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Identification of genes expressed in response to acid stress in *Synechocystis* sp. PCC 6803 using DNA microarrays

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Abstract

Plant cells are always exposed to various environmental stresses such as high light, low temperature and acid rain, and thus have to respond in order to survive these stresses. Although some mechanisms of responses to high light and low temperature etc., have been clarified, there is little information about the acclimation process to acid stress. In this study, the gene expression changes of *Synechocystis* sp. PCC 6803 in response to acid stress were examined using DNA microarrays (CyanoCHIP). We compared gene expression profiles of the cells treated at pH 8 (control) and pH 3 for 0.5, 1, 2 or 4 h. As a result, we found that 32 genes were upregulated by more than 3-fold, and 29 genes were downregulated by at least 3-fold after the acid treatment. Among these upregulated genes, expressions of *slr0967* and *sll0939* kept-increasing until 4 h under the acid stress and increased by 7 to 16-fold after the 4 h treatment. This suggests that the products of these two genes play important roles in the acid acclimation process.

Introduction

Plants have superior acclimation ability to environmental changes because it is impossible for them to move to avoid cell damages that may be caused by the environmental variations such as temperature, ionic strength, light and drought. Among these stresses, some acclimation mechanisms have been intensively studied in *Arabidopsis thaliana*, *Synechocystis* sp. PCC 6803 and other photosynthetic organisms. For instance, Wada et al. (1990) have reported that fatty acid desaturase (*des* A) plays an important role in chilling tolerance of a cyanobacterium, and Yamamoto (2001) reviewed the molecular mechanism for photoinhibition of PSII in the past decade.

Acid rain is one of the most serious threats to the environment. There is, however, little information about the acclimation process to acid stress in plants, algae and cyanobacteria, though acid stress responses have been studied in some bacteria (Olson 1993; Foster 1995; Bearson et al. 1997).

Cyanobacteria are prokaryotic organisms that perform oxygenic photosynthesis with machinery similar to green plants. With the widely accepted endosymbiotic origin of plastids and ease of genetic manipulations, cyanobacteria have been extremely useful in elucidating structural and mechanistic details of oxygenic photosynthesis. Furthermore, because of the ability of cyanobacteria to survive extreme environments, these organisms represent an excellent system for the

understanding of mechanisms of response to various stresses.

DNA microarrays of *Synechocystis* sp. PCC 6803 (CyanoCHIP) have, recently, been commercially available and are a valid tool for extensive analysis of the expression of all genes in the cyanobacterium. Microarrays are powerful tools for systematically analyzing expression profiles of large numbers of genes, especially for stress-inducible gene expression, tissue-specific gene expression and changes in the expression profiles of mutants and transgenic plants (Eisen and Brown 1999; Richmond and Somerville 2000). This DNA chip-based technology arrays cDNA sequences on a glass slide at a density of >1000 genes cm^{-2}. These arrayed sequences are hybridized simultaneously to a two-color fluorescently labeled cDNA probe pair prepared from RNA samples of different cell or tissue types, allowing direct and large-scale comparative analysis of gene expression.

In this study, we examined acid stress-inducible gene expression in *Synechocystis* sp. PCC 6803 using DNA microarrays. As a result, we found that 32 genes are upregulated by more than 3-fold, and 29 genes are downregulated by at least 3-fold after the acid treatment. The possible involvement of these genes in the acclimation to low pH was discussed.

Materials and methods

Culture

Synechocystis sp. PCC 6803 was cultivated at 30 °C in BG-11 medium (Stanier et al. 1971) that was buffered with 10 mM TES–NaOH (pH 8.0) under bubbling with 3% CO_2-containing air and illuminated at 30 μmol photons m^{-2} s^{-1} from incandescent bulbs. Cell growth was measured at 730 nm using a Pharmacia spectrophotometer.

Examination for acid stress conditions

Exponentially growing cells were collected by centrifugation and suspended in pH-adjusted BG-11 medium which was prepared with glycine (pH 2.2–3.6), MES (pH 5.5–7.0) or TES (pH 8.0) buffers. The cells were treated for 0.5–24 h under illumination at 30 μmol photons m^{-2} s^{-1} without CO_2 bubbling. After the treatment, the cells (OD_{730nm}=50) were spotted onto BG-11 plates at pH 8.0 after dilution of the cells to 1/1, 1/3, 1/10 or 1/30, and cultured for 7 days. Experiments at each pH were done in duplicate for at least three times.

DNA microarray analysis

Cells treated at pH 8.0 (control) or pH 3.0 (acid stress) for 4 h were collected by centrifugation at $4000 \times g$ for 5 min and then broken immediately using a Mini-Bead Beater (Biospec, Bartlesville, OK). Total RNA was isolated using the RNeasy Midi kit (Qiagen, Hilden, Germany) as described in Hihara et al. (2001). A *Synechocystis* DNA microarray (CyanoCHIP) was obtained from TaKaRa (Kyoto). This microarray covered 3079 of the 3168 ORFs of *Synechocystis*, excluding ORFs for transposases. Synthesis of Cy3-labeled (acid stressed cells) and Cy5-labeled (control cells) cDNAs and the other microarray assays were carried out according to Hihara et al. (2001). Image acquisition with a ScanArray 4000 (GSI Lumonics, Watertown, MA) was performed with the autobalance-autorange feature. With this feature, the sensitivity of the instrument can be automatically adjusted by changing the laser power and photomultiplier gain settings so that the signal is within 90% of maximum to prevent saturation. The raw data obtained with the ScanArray 4000 were analyzed with QuantArray version 2.0 software (GSI Lumonics). The fluorescence intensity of each spot in both Cy3 and Cy5 images was quantified, and local background fluorescence levels were subtracted. Cy3 and Cy5 images were normalized by adjusting the total signal intensities of the two images. The results shown were averages of four to six biologically independent experiments.

Results and discussion

First, we examined the acid pH limitation under which *Synechocystis* sp. PCC 6803 cells can survive, to determine the acid pH conditions for identification of genes expressed in response to acid stress by DNA microarray analysis. Preliminary experiments revealed that cell conditions of pre-culture and cell densities of inoculation on normal BG-11 plates significantly affected the cell

viabilities after acid stress treatments (data not shown). Therefore, the cells of four different concentrations were inoculated on normal BG-11 plates after the acid stress treatments in this study. Figure 1 and Table 1 show the viability of *Synechocystis* sp. PCC 6803 cells which were treated at various pH (2.2–8.0) for 0.5, 1, 2, 4 and 24 h and subsequently cultivated for 5 days in normal BG-11 (pH 8.0). As shown in Figure 1, growth in normal BG-11 plates of the cells treated below pH 3.6 for 24 h was completely suppressed, compared with the cells treated at pH 8.0. In contrast, the cells treated at pH 3.0 or 3.6 for 4 h showed a similar growth as the cells treated at pH 8.0. The acid pH limitation under which *Synechocystis* sp. PCC 6803 cells can survive, is summarized in Table 1. Cell growth was completely suppressed by treatments at pH 2.3 for 2 h and at pH 2.5 for 4 h. Based on these results, we used the cells treated at pH 3.0 for 0.5–4 h as acid-stressed cells for DNA microarray analysis.

Next, we carried out DNA microarray analysis using the cells treated at pH 8.0 (control) and at pH 3.0 (acid stress), and found that 32 genes were upregulated more than 3-fold by acid stress. Table 2 shows these upregulated genes of the cells treated at pH 3.0 for 0.5, 1 and 4 h, together with their gene products, and the upregulated genes by salt and osmotic stress, high light or low temperature. Acid stress induced the expression of genes encoding some known or putative stress-related proteins such as chaperones [*slr0093* (*dnaJ*), *sll1514* (*hspA*), and *sll0170* (*dnaK*)] and superoxide dismutase [*slr1516* (*sodB*)]. Cells might avoid the accumulation of denatured proteins or protect the transcriptional and translational apparatus by synthesizing chaperones under acid stress. In addition, a number of genes which are considered not to be directly related to stress responses were also observed; these include the *slr1558* gene for putative mannose-1-phosphate guanyltransferase, the *slr1675* (*hypA*) gene for hydrogenase expression/formation factor, the *ssr2595* (*hliB*) gene for high light-inducible protein, the *slr0611* (*sds*) gene for solanesyl diphosphate synthase, the *slr1214* gene for two-component response regulator PatA subfamily, *slr1285* gene for two-component sensor histidine kinase, the *sll0306* (*sigB*) and *sll2012* (*sigD*) genes for group 2 sigma factor, and 19 genes for proteins of unknown function. Among these genes, PatA and/or the gene product of *slr1285* might function to sense the acid signal, and SigB and SigD might induce the expression of some proteins which play a role in acid acclimation. The CtpA protein (*slr0008*) is a processing enzyme that cleaves off the C-terminal extension of the D1 protein (Shestakov et al. 1994). The over-expression of this gene may indicate that production of

Table 1. Summary of the viability of acid-treated cells of *Synechocystis* sp. PCC 6803

Acid treatment	*p*H					
	2.2	2.3	2.5	3.0	3.6	8.0
0.5 h	±	+	+	+	+	+
1 h	−	±	+	+	+	+
2 h	−	−	+	+	+	+
4 h	−	−	−	+	+	+
24 h	−	−	−	−	−	+

+, − and ±: the cell growth was not affected (+), remarkably suppressed (−) and slightly affected by acid stress (±), respectively.

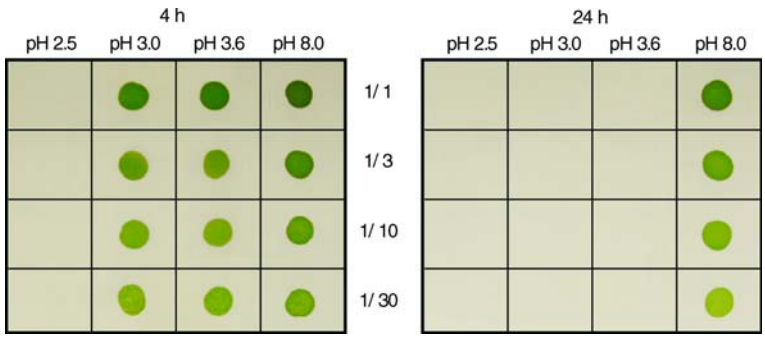

Figure 1. The viability of acid-treated cells of *Synechocystis* sp. PCC 6803. The cells were treated at various pH for 4 h (left) or 24 h (right). The treated cells after dilution of the cells to 1/1, 1/3, 1/10 or 1/30 were spotted onto normal BG-11 plates at pH 8 and cultured for 5 days at 30 °C.

Table 2. Genes whose expression was significantly upregulated by acid stress

ORF number	Gene	Product	Average ± SD			Upregulated genes by other stresses[a]
			0.5 h	1 h	4 h	
sll0170	*dnaK*	DnaK protein	1.93 ± 1.46	5.36 ± 1.87	1.99 ± 0.17	S O HL
sll0306	*sigB*	Group 2 RNA polymerase sigma factor SigB	10.71 ± 8.58	16.74 ± 6.25	4.33 ± 0.90	S O
sll0528		Hypothetical protein	36.82 ± 6.51	40.72 ± 18.71	11.56 ± 0.79	S O HL
sll0549		Hypothetical protein	9.18 ± 2.6	10.56 ± 2.85	8.48 ± 5.10	
sll0846		Hypothetical protein	18.76 ± 7.31	13.89 ± 12.14	41.80 ± 23.75	S O HL
sll0939		Hypothetical protein	7.75 ± 1.89	10.42 ± 3.84	15.69 ± 3.42	S O
sll1086		Unknown protein	3.69 ± 0.85	5.01 ± 1.72	5.28 ± 0.74	S
sll1483		Hypothetical protein	5.36 ± 2.55	6.89 ± 2.44	6.06 ± 1.18	S O HL
sll1514	*hspA*	16.6 kDa small heat shock protein, molecular chaperon	23.53 ± 8.86	16.41 ± 10.81	17.93 ± 3.93	S O HL
sll1558		Mannose-1-phosphate guanyltransferase	3.51 ± 0.59	6.94 ± 3.32	4.05 ± 0.73	
sll2012	*sigD*	Group 2 RNA polymerase sigma factor SigD	7.77 ± 1.04	10.60 ± 5.57	6.17 ± 1.42	HL
slr0008	*ctpA*	Carboxyl-terminal processing protease	3.83 ± 0.96	6.25 ± 3.00	2.85 ± 0.30	
slr0093	*dnaJ*	DnaJ protein	5.88 ± 1.49	6.13 ± 1.67	3.89 ± 0.51	S O
slr0270		Hypothetical protein	4.64 ± 0.87	3.59 ± 1.25	3.52 ± 0.68	
slr0611	*sds*	Solanesyl diphosphate synthase	5.45 ± 1.04	3.66 ± 1.69	2.69 ± 0.68	O LT
slr0967		Hypothetical protein	3.60 ± 0.63	4.09 ± 1.54	6.96 ± 0.33	S
slr1214		Two-component response regulator Pat A subfamily	9.85 ± 3.61	16.23 ± 9.21	7.49 ± 2.53	
slr1285		Two-component sensor histidine kinase	3.58 ± 1.71	8.89 ± 4.77	4.36 ± 1.07	
slr1413		Hypothetical protein	4.80 ± 2.18	7.38 ± 4.71	3.37 ± 0.61	
slr1516	*sodB*	Superoxide dismutase	2.45 ± 0.71	5.07 ± 1.81	2.39 ± 0.13	S O HL
slr1544		Unknown protein	24.46 ± 10.47	26.72 ± 14.56	15.60 ± 1.33	S O HL
slr1573		Hypothetical protein	2.97 ± 0.35	3.61 ± 0.83	2.35 ± 0.23	
slr1674		Hypothetical protein	22.07 ± 2.74	20.27 ± 7.90	9.23 ± 0.93	S O HL
slr1675	*hypA*	Hydrogenase expression/ formation protein HypA	13.51 ± 3.37	9.13 ± 4.43	6.71 ± 0.99	S O HL
slr1676		Hypothetical protein	4.77 ± 1.87	3.69 ± 2.11	2.56 ± 0.54	
slr1687		Hypothetical protein	16.57 ± 3.03	11.79 ± 6.14	6.27 ± 0.93	S O
slr1915		Hypothetical protein	11.16 ± 1.88	3.90 ± 2.08	2.09 ± 0.20	S
slr1916		Probable esterase	3.48 ± 0.46	2.75 ± 1.51	1.45 ± 0.18	
ssl3044		Probable ferredoxin	9.94 ± 1.09	7.91 ± 4.05	6.37 ± 0.57	S O HL
ssl3769		Unknown protein	4.51 ± 2.64	5.86 ± 2.79	2.16 ± 0.63	
ssr2016		Hypothetical protein	46.38 ± 12.88	40.31 ± 21.16	18.42 ± 3.07	S O
ssr2595	*hliB*	High light inducible protein HliB	34.78 ± 9.53	34.53 ± 16.27	24.66 ± 1.65	S O LT

[a] S, O, HL and LT indicate the upregulated genes by salt, osmotic, high light and low temperature stresses, respectively (Kanesaki et al. 2002; Hihara et al. 2001; Suzuki et al. 2001).

the D1 protein from its precursor is stimulated to make up the damaged D1 protein by acid stress. It is of interest to note that the expression of *slr0967* and *sll0939* kept-increasing until 4 h under the acid stress and increased by about 7- to 16-fold after the treatment for 4 h. These genes have also

been reported to be upregulated by osmotic and salt stresses (Kanesaki et al. 2002). The expressions of these genes were verified by real-time PCR in which *slr0967* and *sll0939* increased by 12 to 20-fold after 4 h under acid stress (data not shown). These results suggest that the products of these two genes play important roles in acid acclimation process. Interestingly, the two genes are located close to each other in the *Synechocystis* genome. We will report the characterization of their deletion mutants and functions of these genes (in preparation).

Table 3 shows the genes whose levels of expression were reduced by more than 3.0-fold, upon treatment of the cells at pH 3.0 for 0.5, 1 and 4 h. Interestingly, repression of the genes by acid

Table 3. Genes whose expression was significantly downregulated by acid stress

ORF number	Gene	Product	Average ± SD		
			0.5 h	1 h	4 h
sll0166	*cobA/hemD*	A fusion protein cobA and hemD	0.23 ± 0.05	0.26 ± 0.07	0.21 ± 0.04
sll0218		Hypothetical protein	0.19 ± 0.07	0.44 ± 0.19	0.49 ± 0.23
sll0219		Flavoprotein	0.21 ± 0.07	0.44 ± 0.21	0.49 ± 0.60
sll0947	*lrtA*	Light repressed protein A homolog	0.31 ± 0.03	0.63 ± 0.36	0.14 ± 0.04
sll0990		Glutathione-dependent formaldehyde dehydrogenase	0.67 ± 0.18	0.73 ± 0.24	0.22 ± 0.05
sll1099	*tufA*	Elongation factor Tu	0.18 ± 0.01	0.37 ± 0.63	0.40 ± 0.08
sll1194	*psbU*	Photosystem II 12 kDa extrinsic protein	0.95 ± 0.07	0.22 ± 0.04	0.43 ± 0.09
sll1286		Transcriptional regulator	0.73 ± 0.29	0.33 ± 0.10	0.23 ± 0.06
sll1501	*cobB*	Cobyrinic acid a,c-diamide synthase	0.42 ± 0.09	0.19 ± 0.04	0.40 ± 0.13
sll1732	*ndhF3*	NADH dehydrogenase subunit 5	0.21 ± 0.07	0.22 ± 0.05	0.44 ± 0.17
sll1733	*ndhD3*	NADH dehydrogenase subunit 4	0.13 ± 0.03	0.34 ± 0.14	0.18 ± 0.06
sll1734	*cupA*	Protein involved in low CO_2-inducible, high affinity CO_2 uptake	0.21 ± 0.04	0.31 ± 0.14	0.58 ± 0.23
sll1745	*rpl10*	50S ribosomal protein L10	0.16 ± 0.02	0.54 ± 0.19	1.25 ± 0.20
sll1808	*rpl5*	50S ribosomal protein L5	0.19 ± 0.06	1.77 ± 1.67	1.06 ± 0.27
sll1810	*rpl6*	50S ribosomal protein L6	0.19 ± 0.06	1.71 ± 1.53	0.84 ± 0.14
sll1812	*rps5*	30S ribosomal protein S5	0.23 ± 0.05	1.15 ± 1.00	0.78 ± 0.10
sll1818	*rpoA*	RNA polymerase alpha subunit	0.23 ± 0.03	1.32 ± 1.14	0.94 ± 0.31
sll1819	*rpl17*	50S ribosomal protein L17	0.22 ± 0.01	0.58 ± 0.35	0.78 ± 0.33
sll1820		tRNA pseudouridine synthase 1	0.19 ± 0.03	1.04 ± 0.74	0.63 ± 0.11
slr0009	*rbcL*	Ribulose bisphosphate carboxylase large subunit	0.24 ± 0.04	2.85 ± 4.32	0.23 ± 0.03
slr0011	*rbcX*	Possible Rubisco chaperonin	0.20 ± 0.02	1.24 ± 1.01	0.27 ± 0.03
slr0040	*cmpA*	Bicarbonate transport system substrate-binding protein	0.17 ± 0.08	0.62 ± 0.39	0.15 ± 0.05
slr0041	*cmpB*	Bicarbonate transport system permease protein	0.15 ± 0.04	0.64 ± 0.36	0.63 ± 0.27
slr0042		Probable porin; major outer membrane protein	0.23 ± 0.12	0.15 ± 0.05	0.13 ± 0.03
slr0144		Hypothetical protein	0.56 ± 0.14	0.80 ± 0.29	0.22 ± 0.03
slr0373		Hypothetical protein	1.74 ± 0.62	1.17 ± 0.60	0.15 ± 0.03
slr0374		Hypothetical protein	1.84 ± 0.62	1.66 ± 1.27	0.21 ± 0.04
slr1512	*sbtA*	Sodium-dependent bicarbonate transporter	0.11 ± 0.04	0.15 ± 0.07	0.06 ± 0.02
slr1513		Periplasmic protein, function unknown	0.14 ± 0.04	0.35 ± 0.21	0.22 ± 0.08

stress showed a profile quite different from that of osmotic and salt stresses (Kanesaki et al. 2002), although some of the upregulated genes which seem not to be directly related to stress were common not only in the case of acid stress but also in other stresses. Acid stress specifically depressed the expression of the genes for proteins involved in CO_2 uptake, CO_2 fixation and bicarbonate transport [*sll1732* (*ndhF3*), *sll1733* (*ndhD3*), *sll1734* (*cupA*), *slr0009* (*rbcL*), *slr0011* (*rbcX*), *slr0040* (*cmpA*) *slr0041* (*cmpB*) and *slr1512* (*sbtA*)]. The bicarbonate transport system might be made redundant in the acid-stressed cells, because most of the bicarbonate species exist in the form of CO_2 which diffuses through the cell membranes freely and the bicarbonate concentration is extremely low at pH 3.0. The genes for ribosomal proteins were also significantly repressed by acid stress [*sll1745* (*rpl10*), *sll1808* (*rpl5*), *sll1810* (*rpl6*), *sll1812* (*rps5*) and sll1819 (*rpl17*)], suggesting an overall repression of the translation machinery in the acid-stressed cells. Moreover, the transcript level of *tufA* which encodes protein synthesis elongation factor Tu, was down-regulated by more than 2.5-fold. The repression of *sll0166*, (*cobA*/*hemD*) and *sll1501* (*cobB*) were also observed, suggesting that biosyntheses of tetrapyrrole precursors for phycobilin and chlorophyll is slowed down by acid stress.

In summary, we performed a comprehensive analysis of global gene expression in response to acid stress using a cyanobacterium *Synechocystis* sp. PCC 6803, and found that 32 genes were upregulated by more than 3-fold, and 29 genes were downregulated at least 3-fold after the acid treatment. Characterization of the hypothetical proteins whose genes were upregulated by acid stress is in progress, which may help to understand the acclimation mechanisms in response to acid stress in plants.

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