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Selection and Characterization of Transposon Tagging Mutants of *Synechocystis* sp. PCC 6803 Sensitive to High-Light and Oxidative Stresses

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We compared several analytical tools to identify which were most applicable for the selection and characterization of specific transposon-tagged mutant strains of *Synechocystis* sp. PCC 6803 that are sensitive to high light and oxidative stresses. Our primary parameter was the maximum photochemical efficiency of dark-adapted cells, a very sensitive factor that can be determined in a non-destructive manner. Using this as a tool for primary selection, we identified five mutant strains with different sensitivities to photoinhibition and photooxidation. For further characterization, we obtained data describing the absorption spectra for pigment contents, the 77 K fluorescence spectra, non-photochemical quenching (as a down-regulation process), and the photosynthetic electron transfer rate. Based on these results, we were able to design a strategy for selecting mutants with specific phenotypes. Here, we also discuss the strengths and weaknesses of each selection and characterization tool.

Keywords: chlorophyll fluorescence, cyanobacterium, high light, oxidative stress, photoinhibition

Cyanobacteria are prokaryotic organisms that perform oxygenic photosynthesis in a manner remarkably similar to green plants (Gantt, 1994). Thus, these prokaryotes provide a simple cellular model for examining photosynthetic processes. *Synechocystis* sp. PCC 6803 is the first completely sequenced genome of a photosynthetic organism (Kaneko et al., 1996), and offers useful experimental guides for functional studies of higher plants, from photosynthesis to gene expression (Simpson and Stern, 2002; Vothknecht and Soll, 2002).

Activity of the photosynthetic apparatus is strongly affected by environmental parameters, such as light intensity and temperature (Demmig-Adams and Adams, 1992). As a short-term response to excess absorbed light, photosynthetic activity can be modulated either by redistributing excess light energy between the two photosystems or by quenching that excess energy into heat within antenna complexes (Bennett, 1991; Demmig-Adams and Adams, 1996; Gal et al., 1997). Long-term responses involve alterations in the pigment and protein composition of the photosynthetic apparatus and, usually, irreversible inactivation of the photosystems via photoinhibition or photooxidation (Kim and Lee, 2003).

To understand the function of genes involved in stress adaptation or defense mechanisms, it is useful to select mutants that are sensitive or resistant to those stresses. However, identifying the gene(s) responsible for specific phenotypic characteristics has been an obstacle. This problem could be overcome by the generation of transposon-tagged mutant libraries of *Synechocystis* sp. PCC 7942 (Katayama et al., 1999) and PCC 6803 (Chung et al., 2001). Using these mutant pools, the impaired gene in a selected mutant strain is then easily identified by inverse polymerase chain reaction (PCR) and direct sequencing of the transposon-flanking target sequence.

Another obstacle is that, if the selection criteria are not very specific, the functions of the genes identified are generally too broad. Therefore, one must develop a process to find only specifically useful strains. Although many analytical tools can be used for this purpose, most are time-consuming and laborious when handling several thousand mutant strains, and some require very expensive instrumentation.

Here, we describe specific tools, e.g., monitoring changes in photosynthetic efficiency (Fv_{dark}/Fm_{dark}), for selecting sensitive or tolerant strains against photoinhibition and photooxidation. We also investigate other possibilities that narrowed the specified range of gene

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functioning.

MATERIALS AND METHODS

Strains and Culture Conditions

We used wild type (WT) and transposon (Tn5)-tagged mutant strains of *Synechocystis* sp. PCC 6803, generated in the Korean SMCC (*Synechocystis* PCC 6803 Mutant Culture Collection), as described by Chung et al. (2001). These strains were grown in BG-11 media (Kratz and Myers, 1955) supplemented with 5 mM HEPES-NaOH (pH 7.5). Solid media were prepared by the addition of 1.5% (w/v) Bacto-agar (Difco, USA) and 5 mM sodium thiosulfate. To promote photomixotrophic growth, the cultures were grown in the presence of 10 mM glucose at 30°C under continuous illumination (25 to 35 μmol photons m^{-2} s^{-1} from fluorescent lamps). All strains were prepared as 50-mL cultures in 250-mL Erlenmeyer flasks. Their cell densities were estimated by measuring the optical density (OD) of the suspensions at 730 nm (OD_{730}) with a UV-visible spectrophotometer (CARY-4E; Varian, Australia).

Primary Selection of Transposon-Tagged Mutants Sensitive to High-Light and Oxidative Stresses

Cells were either incubated under high-light intensity (600 μmol photons m^{-2} s^{-1}) for 1 h at 30°C to induce photoinhibition or treated with 0.5 mM H_2O_2 (50 μmol photons m^{-2} s^{-1}) to induce oxidative stress. To screen for sensitive mutants, we used a pulse-modulated (PAM) fluorometer (PAM 101/102/103; Walz, Germany) to measure the maximal photochemical efficiency (Fv_{dark}/Fm_{dark}) of the treated cells after 10 min of dark-adaptation.

Identification of Transposon-Flanking Sequences by Inverse PCR

Genomic DNA was isolated according to Porter (1988), and completely digested by Taq I restriction enzyme (New England BioLabs, USA). The digested DNA products were self-ligated using DNA T4 ligase (Takara Bio, Japan). These products then served as templates to amplify the transposon-flanking sequences, using the sense primer (5′-CCGCACGATGAAGAGCAGAA-3′) and the antisense primer (5′-CACTTACCGATTTTACCGCA-3′). Inverse PCR was conducted as previously described for *Escherichia coli* (Ochman et al., 1988), with minor modifications, and included 32 cycles that each comprised 30 s at 94°C, 30 s at 56°C, and 72°C for 1 min. Afterward, the reactions were incubated at 72°C for an additional 7 min. Sequences for the amplified PCR products were determined with a 3700 DNA sequencer (Applied Biosystems, USA).

Measurement of Chlorophyll Fluorescence

Chlorophyll (Chl) fluorescence was continuously monitored with a PAM fluorometer, as described by Bissati et al. (2000). Cell suspensions containing 5 μg Chl mL^{-1} were stirred in cuvettes at 30°C. After dark-adaptation for 5 to 10 min (State 2), the cells were brought to State 1 through illumination with white light (200 μmol photons m^{-2} s^{-1}) in the presence of 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

The minimal fluorescence level (Fo_{dark}) was determined by illuminating the dark-adapted cells with a low intensity of red-modulated light (pulses of 1 ms, 100 kHz, 0.024 μmol photons m^{-2} s^{-1}). To obtain that minimal level in the light-adapted state (Fo′), the continuous exposure to white light was briefly interrupted. Maximum fluorescence of the dark-adapted (Fm_{dark}) or light-adapted (Fm′) cells was induced by an 800-ms pulse of 3200 μmol photons m^{-2} s^{-1} from a light source (KL 1500LCD; Scott, Germany). The variable fluorescence level (Fv_{dark}) in the dark-adapted state was achieved by subtracting Fo_{dark} from Fm_{dark}. NPQ (non-photochemical energy quenching) was calculated according to the Stern-Volmer formulation: NPQ = (Fm-Fm′)/Fm′, where Fm′ was measured after keeping the cells for 5 min in the presence of actinic light (i.e., light used for growth; 25 to 35 μmol photons m^{-2} s^{-1}) and the saturating-light pulse, as described by Campbell et al. (1998).

Measurement of 77 K Fluorescence Emission Spectra

Low-temperature (77 K) fluorescence emission spectra were measured with a fluorescence spectrophotometer (F-4500; Hitachi, Japan), with excitation at 580 nm. Cell suspensions were diluted to a concentration of 5 μg Chl mL^{-1} in the BG-11 media and injected into 4-mm-diameter glass tubes. After 10 min of dark-adaptation, the suspensions were rapidly frozen in liquid nitrogen for use at State 2. DCMU blocks electron transport from Q_A^- (the primary plastoquinone acceptor of PSII) to Q_B (the secondary

plastoquinone acceptor of PSII) and closes the reaction centers, nullifying qP (photochemical quenching). Therefore, at State 1, the dark-adapted cells were illuminated with white light in the presence of 10 μM DCMU. The slit width for both excitation and emission beams was 5 nm. At the concentration used, reabsorption of emitted fluorescence was negligible.

Measurement of Photosynthetic Electron Transport Rates

The rate of oxygen evolution and consumption from whole-cell suspensions diluted to 20 μg Chl mL^{-1} with BG-11 media was determined with a Clark-type O_2 electrode (YSI 5300; YSI, USA). To measure the whole-chain electron transfer from H_2O to CO_2, 1.0 mM $NaHCO_3$ was added to the cell suspensions. Photosystem II (PSII)-mediated electron transfer was measured in the presence of 2 mM phenyl-p-benzoquinone as the final electron acceptor. Photosystem I (PSI)-mediated electron transfer was assessed by monitoring oxygen consumption after the addition of 1 mM sodium ascorbate, 2 mM methyl viologen, 50 μM DCMU, and 1 mM N,N,N,N-tetramethyl-p-phenylenediamine. The reaction mixtures were maintained at 30°C and continuously stirred during the experiments. Saturating heat-filtered actinic light (1500 μmol photons m^{-2} s^{-1}) was provided from a 500-W slide projector. Photosynthetic electron transport rates (ETRs) were expressed as μmol O_2 mg Chl^{-1} h^{-1}.

Determination of Pigment Contents

Chl/phycocyanin (PC) ratios were calculated from the *in vivo* absorption spectra of WT and transposon-tagged mutant cells in BG-11 media with a UV-visible spectrophotometer (CARY-4E; Varian), according to the equation of Myers et al. (1980). PC and Chl contents were estimated from the corrected absorbance at 625 and 678 nm by simultaneous equations:

$A_{625}^{PC} = 1.0162\ A_{625} - 0.2612\ A_{678}$

$A_{678}^{Chl} = 1.0162\ A_{678} - 0.0630\ A_{625}$

PC content was calculated from A_{625}^{PC} in terms of the phycocyanobilin chromophore, using an absorption coefficient of 111 mM^{-1} cm^{-1}; Chl content was obtained from A_{678}^{Chl}, using an absorption coefficient of 68 mM^{-1} cm^{-1}, as previously determined by calibrating against Chl via extraction.

Chl content was also determined by the equation of Williams (1988), using the whole-cell spectra. To calculate its concentration, the optical density of a cell suspension was measured at 678, 720, and 750 nm:

μg Chl mL^{-1} = 14.96 (A_{678} - A_{750}) - 0.616 (A_{720} - A_{750})

After the Chl determination, cells were harvested and re-suspended in fresh BG11 media. A final Chl concentration of 5 or 20 μg Chl mL^{-1} was achieved, as described above, for the measurement of 77 K fluorescence emission spectra and photosynthetic ETRs.

The relative content of carotenoid was estimated from the ratio of A_{520}/A_{680}. Carotenoid pigment contents were also analyzed in detail using a HPLC system (HP 1100 Series; Hewlett-Packard, Germany) on a Zorbax ODS-1 column protected by a guard column (Lichrosorb, RP18). To extract the pigments, a 100-μL aliquot of the cells was vacuum-dried at room temperature, then vortexed for 30 s in the presence of 100% methanol with the same volume of silica sand. The extracts were filtered through a 0.45-μm membrane filter. Pigments were completely separated and eluted within about 25 min at a flow rate of 2 mL min^{-1} in the column, first using an 85:15 (v/v) solvent mixture of acetonitrile:methanol for 6 min, followed by a 20-min linear gradient of 66:34 (v/v) solvent mixture of methanol:ethyl acetate. The eluted pigments were automatically monitored at 450 nm and quantified according to Lagarde et al. (2000), with some modifications. The content of each carotenoid was determined using extinction coefficients reported previously (Steghens et al., 1997), and according to the following equation:

$C_{car} = C_{chl} \times [(\varepsilon_{chl} \times A_{car})/(\varepsilon_{car} \times A_{chl})]$,

where C_{chl} is the Chl concentration in the pigment extract and ε_{chl} and ε_{car} are the specific extinction coefficients of the chlorophyll and the carotenoids, respectively, at 450 nm.

RESULTS

Screening and Genetic Characterization of Mutants Sensitive to High-Light and Oxidative Stresses

Out of 200 transposon-tagging lines, we were able to select mutants sensitive to high-light or oxidative stress by examining changes in their Fv_{dark}/Fm_{dark} values after treatment with intense illumination or H_2O_2. Inverse PCR was performed to determine the Tn5 insertion sites of the selected mutants; its products were then used for direct sequencing. Full sequences of those genes containing the resulting partial sequences were identified through a BLAST search of Cyanobase on the web (http://www.kazusa.or.jp/cyano). Five of

the selected mutants contained the Tn5 sequences in their structural genes (Table 1).

All five mutants were sensitive to high-light intensity. Their mean values of Fv_{dark}/Fm_{dark} decreased to about 25~35% of those from the untreated controls after treatment, while values from the stressed WT decreased to about 40% of the control (Fig. 1). When the mutants were treated with 0.5 mM H_2O_2 in the light, all except slr1050 were found to be sensitive to this oxidative stress.

Photosynthetic Pigment Contents

Because photosynthetic pigment contents are closely related with the function of photosynthesis, we investigated *in vivo* changes in the pigment composition of *Synechocystis* cells after 1 h of treatment with either high light or oxidative stress. In those absorption spectra, Chls showed peaks at 438 nm and 680 to 681 nm; phycobilisomes, at 630 to 631 nm (Wilde et al., 1995); and carotenoids, at 460 to 520 nm (Shen

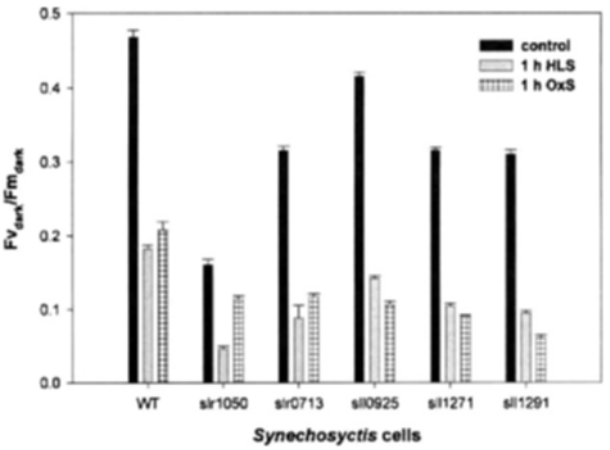

Figure 1. Changes in Fv_{dark}/Fm_{dark} of wild type (WT) and transposon-tagged mutants of *Synechocystis* sp. PCC 6803 after highlight and oxidative-stress treatments. All cells were either incubated under high-light stress (HLS; 600 μmol photons m^{-2} s^{-1}) for 1 h at 30°C or treated with 0.5 mM H_2O_2 (50 μmol photons m^{-2} s^{-1}) for oxidative stress (OxS). Values represent means ± S.E. (n = 3).

Table 1. Genes inserted with a tranposon, Tn5; location of insertion from start codons; and gene products of five *Synechocystis* sp. PCC 6803 transposon-tagged mutant cells.

Gene	Tn5 insertion site from start codon	Product
slr1050	118th bp	Hypothetical protein
slr0713	1106th bp	tRNA-guanine transglycosylase
sll0925	506th bp	Hypothetical protein
sll1271	306th bp	Probable porin, major outer membrane protein
sll1291	63rd bp	Two-component response regulator PatA subfamily

Table 2. Chlorophyll and phycocyanin contents of wild-type and *Synechocystis* sp. PCC 6803 transposon-tagged mutant cells. Values represent mean values from three independent experiments; those in parentheses indicate S.E. WT, wild type; Chl, chlorophyll; HLS, high-light stress; OxS, oxidative stress; PC, phycocyanin.

	Control			HLS			OxS		
	Chl (mM)	PC (mM)	Chl/PC	Chl (mM)	PC (mM)	Chl/PC	Chl (mM)	PC (mM)	Chl/PC
WT	0.198 (0.047)	0.079 (0.029)	2.51	0.191 (0.049)	0.066 (0.026)	2.89	0.294 (0.031)	0.136 (0.018)	2.16
slr1050	0.161 (0.044)	0.067 (0.028)	2.40	0.269 (0.047)	0.123 (0.032)	2.19	0.263 (0.038)	0.126 (0.023)	2.09
slr0713	0.255 (0.040)	0.114 (0.019)	2.24	0.286 (0.036)	0.127 (0.020)	2.25	0.211 (0.039)	0.113 (0.012)	1.87
sll0925	0.247 (0.025)	0.105 (0.026)	2.35	0.325 (0.023)	0.135 (0.012)	2.41	0.283 (0.042)	0.131 (0.024)	2.16
sll1271	0.230 (0.024)	0.105 (0.007)	2.19	0.278 (0.037)	0.132 (0.008)	2.11	0.250 (0.035)	0.116 (0.026)	2.16
sll1291	0.218 (0.031)	0.093 (0.012)	2.34	0.235 (0.026)	0.101 (0.002)	2.33	0.271 (0.025)	0.122 (0.014)	2.22

and Vermaas, 1994). Although the absorption spectra for both WT and mutant strains were roughly similar (data not shown), their Chl/PC ratios differed (Table 2), with ratios in the latter being slightly higher. For example, the Chl/PC ratio for the WT increased from 2.45 to 2.89 after 1 h of high-light stress, but decreased to 2.16 after the same period of oxidative stress. Although that fluctuation in ratios was also similar to that of mutant sll1291, the change in sll1050 was the opposite. No increase in ratio was observed with the other three mutants after either stress treatment. Using the absorbance ratio of A_{520}/A_{680} as an estimate of the relative content of total carotenoids to Chl, we found that those values decreased in all samples after either treatment type, except for oxidative-stressed sll0925 (Table 3).

HPLC also was performed to detail these changes in carotenoid content by separating the peaks for myxoxanthophyll, zeaxanthin, Chl a, echinenone, and β-carotene. Under normal growing conditions, the carotenoid composition (except for myxoxanthophyll) was already high in the control state, but decreased after high-light treatment in all selected transposon-tagged *Synechocystis* mutant cells except the sll1291 strain, which behaved more like the wild type. There, the contents for all WT carotenoids increased after the illumination treatment. In contrast, oxidative-stressed WT showed increased relative contents of myxoxanthophyll and β-carotene, while those of the other two carotenoids declined under such conditions. However, for most mutant cells (except those from sll1271 and sll1291), carotenoid contents either rose or maintained their high control-state values following oxidative treatment.

Energy Distribution and Relative Ratios for Two Photosystems

To determine whether structural modifications in the photosynthetic apparatus of *Synechocystis* cells were accompanied by functional alterations after high-light or oxidative stress, we examined the distribution of energy between the phycobilisomes and the two photosystems via 77 K fluorescence emission spectroscopy upon excitation at 580 nm (Fig. 2). Six spectra were measured at States 1 and 2, under normal conditions as well as after either stress treatment. The broad peak at 650 nm is believed to have resulted from the spectra of two components, one with its peak at 645 nm from PC, the other at 655 nm from allophycocyanin (Mullineaux, 1994; Ashby and Mullineaux 1999). The peak at approximately 690 nm was also composed of two components, one peaking at 685 nm, from the terminal emitters of phycobilisome; the other at 695 nm, from PSII. The peak at 720 to 722 nm came from PSI.

PSII fluorescence increased by the transition of WT cells from State 2 to State 1 without significant changes in phycobilisome fluorescence (Fig. 2A). Treatment of cells at State 2 with high light for 1 h was accompanied by a decrease in PSII emission relative to the PSI peak. Accordingly, the F720/F690 ratio of cells at State 2 increased from 0.66 to 0.72, but this ratio at State 1 decreased from 0.56 to 0.41. After high-light stress, phycobilisome fluorescence from cells at State 1 increased two-fold. In contrast, oxidative-stress treatment for 1 h caused PSII-related fluorescence to decline in cells at both states.

The slr1050 cells at both states showed low F720/F690 ratios compared with the WT and the other four mutants (Fig. 2B). Interestingly, this mutant showed trivial changes in its fluorescence emission spectra upon state transition under normal conditions. This transitional change was also observed in mutant cells under high light, but not under oxidative stress. At both states, the high light stress-induced changes in mutant cells were generally the same as those observed in WT, whereas phycobilisome fluorescence increased in cells at State 2. However, after 1 h of oxidative stress, both the phycobilisome- and PSII-related

Table 3. Relative contents of total carotenoids to chlorophyll in wild-type and *Synechocystis* sp. PCC 6803 transposon-tagged mutant cells, expressed as absorbance ratio of A_{520}/A_{680}. Values represent means from three independent experiments; those in parentheses indicate S.E. WT, wild type; HLS, high-light stress; OxS, oxidative stress.

	WT	slr1050	slr0713	sll0925	sll1271	sll1291
Control	0.626 (0.018)	0.633 (0.021)	0.594 (0.017)	0.569 (0.022)	0.552 (0.015)	0.586 (0.018)
HLS	0.500 (0.021)	0.531 (0.022)	0.480 (0.019)	0.473 (0.024)	0.500 (0.017)	0.556 (0.018)
OxS	0.474 (0.020)	0.470 (0.024)	0.551 (0.017)	0.587 (0.031)	0.482 (0.026)	0.476 (0.022)

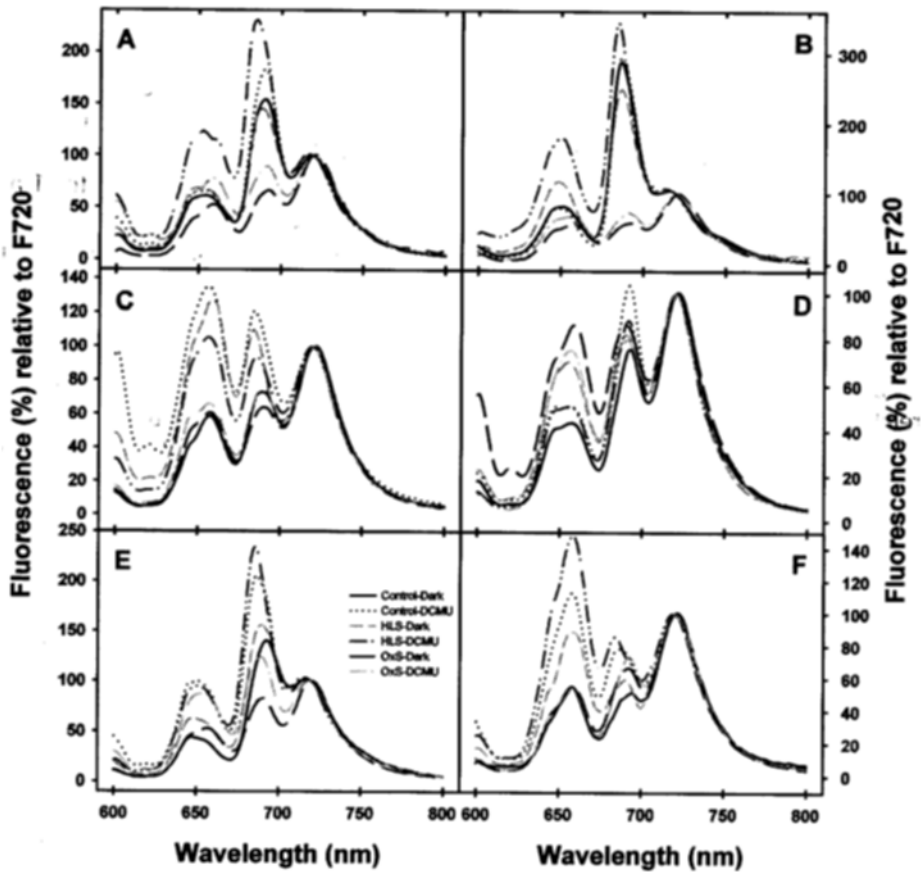

Figure 2. 77 K fluorescence emission spectra for **A**, wild type, or selected mutant strains: **B**, slr1050; **C**, slr0713; **D**, sll0925; **E**, sll1271; **F**, sll1291. Fluorescence was measured from whole cells pre-adapted to State 2 (dark-adapted) or State 1 (light-adaptec in presence of DCMU) before and after high-light and oxidative stresses. All spectra are expressed as percent values of fluorescences at 720 nm after samples were excited at 580 nm.

fluorescence emissions dramatically decreased, and PSI-related fluorescence emission was red-shifted.

The slr0713 mutant changed significantly in its fluorescence emission upon state transition under normal conditions (Fig. 2C). Furthermore, at State 2, both its phycobilisome- and PSII-related fluorescences were two-fold higher after high-light stress compared with cells under normal conditions. In contrast, the F720/F690 ratios decreased in cells at State 1. Following oxidative stress, the fluorescence emission of cells at both states was similar to that of cells at State 2, again under normal conditions.

Mutant cells of sll0925 showed significant changes in their fluorescence emission spectra upon the state transition under both normal conditions and after either stress treatment (Fig. 2D). The only exception was for the phycobilisome-related emission in cells at State 1 under high-light stress. In this mutant, the fluorescence characteristics of dark-adapted cells were similar to those in slr0713, but the phycobilisome-related fluorescence emission was very low compared with that from slr0713 cells. In contrast to the WT and the other mutant cells, this mutant at State 2 showed a higher phycobilisome-related fluorescence emission after oxidative stress. Phycobilisome fluorescence in cells at State 2 increased two-fold after both stresses, but PSII-related fluorescence in cells at State 1 decreased.

The sll1271 mutant showed significant changes in its spectra upon the state transition under normal conditions (Fig. 2E). Although its fluorescence spectra were similar to those of WT, phycobilisome-related fluorescence emission from cells at State 1 did not increase. After high-light stress, cells at State 2 showed an approximately 35% increase in their phycobilisome fluorescence compared with cells under normal conditions. Following oxidative stress, PSII-related fluorescence significantly decreased in cells at both states, while phycobilisome-related fluorescence increased. In general, the former rose after illumination treat-

Table 4. Relative contents of four different carotenoids to chlorophyll in wild-type and *Synechocystis* sp. PCC 6803 transposon-tagged mutant cells. Pigment contents were measured by HPLC. All values represent means± S.E. (n = 3). WT, wild type; HLS, high-light stress; OxS, oxidative stress.

		myxoxanthophyll	zeaxanthin	echinenone	β-carotene
WT	Control	0.017 ± 0.001	0.271 ± 0.002	0.048 ± 0.001	0.066 ± 0.001
	HLS	0.034 ± 0.002	0.398 ± 0.013	0.074 ± 0.005	0.131 ± 0.012
	OxS	0.030 ± 0.006	0.045 ± 0.003	0.016 ± 0.002	0.582 ± 0.032
slr1050	Control	0.016 ± 0.011	0.348 ± 0.010	0.063 ± 0.002	0.221 ± 0.002
	HLS	0.017 ± 0.002	0.188 ± 0.003	0.038 ± 0.002	0.138 ± 0.005
	OxS	0.024 ± 0.003	0.497 ± 0.027	0.071 ± 0.003	0.344 ± 0.033
slr0713	Control	0.017 ± 0.002	0.419 ± 0.019	0.061 ± 0.002	0.209 ± 0.005
	HLS	0.013 ± 0.002	0.201 ± 0.006	0.052 ± 0.003	0.204 ± 0.004
	OxS	0.022 ± 0.001	0.473 ± 0.006	0.052 ± 0.001	0.182 ± 0.002
sll0925	Control	0.016 ± 0.001	0.329 ± 0.005	0.055 ± 0.002	0.186 ± 0.002
	HLS	0.019 ± 0.002	0.311 ± 0.005	0.055 ± 0.004	0.187 ± 0.004
	OxS	0.031 ± 0.003	0.825 ± 0.049	0.106 ± 0.006	0.424 ± 0.021
sll1271	Control	0.029 ± 0.001	0.402 ± 0.009	0.085 ± 0.003	0.205 ± 0.006
	HLS	0.021 ± 0.002	0.375 ± 0.007	0.067 ± 0.002	0.192 ± 0.006
	OxS	0.024 ± 0.001	0.209 ± 0.004	0.040 ± 0.002	0.150 ± 0.002
sll1291	Control	0.015 ± 0.001	0.253 ± 0.017	0.048 ± 0.006	0.193 ± 0.004
	HLS	0.015 ± 0.001	0.388 ± 0.003	0.061 ± 0.003	0.235 ± 0.002
	OxS	0.023 ± 0.001	0.209 ± 0.011	0.045 ± 0.002	0.165 ± 0.002

ment but decreased because of oxidative stress. In cells at State 1, phycobilisome-related fluorescence emission was always higher than that from cells at State 2.

Finally, the spectra for cells of the sll1291 mutant also changed significantly upon the state transition under normal conditions (Fig. 2F). Phycobilisome-related fluorescence was significantly higher in cells at State 1 under both normal conditions and after exposure to high light. After that stress treatment, the phycobilisome fluorescence in cells at both states was roughly two-fold higher than that for normal conditions. In cells at State 1, the fluorescence emission peak at 690 nm was blue-shifted to 685 nm after the high-light stress, but red-shifted to 695 nm after the oxidative stress. After the latter, PSII fluorescence slightly increased after the transition from State 2 to State 1, but no changes were revealed in phycobilisome fluorescence. The same observation was made for cells at State 2 under normal conditions.

Non-Photochemical Quenching

Although intense saturating-light pulses can close all PSII reaction centers, a large NPQ can be observed in dark-adapted cyanobacterial cells. Therefore, the maximum yield of Chl fluorescence, Fm, is determined in the presence of DCMU and white light. The mechanism for NPQ in cyanobacteria is not clearly understood, yet this parameter is often used for estimating photoinhibition. To obviate photooxidative damage under high light intensities, most photosynthetic organisms have developed a non-radiative pathway, which dissipates excess light energy absorbed by the antenna system as heat. Here, NPQ increased following high-light stress in WT, slr0713, and sll1291, but decreased in the other three mutants, most prominently slr1050 (Table 5). After the oxidative stress treatment, NPQ dropped significantly to values near zero in all mutant strains except slr1050, where the value decreased 52% compared with that of the untreated control.

Electron Transport Rate (ETR)

The fluorometric estimation of photosynthetic electron transport efficiency is complicated in the case of cyanobacteria, because the Fo level contains variable contributions from PSII, PSI, and phycobilisome. Therefore, we measured photosynthetic oxygen evolution with a Clark-type oxygen electrode (Table 6). After treatment with high light, PSI-mediated ETRs were accelerated in all strains, whereas their PSII-mediated ETRs were rather depressed. Following oxidative stress, both PSI- and PSII-mediated ETRs declined. Interest-

Table 5. Non-photochemical quenching (NPQ) in *Synechocystis* cells. Values are means ± S.E. from three independent experiments; those in parentheses represent percentage of controls. WT, wild type; HLS, high-light stress; OxS, oxidative stress.

	Control	1 h HLS	1 h OxS
WT	0.178 ± 0.010 (100)	0.202 ± 0.011 (114)	0.138 ± 0.012 (77)
slr1050	0.032 ± 0.008 (100)	0.003 ± 0.003 (8)	0.015 ± 0.006 (48)
slr0713	0.023 ± 0.009 (100)	0.081 ± 0.009 (360)	0.001 ± 0.003 (4)
sll0925	0.134 ± 0.012 (100)	0.045 ± 0.012 (33)	0.001 ± 0.003 (0)
sll1271	0.055 ± 0.007 (100)	0.050 ± 0.009 (91)	0.001 ± 0.002 (2)
sll1291	0.050 ± 0.009 (100)	0.097 ± 0.012 (194)	0.001 ± 0.002 (2)

Table 6. Electron transport rate in *Synechocystis* cells. ETRs were calculated from oxygen evolution or uptake by electron flow from H_2O to HCO_3 through the whole chain (WC), from H_2O to phenylbenzoquinone through PSII, and from ascorbate/tetramethyl-*p*-phenylene diamine to methyl viologen through PSI. Values represent means ± S.E. from three independent experiments. WT, wild type; Chl, chlorophyll; HLS, highlight stress; OxS, oxidative stress.

		WT	slr1050	slr0713	sll0925	sll1271	sll1291
				ìmol O_2 / mg Chl hr			
Control	WC	157 ± 4	302 ± 10	89 ± 4	66 ± 2	60 ± 3	127 ± 5
	PSII	1218 ± 37	1926 ± 25	1015 ± 26	1282 ± 3	995 ± 35	670 ± 30
	PSI	242 ± 15	330 ± 43	100 ± 6	130 ± 5	90 ± 3	135 ± 4
HLS	WC	94 ± 3	168 ± 6	81 ± 3	29 ± 2	47 ± 2	107 ± 4
	PSII	1015 ± 39	1071 ± 10	748 ± 10	476 ± 17	891 ± 11	554 ± 17
	PSI	296 ± 11	340 ± 10	130 ± 4	162 ± 10	104 ± 2	147 ± 4
OxS	WC	90 ± 2	148 ± 1	50 ± 3	46 ± 1	45 ± 1	28 ± 3
	PSII	836 ± 4	1517 ± 12	625 ± 7	843 ± 6	752 ± 21	247 ± 4
	PSI	131 ± 4	145 ± 3	79 ± 3	128 ± 4	70 ± 2	61 ± 1

ingly, ETRs for slr1050 were not lower than those of WT. In the case of sll0925, PSII-mediated ETR was not decreased by oxidative stress, but it did drop significantly due to intense illumination. Changes in whole-chain ETR were essentially the same as those for PSII-mediated ETR. Results from our oxygen-evolution measurements after high-light stress somewhat confirmed those that had been obtained when we measured maximal photochemical efficiency.

DISCUSSION

To identify an efficient selection tool for selecting useful strains for specific purposes, we applied several analytical procedures for screening and characterizing high light- or oxidative stress-sensitive mutants among a transposon-tagged mutant library of *Synechocystis* sp. PCC 6803. Most of the parameters examined here require only non-destructive techniques, and each has inherent strong and weak points.

For preliminary screening of sensitive mutants, we measured the maximum photochemical efficiency of cells in the dark-adapted state (Fv_{dark}/Fm_{dark}). Eu et al. (1998) have demonstrated that the absolute fidelity of the Chl fluorescence assay provides a quick, non-destructive method for screening transgenic plants. This parameter, which can easily be measured with a portable Chl fluorometer, is informative, can efficiently handle thousands of samples in a short time period, and does not require expensive instrumentation. In higher plants, it is considered a good measure of PSII activity because it is correlated with the quantum yield of O_2 evolution and the actual number of functional PSII units, as measured by the yield of O_2 due to single turnover flashes (Chow et al., 1989; Öquist et al., 1992). In this study, we were able to identify five mutants sensitive to high-light stress. All but one, slr1050, were sensitive to oxidative stress. Our results were well-correlated with the photosynthetic ETRs of PSII. Nevertheless, this measure of photosynthetic activity is influenced by too many factors to specify the lesion sites of mutants within a narrow range.

As an alternative, more useful parameter, we were able to easily assess changes in the amounts or structures of most biomolecules by measuring their

absorption spectra. Specifically, photosynthetic pigment contents were monitored by determining fluctuations in their absorbance values at several specified wavelengths. This parameter also requires little time or expensive instruments, so that one could easily design a tool for high throughput measurements. Here, we evaluated the contents of Chls, PCs, and carotenoids to understand the adaptation mechanisms that cyanobacteria possess against high-light and oxidative stresses. In general, the mechanisms for regulating light-harvesting in response to high light include a decrease in the cellular contents of Chl and biliprotein complexes as well as changes in those of carotenoid pigments (Nomsawai et al., 1999; Miskiewicz et al., 2002). However, our results did not follow this general trend, perhaps because of dynamic fluctuations seen with several different carotenoids that are involved in these mutations. Moreover, the effect of applying our particular stresses had been demonstrated by our HPLC results.

Photosystem stoichiometry adjustments occur in cyanobacteria, red algae, and higher plants during growth under various light qualities and irradiances (Anderson and Osmond, 1987; Fujita and Murakami, 1987; Fujita et al., 1987; Chow et al., 1990; Kim et al., 1993). These changes are regulated through the balancing of excitation energy received by the accessory pigments of the two photosystems (Mullineaux, 1994). Although the measurement of 77 K fluorescence emission spectra is time-consuming, difficult to conduct when handling bulk samples, and requires trained hands, this tool provides a sensitive way to monitor in detail the energy transfer to and between photosystems as well as changes in the PSII/PSI ratio. In general, light-adapted cells (State 1) emit relatively more PSII-related fluorescence (F684 and F696) and less PSI-related fluorescence (F720-725) compared with dark-adapted cells (State 2). Therefore, the F720/F690 fluorescence ratio may also serve to diagnose the state transition of *Synechocystis* cells (Murakami, 1997).

Most of the data pertaining to F720/F690 ratios can also be easily obtained by monitoring changes in NPQ after the state transition at room temperature. Although NPQ may not reflect membrane energization in cyanobacteria (Campbell and Öquist, 1996), it still provides meaningful knowledge on the down-regulation and photooxidative damage of photosystems.

By basing the assessment of photosynthetic ETRs on oxygen evolution and/or uptake we are able to directly obtain information concerning the functions of PSII and PSI as well as whole-chain electron transport efficiency in photosynthetic membranes. Such measurements require an instrument to monitor the relative dissolved-oxygen content in a solution. Likewise, higher concentrations are necessary, which makes it difficult to handle bulkier samples than those needed for Chl fluorescence measurements.

As shown in this study, Fv_{dark}/Fm_{dark} proved to be the most convenient tool for screening *Synechocystis* mutants with different sensitivities to high-light or oxidative stress. Because four out of five high light-sensitive mutants were also sensitive to oxidative stress (slr1050 being rather resistant), we believe that when the similar results from our oxygen evolution measurements are also considered, we can conclude that the mutant strains slr1050, slr0713 and sll0925 are indeed sensitive to high light, while slr0713 and sll1291 are greatly affected by oxidative stress (sll1271 being rather less sensitive to either treatment). The partial inconsistency noted between the maximum photochemical efficiencies and PSII-mediated ETRs might be explained by the different mechanisms for photooxidative damage, e.g., donor-side (Powles, 1984; Asada, 1994) and/or acceptor-side inhibitions (Aro et al., 1993; Ke, 2001).

In *Synechocystis*, the fluorescence emission from phycobilisomes is higher in State 1, because those components are energetically decoupled from the photosystems. Interestingly, our oxidative stress-sensitive mutant strains (slr0713 and sll1291) at State 1 had a higher level of phycobilisome fluorescence under normal conditions than did the WT. Although very sensitive, the former showed little change in their fluorescence following that stress treatment. Likewise, carotenoid pigment contents showed only minor fluctuations. As shown in the F720/F690 ratio, the lower fluorescence emission from PSI in the slr1050 cells was probably due to the reduced absorption cross-section of the phycobilisomes or to an increased PSII content. Another possible explanation may be the irreversible photoreduction of pheophytin, as has been described by Nikitishena et al. (2002).

Hydrogen peroxide inhibited photosynthetic electron transport in all our selected mutant strains except for slr1050. Samuilov et al. (2001) have also observed such a decrease in *cyanobacteria* after treatment with exogenous H_2O_2. In the present study, we noted a decline in maximum photochemical efficiency, as well as in whole-chain photosynthetic ETR and NPQ after oxidative stress. Singlet oxygen, a reactive oxygen species generated by high-light intensities, is thought to cause damage to D1 proteins because it is locally produced in the reaction centers of PSII. In contrast,

H_2O_2, another reactive oxygen species, contacts and reacts mainly with the lumenal side of the thylakoid membranes by diffusing from outside the cells when exogenously added. Therefore, we suggest that H_2O_2 acts on Mn-clusters in the oxygen-evolving complexes of PSII.

Maximum photochemical efficiency is still a good parameter for conducting primary selections if no other tool is available. Although it is less sensitive than Chl fluorescence analysis, determining absorbance at certain meaningful wavelengths can be an alternative to estimating pigment content. The measurement of photosynthetic ETR from oxygen evolution or uptake is somewhat labor- and time-consuming but, when necessary, it helps to increase the accuracy of the intended selection. We were able to collect much detailed information by examining the 77 K fluorescence emission spectra, although that technique was not efficient for high-throughput selection of mutants. During state transition, the 77 K fluorescence spectra changed significantly, and some data were instead obtained by measuring NPQ from cells at two different states. Therefore, NPQ analysis may be the best tool for primary selection of mutants with specific phenotypes that are affiliated with the defensive or regulatory mechanisms of Cyanobacteria following stress treatment.

In conclusion, we have adopted a novel approach that utilizes various analytical tools to identify strains of *Synechocystis* sp. PCC 6803 with different degrees of sensitivity to photoinhibition and oxidative stresses. These five strains have very distinctive phenotypes that can provide useful tagging mutants to aid in the selection process for characterizing the structure and function of the photosynthetic apparatus.

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