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Salt Stress and Hyperosmotic Stress Regulate the Expression of Different Sets of Genes in *Synechocystis* sp. PCC 6803

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Acclimation of microorganisms to environmental stress is closely related to the expression of various genes. We report here that salt stress and hyperosmotic stress have different effects on the cytoplasmic volume and gene expression in *Synechocystis* sp. PCC 6803. DNA microarray analysis indicated that salt stress strongly induced the genes for some ribosomal proteins. Hyperosmotic stress strongly induced the genes for 3-ketoacyl-acyl carrier protein reductase and rare lipoprotein A. Genes whose expression was induced both by salt stress and by hyperosmotic stress included those for heat-shock proteins and the enzymes for the synthesis of glucosylglycerol. We also found that each kind of stress induced a number of genes for proteins of unknown function. Our findings suggest that *Synechocystis* recognizes salt stress and hyperosmotic stress as different stimuli, although mechanisms common to the responses to each form of stress might also contribute to gene expression.

Key Words: **DNA microarray; cyanobacteria; salt stress; hyperosmotic stress.**

Microorganisms including cyanobacteria acclimate to various kinds of environmental stress by regulating the expression of numerous stress-inducible genes (1–3). For example, when the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is exposed to salt stress, expression of the following genes is induced: the *ggpS* gene for glucosylglycerolphosphate synthase (4); the *crh* gene for RNA helicase (3); the *isiA* gene for iron-stress-inducible protein A (5); the *isiB* gene for flavodoxin (5) and the *petH* gene for ferredoxin:$NADP^+$ reductase (6).

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The terms salt stress and hyperosmotic stress have often been used in a confusing manner, such that genes induced upon exposure of organisms to high concentrations of NaCl have sometimes been defined as osmostressed genes (7, 8). However, the accumulated evidence suggests that the two kinds of stress are perceived as different signals. Incubation of the cyanobacterium *Synechococcus* sp. PCC 7942 in medium supplemented with 1 M sorbitol decreases the cytoplasmic volume to 45% of the original value (9), whereas incubation in 0.5 M NaCl only decreases the volume to 85% of the original value (10). These findings suggest that cyanobacterial cells might respond to salt stress and hyperosmotic stress in different ways. We wondered whether these two kinds of stress might induce the same set or different sets of genes. To examine this issue, we used a DNA microarray to investigate gene expression in *Synechocystis* in response to salt stress and to hyperosmotic stress.

DNA microarrays allow monitoring of changes in levels of transcripts of almost all genes in specific organisms (11, 12). Such arrays have been used to examine gene expression in response to various kinds of stress in *Saccharomyces cerevisiae* (13) and in *Synechocystis* (14, 15).

In the present study, we obtained clear evidence that salt stress and hyperosmotic stress regulate different sets of genes, although expression of some genes was induced in common by both kinds of stress. Furthermore, we found that expression of a number of genes for proteins of unknown function was induced or repressed by salt stress and hyperosmotic stress.

MATERIALS AND METHODS

Strain and culture conditions. 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). Cells were grown at 34°C in 50 ml of BG-11 medium (16) buffered with 20 mM Hepes–

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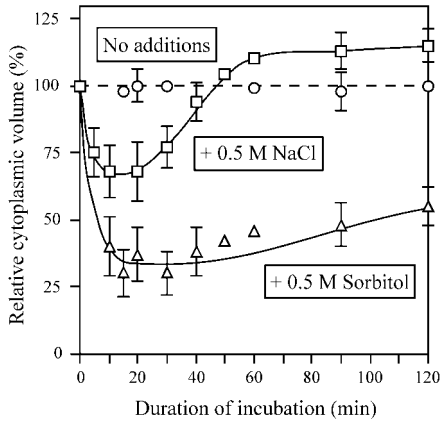

FIG. 1. Effects of NaCl and sorbitol on the volume of *Synechocystis* cells. Circles, control (no additions); squares, 0.5 M NaCl was added at time 0; triangles, 0.5 M sorbitol was added at time 0. The volume of cells was monitored by EPR spectrometry (see text). Each point represents the mean ± SE of results of six independent experiments.

NaOH (pH 7.5) under continuous illumination from incandescent lamps, as described previously (17). Cells at the exponential growth phase (OD_{730} = 0.2) were incubated with 0.5 M NaCl, sorbitol, or mannitol, after addition of an appropriate volume of a 5 M solution of NaCl or sorbitol or a 2 M solution of mannitol to the culture. Cells were incubated under the standard growth conditions for 30 min unless otherwise noted.

Measurement of cytoplasmic volume. The cytoplasmic volume was determined by electron paramagnetic resonance (EPR) spectrometry as described previously (9, 18). Cells were harvested by centrifugation at 30°C at 8000*g* for 10 min and were resuspended at 400 μg Chl ml^{-1} in a solution of 1.0 mM 2,2,6,6-tetramethyl-4-oxopiperidinooxy free radical (TEMPO; a spin probe), 20 mM $K_3[Fe(CN)_6]_3$ and 75 mM Na_2Mn–EDTA. TEMPO that was oxidized by $K_3[Fe(CN)_6]_3$ penetrated plasma membranes rapidly and reached an equilibrium in all phases of the suspension of cells. The addition of the paramagnetic quencher Na_2Mn–EDTA, which cannot penetrate the plasma membrane, broadened all the EPR signals except those that originated within the space bounded by the plasma membrane. The internal volume of cells could be calculated from the difference between the EPR spectrum obtained from the treated cells and that from control cells. Cells were enclosed in a sealed glass capillary tube (i.d., 0.02 cm) in a final volume of 40 μl and EPR spectra were recorded at room temperature in an EPR spectrometer (Model ESP 300E; Bruker, Karlsruhe, Germany). The EPR signal from the 40-μl capillary filled with 1.0 mM TEMPO alone was measured as a blank control. Measurements were made in darkness under the following conditions: 100 kHz field modulation at a microwave frequency of 11.72 GHz; a modulation amplitude of 0.4 mT; microwave power of 10 mW; a time constant of 80 ms; and a scan rate of 0.4 Gs^{-1}.

Profiles of transcriptional activity. Cells that had been exposed to salt stress or hyperosmotic stress were killed instantaneously by the addition of 50 ml of a mixture of phenol and ethanol (1:10, w/v) to 50 ml of the cell suspension and then total RNA was extracted as described previously (19). The RNA was treated with DNase I (Nippon Gene, Tokyo, Japan) to remove contaminating DNA (19).

A *Synechocystis* DNA microarray (CyanoCHIP) was obtained from TaKaRa Co. Ltd. (Kyoto, Japan). This microarray covered 3079 of the 3168 open reading frames of *Synechocystis* (20). We used Cy3 dye-labeled and Cy5 dye-labeled cDNA, synthesized by reverse transcription of 20 μg of total RNA, for hybridizations, which were allowed to proceed at 65°C for 16 h. After the incubation, the microarrays were rinsed with 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate) at room temperature. They were washed with 2× SSC at 60°C for 10 min and 0.2× SSC 0.1% SDS at 60°C for 10 min and then rinsed with distilled water at room temperature for 2 min. Moisture was removed with an air spray prior to analysis with the array scanner (GMS418; Affimetrix, Woburn, MA). Each signal was quantified with the ImaGene ver. 4.0 program (BioDiscovery, Los Angeles, CA). The signal from each gene on the microarray was normalized by reference to the total intensity of signals from all genes with

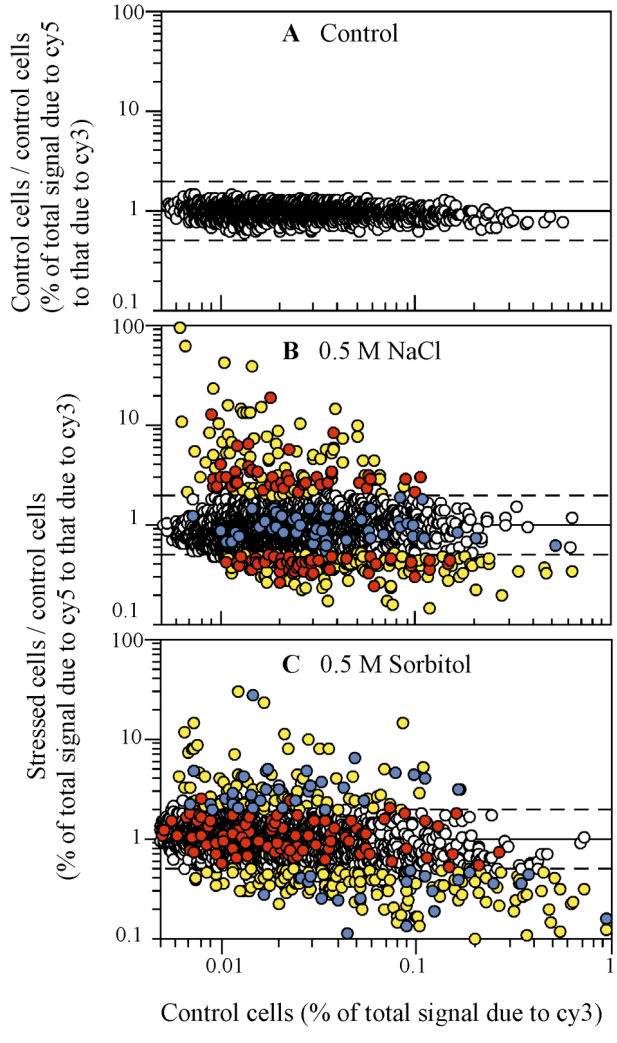

FIG. 2. DNA microarray analysis of salt stress-induced and hyperosmotic stress-induced gene expression. (A) Internal-control experiment (control experiment). RNA extracted from unstressed cells was used to synthesize both Cy3-labeled cDNA and Cy5-labeled cDNA. Dashed lines correspond to reference lines, which indicate the limit of experimental deviations. (B) RNA, extracted from cells that had been exposed to 0.5 M NaCl for 30 min, was compared with RNA that from unstressed cells. (C) RNA, extracted from cells that had been exposed to 0.5 M sorbitol for 30 min, was compared with RNA from unstressed cells. Red circles correspond to genes whose expression was regulated only by NaCl stress. Blue circles correspond to genes whose expression was regulated only by sorbitol. Yellow circles correspond to genes whose expression was affected both by NaCl and by sorbitol. The assay was repeated six times in independent experiments and essentially the same results were obtained in every case.

the exception of genes for rRNAs. Then we calculated changes in the level of the transcript of each gene relative to the total level of mRNA.

RESULTS AND DISCUSSION

Effects of NaCl and Sorbitol on Cytoplasmic Volume

Salt stress and hyperosmotic stress depressed the growth of *Synechocystis* cells. Addition to the standard culture medium of 0.5 M NaCl or 0.5 M sorbitol decreased the growth rate to about 50% of the original level (data not shown). In our subsequent analysis, we used 0.5 M NaCl and 0.5 M sorbitol to induce salt stress and hyperosmotic stress, respectively.

We examined the effects of NaCl and sorbitol on the cytoplasmic volume of *Synechocystis* cells by monitoring the EPR spectrum of TEMPO, as described under Materials and Methods (Fig. 1). When cells were incubated with 0.5 M sorbitol, the cytoplasmic volume decreased to 30% of the original level within 10 min and then remained at this level subsequently. When cells were incubated with 0.5 M NaCl, the cytoplasmic volume decreased to 70% of the original level within 10 min but returned to the original level within 45 min of the start of exposure to salt stress. These observations were consistent with our previous finding that incubation of *Synechococcus* sp. PCC 7942 with 1 M sorbitol and with 0.5 M NaCl decreased the cytoplasmic volume to 45 and 85% of original level, respectively, within 30 min (9, 10). The previous reports that Na^+ ions penetrate the plasma membrane might explain why NaCl is less effective than sorbitol in exerting hyperosmotic pressure (21, 22).

The results in Fig. 1 suggested that the inhibitory effect of 0.5 M NaCl on the cell growth might be due mainly to ionic stress. Therefore, we postulated that an increase in the concentration of Na^+ ions in the cytoplasm, due to the penetration of ions through the cell membrane, might be the main feature of salt stress, with osmotic pressure across the plasma membrane being a minor feature of such stress. By contrast, osmotic pressure due to sorbitol results from the fact that the plasma membrane is impermeable to this compound (9). However, sorbitol might also have an ionic effect, albeit a minor one, because a decrease in cytoplasmic volume to 30% of the original value would lead to a three-fold increase in the concentrations of salts in the cytosol. Thus, our findings suggest that the stress due to NaCl and the stress due to sorbitol might have different effects on cell physiology and might be recognized as different stimuli by cyanobacterial cells.

Analysis of Gene Expression with a DNA Microarray

To study the responses of *Synechocystis* cells to salt stress and hyperosmotic stress in terms of gene expression, we used a DNA microarray. First, we performed an internal-control experiment in which Cy3- and Cy5-labeled cDNAs were synthesized from total RNA that had been extracted from unstressed cells. Figure 2A shows that all the data points appeared between the two reference lines, which corresponded to ratios of expression of 2.0 and 0.5. This control experiment suggested that data points that appeared above the upper reference line or below the lower reference line could be regarded as representing those genes whose expression was induced or repressed, respectively.

Figure 2B shows the effects of the incubation of *Synechocystis* cells with 0.5 M NaCl for 30 min on the expression of individual genes. Upon exposure to the salt stress, 147 genes appeared above the upper reference line and 228 genes appeared below the lower reference line. However, most of the genes appeared between the two reference lines, indicating that the expression of most genes was unaffected by salt stress. Figure 2C shows the effects of incubating *Synechocystis* cells with 0.5 M sorbitol on the expression of individual genes. Upon exposure to the hyperosmotic stress, 113 genes appeared above the upper reference line and 161 genes appeared below the lower reference line. We also examined the effect of 0.5 M mannitol on *Synechocystis* cells; the profile of gene expression was essentially the same as that obtained with 0.5 M sorbitol. This observation indicated that the changes in gene expression due to the sorbitol were caused by hyperosmotic stress and not specifically by sorbitol. We also examined the effects of extended incubation for 2 h with 0.5 M NaCl or 0.5 M sorbitol on the pattern of gene expression. We obtained essentially the same results as those obtained after a 30-min incubation (data not shown).

The genes, whose expression was highly enhanced or was strongly repressed to an extent that corresponded to a ratio greater than 3:1, are listed in Tables 1 and 2. We found that half of genes, whose expression was enhanced or was repressed by each kind of stress, encode proteins of unknown functions. It suggests that a number of proteins of unknown functions may be related to the known or unknown mechanisms for the acclimation to these kinds of stress. We also found that salt stress and hyperosmotic stress regulate the expression of different sets of genes (see red spots or blue spots in Figs. 2B and 2C). It should be noted here, however, that salt stress tends to depress gene expression in general (23). Eventually, gene expression was reduced to 75 and 90% of the control level in response to 0.5 M NaCl and 0.5 M sorbitol, respectively. The results in Figs. 2B and 2C reveal the changes in the contribution of the expression of individual genes relative to the total level of gene expression, which was estimated from signals that reflected the extent of hybridization.

TABLE 1

Genes Whose Expression Was Enhanced by Salt and/or Hyperosmotic Stress

ORF number	Gene	Product	Extent of enhancement	
			0.5 M NaCl	0.5 M sorbitol
Genes whose expression was strongly enhanced by salt stress (fold)				
slr1704		Protein of unknown function	17.4 ± 1.2	2.3 ± 0.6
slr0967		Protein of unknown function	16.0 ± 3.6	2.5 ± 0.6
sll1621		Membrane protein	8.3 ± 1.1	1.5 ± 0.3
sll1515		Protein of unknown function	7.6 ± 1.1	0.8 ± 0.2
slr1501		Protein of unknown function	7.0 ± 1.2	1.4 ± 0.5
sll1864		Chloride channel protein	6.1 ± 1.9	1.1 ± 0.2
slr1932		Protein of unknown function	5.4 ± 0.6	1.2 ± 0.1
sll1022		Protein of unknown function	4.7 ± 0.4	1.6 ± 0.6
ssr2194		Protein of unknown function	4.7 ± 1.4	1.4 ± 0.4
slr1738		Protein of unknown function	4.4 ± 0.2	1.9 ± 0.4
slr0095		*O*-Methyltransferase	4.2 ± 0.1	1.4 ± 0.2
sll1086		Protein of unknown function	4.0 ± 0.6	1.6 ± 0.2
slr0529		Protein of unknown function	3.9 ± 0.7	1.3 ± 0.2
sll1167	*pbp*	Penicillin-binding protein	3.8 ± 0.2	1.3 ± 0.5
slr1259		Protein of unknown function	3.7 ± 0.7	1.1 ± 0.2
sll0905	*maf*	maf protein	3.6 ± 0.7	1.3 ± 0.4
sll1236		Protein of unknown function	3.2 ± 0.6	1.1 ± 0.4
slr1916		Esterase	3.2 ± 0.5	1.5 ± 0.4
sll1594	*rbcR*	Rubisco operon regulator	3.2 ± 0.4	1.2 ± 0.2
slr1971		Protein of unknown function	3.1 ± 0.7	1.7 ± 0.5
sll1723		Protein of unknown function	3.1 ± 0.6	1.6 ± 0.4
Slr1604	*ftsH*	Cell division protein	3.1 ± 0.0	1.6 ± 0.3
sll1491		Protein of unknown function	3.0 ± 0.4	1.4 ± 0.3
slr1751	*prc*	Carboxy-terminal protease	3.0 ± 0.2	1.4 ± 0.2
sll0188		Protein of unknown function	3.0 ± 0.2	1.7 ± 0.5
slr1608	*gdhB*	Glucose dehydrogenase-B	3.0 ± 0.4	1.4 ± 0.3
sll0938		Aspartate transaminase	3.0 ± 0.6	1.3 ± 0.4
sll1799	*α rp13*	50S ribosomal protein L3	3.0 ± 0.2	0.8 ± 0.2
Genes whose expression was strongly enhanced by hyperosmotic stress				
sll0330	*fabG*	3-Ketoacyl-ACP reductase	1.2 ± 0.2	23.9 ± 6.5
slr0423	*rlpA*	Rare lipoprotein A	1.2 ± 0.1	6.2 ± 0.4
slr1748		Protein of unknown function	1.7 ± 0.4	4.6 ± 0.6
slr1119		Protein of unknown function	1.3 ± 0.2	4.2 ± 1.1
slr0381		Protein of unknown function	0.9 ± 0.1	3.9 ± 0.9
sll0430	*htpG*	Heat-shock protein Hsp90	1.9 ± 0.1	3.8 ± 0.7
ssr1256		Protein of unknown function	0.7 ± 0.1	3.7 ± 0.3
ssl3177	*repA*	Rare lipoprotein A	1.6 ± 0.2	3.6 ± 0.5
sll0185		Protein of unknown function	1.3 ± 0.4	3.3 ± 0.5
sll0293		Protein of unknown function	0.9 ± 0.1	3.1 ± 0.6
slr0753	*p*	P protein	0.6 ± 0.1	3.0 ± 1.0
Genes whose expression was strongly enhanced by salt and hyperosmotic stress				
sll1862	*ϵ*	Protein of unknown function	93.8 ± 7.5	17.2 ± 5.3
sll1514	*hspA*	Heat-shock protein Hsp17	56.2 ± 10.7	26.5 ± 4.5
sll1863	*ϵ*	Protein of unknown function	52.7 ± 10.1	8.3 ± 1.8
sll0528		Protein of unknown function	40.0 ± 6.5	20.0 ± 6.0
slr1544		Protein of unknown function	20.3 ± 3.3	7.8 ± 0.9
slr1516	*sodB*	Superoxide dismutase	16.5 ± 1.6	4.9 ± 0.7
sll0939		Protein of unknown function	15.3 ± 3.2	3.5 ± 0.9
sll0846		Protein of unknown function	14.9 ± 1.5	10.1 ± 1.4
slr1674	*ϵ*	Protein of unknown function	14.2 ± 0.8	10.6 ± 1.2
ssr2595		High light-inducible protein	13.4 ± 1.6	7.0 ± 1.4
sll1106		Protein of unknown function	12.6 ± 2.8	3.4 ± 0.8
sll1085	*ϵ glpD*	Glycerol-3-P dehydrogenase	11.8 ± 2.1	3.2 ± 0.5
sll0306	*rpoD*	RNA polymerase sigma factor	11.5 ± 0.3	7.2 ± 2.3
sll0170	*dnaK*	DnaK protein	11.5 ± 2.8	4.1 ± 0.9

TABLE 1—*Continued*

ORF number	Gene	Product	Extent of enhancement	
			0.5 M NaCl	0.5 M sorbitol
sll1566	ϵ *ggpS*	Glucosylglycerol-P synthase	10.7 ± 1.5	3.7 ± 0.5
slr1675	ϵ *hypA*	Hydrogenase-related protein	10.1 ± 1.5	7.8 ± 2.1
slr1963		Protein of unknown function	10.0 ± 1.4	9.1 ± 2.4
slr0093	*dnaJ*	DnaJ protein	9.6 ± 1.6	3.2 ± 0.8
sll1884		Protein of unknown function	9.4 ± 1.1	3.9 ± 0.3
slr1687	γ	Protein of unknown function	9.4 ± 2.2	3.2 ± 0.9
slr1641	*clpB*	ClpB protein	8.4 ± 1.2	3.5 ± 1.2
sll0788	β	Protein of unknown function	8.3 ± 1.3	7.0 ± 0.6
sll1483		Protein of unknown function	7.8 ± 1.3	22.9 ± 0.6
slr1603		Protein of unknown function	7.2 ± 0.8	3.9 ± 1.1
slr0959		Protein of unknown function	6.6 ± 1.2	4.2 ± 1.1
ssl1633		CAB/ELIP/HLIP superfamily	6.5 ± 0.9	5.1 ± 0.5
sll0416	*groEL2*	60-kDa chaperonin	6.2 ± 0.4	3.2 ± 1.1
ssl3044	γ	Hydrogenase component	5.7 ± 1.5	3.5 ± 1.2
ssr2016		Protein of unknown function	5.5 ± 0.9	4.6 ± 0.5
ssl2542	*hliA*	HLIP	5.0 ± 0.8	4.8 ± 1.8
slr1204	*htrA*	Serine protease	4.8 ± 0.3	4.6 ± 0.7
sll0789	β *copR*	Response regulator	4.4 ± 0.9	3.9 ± 0.9
ssl2971		Protein of unknown function	4.3 ± 0.7	4.0 ± 0.2
slr0581		Protein of unknown function	3.9 ± 0.7	3.3 ± 0.3

Note. Cells grown at 34°C (to OD_{730} = 0.2) were incubated with 0.5 M NaCl or 0.5 M sorbitol for 30 min. Each value indicates the ratio of the level of expression in stressed cells to that in control cells. Values shown are means ± SE of results of three independent experiments. The numbering of ORFs corresponds to that of Kaneko *et al.* (20, 29). Genes marked by the same Greek letter are located in tandem on the *Synechocystis* genome.

Genes Whose Expression Was Strongly Enhanced by Salt Stress

Figure 3 shows that salt stress due to 0.5 M NaCl strongly induced the expression of genes for proteins involved in translation (*rpl3*), the modification and degradation of proteins (*prc* and *ftsH*). The expression of these genes was not induced by osmotic stress due to 0.5 M sorbitol.

In *Synechocystis*, genes for ribosomal proteins, namely, *rpl2*, *rpl3*, *rpl4*, and *rpl23* are located in a putative ribosomal-protein operon (20). Salt stress enhanced the expression of these genes by factors of 2.0, 3.0, 2.3, and 2.7, respectively. The crystallographic structure of the large subunit of ribosomes has revealed that the ribosomal proteins, L2, L3 and L4, encoded by *rpl2*, *rpl3*, and *rpl4*, respectively, are located near the peptidyltransferase center (24, 25). These observations suggest that salt stress might destabilize ribosomes and that the synthesis *de novo* of L2, L3, and L4 might be necessary for maintenance of the activities of ribosomes.

The D1 protein at the photochemical reaction center of the photosystem II complex is rapidly damaged under strong light (26). The rapid degradation of photodamaged D1 and regeneration of D1 by synthesis *de novo* are important if photosynthetic organisms are to maintain the activity of photosystem II. We found that the proteins that are involved in the degradation of photodamaged D1 are also induced by salt stress. Lindahl *et al.* demonstrated that an ATP-dependent metalloprotease, FtsH, located in the thylakoid membrane in *Arabidopsis thaliana*, degrades photodamaged D1 (27, 28). *Synechocystis* has four open reading frames (ORFs) that encode homologs of FtsH, namely, *sll1463*, *slr0228*, *slr1390*, and *slr1604* (20, 29). We found that the expression of *slr1390* and *slr1604* was enhanced 2.1- and 3.1-fold by salt stress, respectively, whereas levels of expression of the other proteins were basically unchanged.

The CtpA protein catalyzes the cleavage of the carboxy-terminal region of the precursor to the D1 protein to generate the mature D1 protein (30). The expression of the *ctpA* gene and that of a homologous gene, *prc*, were also enhanced 2.7- and 3.0-fold by salt stress but were unaffected by hyperosmotic stress. These results suggest that salt stress might inhibit the turnover of D1 not only at the transcriptional and translational levels, but also at the levels of degradation of photodamaged D1 and the processing of the precursor to D1. The enhanced expression of the *ftsH*, *prc* and *ctpA* genes might compensate for the inhibition via the synthesis of FtsH, Prc, and CtpA.

Genes Whose Expression Was Strongly Enhanced by Hyperosmotic Stress

Hyperosmotic stress strongly induced the expression of the *fabG* gene for 3-ketoacyl-ACP reductase, the *rlpA* and *repA* genes for rare lipoproteins. Salt stress

TABLE 2

Genes Whose Expression Was Repressed by Salt and/or Hyperosmotic Stress

ORF number	Gene	Product	Extent of repression	
			0.5 M NaCl	0.5 M sorbitol
		Genes whose expression was strongly repressed by salt stress (fold)		
slr0294		Protein of unknown function	3.7 ± 0.9	1.3 ± 0.2
sll1453	*nrtD*	Nitrate transport protein	3.5 ± 0.4	1.3 ± 0.6
ssr0536		Protein of unknown function	3.4 ± 0.5	1.7 ± 0.2
slr1658		Protein of unknown function	3.3 ± 0.5	1.3 ± 0.2
sll1693		Protein of unknown function	3.2 ± 0.6	1.7 ± 0.5
slr0213	*guaA*	GMP synthetase	3.1 ± 0.8	1.7 ± 0.2
ssl2084	*acp*	Acyl carrier protein	3.1 ± 0.5	1.4 ± 0.6
slr0591		Protein of unknown function	3.0 ± 0.8	0.9 ± 0.2
sll0262	*desD*	Delta-6 desaturase	3.0 ± 0.3	1.3 ± 0.3
ssl2874		Protein of unknown function	3.0 ± 0.7	1.2 ± 0.5
		Genes whose expression was strongly repressed by hyperosmotic stress		
slr1634		Protein of unknown function	1.5 ± 0.7	7.2 ± 1.0
sll0381	*ζ*	Protein of unknown function	0.5 ± 0.1	5.9 ± 1.1
sll0382	*ζ*	Protein of unknown function	0.5 ± 0.1	3.7 ± 0.6
slr1272		Protein of unknown function	1.4 ± 0.1	3.7 ± 0.7
sll1626	*lexA*	SOS regulatory protein	1.2 ± 0.5	3.4 ± 1.1
sll0383	*ζ cbiM*	CbiM protein	0.5 ± 0.0	3.0 ± 0.9
		Genes whose expression was strongly repressed by salt and hyperosmotic stress		
sll1783	*η*	Protein of unknown function	8.9 ± 2.0	6.8 ± 0.2
slr1854	*η*	Protein of unknown function	8.9 ± 1.6	10.3 ± 2.0
sll1785	*η*	Protein of unknown function	8.0 ± 2.1	5.4 ± 0.5
slr1852	*η*	Protein of unknown function	7.7 ± 1.3	5.5 ± 1.0
sll1784	*η*	Protein of unknown function	7.2 ± 1.6	5.0 ± 0.5
slr0737	*psaD*	Photosystem I subunit II	5.8 ± 0.9	3.6 ± 0.1
slr1855	*η*	Protein of unknown function	5.5 ± 1.2	5.0 ± 0.7
slr2051	*cpcG*	Phycobilisome rod-core linker	5.5 ± 1.1	4.3 ± 0.3
slr1277	*gspD*	Secretion pathway protein D	4.9 ± 0.7	3.7 ± 0.4
sll0819	*psaF*	Photosystem I subunit III	4.5 ± 1.3	4.5 ± 0.8
sll1091		Bacteriochlorophyll synthase	3.9 ± 0.3	3.0 ± 0.5
slr0335	*apcE*	Phycobilisome LCM linker	3.8 ± 0.6	4.3 ± 0.1
slr0653	*rpoDI*	RNA polymerase sigma factor	3.8 ± 1.2	4.0 ± 0.5
slr1459	*apcF*	Phycobilisome core component	3.8 ± 0.5	3.6 ± 1.0
sll1305	*η*	Protein of unknown function	3.7 ± 0.8	3.0 ± 0.6
slr2067	*ι apcA*	Allophycocyanin a chain	3.6 ± 0.7	4.1 ± 0.1
slr1986	*ι apcB*	Allophycocyanin b chain	3.6 ± 0.5	3.3 ± 0.4
sll0427	*psbO*	Mn-stabilizing polypeptide	3.5 ± 0.8	3.5 ± 0.3
sll1580	*θ cpcC*	Phycocyanin-associated linker	3.4 ± 0.8	8.2 ± 0.4
sll1577	*θ cpcB*	Phycocyanin b subunit	3.4 ± 1.1	7.2 ± 0.8
sll1712		DNA-binding protein HU	3.4 ± 1.0	3.7 ± 1.1
slr1859		Protein of unknown function	3.3 ± 0.4	3.0 ± 0.6
sll0661	*ycf35*	Protein of unknown function	3.3 ± 0.8	3.6 ± 0.8
slr1276		Protein of unknown function	3.2 ± 0.1	3.4 ± 0.6
slr0011	*rbcX*	Protein of unknown function	3.1 ± 0.8	3.5 ± 1.0
slr1856	*η*	Protein of unknown function	3.0 ± 0.3	4.2 ± 0.6

Note. See Table 1 for details.

did not induce the expression of any of these genes, the result suggests again that salt stress and hyperosmotic stress affect the expression of different sets of genes.

The efflux of water from cells due to hyperosmotic stress reduces the volume of the cytoplasmic space and such reduction can lead to plasmolysis and changes the state of the periplasmic space (31). A decrease in the cytoplasmic space might cause close packing of membrane lipids and membrane proteins in the plasma membrane (31). Such changes in the periplasm and/or the plasma membrane might be the first signal of hyperosmotic stress that is perceived by the cell and that

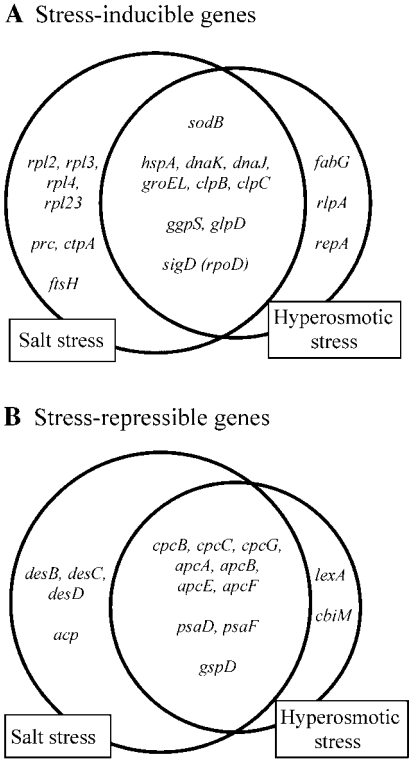

FIG. 3. Categorization of salt stress-regulated and hyperosmotic stress-regulated genes by reference to the specific stress that affected gene expression. Each of the two overlapping circles in A and B includes genes whose expression was altered by salt stress or hyperosmotic stress. The overlapping regions of the circles include those genes whose expression was affected by both kinds of stress. The area of each circle and of each overlapping region reflects the number of genes whose expression was affected by each kind of stress. (A) Stress-inducible genes. (B) Stress-repressible genes. Genes annotated by Cyanobase (http://www.kazusa.or.jp/cyano/) are indicated (20).

results in changes in the levels of expression of certain genes.

The FabG protein, 3-ketoacyl-ACP reductase, which catalyzes the production of 3-hydroxyacyl-ACP (ACP, acyl carrier protein) from 3-ketoacyl-ACP, is required for the elongation of carbon chains in the synthesis of fatty acids. *Synechocystis* has three *fabG* genes: *sll0330*, *slr1994*, and *slr0886*. However, of these three, only sll0330 was induced by hyperosmotic stress, suggesting that synthesis *de novo* of lipids might be related to the acclimation of the cyanobacterium to hyperosmotic stress. If the cytoplasm shrinks and membranes become closely packed under hyperosmotic stress, synthesis *de novo* of membrane lipids might assist in restoration of the original volume of the cytoplasm.

The RlpA protein, rare lipoprotein A, in *Escherichia coli* is exported to the periplasm where it is fatty-acylated to allow anchorage in the plasma membrane (32). It is previously demonstrated that the gene for this protein is located in a cluster of *mrdAB* genes for peptidoglycan synthesis in *E. coli* (33). *Synechocystis* has two genes for homologs of rare lipoprotein A (*rlpA* and *repA*) and both were specifically induced by hyperosmotic stress. Shrinkage of the cytoplasm by plasmolysis might damage the peptidoglycan layer. Thus, it is possible that the cell might strengthen or reconstruct the cell wall under hyperosmotic stress.

Genes Whose Expression Was Enhanced by Both Kinds of Stress

Both salt stress and hyperosmotic stress induced the expression of genes for heat-shock proteins (*hspA*, *dnaK*, *dnaJ*, *htrA*, *groEL2*, and *clpB*), superoxide dismutase (*sodB*), proteins for the synthesis of glucosylglycerol (*glpD* and *ggpS*), and sigma 70 factors (two *rpoD* genes). The efflux of water from cells due to hyperosmotic stress reduces the volume of the cytoplasmic space and, thus, increases the concentration of ions in the cytosol. Therefore, hyperosmotic stress might be expected to have effects similar to those of salt stress. Such effects might explain why a large number of genes were induced both by salt stress and by hyperosmotic stress.

The *glpD* and *ggpS* genes for proteins involved in the synthesis of glucosylglycerol are located in tandem on the chromosome of *Synechocystis* (20). Hagemann *et al.* demonstrated that GgpS is a key enzyme of the synthesis of glucosylglycerol, which is synthesized as a major compatible solute upon exposure of *Synechocystis* to salt stress (34).

The expression of genes for heat-shock proteins (*hspA*, *dnaK*, *dnaJ*, *htrA*, *groEL2*, and *clpB*) was induced both by salt stress and by hyperosmotic stress. It is demonstrated that, in *Bacillus subtilis*, the expression of these genes is induced not only by heat shock but also by salt stress, by ethanol and by starvation (1). In *Synechocystis*, heat shock induces the expression of these heat-shock genes (35, 36). In the present study, we found that a group of heat-shock proteins was also induced both by salt stress and by hyperosmotic stress in *Synechocystis*. The HtrA protein (DegP), a serine protease that is localized on the thylakoid membrane, degrades membrane-bound proteins that include photodamaged D1 (37). Török *et al.* demonstrated that HspA stabilizes proteins that have been heat-denatured *in vitro* for subsequent refolding by the DnaK/DnaJ/GrpE and GroEL/ES chaperone network (38). These observations suggest that the effect of both salt stress and hyperosmotic stress might be related to the stability of proteins. Cells might avoid the accumulation of denatured proteins by synthesizing chaperones and proteases under salt stress and hyperosmotic stress. Furthermore, It is demonstrated that both transcription and translation in *Synechocystis* cells might be inhibited when cells are incubated in medium supplemented with 684 mM NaCl (23). Thus, chaperones

might protect the transcriptional and translational apparatus under salt stress, as well as under hyperosmotic stress.

Both salt stress and hyperosmotic stress induced the expression of the two *rpoD* genes (*sll2012* and *sll0306*) that encode sigma 70 factors. These findings suggest that transcription might be one of the targets of salt stress and hyperosmotic stress. The induction of these genes might represent the strategy whereby *Synechocystis* compensates for the salt stress-induced depression of transcription. In *E. coli* and *B. subtilis,* the stress-dependent expression of genes for heat-shock proteins is regulated by specific sigma factors, namely, SigH and SigB, respectively (39). However, it is unclear which sigma factor regulates the expression of genes for heat-shock proteins in *Synechocystis.* The induction both by salt stress and by hyperosmotic stress of two *rpoD* genes (*sll2012* and *sll0306*), which are homologous to genes for SigB of *B. subtilis,* suggests that these sigma factors might be involved in regulation of the expression of heat-shock proteins.

Genes Whose Expression Was Strongly Repressed by Salt Stress

Figure 3B shows the genes whose levels of expression were reduced by more than 3.0-fold upon incubation of cells with 0.5 M NaCl or 0.5 M sorbitol for 30 min. Salt stress specifically depressed the expression of the genes for a desaturase (*desD*) and acyl carrier protein (*acp*).

The genome of *Synechocystis* includes the genes for four acyl-lipid desaturases (*desA, desB, desC,* and *desD*), which introduce double bonds into fatty-acyl chains (40). These enzymes play an important role in the maintenance of the structure and function of biological membranes (2). We found that expression of *desB, desC,* and *desD* genes was specifically reduced by salt stress, with reductions of 2.0-, 2.7-, and 3.0-fold, respectively.

Genes Whose Expression Was Strongly Repressed by Hyperosmotic Stress

Hyperosmotic stress specifically depressed the expression of the *cbiM* gene, the *lexA* gene. The *cbiM* (*sll0383*) genes are included in the gene cluster that encodes components of ABC-type cobalt transporter. Cobalt ions are necessary for the biosynthesis of cobalamin. The *lexA* gene encodes a transcriptional repressor of SOS function. The SOS response has been well characterized in *E. coli,* in which the reduce expression of the *lexA* gene under severe hyperosmotic stress results in damage to DNA (41).

Genes Whose Expression Was Repressed by Both Kinds of Stress

Both salt stress and hyperosmotic stress depressed the expression of genes for components of photosystem I (*psaD* and *psaF*), components of phycobilisomes (*cpcB, cpcC, cpcG, apcA, apcB, apcE,* and *apcF*), a sigma factor (*rpoDI*), a general secretion protein (*gspD*) and proteins of unknown function.

Most of the genes for components of photosystem I and phycobilisomes are located in tandem on the *Synechocystis* chromosome (20). We demonstrated previously that salt stress and hyperosmotic stress have inhibitory effects on the electron-transfer activity of photosystem I (9, 10). Repression of the expression of genes for photosystem I and phycobilisomes might be important for maintenance of a certain level of photosynthetic activity.

Synechocystis has five homologs of sigma-70 factors. The *rpoD1* gene (*slr0653*) encodes an indispensable sigma-70 factor, whereas the other *rpoD* genes encode sigma-70 factors that are not essential for growth under normal conditions (42). Both salt stress and hyperosmotic stress induced the enhanced expression of sigma-70 factors, slr0306 and sll2012, which are not essential for normal growth but might be involved in regulation of the expression of stress-inducible genes in *Synechocystis.*

Expression of the *gspD* gene for a protein in the general secretion pathway was also depressed by both salt stress and hyperosmotic stress. The GspD protein is located in the outer membrane in *Pseudomonas aeruginosa* (43). The repressed expression of the *gspD* gene suggests again that the outer membrane and plasma membrane are the targets of salt stress and hyperosmotic stress.

The regulation by salt stress and hyperosmotic stress of the expression of different sets of genes suggests that different mechanisms are operative in *Synechocystis* for acclimation to salt stress and hyperosmotic stress and, moreover, that *Synechocystis* recognizes salt stress and hyperosmotic stress as different kinds of stress. However, the components of the signaling pathways for salt stress and hyperosmotic stress remain to be identified.

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