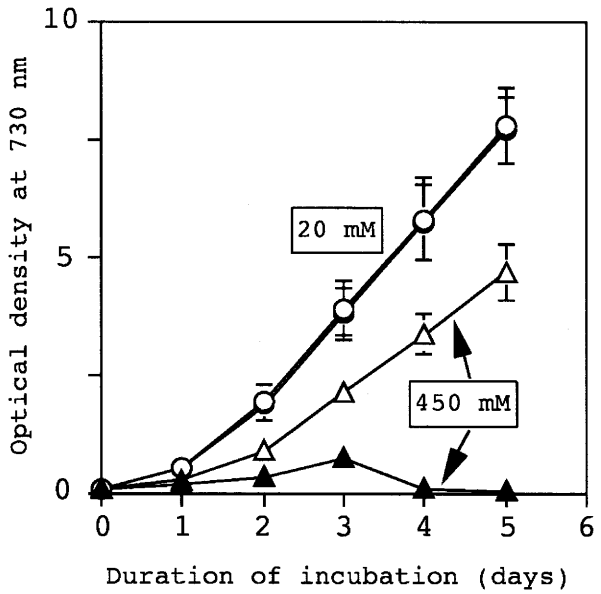

Figure 1. Effects of 450 mM NaCl on the growth of wild-type and Δ*ggpS* cells. Cells that had been grown under normal conditions in BG-11 medium that contained 20 mM NaCl were seeded at an A_{730} of 0.1 in BG-11 medium (abbreviated as 20 mM NaCl) or in BG-11 medium that had been supplemented with NaCl to a final concentration of 450 mM NaCl (abbreviated as 450 mM NaCl). Growth was monitored in terms of A_{730}. Graphs show growth of wild-type (○) and Δ*ggpS* mutant (●) cells in 20 mM NaCl and growth of wild-type (△) and Δ*ggpS* (▲) cells in the presence of 450 mM NaCl. Data and error bars were calculated from the results of at least five independent experiments.

Effects of Salt Stress on the Size of Cells

We examined the changes in shape of wild-type and Δ*ggpS* cells during incubation with 450 mM NaCl by optical microscopy (Fig. 2), and we detected no significant changes in the size of wild-type cells during incubation with 450 mM NaCl (Fig. 2, A and B). However, a significant increase in the diameter of wild-type cells was observed at 800 mM NaCl (Fig. 2C). On the other hand, the diameter of Δ*ggpS* cells almost doubled during incubation for 3 d in medium supplemented with 450 mM NaCl (Fig. 2, D and E). Furthermore, after 3 d, all of the Δ*ggpS* cells seemed to be in the process of dividing. These observations suggested that the increase in cell size might have been caused by inhibition of cell division. Salt stress due to 800 mM NaCl totally inhibited the growth of Δ*ggpS* cells (Fig. 2F). Therefore, to investigate the protection of Δ*ggpS* cells by GG, we used 450 mM NaCl as the salt stress in our subsequent experiments.

Effect of Salt Stress on Cell Density of Cultures

Flow cytometry analysis revealed that the cell density of culture of Δ*ggpS* cells did not increase during a 3-d incubation in the presence of 450 mM NaCl (Table I). This result confirmed that Δ*ggpS* cells were unable to divide under salt stress due to 450 mM NaCl. After more than 3 d, the cell density of the culture decreased rapidly (Table I, fifth column), suggesting that most of the Δ*ggpS* cells lysed.

We also examined the distribution of cell size by flow cytometry (Fig. 3). Whereas the size of wild-type cells increased slightly during incubation with 450 mM NaCl (Fig. 3, A and B), the size of Δ*ggpS* cells increased gradually, and the apparent diameter had approximately doubled at 3 d (Fig. 3, C and D). These observations suggest that 450 mM NaCl arrested cell division, with significant resultant enlargement of Δ*ggpS* cells. It is noticeable that the increase in cell size of Δ*ggpS* cells represented an increase in average cell diameter plus the formation of cell duplets.

Ionic stress due to NaCl disturbs ion homeostasis but NaCl also has an osmotic effect (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998). Therefore, we attempted to examine the effects of osmotic stress on the growth, division, and size of *Synechocystis* sp. PCC 6803 cells. Osmotic stress due to 900 mM sorbitol, which has approximately the same osmotic effect as 450 mM NaCl, arrested the growth of both wild-type and Δ*ggpS* mutant cells, which was measured by A_{730}. In addition, the cell density of cultures of wild-type and Δ*ggpS* cells, as determined by flow cytometry, did not increase in response to 900 mM sorbitol, suggesting that cell division was inhibited. However, whereas 450 mM NaCl caused a clear increase in the size of Δ*ggpS* cells, we observed a slight decrease (about 10%~20%) in the size of wild-type and Δ*ggpS* cells when they were incubated

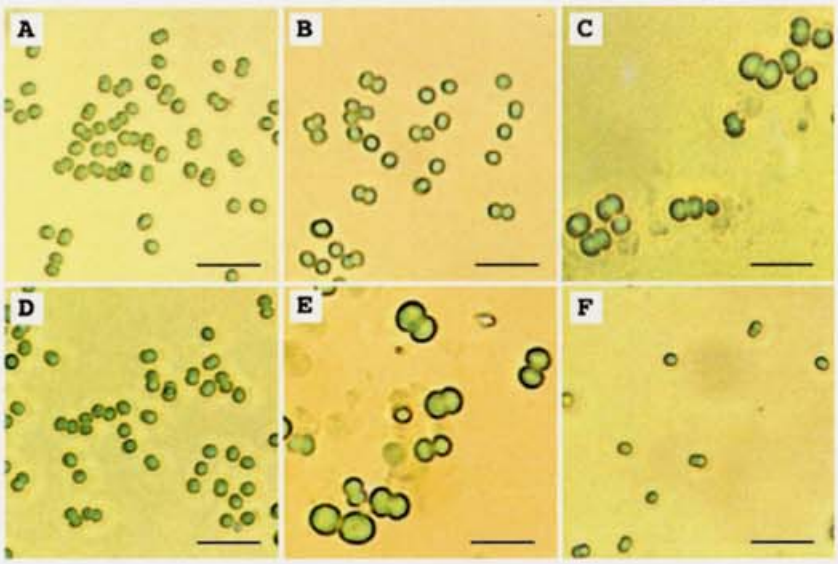

Figure 2. Effects of NaCl on the size of *Synechocystis* sp. PCC 6803 cells. Wild-type and Δ*ggpS* cells that had been grown in 20 mM NaCl were cultured in the presence of 20, 450, or 800 mM NaCl for 3 d. The other experimental conditions were the same as those described in the legend to Figure 1. Light micrographs show wild-type cells in 20 mM NaCl (A), wild-type cells in 450 mM NaCl (B), wild-type cells in 800 mM NaCl (C), Δ*ggpS* cells in 20 mM NaCl (D), Δ*ggpS* cells in 450 mM NaCl (E), and Δ*ggpS* cells in 800 mM NaCl (F). Bars = 10 μm.

with 900 mM sorbitol when we examined cells by optical microscopy and by flow cytometry.

Effects of Salt Stress on the Ultrastructure of *Synechocystis* Cells

To clarify the effects of salt stress in greater detail, we examined the ultrastructure of cells by transmission electron microscopy. The size and the ultrastructure of wild-type cells were not significantly affected by salt stress (Fig. 4, A and B). However, after incubation for 3 d in the presence of 450 mM NaCl, Δ*ggpS* cells were much larger than wild-type cells, and a structure that resembled a division ring was visible at the equator of cells (Fig. 4, C and D). Moreover, the presence of division ring-like structures, as shown in Figure 4D, suggested that NaCl might specifically inhibit the cell division machinery in Δ*ggpS* cells.

We next examined Δ*ggpS* cells that had been incubated with 450 mM NaCl for 4 d. Figure 4, E and F, shows that Δ*ggpS* cells were unable to complete cell division and lysed, leaving a division ring that adhered closely to the cell envelope. Figure 4G shows a cell that appears to have burst during preparation for electron microscopy. Figure 4H shows a triplet with an unusual division pattern, demonstrating again the dramatic effects of NaCl on the cell division machinery.

Effects of Salt Stress on Levels of Proteins, DNA, and Chlorophyll in Δ*ggpS* Cells

To determine the effects of salt stress on the biosynthesis of proteins, DNA, and chlorophyll, we examined the levels of these macromolecules during incubation with 450 mM NaCl. The results in Table II show that in 20 mM NaCl, levels of these macromolecules in Δ*ggpS* cells remained almost constant during incubation for 3 d. However, when Δ*ggpS* cells were incubated in 450 mM NaCl, levels of proteins, DNA, and chlorophyll per cell increased approximately 7-, 4-, and 4-fold, respectively (Table II). Under salt-stress conditions, Δ*ggpS* cells almost doubled in diameter, an increase that is equivalent to an ap-

Table I. *Effects of exogenous GG on the cell count of Δggps cells under salt stress*

Δ*ggpS* cells that had been grown with 20 mM NaCl were cultured with 20 or 450 mM NaCl in the absence or presence of 1 mM GG that was added to the medium 1, 2, or 3 d after the start of salt stress. Means and SDS were calculated from the results of three independent experiments.

Days of Incubation	Cell Count of Δ*ggpS* Cells				
	20 mM NaCl	450 mM NaCl			
	No GG	1 mM GG Added after			No GG
		1 d	2 d	3 d	
		10^7 mL^{-1}			
0	0.80 ± 0.10	0.81 ± 0.08	0.80 ± 0.08	0.79 ± 0.05	0.80 ± 0.03
1	3.76 ± 0.12	0.87 ± 0.04	0.82 ± 0.05	0.78 ± 0.04	0.79 ± 0.03
2	13.44 ± 1.31	1.10 ± 0.11	0.90 ± 0.11	0.83 ± 0.03	0.82 ± 0.02
3	22.48 ± 2.11	3.03 ± 0.01	1.82 ± 0.01	1.00 ± 0.02	0.92 ± 0.03
4	29.53 ± 2.54	7.85 ± 0.33	6.11 ± 0.13	0.17 ± 0.30	0.055 ± 0.008
5	37.92 ± 2.75	11.0 ± 0.10	9.17 ± 0.14	0.62 ± 0.05	0.016 ± 0.009

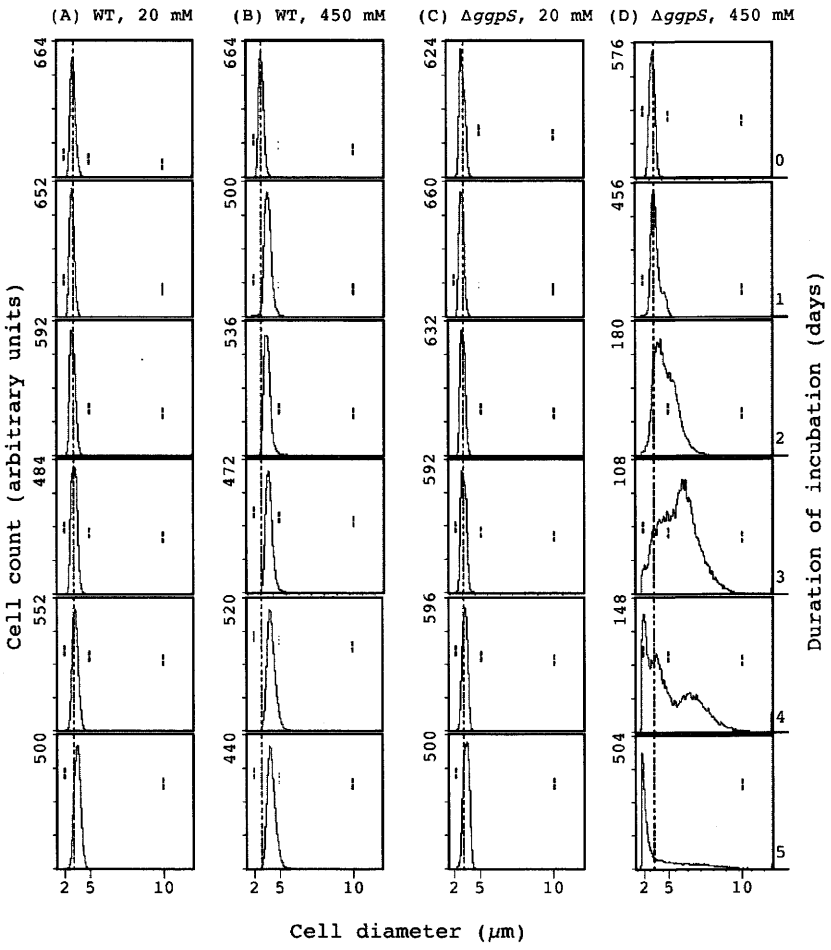

Figure 3. Effects of 450 mM NaCl on the distribution of the sizes of *Synechocystis* sp. PCC 6803 cells, as determined by flow cytometry. Wild-type and Δ*ggpS* cells that had been grown with 20 mM NaCl were cultured in the presence of 20 or 450 mM NaCl. Aliquots of both types of cell were withdrawn, and the distribution of cell sizes was analyzed by flow cytometry after appropriate dilutions. A, Wild-type cells in 20 mM NaCl; B, wild-type cells in 450 mM NaCl; C, Δ*ggpS* cells in 20 mM NaCl; and D, Δ*ggpS* cells in 450 mM NaCl. Arbitrary scales have been used to provide a better view of the distribution of cell sizes. Numbers in the top left part of plots represent the cell count of each plot but do not have a quantitative meaning for the cell density of cultures during the 5-d time course. Vertical dashed lines represent the initial sizes of wild-type and Δ*ggpS* cells before salt stress. The three pairs of vertical dashes represent the positions of size markers with diameters of 2, 5, and 10 μm.

proximately 8-fold increase in cell volume. Taken together, the results suggest that the concentration of proteins in each cell remained almost constant, whereas concentrations of DNA and chlorophyll were reduced to about 50% under salt stress (Table II). It is noteworthy that the amount of DNA in each cell increased only 4-fold during incubation with 450 mM NaCl for 3 d, whereas the level of proteins increased in parallel with the increase in cell volume. Thus, although NaCl totally arrested the division of Δ*ggpS* cells, the biosynthesis of proteins, DNA, and chlorophyll was not totally inhibited.

Accumulation of GG and Suc under Salt Stress

Synechocystis sp. PCC 6803 cells accumulate GG and Suc in response to salt stress (Reed and Stewart, 1985; Marin et al., 1998; Hagemann and Marin, 1999). We examined the effects of the mutation in Δ*ggpS* cells on levels of GG and Suc during incubation in 450 mM NaCl. Figure 5A shows that the concentration of GG reached a maximum in wild-type cells 8 h after the onset of salt stress and remained constant thereafter. However, Suc accumulated transiently and at only a low level and had almost disappeared within 10 h. By contrast, Δ*ggpS* cells were unable to accumulate GG but synthesized and accumulated a much higher level of Suc than wild-type cells (Fig. 5B).

Effects of Exogenous GG on Cell Division

Although Δ*ggpS* cells are unable to synthesize GG in response to salt stress, they are capable in taking up GG from the medium via an ABC-type transport system, which is encoded by the *ggtA* and *ggtBCD* gene cluster (Mikkat et al., 1996, 1997; Hagemann et al., 1997a; Mikkat and Hagemann, 2000). Thus, we examined the effect of exogenously supplemented GG on both cell size and cell count of Δ*ggpS* cells.

Flow-cytometric analysis revealed that exogenously supplied GG at 1 mM reversed the effects of 450 mM NaCl on cell size (Fig. 6) and cell count (Table I). Figure 6D shows the distribution of Δ*ggpS* cells incubated with 450 mM NaCl for 5 d. When GG was added to the medium 24 and 48 h after the onset of salt stress, cells decreased significantly in size over the course of the next few days (Fig. 6, A and B). GG also increased cell count for as long as 5 d (Table I), and the results suggested that GG accelerated cell division and prevented cell lysis. How-

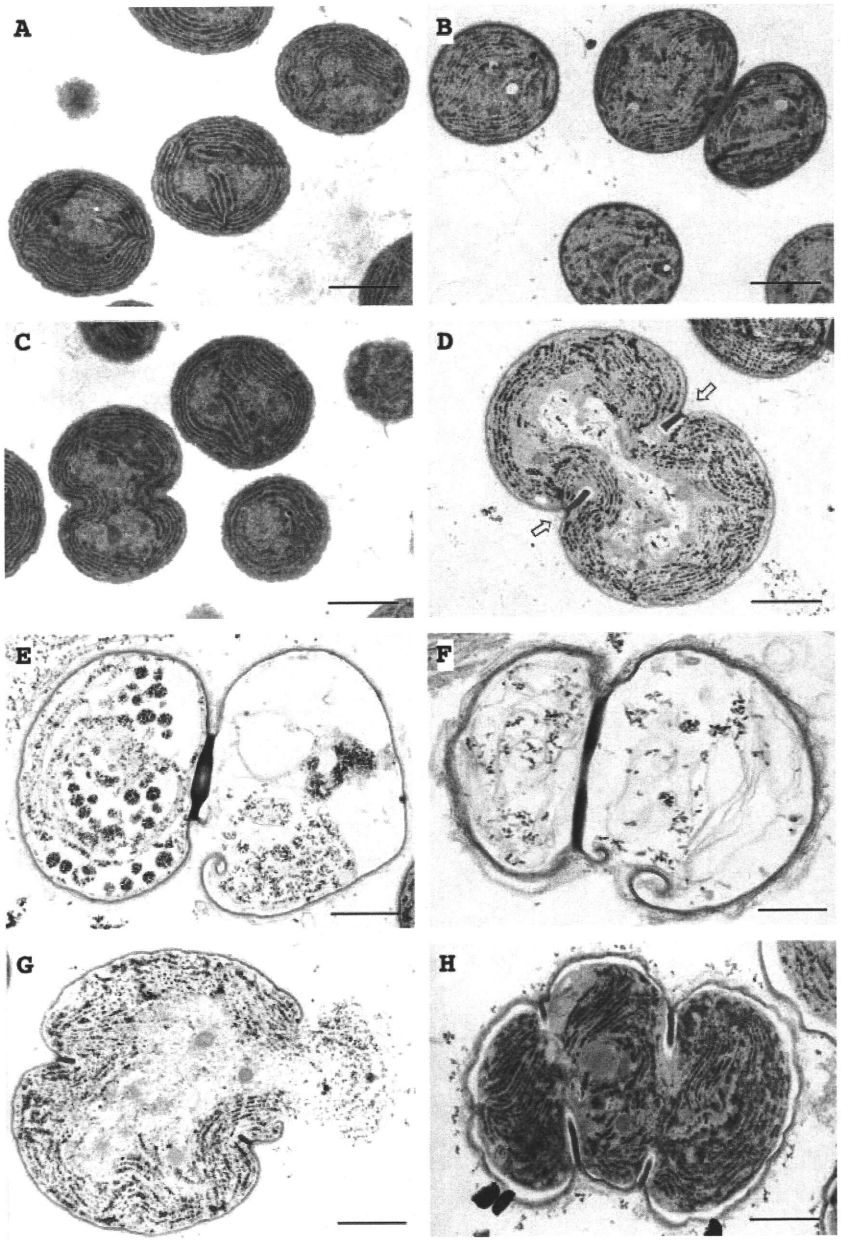

Figure 4. Effects of 450 mM NaCl on the ultrastructure of wild-type and Δ*ggpS* cells of *Synechocystis* sp. PCC 6803, as examined by electron microscopy. Wild-type and Δ*ggpS* cells that had been grown with 20 mM NaCl were cultured in the presence of 20 or 450 mM NaCl. A, Wild-type cells in 20 mM NaCl for 3 d; B, wild-type cells in 450 mM NaCl for 3 d; C, Δ*ggpS* cells in 20 mM NaCl for 3 d; and D, Δ*ggpS* cells in 450 mM NaCl for 3 d. Arrows in D indicate a division ring-like structure at the cell's equator. E through H, Δ*ggpS* cells in 450 mM NaCl for 4 d. Bars = 1 μm.

ever, when added at 3 d after the onset of salt stress, GG was only partially effective (Fig. 6C; Table I). Nonetheless, even in this case, cell lysis was somewhat retarded and Δ*ggpS* cells began to grow again 2 d after the addition of GG to the medium (Table I). Finally, we found that the presence of GG during incubation of wild-type cells with 450 mM NaCl had no effect at all.

We also examined the effects of exogenous GG on Δ*ggpS* cells by electron microscopy. When GG was added simultaneously with exposure of cells to salt stress (450 mM), the size and ultrastructure of Δ*ggpS* cells were unaffected (Fig. 7, A and B). These results suggested that the uptake of exogenous GG counteracted the effects of salt stress. Figure 7C shows that incubation with 450 mM NaCl for 48 h caused a significant increase in the size of Δ*ggpS* cells as compared with Δ*ggpS* cells that had been grown in the presence of 20 mM NaCl (see Fig. 7A). After addition of GG to medium that contained 450 mM NaCl for 2 d, cell size decreased significantly (Fig. 7D), suggesting that cell division resumed. This observation was in good agreement with the effect of exogenous GG on cell size, as determined by flow cytometry (Fig. 6; Table I). Finally, upon addition of GG to a culture of Δ*ggpS* cells that had been incubated with 450 mM NaCl for 3 d, we observed very large tetrads (Fig. 7, E and F). Although most cells lysed subsequently, cell lysis was retarded by the addition of GG, and tetrads might have represented an intermediate stage before the reinitiation of cell division.

Table II. *Effects of 450 mM NaCl on levels of proteins, DNA, and chlorophyll in ΔggpS cells*

ΔggpS cells that had been grown with 20 mM NaCl were cultured with 20 or 450 mM NaCl for 3 d. Aliquots of cultures were withdrawn at designated times, and levels of proteins, DNA, and chlorophyll were determined. Means and SDS were calculated from the results of three independent experiments.

Days of Incubation	Proteins		DNA		Chlorophyll	
	20 mM	450 mM	20 mM	450 mM	20 mM	450 mM
			$\mu g\ 10^{-7}$ cells			
0	0.98 ± 0.10 (1.00)[a]	0.98 ± 0.10 (1.00)	1.21 ± 0.30 (1.00)	1.33 ± 0.19 (1.00)	0.48 ± 0.02 (1.00)	0.48 ± 0.02 (1.00)
1	1.25 ± 0.18 (1.27)	1.42 ± 0.08 (1.44)	1.36 ± 0.52 (1.12)	1.61 ± 0.40 (1.21)	0.44 ± 0.03 (0.91)	0.47 ± 0.08 (0.97)
2	1.73 ± 0.08 (1.76)	1.83 ± 0.13 (1.86)	1.15 ± 0.50 (0.95)	3.08 ± 0.22 (2.31)	0.44 ± 0.04 (0.91)	0.79 ± 0.05 (1.64)
3	1.34 ± 0.14 (1.36)	6.96 ± 1.31 (7.10)	1.32 ± 0.47 (1.09)	5.71 ± 0.50 (4.29)	0.55 ± 0.05 (1.14)	2.16 ± 0.02 (4.50)

[a]Numbers in parentheses indicate increases relative to d 0.

DISCUSSION

Inhibition of Cell Division and Induction of Cell Lysis by Salt Stress

In wild-type cells, cell division and cell size were unaffected by NaCl at 450 mM, which completely inhibited cell division of Δ*ggpS* cells (Fig. 2, B and E). However, at high concentrations of NaCl, such as 800 mM, cell division was impaired, and the apparent cell size significantly increased in wild-type cells (Fig. 2C), which synthesize GG at about 110 mg mL^{-1} (Ritchie and Islam, 2001; Marin et al., 2002). These observations suggest that the high concentrations of NaCl inhibit cell division without significantly affecting cell growth in wild-type cells.

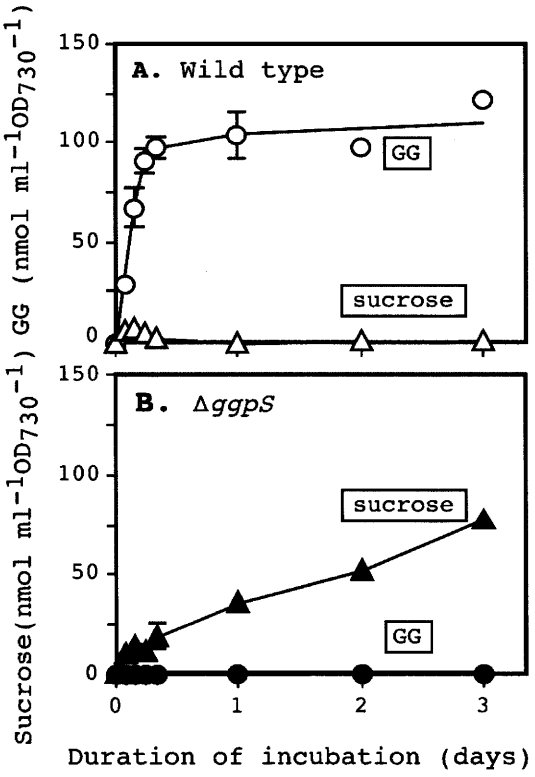

Figure 5. Effects of 450 mM NaCl on levels of GG and Suc in wild-type and Δ*ggpS* cells. Wild-type and Δ*ggpS* cells that had been grown in the presence of 20 mM NaCl were transferred to medium that contained 450 mM NaCl. A, Levels of GG (○) and Suc (△) in wild-type cells. B, Levels of GG (●) and Suc (▲) in Δ*ggpS* cells.

We found that Δ*ggpS* mutant cells, which were unable to synthesize GG, were sensitive to salt stress due to 450 mM NaCl. At this concentration, NaCl arrested cell division but did not inhibit cell growth. As a result, Δ*ggpS* cells increased significantly in size. After 3 d of salt stress, cells started to lyse. Osmotic stress due to 900 mM sorbitol also prevented cell division by inhibiting cell growth but not inducing cell lysis. Thus, salt stress and osmotic stress had different effects on the proliferation of Δ*ggpS* cells of *Synechocystis* sp. PCC 6803. In a previous study, our DNA microarray analysis indicated that salt stress and osmotic stress are recognized by *Synechocystis* sp. PCC 6803 as different stimuli and induce the regulation of expression of different sets of genes (Kanesaki et al., 2002).

To date, little attention has been paid to the morphological changes that occur in *Synechocystis* sp. PCC 6803 cells under stress conditions. After careful examination of cellular ultrastructure, we identified previously unreported structures that we named "division ring-like structures," the formation of which was specifically induced by salt stress (Fig. 4).

Although we have no direct experimental evidence for a specific mechanism of cell lysis, it is possible that NaCl might somehow change the structure of peptidoglycans. In fact, 2.5 M NaCl shortened peptidoglycan interpeptide bridge of *Staphylococcus aureus* (Vijaranakul et al., 1995). However, because after 3 d of salt stress, Δ*ggpS* cells had dramatically increased in size (Figs. 2E and 3D), it is likely that the lysis of Δ*ggpS* cells might be attributable in part to weakened cell walls and the swelling of Δ*ggpS* cells that was induced by salt stress.

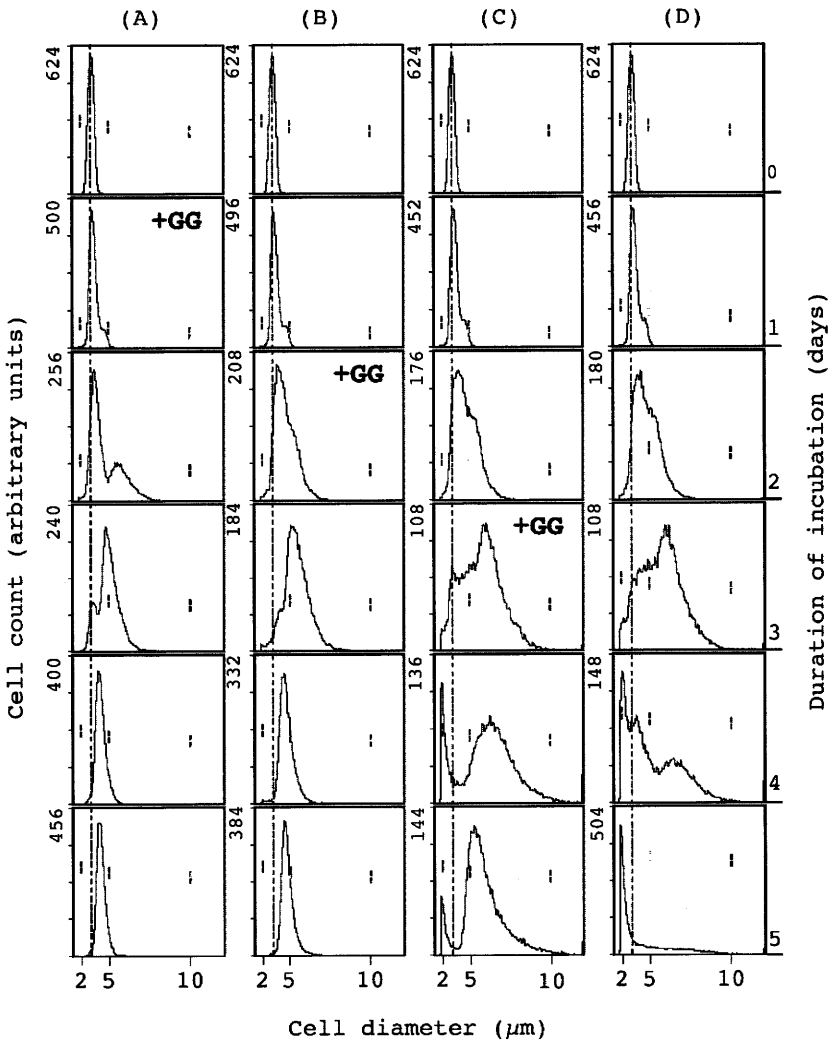

Figure 6. Effects of exogenous GG on the distribution of sizes of Δ*ggpS* mutant cells, as determined by flow cytometry. Δ*ggpS* cells that had been grown with 20 mM NaCl were cultured with 450 mM NaCl in the presence of exogenous GG, which was added to independent cultures 1 d (A), 2 d (B), or 3 d (C) after the onset of salt stress, or in the absence of GG (D). The sizes of cells were analyzed by flow cytometry, as described in the legend to Figure 3. Numbers in the top left part of plots represent the cell count of each plot but do not have a quantitative meaning for the cell density of cultures during the 5-d time course. Vertical dashed lines represent initial sizes of Δ*ggpS* cells before the onset of salt stress. The three pairs of vertical dashes represent the positions of size markers of 2, 5, and 10 μm in diameter.

Bacterial cells usually divide by generating a central septum across the middle of the mother cell (for reviews, see Bramhill, 1997; Rothfield et al., 1999). Recent studies indicate that the assembly of a fairly complicated protein complex is required at the site of division for orchestration of the division into daughter cells. Several of our electron micrographs revealed the formation of incomplete septa (Fig. 4), suggesting that NaCl might arrest the formation of the septum and the separation of daughter cells. It is also likely that the high concentration (450 mM) of NaCl inhibited the correct assembly of such a protein complex, thereby inhibiting cell division.

The results of flow cytometry demonstrated clearly that Δ*ggpS* cells under salt stress were highly heterogeneous with respect to size, ranging from 2 to 10 μm in diameter (Fig. 3D). Because we used nonsynchronized cultures of Δ*ggpS* cells, this heterogeneity might have been due to differential effects of NaCl on cells at different stages of the cell cycle. It will be of interest to identify the stage in the cell cycle at which stress exerts its effects.

GG Counteracts the Effects of Salt Stress

Ionic homeostasis within cells is disturbed by salt stress and such stress may be ultimately toxic. Upon exposure to salt stress, *Synechocystis* sp. PCC 6803 synthesizes GG, which reaches a maximal level within 8 h (Marin et al., 1998, 2002; Fig. 5). This rapid response to NaCl stress in combination with the exclusion of Na^+ ions by Na^+/H^+ antiporters (Inaba et al., 2001; Elanskaya et al., 2002) protects wild-type cells from the toxic effects of excess Na^+ ions. Thus, wild-type cells are able to proliferate in the presence of high concentrations of NaCl (Reed and Stewart, 1985). However, the Δ*ggpS* null mutant lacked this acclimative response to NaCl stress. The negative effects of NaCl on several metabolic pathways, including photosynthesis (Marin et al., 1998; Allakhverdiev et al., 2002), are much more serious in Δ*ggpS* cells than in wild-type cells.

The structural aberrations and morphological abnormalities induced by NaCl stress (Figs. 2 and 4) were efficiently reversed by the addition of GG to the

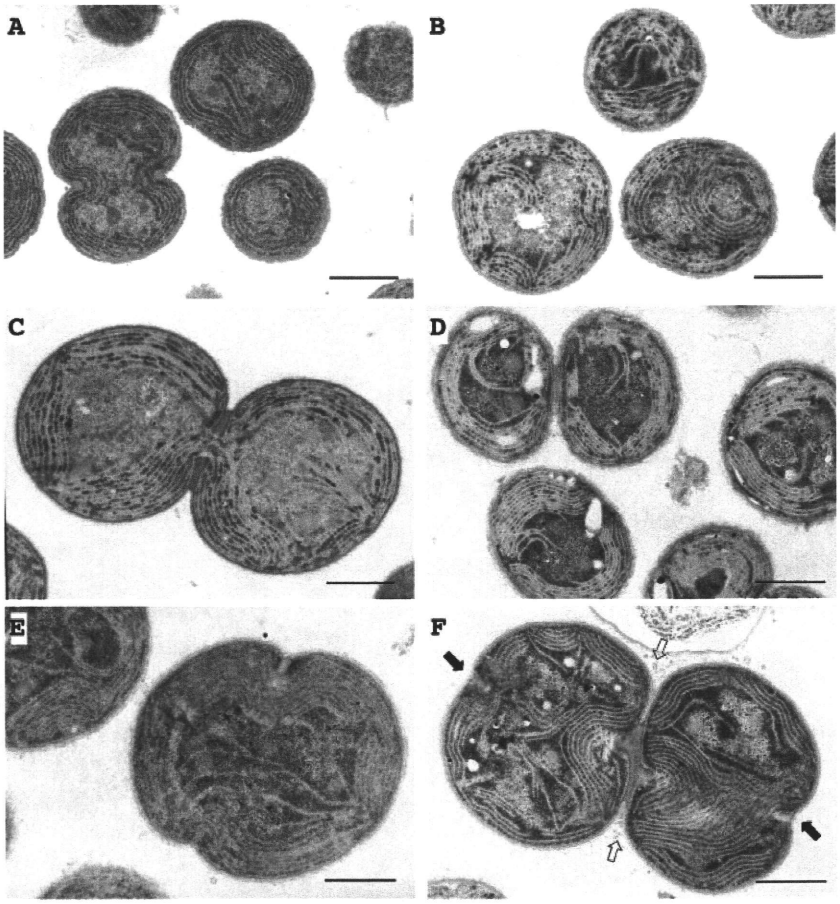

Figure 7. Effects of exogenous GG on the ultrastructure of Δ*ggpS* cells cultured with 450 mM NaCl. Δ*ggpS* cells that had been grown with 20 mM NaCl were incubated for 3 d with 20 mM NaCl (A) or with 450 mM NaCl plus 1 mM GG (B). Δ*ggpS* cells that had been cultured for 2 d with 450 mM NaCl (C) were cultured for 2 d further in the presence of 1 mM GG (D). Δ*ggpS* cells that had been cultured for 3 d with 450 mM NaCl (E) were cultured for a further 2 d in the presence of 1 mM GG (F). White arrows indicate the first septation site, and black arrows indicate the starting positions of the second septation site. Bars = 1 μm.

growth medium (Fig. 7). In Δ*ggpS* cells, GG had an "anti-NaCl effect," returning cell size to close to normal and stimulating both normal cell growth and cell division (Figs. 6 and 7; Table I). The mechanism(s) whereby GG sustains cell division and regulates cell size remains to be elucidated.

The timing of inclusion of GG in the growth medium after the onset of salt stress was also critical in the protection of Δ*ggpS* cells (Figs. 6 and 7), suggesting that GG is able to rescue *Synechocystis* sp. PCC 6803 until a certain amount of NaCl-induced damage has occurred. However, when most cell functions have been seriously damaged after a long incubation with NaCl, GG can no longer efficiently reverse the damage caused by NaCl.

The effects of salt stress on cell size and cell division of the halotolerant bacterium *S. aureus* was investigated in detail (Vijaranakul et al., 1995, 1997). Salt shock due to 2.5 M NaCl increased the cell size and inhibited separation of daughter cells. The addition of Gly betaine to the culture medium alleviated the adverse effects of salt stress by reducing cell size and accelerating cell division. These effects of Gly betaine on cell size and cell division are similar to that of GG in *Synechocystis* sp. PCC 6803 cells, as described herein. Thus, GG and Gly betaine might have similar effects on the regulation of cell size and might play similar roles in the protection of cell division. However, such does not seem to be the case for all compatible solutes. Thus, despite the high concentration of Suc accumulated in Δ*ggpS* cells under salt stress (Fig. 5), protection from salt stress was insufficient. These observations demonstrate a qualitative difference between the protective effects of Suc and those of GG and suggest that these compounds play different roles in protection against salt stress.

MATERIALS AND METHODS

Growth Conditions and Salt Stress

A Glc-tolerant strain of *Synechocystis* sp. PCC 6803 was kindly provided by Dr. J.G.K. Williams (DuPont de Nemours and Co., Wilmington, DE). The Δ*ggpS* mutant (previously designated ΔGK2) was produced as described previously (Marin et al., 1998). Wild-type and Δ*ggpS* mutant strains were cultured at 34°C in BG-11 medium (Stanier et al., 1971), buffered with 20 mM HEPES-NaOH (pH 7.6), which contained 20 mM NaCl. Cell cultures were bubbled with air containing 1% (v/v) CO_2 and under constant illumination at 70 $\mu E\ m^{-2}\ s^{-1}$ from incandescent lamps (Ono and Murata, 1981).

Salt stress was applied by adding an appropriate volume of a stock solution of 5 M NaCl to cultures to give a final concentration of 450 mM. Growth of cells was monitored by measuring changes in A_{730} using a spectrophotometer (model 200–20, Hitachi, Tokyo) after suitable dilution of aliquots from cell cultures.

Optical and Electron Microscopy

Optical microscopy was performed with a microscope (Axioskop FL, Carl Zeiss, Gottingen, Germany) that was equipped with a high-definition image-capture camera (model HC-1000, Fujix, Tokyo).

For electron microscopy, cells were pelleted by centrifugation at 3,000*g* for 5 min and then immediately fixed for 1 h with 2% (v/v) glutaraldehyde in 100 mM sodium phosphate (pH 7.2). After rinsing overnight in sodium phosphate buffer, samples were post-fixed in 1% (v/v) osmium tetroxide for 1 h before dehydration by passage through a graded ethanol series (50%–100%, v/v). Then samples were infiltrated with and embedded in resin (Araldite CY-212, Ouken, Tokyo). Thin sections were mounted on copper grids, stained with uranyl acetate, and examined under an electron microscope (model 1200EX, JEOL, Tokyo).

Flow Cytometry

For flow cytometry, aliquots of culture (1 mL) were withdrawn at 24-h intervals. Samples were analyzed with a flow cytometer (EPICS XL, Beckman Coulter, Miami). As size standards, we used 2-, 5-, and 10-μm polystyrene latex beads (Beckman Coulter), and excitation at 488 nm was provided by an argon-ion laser. For the determination of cell density of cultures, count fluorospheres (Beckman Coulter) were mixed with samples (1:1, v/v), and the system was programmed to stop the cell count at 30,000. All data were collected and analyzed with the cytometer's software (v3.0 EPICS XL System II, Beckman Coulter).

Quantitation of GG and Suc

Aliquots (4 mL) were withdrawn from cultures (A_{730} = 0.6) and cells were collected by centrifugation at 3,000*g* for 10 min at 4°C. Absolute ethanol (1 mL) was added to each pellet, and tubes were shaken vigorously for extraction of sugars. The ethanol was then evaporated on a centrifugal concentrator (model CC-101, Tomy, Tokyo). Dried pellets were suspended in distilled water. Sugars, amino acids, and organic acids were separated, and the sugar fraction was analyzed by gas chromatography, as described previously by Adams et al. (1999). Minor modifications and additions to the protocol were made to improve the separation of the various sugars.

Quantitation of Total Proteins, DNA, and Chlorophyll

For quantitation of proteins, 1 mL of cell suspension was supplemented with 0.1 g of trichloroacetic acid, and then the precipitate was collected by centrifugation at 15,000*g* for 10 min at 4°C. The pellet was suspended in 1 N NaOH. The suspension was boiled for 30 min, cooled, and then centrifuged at 15,000*g* for 5 min. The protein in the supernatant was quantitated as described by Lowry et al. (1951) with bovine serum albumin as the standard.

Concentrations of DNA were estimated as described by Labarca and Paigen (1980). For the quantitation of chlorophyll, cells in 1 mL of culture were collected by centrifugation at 15,000*g* for 10 min at 4°C. Pigments were extracted by suspending cells in 1 mL of a mixture of methanol:water (9:1, v/v). After removal of the precipitate by centrifugation at 15,000*g* for 5 min, chlorophyll in the supernatant was quantified in terms of A_{665} (Talling and Driver, 1961; Porra, 1991).

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Transgenics of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress

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Abstract Transgenic lines of *indica* rice were generated by *Agrobacterium*-mediated transformation with the choline oxidase (*codA*) gene from *Arthrobacter globiformis*. Choline oxidase catalyses conversion of choline to glycine betaine. Glycine betaine is known to provide tolerance against a variety of stresses. Molecular analyses of seven independent transgenic lines as performed by Southern, Northern and Western hybridization revealed integration and expression of the transgene as well as inheritance in the progeny plants. A good correlation was observed between levels of mRNA and protein accumulation, and a significant amount of choline oxidase product, i.e. glycine betaine, accumulated in R0 as well as R1 plants. Mendelian as well as non-Mendelian segregation patterns were obtained in the progeny plants. Challenge studies performed with R1 plants by exposure to salt stress (0.15 M NaCl) for 1 week, followed by a recovery period, revealed that in some cases more than 50% of the transgenic plants could survive salt stress and set seed whereas wild-type plants failed to recover.

Keywords Abiotic stress tolerance · Glycine betaine · *Indica* rice · Transgenics

Introduction

Increased tolerance against various osmotic stresses is one of the major objectives of plant biotechnology (Holmberg and Bülow 1998; Tyagi et al. 1999; Sakamoto and Murata 2001). Among various osmotic abiotic stresses such as drought, salinity and freezing, salinity is the most important factor limiting crop productivity. According to the United Nations Environment Program, nearly 20% of the World's agricultural lands are salt-stressed, especially in countries like India, Pakistan, sub-Saharan Africa and Australia where approximately one-third of the land may be adversely affected. Worldwide, about 10 million ha of irrigated land is abandoned because of excess salt deposition each year (Nelson et al. 1998). Higher plants from several families (e.g. Chenopodiaceae, Asteraceae) have evolved mechanisms such as the accumulation of osmoprotectants including amino acids, ammonium compounds and polyols/sugars to protect themselves under stress conditions. Glycine betaine, a quaternary ammonium compound, is a compatible solute which accumulates under periods of stress in higher plants (Robinson and Jones 1986; Rhodes and Hanson 1993; Yeo 1998; Sakamoto and Murata 2000, 2001; Tyagi and Mohanty 2000), animals (Garcia-Perez and Burg 1991) as well as bacteria (Csonka 1989). This is known to provide tolerance to the cells under stress by stabilizing the quaternary structure of the complex proteins and adjusting the osmotic potential in their cytoplasm to maintain water content. In photosynthesis it stabilizes both PSII complexes (Papageorgiou and Murata 1995), as well as Rubisco, at a high concentration of NaCl and at extreme temperatures (Gorham 1995). A two-step enzymatic process accomplishes production of glycine betaine in plants. The first step involves conversion of choline to betaine aldehyde by choline monoxygenase, a stromal enzyme with a Rieske-type [2Fe-2S] centre (Brouquisse et al. 1989), and the second step involves betaine aldehyde dehydrogenase, a nuclear-encoded chloroplast stromal enzyme, which converts it to glycine betaine (Weigel et al. 1986). However, an endogenous supply of choline is also important as shown by Nuccio et al. (1998) with transgenic tobacco plants expressing genes encoding choline monoxygenase enzyme. Earlier, transgenic *Arabidopsis*

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plants were obtained with the *codA* gene and it was shown to provide tolerance, not only against salt stress (Hayashi et al. 1997), but also against low temperature, freezing, high temperature as well as light stress (Alia et al. 1998, 1999; Sakamoto et al. 2000). The same group reported transformation of *japonica* rice with the *codA* gene (Sakamoto et al. 1998). For this purpose, the gene construct originally used for *Arabidopsis* transformation was modified by incorporating a rice *RbcS* transit peptide for chloroplast targeting (chl-COD). The other construct (cyt-COD) would result in accumulation of glycine betaine in the cytosol. PSII activity of both types of transgenic plants was tolerant to low temperature as well as salt stress. The potential of the gene, therefore, needs to be evaluated in other crops, as engineering of complex traits for abiotic stress resistance would be of great value (Christou 1994; Holmberg and Bülow 1998; Bajaj et al. 1999; Grover et al. 1999; McNeil et al. 1999; Sakamoto and Murata 2000, 2001; Tyagi and Mohanty 2000).

Rice is a salt-sensitive cereal crop and it cannot produce glycine betaine in response to abiotic stress. *Indica* rice contributes 80% of total rice production (Christou 1994). Earlier, we have reported an efficient protocol for generation of *Agrobacterium*-mediated transgenic *indica* rice of an elite variety Pusa Basmati 1 (Mohanty et al. 1999, 2000). In the present study, we report *Agrobacterium*-mediated transformation of Pusa Basmati 1 with the *codA* gene. A large number of morphologically normal and fertile transgenic plants was obtained. Detailed molecular, biochemical, genetic and physiological analyses were carried out with selected R0 as well as R1 transgenic plants.

Materials and methods

Agrobacterium-mediated transformation of Pusa Basmati 1

Agrobacterium-mediated transformation was carried out as described earlier (Mohanty et al. 1999, 2000). Briefly, scutella-derived calli were used for co-cultivation with *Agrobacterium tumefaciens* strain EHA101 (pGAH/*codA*, Hayashi et al. 1997). After removing excess bacterial cells, calli were selected on call-using medium containing hygromycin (50 mg/l) and cefotaxime (250 mg/l) or timentin (125 mg/l). It was observed that for removing EHA101 (pGAH/*codA*) cells, timentin was more effective. Further, calli growing on selection medium were excised and transferred to fresh selection medium. Proliferating calli were transferred to regeneration medium and incubated at 26 ± 2 °C, 16-h photoperiod for 2–3 weeks. Regenerated plants of 2–3 cm height were transferred to MSBH medium (Mohanty et al. 1999) for root formation. Rooted plants were transferred to pots containing a mixture of soil and soilrite (1:1) and were incubated in a growth chamber or a green house operating at 24–28 °C, 14–16 h light at 100–125 µmol m^{-2} s^{-1} and 70–75% relative humidity. The plants were regularly supplied with rice growth medium (Mohanty et al. 1999) and were grown to maturity.

Molecular analysis

Genomic DNA isolation and Southern hybridization

Genomic DNA isolation and Southern hybridization were performed as described earlier (Sambrook et al. 1989; Mohanty et al. 1999). Briefly, 5 µg of genomic DNA was digested with *Eco*R1 and resolved on a 1.2% agarose gel. The probe used was a 1.2-kb *Bam*H1/*Sac*I fragment of pGAH/*codA* encompassing the coding sequence of the *codA* gene. The probe was labeled with α-^{32}P [dATP] using a Megaprime labeling kit (Amersham Pharmacia Biotech UK limited, England). Hybridization was carried out at 42 °C in a solution containing 50% formamide, 5% SSC [20 × SSC (saline sodium citrate, 175.3 g l^{-1} of NaCl; 88.2 g l^{-1} of sodium citrate; pH 7.0)], 1 × Denhardt's solution, 10% dextran sulphate and denatured herring sperm DNA (200 µg/ml) for 24 h. Filters were washed at high stringency by gradually reducing the salt from 5% SSC and 0.1% SDS to 0.1% SSC and 0.1% SDS. The filters were wrapped in cling film and exposed to X-ray film for a suitable period before developing.

Northern analysis

RNA was isolated from green leaves essentially as described by Logemann et al. (1987) with minor modifications. RNA samples (20 µg) were run on a 1.2% agarose-formaldehyde gel using 1 × MOPS buffer (3[N-Morpholino]propanesulfonic acid, 20 mM of MOPS, pH 7.0; 2 mM of sodium acetate; 1 mM of EDTA, pH 8.0) and were transferred to Hybond-C membrane using 20 × SSC. The pre-hybridization solution contains 50% formamide (deionized); 5 × SSC; 50 mM of sodium phosphate buffer, pH 6.5; 250 µg ml^{-1} of herring sperm DNA (denatured); 10 × Denhardt's solution and the hybridization solution contains an additional 0.2 vol of 50% dextran sulphate (final concentration of dextran sulphate is 10%). Other steps such as preparation of a radiolabeled probe and its use, hybridization time and temperature, washing of the blot and exposure to X-ray film, essentially remained the same as for Southern analysis.

Western analysis

Western analysis was performed according to Arakawa et al. (1997) with suitable modifications. Clarified extract was prepared from leaf tissue (500 mg) from 6-month-old R0 plants and 1-month-old R1 plants. An aliquot of 5 µl of clarified extract was used for protein estimation (Bradford 1976). A total of 30 µg of total soluble protein was separated on 10% sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) at 25 mA, constant current, for 60–90 min in Tris-glycine-SDS buffer (25 mM of Tris; 250 mM of glycine, pH 8.3; 0.1% SDS). The separated proteins were blotted onto a PVDF membrane pre-wetted in 100% methanol and then equilibrated in protein transfer buffer, using a Hoeffer electroblotter at a 100 mA current overnight. Subsequently, the membrane was incubated in blocking solution [5% non-fat dry milk prepared in TBST buffer (10 mM of Tris, pH 7.5; 500 mM of NaCl; 0.05% Tween 20)] for 1 h at 40 rpm and room temperature. The membrane was given 3-washes with TBST buffer for 5 min each. The blot was incubated in TBST buffer containing 1% non-fat dry milk and a 1:3000 dilution of anti-rabbit antisera of choline oxidase (Hayashi et al. 1997) for 1 h at RT at 40 rpm. The membrane was washed with TBST buffer three times for 5 min each at 40 rpm and room temperature. The blot was probed with a secondary antibody (anti-rabbit HRP 1:7000 dilution) in TBST buffer containing 1% non-fat dry milk for 1 h at room temperature at 40 rpm, followed by 3-washes with TBST for 5 min each at 40 rpm and room temperature. The blot was developed using the ECL Plus chemiluminiscence kit from Amersham Pharmacia Biotech UK limited, England, as per manufacturer's instructions.

Salt stress studies

R1 seeds of the transgenic lines were grown on hygromycin selection medium. The resistant seedlings were transferred to rice growth medium (Mohanty et al. 1999) after they were 1-week-old under selection. Only hygromycin-resistant seedlings were assayed for salt sensitivity. Seedlings were grown on rice growth

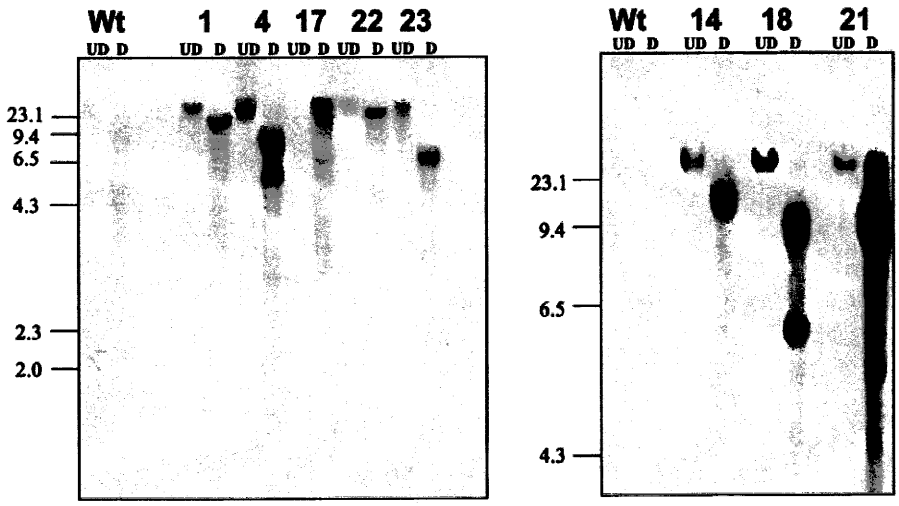

Fig. 1 Southern analysis of the R0 generation of PB1/*codA* transgenic rice plants. Undigested DNA (UD) or genomic DNA (5 μg) digested with *Eco*RI (D) was resolved on the gel. *Wt* represents the wild-type plant and *numbers* represent the identity of transgenic lines

medium for another 2 weeks at the end of which they were transferred to rice growth medium having 150 mM of NaCl. This salt stress was given for 1 week after which seedlings were allowed to grow on normal rice growth medium for another 3 weeks. They were subsequently transferred to soil for further growth and seed harvesting.

Analysis of glycine betaine content by NMR

The levels of glycine betaine and choline were determined in leaves of unstressed mature plants by NMR spectrometric analysis as described by Sakamoto et al. (1998). The analysis was performed in duplicate.

Genetic analysis

For genetic analysis of hygromycin resistance in the progeny, seeds of R0 and R1 plants were de-husked and sterilized, and were inoculated on MSB medium for 2 days (Mohanty et al. 1999). Germinated seedlings were transferred to MSB medium containing 50 mg l^{-1} of hygromycin. Hygromycin resistance/sensitivity was scored after 7 days.

Results

Rice transformation

From six independent transformation experiments with EHA101 (pGAH/*codA*), a total of 53 transgenic plants were obtained. Some of these plants were analyzed in detail, grown to maturity in the greenhouse and seeds collected.

Characterization of R0 plants

A total of eight independent transgenic plants were analyzed by Southern hybridization using the *codA* gene as a probe. Both single as well as multi-copy insertions were revealed (Fig. 1).

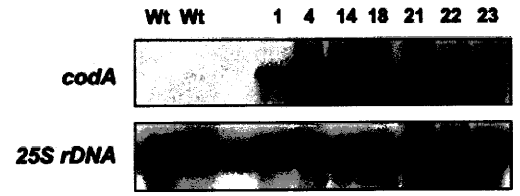

Fig. 2 Northern analysis of the PB1/*codA* gene in the leaves of R0 transgenic plants. The lower panel depicts RNA samples probed with 25S *rDNA* as a loading control. *Wt* represents the wild-type plant and the *numbers* represent the identity of transgenic lines

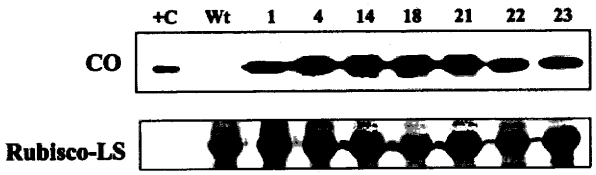

Fig. 3 Western analysis of R0 generation PB1/*codA* transformed rice lines. +C depicts choline oxidase from *Arthrobacter globiformis* as the positive control. *Wt* represents wild-type plants as a negative control. Numbers on the top depict the identity of transgenic lines. The CO panel shows a band of choline oxidase and the lower panel depicts a Rubisco large subunit as a loading control

Expression of the codA gene in transgenic plants

Northern-blot analysis of RNA samples from leaves of seven representative plants revealed variable but significant levels of the *codA* transcript. Transgenic lines 4, 14, 18 and 21 were found to express the transcript at very high levels, followed by transgenic lines 22 and 23. Transgenic line 1 was found to have low expression of the *codA* transcript. No hybridization was detected in wild-type plants (Fig. 2).

Western analysis with choline oxidase antiserum revealed the presence of a 64-kDa protein which corresponds to the choline oxidase protein (Fig. 3). No such

Table 1 Segregation analysis of hygromycin resistance in R1 progeny of PB1/*codA* transgenic rice plants

Plant identity	Copy no.	No. of seeds inoculated	No. of germinated seeds transferred to hygromycin	No. of seeds resistant to hygromycin	No. of seeds sensitive to hygromycin	χ^2 value
1	1	67	67	36	31	0.373 (1:1)[b]
4	5	69	66	31	35	0.242 (1:1)[b]
14	2	74	69	55	14	0.814 (3:1)[c]
18	5	68	65	49	16	0.005 (3:1)[a]
21	3	43	33	20	13	1.4 (1:1)[c]
22	1	68	66	37	29	0.96 (1:1)[c]
23	1	52	52	35	17	1.61 (3:1)[c]

[a] $0.95>p>0.80$; [b] $0.80>p>0.50$; [c] $0.50>p>0.20$

band was detected in wild-type plants. In addition, we also detected a faint band at the approximately 70-kDa region on long exposure. This could be a precursor of choline oxidase, as has been shown earlier by Hayashi et al. (1997), revealing thereby that the precursor protein containing a transit peptide was targeted to the chloroplast and processed correctly. A good correlation could be established between the transcript levels and protein levels in the respective plants. For example, plant lines 4, 14, 18 and 21, which show high levels of transcript, also show higher protein levels.

Glycine betaine accumulation

Glycine betaine levels were detected by employing proton-NMR studies in six lines (1, 14, 17, 18, 22 and 23). All of these showed accumulation of glycine betaine and also significant levels of choline. Similar data collected for R1 progeny plants are subsequently provided in this paper.

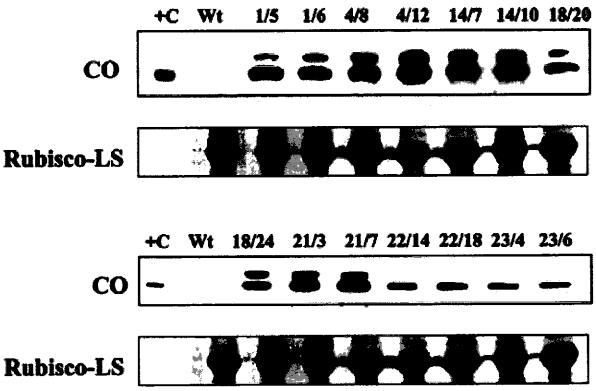

Fig. 4 Western analysis of R1 generation PB1/*codA* transgenic rice plants. +C is choline oxidase from *Arthrobacter globiformis* loaded as a positive control. *Wt* represents wild-type plants as a negative control. Numbers on the top depict the identity of the R1 generation transgenic plants from transgenic lines 1, 4, 14, 18, 21, 22 and 23. CO represents choline oxidase and Rubisco-LS represents a large subunit of Rubisco as a loading control

Genetic analysis of hygromycin resistance in the progeny of R0 plants

Seeds obtained from seven R0 plants were analyzed for segregation of the hygromycin resistance trait, as shown in Table 1. Mendelian as well as non-Mendelian segregation ratios were obtained. Interestingly, two single-copy plants segregated in a 1:1 ratio for hygromycin resistance. On the other hand, two transgenic lines with multiple copies of the transgene show a 3:1 segregation ratio for hygromycin resistance.

Analysis of R1 plants and their progeny

Expression of the codA gene in R1 transgenic plants

For Western analysis of progeny plants, we selected two R1 plants that were hygromycin-resistant from each of the seven independent R0 lines. As in R0 lines, the analysis of R1 plants revealed the presence of a 64-kDa protein which corresponds to choline oxidase (Fig. 4). No such band was detected in wild-type plants. In addition, we detected a band at about 70 kDa representing a precursor of choline oxidase, in lines showing higher levels of choline oxidase. Variable levels of protein were detected among various transgenic lines. Overall, the expression levels of R1 lines were similar to that of their parents.

Glycine betaine accumulation

NMR analysis was also performed with leaves of R1 transgenic plants for quantitation of accumulated glycine betaine. A total of seven R1 plants from seven independent lines were selected for NMR analysis. R1 transgenic line 21 accumulated the highest amount of glycine betaine (about 2.12 µmol/g of dry weight). Except for transgenic line 22 and 23, all other lines accumulated about 1 µmol/g dry weight of glycine betaine (Table 2). Interestingly, all lines also accumulated highly significant levels of choline, almost to the level of the wild-type.

Table 2 Determination of the concentration (μmol/g dry weight) of choline and glycine betaine in R1 generation PB1/*codA* transgenic rice plants

Plant identity	Choline in the R1 generation			Glycine betaine in the R1 generation		
	Experiment 1	Experiment 2	Average	Experiment 1	Experiment 2	Average
C	5.73	4.65	5.19 ± 0.54	0.00	0.00	0.00
1	4.06	3.04	3.55 ± 0.51	1.19	1.394	1.29 ± 0.1
4	5.35	7.54	6.45 ± 1.1	1.148	1.007	1.07 ± 0.07
14	5.16	3.04	4.10 ± 1.06	0.96	1.025	0.99 ± 0.03
18	4.33	3.14	3.74 ± 0.59	0.93	1.025	0.97 ± 0.04
21	5.65	4.12	4.89 ± 0.76	1.92	2.333	2.12 ± 0.21
22	5.45	3.93	4.69 ± 0.76	0.66	0.769	0.71 ± 0.05
23	5.06	3.49	4.27 ± 0.79	0.23	0.216	0.22 ± 0.007

Table 3 Agronomic traits of PB1/*codA* R1 generation transgenic plants recovered from salt stress and wild-type (WT) rice. Wild-type plants failed to recover after salt stress

Plant identity	NaCl 150 mM	No. of seedlings evaluated	No. of surviving plantlets	Number of surviving plants in two experiments grown to maturity	Average height (cm)	Average no. of panicles	Average no. of seeds
WT	–	17	17	17	88.3	10	388
WT	+	32	0	0	–	–	–
1	+	22	15	5	63.8	5	172
4	+	15	10	3	80.2	6	200
14	+	33	8	8	69.3	5	102
18	+	27	20	17	70.4	5	174
21[a]	+	10	5	3	55.0	5	100
22	+	21	11	8	71.8	6	166
23	+	17	5	5	72.8	6	172

[a] Result of one experiment only

Agronomic performance of transgenic lines under salt stress

When grown in liquid medium, 150 mM of NaCl is harmful to rice seedlings. However, several *codA* transgenic rice seedlings survived a 150-mM NaCl stress for a week before being transferred to normal rice growth medium. From two independent experiments, approximately 51% (74 out of 145) transgenic plants recovered well after exposure to salt stress. Out of the seven independent transgenic lines analyzed for their performance under salinity stress, four lines (1, 4, 18 and 22) showed high levels of stress tolerance (about 66% survival, 56 out of 85) against salt stress, whereas the wild-type plants failed to recover after exposure to salt stress and died. Some of the surviving seedlings were transferred to soil and have set seed (Table 3). A total of 49 plants were grown to maturity. In comparison to unstressed wild-type plants, transgenic plants exposed to salt stress gave rise to marginally shorter plants with a lesser number of panicles. The number of seeds obtained varied from approximately 100–200 seeds among various lines, whereas the unstressed wild-type plants yielded on average 388 seeds per plant. Obviously there is some degree of penalty on exposure to salt. However, the yield is significant keeping in view of the fact that all wild-type plants failed to recover on exposure to salt stress.

Genetic analysis of hygromycin resistance in progeny of R1 plants

Seeds from 3–5 R1 plants, representing the R2 generation, of six different R0 lines having 1–5 copies of the transgene were grown on hygromycin-containing medium. Among the various transgenic lines analyzed, seed germination was found to be 77.7 to 93.3%. As in the R1 progeny, Mendelian as well as non-Mendelian segregation patterns were obtained (data not shown). These plants are being grown for further analysis.

Discussion

Agrobacterium-mediated transformation of an elite *indica* variety Pusa Basmati 1 has been accomplished. Although the transformation efficiency obtained with binary vectors was less than that achieved with superbinary vectors (Mohanty et al. 1999, 2000), we could still obtain several transgenic plants for analysis of abiotic stress tolerance. This work, together with earlier reports of Hayashi et al. (1997) for *Arabidopsis* and Sakamoto et al. (1998) for *japonica* rice, conclusively proves that engineering for glycine betaine, as accomplished by a gene (*codA*) encoding for the enzyme choline oxidase, is an effective way for imparting stress

tolerance to non-accumulators such as rice. In addition, this work has been carried out utilizing a popular and economically important *indica* variety Pusa Basmati 1.

In contrast to the wild-type, all transgenic lines containing the *codA* gene showed the presence of glycine betaine, reflecting the fact that all the transgenic lines have acquired the ability to synthesize this compatible solute. Effectiveness of glycine betaine for salt and heat tolerance has also been shown by genetic mutation in maize by Saneoka et al. (1995) and Yang et al. (1996). Stable integration and faithful transmission of the transgene are of paramount importance in transgene technology. We have obtained Mendelian as well as non-Mendelian segregation ratios for the transgene(s). Similar observations have been made earlier (Komari et al. 1996; Mohanty et al. 1999). Interestingly, transgenic line 18 with five copies of the transgene segregated in a 3:1 ratio for hygromycin resistance, indicating single-locus integration of the transgenes.

Although the glycine betaine level obtained (0.22–2.12 μmol per gram of dry weight) for R1 lines was much lower than that of natural accumulators such as spinach and sugar beat, it was more than that reported earlier for transgenic tobacco (Holmström et al. 2000; see Sakamoto and Murata 2000, 2001), and even this much glycine betaine was capable of imparting a high level of salt tolerance as revealed by challenge studies. Holmstörm et al. (2000) reported that even 0.035 μmol/g fresh weight of glycine betaine was capable of imparting salinity and low temperature tolerance to transgenic tobacco plants. Unlike the apprehensions raised (Nuccio et al. 1998, 1999; McNeil et al. 1999), the availability of choline does not seem to be a problem in rice. Interestingly, the level of choline in transgenic plants was found to be only marginally different from wild-type plants even though part of it would have been used for the synthesis of glycine betaine. Probably the reason for this is the fact that the transgenic plants have started to produce glycine betaine, and the drive to reach homeostasis has resulted in more choline synthesis in the transgenic plants to be able to replenish choline utilized for glycine betaine synthesis. The level of expression of glycine betaine in transgenic plants points towards the stabilization of cellular structures and macromolecules rather than osmotic adjustments alone (Holmberg and Bülow 1998; Sakamoto et al. 1998; Sakamoto and Murata 2001). The agronomic performance of the transgenic plants under stress is significant. More than 50% plants of some transgenic lines survived on exposure to salt stress and yielded seeds, which could provide a significant yield gain under stress conditions. This could be of use for crop productivity in saline areas. To the best of our knowledge this is the first report involving transgenic *indica* rice plants with a *codA* gene and providing data on yield under salt stress.

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Characterization of the stromal protease(s) degrading the cross-linked products of the D1 protein generated by photoinhibition of photosystem II

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Abstract

When photosystem (PS) II-enriched membranes are exposed to strong light, cross-linking of the intrinsic D1 protein with the surrounding polypeptides and degradation of the D1 protein take place. The cross-linking of the D1 protein with the α-subunit of cytochrome b_{559} is suggested to be an early event of photoinduced damage to the D1 protein (Barbato et al., FEBS Lett. 309 (1992) 165–169). The relationship between the cross-linking and the degradation of the D1 protein, however, is not yet clear. In the present study, we show that the addition of stromal extract from chloroplasts degrades the 41 kDa cross-linked product of D1/cytochrome b_{559} α-subunit and enhances the degradation of the D1 protein. Incubation of the preilluminated PS II-enriched membranes with the stromal extract at 25°C causes the degradation of the cross-linked product by more than 70%. The activity of the stromal extract showed a pH optimum at 8.0, and was enhanced by the addition of ATP or GTP. Consistent with the nucleotide effect, this stromal activity was eliminated by the preincubation of the stromal extract with apyrase, which hydrolyzes nucleotides. Also, the stromal activity was nearly fully inhibited by a serine-type protease inhibitor, 3,4-dichloroisocoumarin, which suggests participation of a serine-type protease(s).

Keywords: D1 protein; Cytochrome b_{559}; Cross-linked product; Stromal protease; Photoinhibition; Photosystem II

1. Introduction

The intrinsic D1 and D2 proteins of the photosystem (PS) II complex play a central role in the photochemistry of PS II as the reaction center-binding proteins. The D1 protein, however, is labile

Abbreviations: PS II, photosystem II; D1 and D2, the reaction center-binding proteins of photosystem II; P680, the primary electron donor of PS II; Pheo, the primary electron acceptor of PS II; Tyr_Z, the secondary electron donor of PS II; ClpP and ClpA, prokaryotic ATP-dependent proteases; FtsH, a membrane-bound protease in bacteria; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; Bis-tris, bis(2-hydroxyethyl)imino-tris(hydroxyl)methane; tricine, *N*-tris(hydroxymethyl)methylglycine; CHES, cyclohexylaminoethanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid; 3,4-DCI, 3,4-dichloroisocoumarin; E-64, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido(4-guanidino)butane; ECL, enhanced chemiluminescence; CP43, the antenna chlorophyll-binding protein of photosystem II with molecular mass of 43 kDa

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under illumination where it is the main target of photoinhibition. The D1 protein is continuously damaged, degraded, and replaced by newly synthesized protein [1,2]. Consequently, its efficient turnover is crucial for maintaining functional PS II activity.

In regard to photoinhibition of PS II, two mechanisms have been proposed, namely acceptor-side photoinhibition and donor-side photoinhibition [1]. It has been suggested that singlet oxygen produced by the photochemical reactions in PS II under excessive illumination is responsible for the damage in the D1 protein in the acceptor-side mechanism [3]. This toxic oxygen species is generated by the reaction of molecular oxygen with the primary electron donor P680 in its triplet state, which is formed by the charge recombination between $P680^+$ and the reduced primary electron acceptor $Pheo^-$. Actually, the singlet oxygen was detected by the illumination of isolated PS II reaction center complexes with steady-state and time-resolved luminescence measurements [4] and chemical methods [5]. In the donor-side photoinhibition mechanism, endogenous cationic radicals such as $P680^+$ or Tyr_Z^+ are responsible for the damage to the D1 protein [6]. These radicals are generated by illumination under the conditions where the water oxidation system is not functioning properly for some reasons. Irrespective of the inhibition mechanism, specific proteases may degrade the photodamaged D1 protein [2]. As chloroplasts have a prokaryotic origin, they may have bacterial type proteases. Homologues of ClpP and ClpA proteases, and FtsH protease in *Escherichia coli*, have been identified in chloroplasts [7,8] and a possible role of the FtsH protease in the degradation of the D1 protein was suggested [9].

In addition to the degradation of the D1 protein, cross-linking of the D1 protein with nearby polypeptides takes place during photoinhibition of PS II by excessive illumination [10–13]. Among the photoinduced cross-linked adducts of the D1 protein, a 41 kDa band detected by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis with specific antibodies was shown to be the cross-linked product between the D1 protein and the α-subunit of cytochrome b_{559} [14]. In a later study, Barbato et al. showed that cross-linking takes place between the N-terminal serine of the α-subunit of cytochrome b_{559} and the 239Phe-244Glu region in the DE loop of the D1 protein [15]. In a more recent study, they used mutants of *Synechocystis* sp. PCC6803 having deletions in the DE loop of the D1 protein and observed that the D1 cross-linking site was different [16]. Although the cross-linking site of the D1 protein and the α-subunit of cytochrome b_{559} is not clearly established yet, the nature of the 41 kDa cross-linked product is characterized more than that of the other D1 adducts.

In our previous study, we showed that the cross-linked products of the D1 protein in PS II are removed by the incubation of the PS II-enriched membranes with a stromal extract at room temperature. These results indicate the presence of a protease(s) in the stroma that digests the cross-linked products [13]. The putative protease(s) in the stroma was resistant to SDS. Also, it recognized the cross-linked products of the D1 protein but not the native D1 protein. In the present study, we further characterized the stromal protease activity using the 41 kDa cross-linked products between the D1 protein and the α-subunit of cytochrome b_{559} as the substrate.

2. Materials and methods

2.1. Preparation of PS II-enriched membranes and illumination conditions

PS II-enriched membranes were prepared from spinach according to the method of Kuwabara and Murata [17] and stored at −80°C. Tris-treated PS II-enriched membranes were obtained by washing the membranes with a solution containing 0.8 M Tris-HCl and 3 mM EDTA (pH 9.3) as described previously [11]. Chlorophyll was determined in 80% acetone with the absorption coefficient reported by Mackinney [18]. For photoinhibition, the samples were suspended in a solution of 0.4 M sucrose, 10 mM NaCl and 40 mM MES-NaOH (pH 6.5) at a concentration of 0.5 mg chlorophyll ml^{-1} and put into microtubes. The microtubes were incubated in a circulating water bath at 25°C and illuminated with white light from a fluorescent lamp or from a slide projector (light intensity was 100–4000 μE m^{-2} s^{-1}). The optical path length of the sample suspensions was 17 mm. Where indicated, the samples were illu-

minated under anaerobic conditions, which was achieved by the addition to final concentrations of 0.1 mg ml^{-1} catalase, 0.1 mg ml^{-1} glucose oxidase, and 10 mM glucose to the samples.

2.2. Preparation of stromal extracts and protease assay conditions

Intact chloroplasts were isolated from spinach according to the method of Mullet and Chua [19]. Stromal extracts were prepared fresh before use from intact chloroplasts. Intact chloroplasts were suspended and lysed in a solution containing 5 mM $MgCl_2$ and 10 mM MES-NaOH (pH 6.5) at 2.0 mg chlorophyll ml^{-1}, incubated for 5 min on ice, and then centrifuged at $10000 \times g$ for 10 min. The supernatant was re-centrifuged at $40000 \times g$ for 1 h and the final supernatant was collected as the stromal extract. For pH dependence studies of the stromal protease activity, stromal extracts were prepared from intact chloroplasts which were suspended in solutions with different pH values (from pH 5.5 to 11.0) containing 5 mM $MgCl_2$ and 10 mM of Good's buffers (MES, pH 5.5–6.5; Bis-tris, pH 6.5–7.5; tricine, pH 7.5–8.5; CHES, pH 8.5–10.0; CAPS, pH 10.0–11.0). The effects of ATP and GTP were examined at final concentrations of between 2 and 10 mM of added nucleotide to the stromal extracts. Apyrase was added to 2 units/ml final concentration to stromal extracts and incubated for 30 min at 30°C for removal of endogenous nucleotides. In the experiments with protease inhibitors, 2–10 mM final concentration of either 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido(4-guanidino)butane (E-64), or EDTA, was added to stromal extracts, and incubated for 10 min at 30°C prior to use with PS II samples.

2.3. SDS/urea-polyacrylamide gel electrophoresis and Western blot analysis

SDS/urea-PAGE and Western blot analysis were carried out as described previously [11]. Polyclonal antibodies against the C terminus of the D1 protein, and the DE loop (25 amino acids from 225 Arg to 249 Val) of the D1 protein, were kindly provided by Dr. P. Nixon of the Imperial College of Science, Technology and Medicine, UK, and by Dr. M. Miyao-Tokutomi of the National Institute of Agrobiological Resources, Japan, respectively. Polyclonal antibodies against the α-subunit of cytochrome b_{559} were kindly provided by Dr. B. Andersson of the University of Stockholm, Sweden. Immuno-decorated proteins were detected by enhanced chemiluminescence (ECL) (Amersham, Japan) on X-ray film (Fuji, Japan). Densitometric measurements of the chemiluminescently produced bands on the X-ray films were quantified with a Personal Scanning Imager PD110 (Molecular Dynamics, USA).

3. Results

3.1. Formation of the 41 kDa cross-linked product of the D1 protein and the α-subunit of cytochrome b_{559}

Illumination of spinach PS II membranes with strong white light (intensity, 1500 μE m^{-2} s^{-1}) generates a 41 kDa cross-linked product of the D1 protein and the α-subunit of cytochrome b_{559} [14], cross-linked products with larger molecular masses (70–100 kDa) corresponding to the D1/D2 heterodimers and the D1/CP43 adducts [11,13]. Also, several degradation fragments of the D1 protein were generated. These products were detected by SDS/urea-PAGE and Western blot analysis with polyclonal antibodies against the DE loop of the D1 protein (Fig. 1A, left). The 41 kDa cross-linked product was also detected by the antibody against the α-subunit of cytochrome b_{559} (Fig. 1A, right), which confirmed the cross-linked products of the D1 protein and the α-subunit of cytochrome b_{559}. The immunodetected quantity of the 41 kDa adduct increased with increasing illumination times from 20 to 120 min. Formation of the 41 kDa adduct was also dependent on light intensity (Fig. 1B). The 41 kDa adduct was generated even under weak light, 100 μE m^{-2} s^{-1} for 30 min, and its level increased in proportion to the light intensity up to 4000 μE m^{-2} s^{-1}. Similar results were previously reported with PS II reaction center preparations [15], but cross-linking took place within a shorter illumination time.

The 41 kDa cross-linked product was detected only under aerobic conditions (Fig. 2). These results indicate that the adduct is formed by the action of

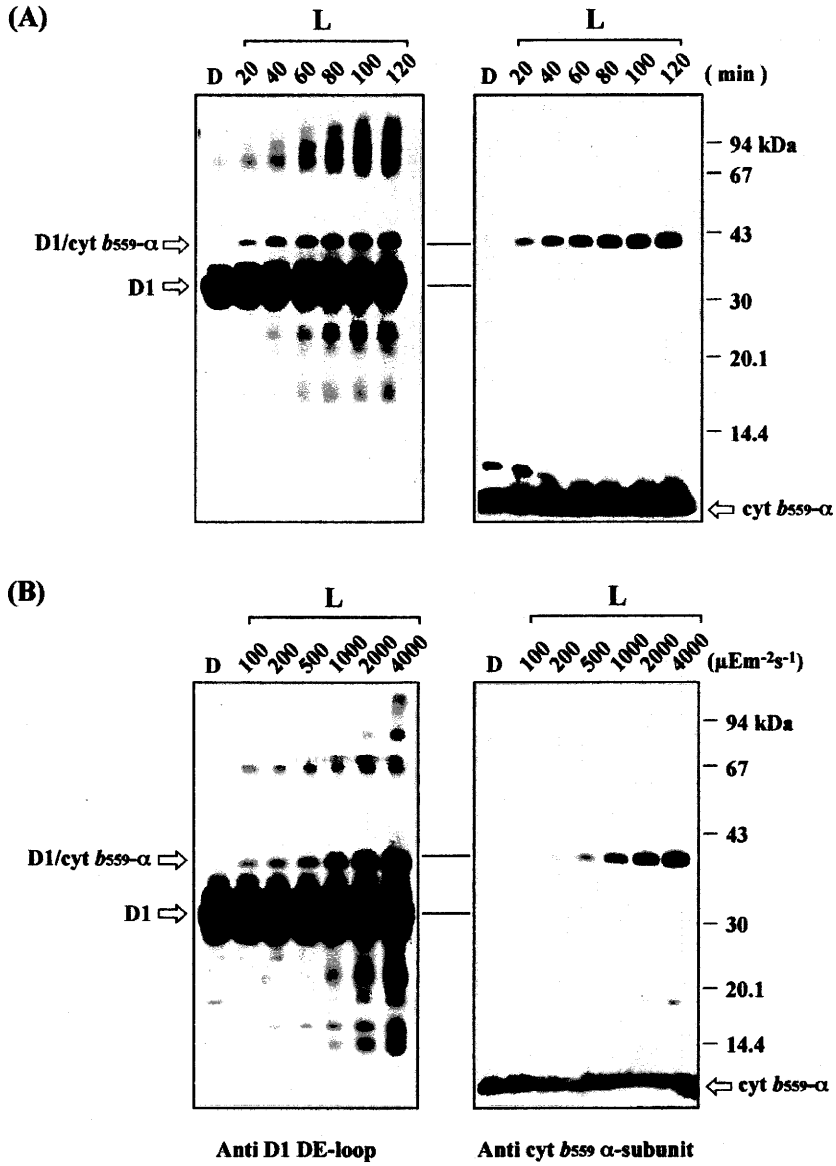


Fig. 1. Light-induced formation of the 41 kDa cross-linked adduct of the D1 protein and the α-subunit of cytochrome b_{559}. The effects of illumination time (A) and light intensity (B) are shown. The PS II-enriched membranes (0.5 mg chlorophyll ml^{-1}) under aerobic conditions were illuminated with white light (1500 μE m^{-2} s^{-1} in A and 100–4000 μE m^{-2} s^{-1} in B, respectively) for the periods indicated at the top of the gels (A) or for 30 min at 25°C at the light intensity shown at the top of the gels (B). The PS II membranes were then subjected to SDS/urea-PAGE. The samples equivalent to 2.5 mg chlorophyll were loaded to each lane of the gels. After that, Western blot analysis was carried out with antibodies against the DE loop of the D1 protein (left) and against the α-subunit of cytochrome b_{559} (right). The immuno-decorated bands were detected by fluorography with ECL. D and L denote the dark control and the illuminated samples, respectively. The bands of the D1 protein, the α-subunit of cytochrome b_{559}, and the 41 kDa cross-linked adduct are shown on both sides of the gels. Molecular markers are shown on the right side of the gels.

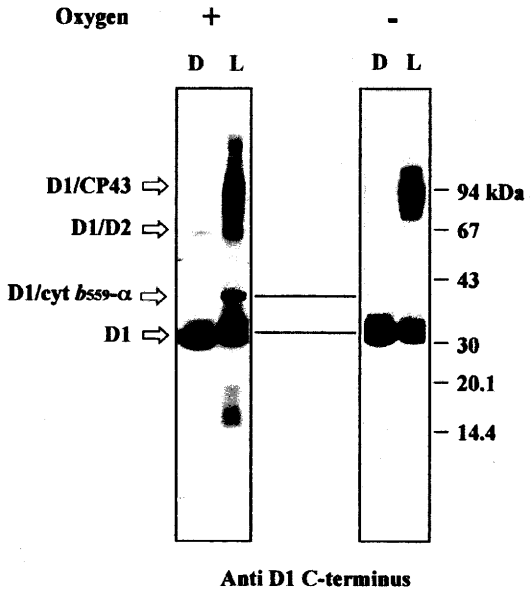


Fig. 2. The effects of oxygen on the light-induced formation of the cross-linked products of the D1 protein and the α-subunit of cytochrome b_{559}. Tris-treated PS II-enriched membranes were illuminated with white light (2500 μE m^{-2} s^{-1}) for 30 min at 25°C under aerobic (denoted +) and anaerobic (denoted −) conditions. Antibodies against the C terminus of the D1 protein were used for Western blot analysis. Other experimental conditions were the same as those described in the legend to Fig. 1.

reactive oxygen molecules produced by the illumination of PS II. In connection with this, it was observed that the light-induced cross-linking of the D1 protein with the α-subunit of cytochrome b_{559} was more prominent in Tris-treated PS II samples compared to that for the non-Tris-treated PS II samples. In Tris-treated samples, the extrinsic proteins of PS II (OEC subunits) and Mn clusters are removed, and the illumination of those samples should induce the donor-side photoinhibition of PS II. Although the reason why the cross-linking reaction was enhanced by the illumination of Tris-treated samples is not yet clear, it is likely that the generation of reactive oxygen is increased in the Tris-treated PS II membranes when illuminated compared to the non-Tris-treated membranes. We therefore used Tris-treated PS II membranes to analyze the light-induced cross-linking of the D1 protein and the stromal protease activity in the following studies.

3.2. *Proteolysis of the 41 kDa cross-linked product by a stromal protease(s)*

When the illuminated Tris-treated PS II membranes were incubated with stromal extracts for 30 min at 25°C, a significant decrease in the amounts of the 41 kDa cross-linked product, as well as those of D1/D2 and D1/CP43, was observed by Western blot analysis (Fig. 3). Densitometric analysis showed that the amount of the 41 kDa adduct in the samples (equivalent to 75 μg chlorophyll) decreased by approx. 40% with the addition of stromal extract (equivalent to 0.3 mg chlorophyll). The loss of the 41 kDa adduct was dependent on temperature [13],

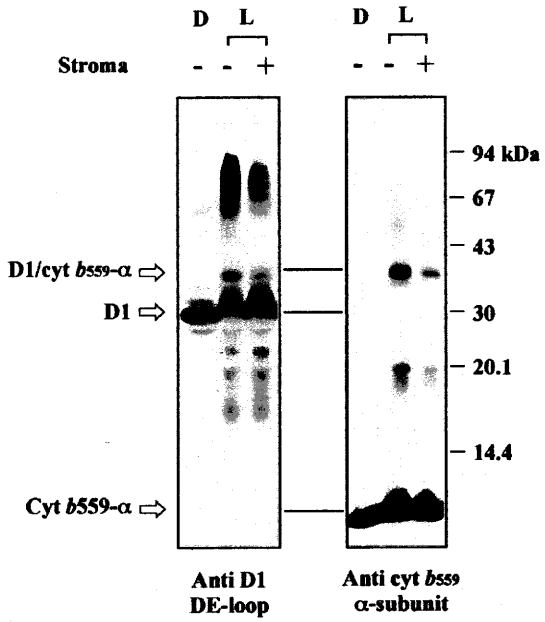


Fig. 3. The effects of stromal extracts on the light-induced 41 kDa cross-linked adduct of the D1 protein and the α-subunit of cytochrome b_{559}. Tris-treated PS II membranes were illuminated with white light (2500 μE m^{-2} s^{-1}) for 30 min at 25°C under aerobic conditions. Illuminated samples containing 75 μg chlorophyll were then incubated in the presence (+) or absence (−) of stromal extracts equivalent to 0.3 mg chlorophyll for 30 min at 25°C in darkness. In the absence of stromal extract addition, a solution containing 10 mM MES-NaOH and 5 mM $MgCl_2$ (pH 6.5) was used. Other experimental conditions were the same as those described in the legend to Fig. 1.

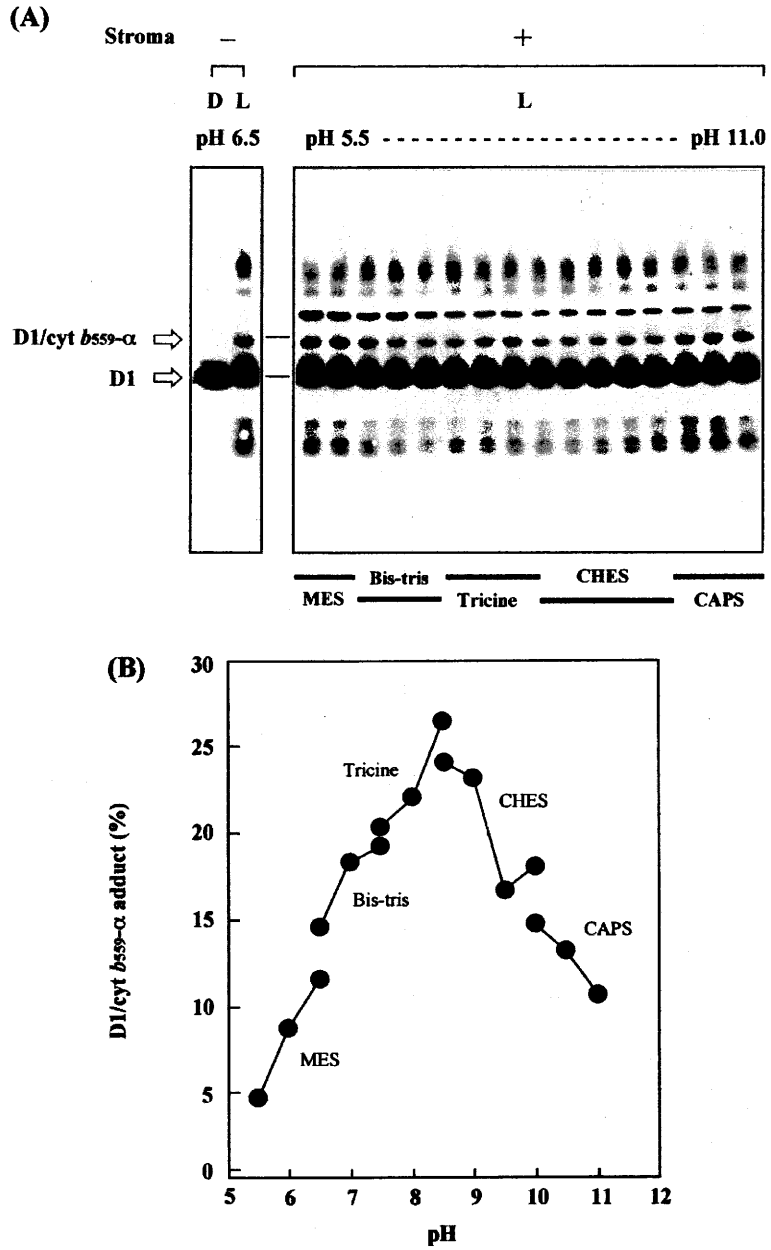


Fig. 4. The effects of pH on the stromal protease activity. (A) A fluorogram showing the results of the stromal protease activity at different pH values. Tris-treated PS II membranes were illuminated with white light (1500 μE m^{-2} s^{-1}) for 30 min under aerobic conditions, followed by incubation in darkness in the presence (+) or absence (−) of stromal extract equivalent to 0.3 mg chlorophyll for 30 min at 25°C. Stromal extracts were prepared from intact chloroplasts equivalent to 2.0 mg chlorophyll that were suspended in five different solutions containing 5 mM $MgCl_2$ and one of the Good's buffers (MES, pH 5.5–6.5; Bis-tris, pH 6.5–7.5; tricine, pH 7.5–8.5; CHES, pH 8.5–10.0; CAPS, pH 10.0–11.0). (B) The pH profile of the stromal protease activity obtained from the data in panel A. Other experimental conditions were the same as those described in the legend to Fig. 1.

and also on the amount of the stromal extract added and incubation time (data not shown).

It was noted that when the illuminated PS II membranes were subjected to Western blot analysis with the antibody against the α-subunit of cytochrome b_{559}, an additional band with a molecular mass of approx. 20 kDa was detected between the bands corresponding to the 41 kDa cross-linked adduct and to the native α-subunit of cytochrome b_{559} (Fig. 3, right). The 20 kDa band may be either a degradation product of the 41 kDa adduct, or a cross-linked product of a D1 fragment and the α-subunit of cytochrome b_{559}. The 20 kDa band becomes less obvious by the addition of stromal extract to illuminated samples, indicating the digestion of this molecular species as well.

3.3. Characterization of the stromal protease(s)

We next looked at the nature of the stromal protease activity responsible for the loss of the 41 kDa cross-linked product more closely. First, the pH dependence of the stromal protease activity was examined (Fig. 4). The optimum pH was 8, and the peak was relatively narrow. It should be noted that not only the 41 kDa adduct was degraded, but the cross-linked products with higher molecular masses, which correspond to D1/D2 and D1/CP43 adducts, and the partially degraded products of the D1 protein were also degraded by the addition of the stromal extracts with a similar pH dependence.

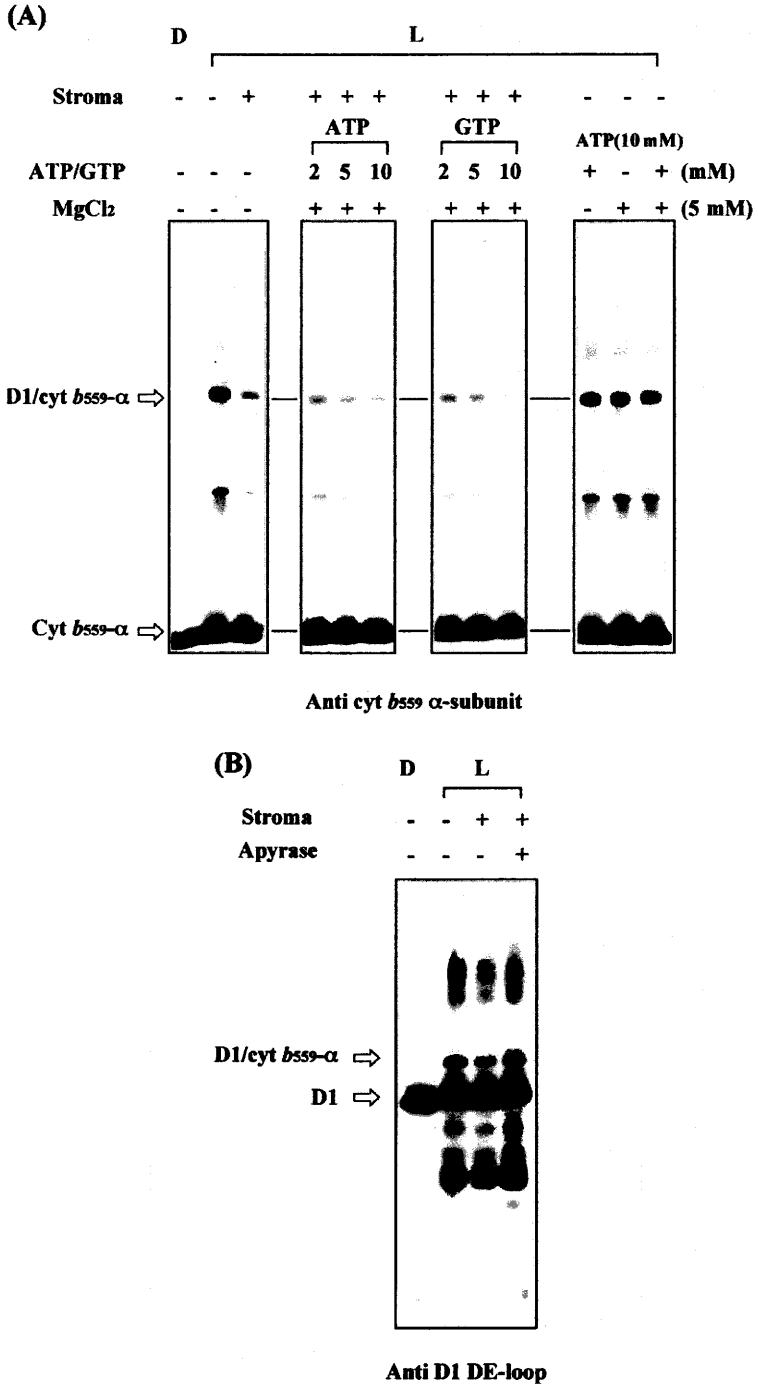
Addition of ATP or GTP stimulated the activity of the stromal protease(s) (Fig. 5A). In the presence of these nucleotides, the amount of the 41 kDa adduct was decreased by 70% or more. In accordance with this result, when preilluminated samples were incubated with stromal extracts, in which endogenous nucleotides were previously hydrolyzed by the addition of apyrase, the D1/cytochrome b_{559} adduct remained intact (Fig. 5B). Thus, the stromal protease activity was eliminated by the destruction of endogenous nucleotides. The stimulatory effects of the nucleotides were not observed with PS II membranes in the absence of stromal extracts (Fig. 5A).

To investigate further whether the effect of the stromal extract on the degradation of the 41 kDa adduct is due to a protease(s), we next examined the effect of protease inhibitors in the assays. The inhibitors 3,4-DCI, a serine-type protease inhibitor, E-64, a cysteine protease inhibitor, or EDTA, a metalloprotease inhibitor, were each added to stromal extracts. Among these inhibitors, 3,4-DCI suppressed the stromal protease activity (Fig. 6).

4. Discussion

In a recent work, we showed that the cross-linked products of the D1 protein are formed not only in the PS II membranes, but also in thylakoids and intact chloroplasts [13]. These results, as well as those reported previously [20], suggest that the adduct formation is a phenomenon that occurs in vivo. It was also obvious from previous results that the extent of cross-linking observed is less in chloroplasts than in thylakoids and PS II membranes. This indicates that in chloroplasts the photoinduced cross-linking of the D1 protein is much reduced by the presence of stromal factor(s), or that the cross-linked products are formed and turned over by stromal factor(s). It is known that the presence of reductants, such as ascorbate or glutathione, in the stroma protects chloroplasts from oxidative damage. However, generation of the reactive oxygen species such as singlet oxygen may be localized within the PS II complexes, and the PS II complexes are enriched in the stacked thylakoid regions. Hence, it is likely that the PS II complex is inaccessible to the soluble reductants of the stroma. Once oxidative stress becomes prominent, especially under strong light intensity, damage to the D1 protein and cross-linking of the photodamaged D1 protein with surrounding polypeptides are observed [13]. It is expected then that a mechanism for turning over the photodamaged cross-linked products of the PS II complex be in effect.

Previously, we showed by Western blot analysis that adducts of the D1 protein and antenna chlorophyll-binding CP43 disappeared by the addition of the stromal extracts [13]. In that study, however, we could not determine whether the loss of the D1/CP43 adduct was due either to digestion of the adducts by a protease(s) or to dissociation of the adducts to reproduce native proteins by the action of a molecular chaperon(s) and/or the related components, because the nature of the binding of the two proteins is not known exactly. In contrast with the D1/CP43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D1
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Fig. 5. The effects of nucleotides and apyrase on the stromal protease activity. (A) Stimulation of the stromal protease activity by ATP or GTP. ATP or GTP was added to the stromal extracts at concentrations of 2, 5 and 10 mM, with 5 mM $MgCl_2$. Tris-treated PS II membranes were preilluminated with white light (2500 μE m^{-2} s^{-1}) for 30 min at 25°C. Preilluminated samples were suspended in a solution containing 10 mM tricine-NaOH and 5 mM $MgCl_2$ (pH 8.0) and incubated in the presence or absence of stromal extract containing ATP or GTP. The symbols + and − at the top of the gels denote the presence and absence of stroma fraction, each nucleotide, and $MgCl_2$. Western blot analysis used the antibody against the α-subunit of cytochrome b_{559}. (B) Inhibition of the stromal protease activity by apyrase. Tris-treated PS II-enriched membranes were illuminated as described above, and then incubated in darkness in the presence (+) or absence (−) of apyrase (2 units/ml) for 30 min at 30°C. Western blot analysis used the antibody against the DE loop of the D1 protein. Other experimental conditions are the same as those shown in the legend to Fig. 1.
←

adduct, the 41 kDa adduct is relatively well characterized. The 41 kDa adduct was detected not only in vitro [14] but also in vivo [20]. The site of cross-linking of the D1 protein and the α-subunit of cytochrome b_{559} was previously shown to be the stroma-exposed DE loop of the D1 protein and the N terminus of the α-subunit of cytochrome b_{559} [15]. More recently, however, the cross-linking site on the DE loop is questioned with the study of *Synechocystis* mutants which have a deletion in the DE loop [16].

The relationship between the formation of the 41 kDa adduct and turnover of the D1 protein has received considerable attention. In the present study, we examined the effects of stromal extracts on the covalently cross-linked 41 kDa product in illuminated PS II membranes. Despite the uncertainty of the cross-linking site, the 41 kDa adduct has merit for use as a substrate to characterize the observed stromal protease activity. The 41 kDa adduct is easily generated even under low light intensities [13], and is easily detected and quantified by SDS/urea-PAGE and Western blot analysis. Our present results indicate that a serine-type protease(s) in the stroma proteolyzes the 41 kDa adduct. The optimum pH of the protease(s) was 8, and is similar to the values reported with other serine-type proteases (pH 7.5–8.5) (Fig. 4) [21]. It is noted that by the addition of stromal extracts to preilluminated PS II membranes, the other D1 cross-linked products, such

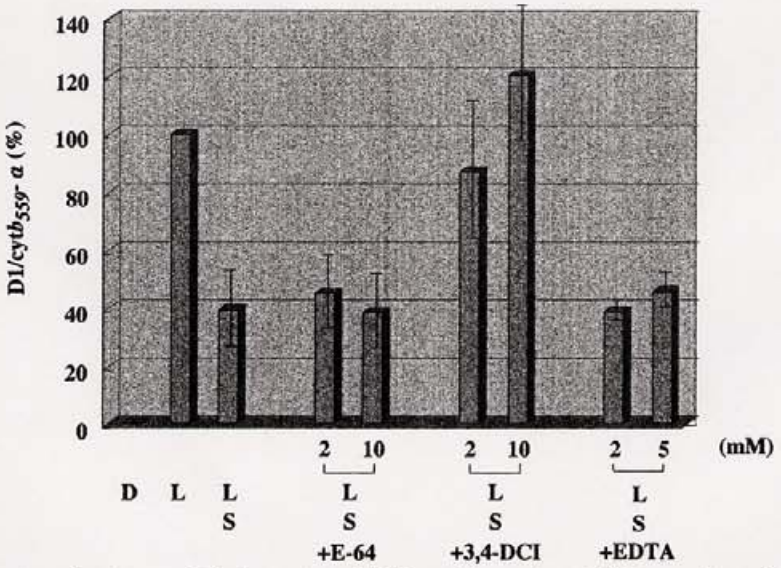

Fig. 6. Inhibition of the stromal protease activity by protease inhibitors. The protease inhibitors used are: E-64 (a cysteine protease inhibitor), 3,4-DCI (a serine-type protease inhibitor), and EDTA (a metalloprotease inhibitor). The 41 kDa adduct was quantified from fluorograms by densitometry as described in Section 2. Averages of data from three independent measurements with standard deviations are shown. Other experimental conditions are the same as those shown in the legend to Fig. 1.

as D1/D2 and D1/CP43 adducts, were removed as well. It is likely that the putative serine protease(s) has a broad substrate specificity functioning as a housekeeping enzyme to digest damaged and unneeded proteins, such as the photoinduced protein adducts.

The results of this study suggest that the stromal protease(s) are energy-dependent, because its (their) activity was enhanced by the addition of ATP or GTP (Fig. 5A). This was demonstrated by depleting the stromal extracts of endogenous nucleotides with apyrase treatment prior to its addition to PS II membranes, which inhibited the proteolytic activity of the extract (Fig. 5B). It was shown recently that GTP bound to thylakoid membranes is required for the light-induced degradation of the D1 protein [22,23]. The relationship between the requirement of GTP in the protease activity in our study and that in thylakoid membranes is currently unknown and should be further investigated.

When incubating preilluminated PS II membranes with stromal extracts, we observed a small increase in the quantity of D1 degradation products, by Western blot analysis (Fig. 3, left). This increase in D1-derived degradation products may be due, in part, to the degradation of the larger cross-linked products. The 20 kDa band detected by both the antibodies against the D1 protein and the α-subunit of cytochrome b_{559}, which may be the degradation products of the 41 kDa adduct or a cross-linked product of a D1 fragment and the α-subunit of cytochrome b_{559}, was also digested by the putative stromal protease(s) (Fig. 3, right). As we could not detect fragments that are newly produced by the addition of the stroma and cross-react with the antibody against the α-subunit of cytochrome b_{559}, it is suggested that the amino acid sequence at or very close to the covalent binding site of the two proteins is recognized by the protease(s), and thereby, the α-subunit of cytochrome b_{559} remains almost intact without being digested. The putative cross-linking site in the DE loop of the D1 protein contains a Phe-Gly-Gln-Glu-Glu-Glu motif [15]. As many serine-type endopeptidases are known to also recognize a Glu residue for substrate cleavage [24], the short poly-Glu sequence may be the site of cross-linking with the N-terminal serine of the α-subunit of cytochrome b_{559}. This stretch of amino acids also may be the cleavage site of the putative stromal protease(s). We must, however, await the determination of the exact cross-linking site between the D1 protein and the α-subunit of cytochrome b_{559} to estimate the cleavage site by the protease(s). These results indicate that the stromal protease activity plays a primary functional role in the dissolution of the 41 kDa cross-linked adduct freeing the α-subunit of cytochrome b_{559} and in the subsequent turnover of the D1 protein.

The digestion of the cross-linked products of the D1 protein and the α-subunit of cytochrome b_{559} appears as one of the quality control mechanisms to maintain the functional integrity of the PS II complex under light stress. Protease systems functioning in chloroplasts are most probably similar to those in prokaryotes. In *E. coli*, several protease families, such as the serine-type Clp endopeptidase [25] and metalloprotease FtsH [26], are well characterized. Proteases homologous to those in *E. coli* were found in chloroplasts and characterized [7–9]. In a previous study, we showed that the cross-linked products of the D1 protein generated by illumination are removed by SDS-resistant protein components in the stroma [13]. A 15 kDa protein was most prominent in the activity. Apparently the 15 kDa protein was able to digest all the cross-linked products of the D1 protein including the 41 kDa D1/cytochrome b_{559} adduct. Purification of the 15 kDa SDS-resistant protease is now in progress.

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