A Specific Single Nucleotide Polymorphism in the ATP Synthase Gene Significantly Improves Environmental Stress Tolerance of *Synechococcus elongatus* PCC 7942

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ABSTRACT In response to a broad range of habitats and environmental stresses, cyanobacteria have evolved various effective acclimation strategies, which will be helpful for improving the stress tolerances of photosynthetic organisms, including higher plants. *Synechococcus elongatus* UTEX 2973 and PCC 7942 possess genomes that are 99.8% identical but exhibit significant differences in cell growth and stress tolerance. In this study, we found that a single amino acid substitution at F_oF₁ ATP synthase subunit α (AtpA), C252Y, is the primary contributor to the improved stress tolerance of *S. elongatus* UTEX 2973. Site-saturation mutagenesis experiments showed that point mutations of cysteine 252 to any of the four conjugated amino acids could significantly improve the stress tolerance of *S. elongatus* PCC 7942. We further confirmed that the C252Y mutation increases AtpA protein levels, intracellular ATP synthase activity, intracellular ATP abundance, transcription of *psbA* genes (especially *psbA2*), photosystem II activity, and glycogen accumulation in *S. elongatus* PCC 7942. This work highlights the importance of AtpA in improving the stress tolerance of cyanobacteria and provides insight into how cyanobacteria evolve via point mutations in the face of environmental selection pressures.

IMPORTANCE Two closely related *Synechococcus* strains showed significantly different tolerances to high light and high temperature but limited genomic differences, providing us opportunities to identify key genes responsible for stress acclimation by a gene complementation approach. In this study, we confirmed that a single point mutation in the α subunit of F_oF₁ ATP synthase (AtpA) contributes mainly to the improved stress tolerance of *Synechococcus elongatus* UTEX 2973. The point mutation of AtpA, the important ATP-generating complex of photosynthesis, increases AtpA protein levels, intracellular ATP synthase activity, and ATP concentrations under heat stress, as well as photosystem II activity. This work proves the importance of ATP synthase in cyanobacterial stress acclimation and provides a good target for future improvement of cyanobacterial stress tolerance by metabolic engineering.

KEYWORDS *Synechococcus elongatus*, ATP synthase, point mutation, abiotic stress, photosynthesis

As the most ancient oxygenic photosynthetic microorganisms, cyanobacteria have survived and emerged from multiple mass extinction events over the past 3.5 billion years (1). They are widely distributed in aquatic, terrestrial, and extreme habitats and are the numerically dominant global primary producers at present (2). Various effective adaptive strategies used by cyanobacteria to combat different environmental stresses have been explored and reported (3), and some of these have been success-

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fully used for improving the stress tolerances of higher plants (4, 5). In recent years, cyanobacterial cells have been genetically engineered to produce many chemicals and fuels directly from carbon dioxide and solar energy by modifying natural metabolic pathways and installing heterologous synthetic pathways (6). Due to the complex environmental implications and serious contamination associated with outdoor scale-up cultivation, it is important to understand and improve the stress tolerance of cyanobacterial cell factories for future industrial applications.

Synechococcus elongatus UTEX 2973 (Sye2973) is a recently isolated fast-growing cyanobacterium with excellent stress tolerance (7), exhibiting its fastest photoautotrophic growth at 42°C and 1,500- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination (8). In contrast, *Synechococcus elongatus* PCC 7942 (Sye7942) cannot grow under the same conditions (8). Interestingly, the genome sequences of these two *Synechococcus* strains are 99.8% identical. A total of 55 single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) between these two genomes have led to amino acid changes in 21 chromosome-encoded and 3 plasmid-encoded proteins (7). Thus, some of these genomic differences may be responsible for the phenotypic differences between the two *Synechococcus* strains, particularly in terms of stress tolerance under high-temperature and high-light conditions. Among the proteins that differ in the two strains, chaperone DnaK, DNA-directed RNA polymerase, F_oF_1 ATP synthase, photosystem I (PSI) assembly protein Ycf4, and glutamate synthase may be related to significant differences in stress tolerance capability. However, there is no experimental evidence to predict which genes are involved and how they contribute to the improved performance of Sye2973 under stress conditions.

In cyanobacteria, photosynthesis involves a series of light-induced electron transfers from water to NADP^+ , accompanied by the acidification of the thylakoid lumen and the generation of oxygen, NADPH, and ATP (9). Moreover, photosynthesis is highly susceptible to environmental changes. Photosystem protection is one of the most important strategies for acclimation to abiotic stresses and involves (i) accelerating repairs of PSII, consisting in replacement of damaged D1 proteins with newly synthesized and matured ones (10), (ii) redistributing the excitation light energy between PSII and PSI (11, 12) and decreasing the light energy transferred to PSII through nonphotochemical quenching (NPQ) (13), (iii) increasing cyclic electron transport and decreasing linear electron transport, and (iv) regulating the transcription of stress-responsive genes encoding heat shock proteins (HSPs), superoxide dismutase (SOD), and the extrinsic PSII oxygen-evolving complex (3, 14).

In this study, a high-throughput plate-based screening method was newly developed to identify specific SNPs contributing to the high-temperature and high-light tolerance of Sye2973. Using this method, we identified a single SNP in *atpA*, encoding the F_oF_1 ATP synthase α subunit (AtpA), as the main contributor. This mutation, introduced into Sye7942, improved its stress tolerance, and a site-saturation mutagenesis experiment was performed to assess SNP plasticity. We demonstrated that point mutations of cysteine 252 to four conjugated amino acids could significantly improve the stress tolerance of Sye7942. Then we confirmed that the C252Y mutation in AtpA served to increase both the protein level and the intracellular activity of F_oF_1 ATP synthase, as well as increasing intracellular ATP abundance, *psbA* transcription, the PSII/PSI ratio, the linear electron transport rate, the oxygen evolution rate, and glycogen accumulation under heat stress conditions. These results elucidate the mechanism leading to the significant difference in stress tolerance between Sye2973 and Sye7942.

RESULTS

A single nonsynonymous SNP in the *atpA* gene is predominantly responsible for the improved stress tolerance of Sye2973. To identify genes indispensable for the improved stress tolerance of Sye2973 over that of Sye7942 (Table 1), a plate-based screening method was established through gene complementation of Sye7942 by introducing different SNP-containing genes of Sye2973 (see Fig. S1A in the supplemental material) rather than constructing traditional gene knockouts (Fig. S1B) of

TABLE 1 Bacterial strains used in this study

Strain name	Genotype description ^a	Source
Sye7942	<i>Synechococcus elongatus</i> PCC 7942, wild type	Xudong Xu
Sye2973	<i>Synechococcus elongatus</i> UTEX 2973, wild type	UTEX ^b
Sye7942-C252Y	Mutant strain of Sye7942 harboring AtpA with the C252Y point mutation	This study
Sye7942-C252H	Mutant strain of Sye7942 harboring AtpA with the C252H point mutation	This study
Sye7942-C252F	Mutant strain of Sye7942 harboring AtpA with the C252F point mutation	This study
Sye7942-C252W	Mutant strain of Sye7942 harboring AtpA with the C252W point mutation	This study

^aAtpA, ATP synthase F₀F₁ subunit α .^bUTEX, the Culture Collection of Algae at The University of Texas at Austin.

Sye2973. It was found that Sye7942 cells loaded onto plates could not survive at 43°C and 220- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination, whereas Sye2973 could survive not only under these conditions but also under even harsher conditions (43°C, 500- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination) (see Fig. S2 in the supplemental material). Therefore, a two-step plate-based screening method was implemented for further transformation experiments, using 43°C and 220- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination for the first round of screening and 43°C and 500- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination for rechecking (see Fig. S3 in the supplemental material).

From an evolutionary perspective, genes containing nonsynonymous SNPs are more likely to lead to phenotypic changes than those harboring synonymous SNPs. As reported previously, there are 55 SNPs that cause nonsynonymous mutations in 24 proteins in Sye2973 (7). The small endogenous plasmid (GenBank accession number [CP006473](#)) harboring three nonsynonymous SNPs was found to be lost in the Sye2973 strain (Table 1) used in this study. Thus, recombinant plasmids (Table 2) harboring each of the 21 chromosome-encoded candidate genes from Sye2973 were used to transform Sye7942. After the two-step plate screening (Fig. S3 in the supplemental material), only high-temperature- and high-light-tolerant colonies could be isolated from the transformed cells of Sye7942. We found that all tolerant colonies isolated contained a SNP in the *atpA* gene, encoding the α subunit of F₀F₁ ATP synthase. The point mutation replacing cysteine 252 with tyrosine (C252Y) in AtpA was first confirmed by Sanger sequencing. This isolated mutant of Sye7942 was named Sye7942-C252Y, and like Sye2973, it was able to grow at 43°C and 500- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination (Fig. 1A). Resequencing of the complete genome further ensured that this point mutation was the only mutation found in the genome of Sye7942-C252Y relative to the original wild-type strain of Sye7942. These findings confirmed that among 21 SNP-containing genes (Table 2), the *atpA* gene is the primary contributor to the increased stress tolerance of Sye2973.

The C252Y mutation in AtpA improves the tolerance of Sye7942 to abiotic stresses. To systematically evaluate the tolerance of Sye7942-C252Y to abiotic stresses, its growth in liquid medium under normal conditions (Fig. 1B) and under high-temperature (Fig. 1C), high-light and high-temperature (Fig. 1D), and high-light (see Fig. S4 in the supplemental material) stress conditions was compared with that of wild-type Sye7942 and Sye2973. Under both normal (30°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) (Fig. 1B) and high-light (Fig. S4) culture conditions, the growth of Sye7942-C252Y was similar to that of Sye2973 and Sye7942. When the temperature was increased to 41°C, however, the Sye7942-C252Y mutant clearly exhibited better growth than Sye7942 (Fig. 1C). Under high-light and high-temperature conditions (41°C, 500 $\mu\text{E m}^{-2} \text{s}^{-1}$), the growth rate of Sye7942-C252Y increased significantly ($P < 0.05$), from 0.03 to 0.05 h^{-1} (Fig. 1D; see also Fig. S5 in the supplemental material), over that under normal conditions. Sye2973 showed a growth rate similar (0.04 h^{-1}) to that of Sye7942-C252Y, while Sye7942 did not grow at all, under the same culture conditions (Fig. 1D; also Fig. S5). These results demonstrate that the C252Y point mutation in AtpA significantly improves the tolerance of Sye7942 to high-light and high-temperature stresses.

TABLE 2 Plasmids and primers used in this study

Purpose and plasmid	Characteristics ^a	Primers used for plasmid construction (5' → 3')
Identification of genes responsible for stress tolerance		
pSK027	Contains the M744_03965 gene, encoding ABC transporter substrate-binding protein; Ap ^r Km ^r	F1, GGGAATCCCGCTAGCTTGGCAACTGCAGC R1, GTTGTGCGGTGCGGAGCAGATCGGACAGG
pSK028	Contains the M744_03975 gene, encoding a hypothetical protein; Ap ^r Km ^r	F2, CTGTCCGATGCTCTCCGACCGCCATCAAC R2, CGCAAGTAGAGCTGCGCTGCAAGCCAGCC
pSK029	Contains the M744_04780 gene, encoding PpnK, inorganic polyphosphate/ATP-NAD kinase; Ap ^r Km ^r	F3, CGGCAATCTGTAGCGGATCGATGACAG R3, GGATCAGGAACCACTGATCCCGCGAGC
pSK030	Contains the M744_06025 gene, encoding molecular chaperone DnaK; Ap ^r Km ^r	F4, CCACAGTCTGATGCCGCAATTGACCGC R4, GGGCAAGAGCGGAGGCGATTCACAAGAGC
pSK031	Contains the M744_06570 gene, encoding hydrolase; Ap ^r Km ^r	F5, GGGCAGGCTTGGAGGGGCTGCGAATG R5, CAGTCAGGCACAGGCTGACATTCACACAC
pSK032	Contains the M744_06650 gene, encoding CTP synthetase; Ap ^r Km ^r	F6, GGGCAGCTTGGCCCGATTGTACTGCTG R6, GAGTGTGCAATGCGAACGACTTGAATCG
pSK033	Contains the M744_05865 gene, encoding a hypothetical protein; Ap ^r Km ^r	F7, GGAATACCTTTACTGAGCTTGAGCAATTAC R7, CATTCTGAGATTTTGAATTATCTCACCCC
pSK034	Contains the M744_06850 gene, encoding chorismate mutase; Ap ^r Km ^r	F8, CTCCTAGCGGGGATTTTACAAATCAGAAG R8, CTCTGCTGTAGCCACATTTGCTCAGTAC
pSK035	Contains the M744_11685 gene, encoding anthranilate synthase, component I; Ap ^r Km ^r	F9, CCCAACAACTGGCGAGATATCCCGAAAAG R9, GAAATGAACGAAGTCTCTCTTCGCGGG
pSK036	Contains the M744_12130 gene, encoding long-chain-fatty-acid CoA ligase; Ap ^r Km ^r	F10, GATCGCTTTGACATTGATCGTCGCTTTAGC R10, CAGGCTGAGACGAGGATGCTCCAGTCG
pSK037	Contains the M744_00705 gene, encoding a hypothetical protein; Ap ^r Km ^r	F11, GTTGTGCAACAGCTGATTTTGTGATCGAG R11, CTAGGCTGAGGATGAGGCGCAGCGATCGC
pSK038	Contains the M744_01335 gene, encoding ATP synthase F _o F ₁ subunit α; Ap ^r Km ^r	F12, GAGAACTTGTCTTGAGCCGACGACCTTG R12, CAGCCAGAAAAAGCGTTCTCAACCAAGTG
pSK039	Contains the M744_02605 gene, encoding chemotaxis protein CheY; Ap ^r Km ^r	F13, CATTAACTGCCCTAGAAAGATGCTGTG R13, CGATCACTGGTGGTATTGAACAGGCTG
pSK040	Contains the M744_02855 gene, encoding a pilus assembly chaperone; Ap ^r Km ^r	F14, GGATGAATGGTAACTCTTGATCGAGTG R14, CAGCAACGCTCGGTGTTGATGGAATTGG
pSK041	Contains the M744_03320 gene, encoding 23S rRNA; Ap ^r Km ^r	F15, CATATTTGATAGCGGTATCAGAGTCAAC R15, CAGTACTACTCAAGCCTACCACTGCCAG
pSK042	Contains the M744_03335 gene, encoding manganese ABC transporter ATP-binding protein; Ap ^r Km ^r	F16, CTCTCAAGATTGCGCTGAACTGTGACACC R16, GAATCAGGACTGATAGTCTTCAAACTG
pSK043	Contains the M744_03855 gene, encoding guanylate cyclase; Ap ^r Km ^r	F17, GGTCTACAGAGATCGCTCACCGATCCATCG R17, GAGTAGCTGCGCGGCTGTGATAAAACC
pSK044	Contains the M744_08615 gene, encoding DNA-directed RNA polymerase subunit B; Ap ^r Km ^r	F18, CGGCGCTAAACGACCGAGGAGCGCATGGC R18, CTCAAAACCATCCCGGATCACTATCGAC
pSK045	Contains the M744_09095 gene, encoding porin; major outer membrane protein; Ap ^r Km ^r	F19, GCTTGCTGACAGGACTTTCATGTGTG R19, GTGACATTGCGCCCGCTTCTTCTAGG
pSK046	Contains the M744_12285 gene, encoding glutamate synthase; Ap ^r Km ^r	F20, CAGGCCATGATGAGCTAACAGCGCAAG R20, GCTTTCTCCATTGCGCGGTTATCGCAGC
pSK047	Contains the M744_13540 gene, encoding photosystem I assembly protein Ycf4; Ap ^r Km ^r	F21, GTTCGAAACGCAATCCGAAATCTGGCTCC R21, GCTGACCACCTATGGCGTTGAGGTGTC

(Continued on next page)

TABLE 2 (Continued)

Purpose and plasmid	Characteristics ^a	Primers used for plasmid construction (5' → 3')
<i>In vivo</i> site-saturation		
mutagenesis of AtpA		
pSK090	Contains the AtpA gene with the C252A mutation; Ap ^r Km ^r	252A-1, GATGCCGAGTACTTTATGGCTAAAGGCAAAGCC
pSK091	Contains the AtpA gene with the C252D mutation; Ap ^r Km ^r	252A-2, AGCCATAAAGTACTCGCGATCGCAGCACCGC
pSK092	Contains the AtpA gene with the C252E mutation; Ap ^r Km ^r	252D-1, GATGCCGAGTACTTTATGGACAAAGGCAAAGCC
pSK093	Contains the AtpA gene with the C252F mutation; Ap ^r Km ^r	252D-2, GTCATAAAGTACTCGCGATCGCAGCACCGC
pSK094	Contains the AtpA gene with the C252G mutation; Ap ^r Km ^r	252E-1, GATGCCGAGTACTTTATGGAAAAGGCAAAGCC
pSK095	Contains the AtpA gene with the C252H mutation; Ap ^r Km ^r	252E-2, TTCATAAAGTACTCGCGATCGCAGCACCGC
pSK096	Contains the AtpA gene with the C252I mutation; Ap ^r Km ^r	252F-1, GATGCCGAGTACTTTATGTTCAAAGGCAAAGCC
pSK097	Contains the AtpA gene with the C252K mutation; Ap ^r Km ^r	252F-2, GAACATAAAGTACTCGCGATCGCAGCACCGC
pSK098	Contains the AtpA gene with the C252L mutation; Ap ^r Km ^r	252G-1, GATGCCGAGTACTTTATGGCAAAGGCAAAGCC
pSK099	Contains the AtpA gene with the C252M mutation; Ap ^r Km ^r	252G-2, GCCATAAAGTACTCGCGATCGCAGCACCGC
pSK100	Contains the AtpA gene with the C252N mutation; Ap ^r Km ^r	252H-1, GATGCCGAGTACTTTATGCACAAAGGCAAAGCC
pSK101	Contains the AtpA gene with the C252P mutation; Ap ^r Km ^r	252H-2, GTGCATAAAGTACTCGCGATCGCAGCACCGC
pSK102	Contains the AtpA gene with the C252Q mutation; Ap ^r Km ^r	252I-1, GATGCCGAGTACTTTATGATCAAAGGCAAAGCC
pSK103	Contains the AtpA gene with the C252R mutation; Ap ^r Km ^r	252I-2, GATCATAAAGTACTCGCGATCGCAGCACCGC
pSK104	Contains the AtpA gene with the C252S mutation; Ap ^r Km ^r	252K-1, GATGCCGAGTACTTTATGAAAAGGCAAAGCC
pSK105	Contains the AtpA gene with the C252T mutation; Ap ^r Km ^r	252K-2, TTTATAAAGTACTCGCGATCGCAGCACCGC
pSK106	Contains the AtpA gene with the C252V mutation; Ap ^r Km ^r	252L-1, GATGCCGAGTACTTTATGCTGAAAGGCAAAGCC
pSK107	Contains the AtpA gene with the C252W mutation; Ap ^r Km ^r	252L-2, CAGCATAAAGTACTCGCGATCGCAGCACCGC
Random library	Contains the AtpA gene with a random C252 mutation; Ap ^r Km ^r	252M-1, GATGCCGAGTACTTTATGATGAAAAGGCAAAGCC

^aAp, ampicillin; Km, kanamycin; AtpA, gene encoding the F₀F₁ ATP synthase α subunit.

FIG 1 Evaluation of stress tolerances of *S. elongatus* strains to different abiotic stresses. (A) *S. elongatus* strains grown on BG11 plates at 43°C with 500- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination. (B through D) Growth of the same strains in liquid BG11 medium at 30°C with 50- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination (B), at 41°C with 50- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination (C), and at 41°C with 500- $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination (D). Error bars indicate standard deviations ($n = 3$).

A point mutation of cysteine 252 to any of four amino acids with conjugated side chains improves the stress tolerance of Sye7942. To further analyze the plasticity of this SNP, an artificial mutation selection approach was undertaken for AtpA evolution as follows. The C252 residue of AtpA was mutated *in vivo* to 19 other amino acids via random or specific mutation, and these mutations were screened using the screening method described above. After Sye7942 was transformed with a random plasmid library containing *atpA* genes with random codons (NNN) at amino acid position 252, the C252W, C252F, and C252H mutations in AtpA were observed among high-temperature- and high-light-tolerant colonies after phenotypic and genotypic confirmation (Table 3). Interestingly, all three of these amino acids and tyrosine together make up the only four conjugated amino acids in nature. Unexpectedly, the C252Y mutation was not found in this screening. This may be due to the small number ($n = 35$) of stress-tolerant colonies available for genotyping or to base composition bias in the plasmid library, which may have led to the lack of tyrosine-specific codons at position 252.

To ensure that all amino acid mutations were screened equally, 19 plasmids (Table 2), each harboring 1 of 19 specific C252 amino acid substitutions, were constructed and were used to transform Sye7942. Although stress-tolerant colonies could be isolated following transformation with eight plasmids, only four amino acid substitutions—C252W, C252F, C252H, and C252Y—were confirmed among the AtpA proteins in these colonies following genotyping (Table 3). Combined with the findings of random codon experiment mentioned above, our results indicate that only mutations of C252 in AtpA to conjugated amino acids (Y, W, F, H) can improve the tolerance of Sye7942 to high-temperature and high-light conditions. This implies that the conjugated side chain of the amino acid at position 252 in the ATP synthase α subunit might be crucial for the stress tolerance of Sye7942 under the conditions tested.

C252 point mutations increase AtpA protein levels, the intracellular enzymatic activity of F_0F_1 ATP synthase, and ATP levels. To determine how the C252Y mutation

TABLE 3 Codons and amino acids appearing at position 252 of AtpA from stress-tolerant colonies

Amino acid expected	Codon used	Amino acid mutation	Codon confirmed by Sanger sequencing
Random	NNN	F W H Y	UUC UGG CAU UAC
A	GCU		
D	GAC		
E	GAA		
F	UUC	F	UUC
G	GGC	F	UUC
H	CAC	H	CAC
I	AUC		
K	AAA	Y	UAC
L	CUG		
M	AUG		
N	AAC		
P	CCC		
Q	CAA		
R	CGC	F	UUC
S	AGC		
T	ACC		
V	GUG		
W	UGG	W	UGG
Y	UAC	Y	UAC

affects AtpA function, the AtpA expression levels, F_0F_1 ATP synthase activities, and intracellular ATP levels in crude extracts of different *Synechococcus* strains grown under both normal and heat stress conditions were determined and compared. As shown in Fig. 2A, AtpA levels were clearly elevated in all three strains under heat stress for 2 or 4 h. This increase in AtpA levels and the observed decrease in F_0F_1 ATPase synthase activity in Sye7942 during heat stress are consistent with previous findings (15, 16). Compared with Sye7942, both Sye7942-C252Y and Sye2973 clearly exhibited higher AtpA protein levels and F_0F_1 ATPase synthase activities under both normal and heat stress conditions (Fig. 2A and B). These results suggest that the C252Y mutation leads to increases in both AtpA accumulation and the F_0F_1 ATP synthase activity of Sye7942 under both normal and heat stress conditions. Furthermore, the F_0F_1 ATPase synthase activities of three other AtpA mutants, Sye7942-C252H, Sye7942-C252F, and Sye7942-C252W, under heat stress conditions (Fig. 2B) were also significantly higher than that of the wild-type Sye7942 strain ($P < 0.05$). The results prove that the mutation of C252 to any of these four conjugated amino acids (Y, W, F, H) could improve intracellular F_0F_1 ATPase synthase activity under heat stress conditions. This strengthened ATP synthesis was supposed to increase intracellular ATP abundance in *Synechococcus*. As shown in Fig. 2C, the ATP concentration in Sye7942-C252Y cells under the heat shock condition was higher than that in Sye7942 ($P > 0.05$), as expected. Additionally, both Sye7942-C252Y and Sye2973 cells contained more ATP when grown under high-light and high-temperature conditions ($P < 0.05$) (Fig. 2D). These results suggest that the C252Y mutation could enhance the intracellular ATP contents of Sye7942 under stress conditions.

C252Y mutation enhances the photosynthetic efficiency of Sye7942. According to previous reports, the ATP supply, controlled by F_0F_1 ATP synthase, regulates the turnover rate of the PSII core protein D1 during the acclimation of cyanobacteria to stress (10). A higher turnover rate of the D1 protein might further affect the photosynthetic efficiency of *Synechococcus*. Thus, multiple photosynthetic parameters, including the excitation energy distribution between PSII and PSI, NPQ, electron transport rate (ETR), and oxygen evolution rate, were measured and analyzed in both Sye7942 and Sye7942-C252Y.

In the fluorescence emission spectra at 77 K shown in Fig. 3A, the PSII/PSI ratios in both Sye7942 and Sye7942-C252Y were lower under heat stress than under normal

FIG 2 Effects of heat and high-light stresses on AtpA protein levels, F₀F₁ ATP synthase activity, and intracellular ATP contents. (A) Determination of AtpA protein levels under both normal and heat shock conditions by Western blotting. Five micrograms of each protein sample was used for Western blotting. To show the normalization of protein samples, SDS-PAGE analysis of the same samples was conducted (Fig. S9 in the supplemental material). (B) F₀F₁ ATP synthase activities of *S. elongatus* strains cultured under normal conditions and at 45°C for 2 h. (C) Determination of ATP contents in *S. elongatus* strains cultured under normal and heat shock conditions. Log-phase cultures of three *Synechococcus* strains were transferred to 45°C and were incubated at 45°C for 3.5 h. The ATP contents in these strains with or without heat shock were determined. (D) *S. elongatus* strains were inoculated at an initial OD₇₃₀ of ~0.05 and were grown under normal conditions (30°C, 50 μE m⁻² s⁻¹) or high-light and high-temperature conditions (43°C, 500 μE m⁻² s⁻¹) for 30 h. The ATP contents in these strains were then determined and compared. Stars indicate significant differences ($P < 0.05$) between Sye7942 and other strains grown under the same culture condition. Error bars indicate standard deviations ($n = 3$).

conditions. Moreover, PSI turnover was found to be significantly higher in all strains under the high-temperature and high-illumination condition (41°C, 200 μE m⁻² s⁻¹) than under the normal condition (see Table S1 in the supplemental material). Sye7942-C252Y also showed a significantly higher PSI turnover rate at 38°C than at 30°C. These results indicate that in response to heat shock, less light energy is transferred to the PSII reaction center to decrease photodamage to the PSII reaction center, and cyclic electron transport is used to generate more ATP in *Synechococcus* cells. The Sye7942-C252Y mutant showed a PSII/PSI ratio of 0.71, which was 3% higher ($P > 0.05$) than that in Sye7942 (0.69), under heat stress (Fig. 3A). This may result in a higher linear electron transport rate and increased PSII activity. As expected, both the ETR and the oxygen evolution rate were found to be significantly higher in Sye7942-C252Y (4.88- and 1.17-fold, respectively) than in Sye7942 under heat stress conditions (Fig. 3B and C). Moreover, there was no significant difference ($P > 0.05$) in the cyclic electron transport rate between Sye7942 and Sye7942-C252Y (Table S1). These results confirm that the Sye7942-C252Y mutant exhibits higher PSII activity than Sye7942 under heat stress. Besides regulating the light energy distribution between PSII and PSI, the NPQ mechanism is generally used by cyanobacteria to decrease the light energy transferred to the PSII reaction center; this depends mainly on an IsiA-related mechanism in Sye7942, which is generally and largely upregulated under stress conditions (17). After heat shock, the NPQ value of Sye7942 was decreased by about 50%, while that of Sye7942-C252Y was maintained at the same level as that under normal conditions (Fig. 3D). These results show that Sye7942-C252Y has a more reliable photoprotection system (NPQ) than Sye7942 in response to photodamage.

FIG 3 Effects of heat shock on photosynthesis in Sye7942 and Sye7942-C252Y. Both *Synechococcus* cultures were grown at 30°C and 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ to the log phase, shifted to 45°C and 50 $\mu\text{E m}^{-2} \text{s}^{-1}$, and then incubated for another 2 h. The 77 K fluorescence emission spectra (excitation at 430 nm) (A), electron transport rate (ETR) (B), oxygen evolution rate (C), and nonphotochemical quenching (NPQ) value (D) were determined for Sye7942 and Sye7942-C252Y with or without a heat shock at 45°C for 2 h. 77 K fluorescence emission spectra were recorded from 674.2 to 750.2 nm, with the PSII peak at 695 nm and the PSI peak at 715 nm. F_{695}/F_{715} , which was calculated by dividing the fluorescence intensity at 695 nm (F_{695}) by that at 715 nm (F_{715}), was used as a relative indicator of the distribution of excitation energy between PSII and PSI (PSII/PSI ratio). Stars indicate significant differences ($P < 0.05$) in photosynthetic parameters between Sye7942 and Sye7942-C252Y grown under the same culture conditions.

To examine the effects of the C252Y mutation on the photorepair process, the transcript abundances of three *psbA* genes during heat shock were determined by quantitative real-time reverse transcription-PCR (qRT-PCR) approaches. After 2 h of heat stress, the *psbA1* transcript levels of strain Sye7942 were 5.1-fold higher than those under normal conditions. Similar upregulation of *psbA1* was observed in strain Sye7942-C252Y. The *psbA2* transcript levels of strain Sye7942 were increased 2.6-fold, while the same gene was upregulated 38.8-fold in strain Sye7942-C252Y, after heat shock. In contrast, under heat stress, *psbA3* transcript levels were significantly lower in the wild-type strain Sye7942, but 3.5-fold higher in Sye7942-C252Y, than those under normal conditions (Fig. 4A). Considering that *psbA2* and *psbA3* encode form II of the D1 protein, D1:2 (18), these results suggest that the C252Y mutation increases the percentages of the D1:2 proteins in the PSII reaction center of Sye7942-C252Y and accelerates replacement of the damaged D1 protein in response to heat stress, relative to those in Sye7942.

Analysis of all these photosynthetic parameters suggests that the strengthened ATP synthesis caused by the C252Y mutation increases the turnover rate of the D1 protein and enhances PSII activity and the NPQ system under heat stress, which accounts for the improved stress tolerance of Sye7942-C252Y.

C252Y mutation enhances photosynthetic carbon fixation. To evaluate the effects of the C252Y mutation in AtpA on photosynthetic carbon fixation, the transcript levels of some key genes encoding subunits of RuBisCO and ADP-glucose pyrophosphorylase in Sye7942 and Sye7942-C252Y were determined and compared through

FIG 4 Effects of the C252Y mutation on *psbA* transcription and photosynthetic carbon fixation. Changes in gene transcript levels of *Synechococcus* strains shocked by heat (45°C) for 2 h were evaluated and compared by real-time PCR. The fold change was calculated as $2^{-[(C_{T_gene_45^{\circ}C} - C_{T_ref_45^{\circ}C}) - (C_{T_gene_30^{\circ}C} - C_{T_ref_30^{\circ}C})]}$, where $C_{T_gene_45^{\circ}C}$, $C_{T_ref_45^{\circ}C}$, $C_{T_gene_30^{\circ}C}$, and $C_{T_ref_30^{\circ}C}$ refer to the C_T values of the tested gene at 45°C, the reference gene (*rnpB*) at 45°C, the tested gene at 30°C, and the reference gene (*rnpB*) at 30°C, respectively. (A) D1 protein-encoding genes (*psbA1*, *psbA2*, and *psbA3*). (B) Genes encoding subunits of RuBisCO (*rbcL* and *rbcS*) and ADP-glucose pyrophosphorylase (*glgC*). (C and D) Glycogen contents of Sye7942 and Sye7942-C252Y grown under normal (30°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) (C) and high-temperature (41°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) (D) conditions. Stars indicate significant differences ($P < 0.05$) in gene transcription or glycogen content between Sye7942 and Sye7942-C252Y grown under the same culture conditions.

qRT-PCR approaches. Under heat stress, *rbcL* and *rbcS*, encoding two subunits of RuBisCO, and *glgC*, encoding ADP-glucose pyrophosphorylase, were repressed in Sye7942 (Fig. 4B). In Sye7942-C252Y, transcript levels of both *rbcL* and *rbcS* were not significantly different from those in Sye7942 (Fig. 4B).

Glycogen is the major carbon storage source in photosynthetic cyanobacteria (19). To further evaluate the effects of the C252Y mutation on glycogen accumulation, both the wild-type strain Sye7942 and the Sye7942-C252Y mutant were grown under normal and stress conditions, and their glycogen contents were analyzed and compared. Under normal culture conditions (30°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$), the glycogen content of strain Sye7942-C252Y was about 0.05 g liter⁻¹ OD₇₃₀⁻¹, which is almost 1.2- to 1.7-fold that of the wild-type strain (Fig. 4C). When these strains were grown under high-temperature stress conditions (41°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) for >90 h, the glycogen content of strain Sye7942-C252Y was increased to 0.08 g liter⁻¹ OD₇₃₀⁻¹, significantly higher than that of the wild-type strain (Fig. 4D). These results prove that the C252Y mutation significantly promotes intracellular glycogen accumulation under both normal and heat stress conditions.

AtpA is highly conserved in cyanobacteria. AtpA is a highly conserved protein among cyanobacterial species (see Table S2 in the supplemental material). Of 353 cyanobacterial AtpA homologs, 88.95% were found to have a conserved tyrosine at the 252nd amino acid position, whereas the AtpA protein of Sye7942 is the only one containing a cysteine at the same position (see Fig. S6a and Table S2 in the supplemental material). By phylogenetic analysis, the AtpA sequences from most *Synechococcus* species, including Sye7942 and Sye2973, as well as all *Prochlorococcus* species, were found to be clustered together (Fig. S6b and S7). However, the AtpA sequences of Sye7942 and Sye2973, as well as other sequences in this clade, clearly evolved from their common ancestor into two divergent groups (Fig. S6b and S7). Interestingly, both

Sye7942 and Sye2973 were isolated from freshwater, while most of the other species analyzed were from marine or brackish environments (see Table S3 in the supplemental material). These findings indicate that the AtpA proteins from Sye7942 and Sye2973 show different evolution directions from their homologs in other *Synechococcus* species living in diverse environments.

DISCUSSION

As the cyanobacterial isolate with the highest known growth rate, Sye2973 has shown great potential for biochemical production and has been considered a promising photosynthetic cell factory (20). Its rapid growth under high-light and high-temperature conditions, and the limited genomic differences between Sye7942 and Sye2973 (7), provided us with an opportunity to gain insight into the latter strain's special stress acclimation mechanisms. In this study, we confirmed that, uniquely among single amino acid substitutions in 21 proteins, the C252Y mutation in the α subunit of the F_oF_1 ATP synthase protein (AtpA) significantly increases AtpA protein levels, intracellular F_oF_1 ATP synthase activity, intracellular ATP contents, and *psbA* transcripts encoding the D1 protein (especially the D1:2 protein), enhances PSII activity, and, finally, results in the promotion of stress tolerance and glycogen accumulation in Sye7942.

F_oF_1 ATP synthase has been demonstrated to modulate various important physiological activities in higher plants, including NPQ of excitation energy (21), photosynthetic electron transport and carbon fixation (22), and abiotic stress tolerance (23). Moreover, the relationships among the intracellular ATP supply, PSII repair, and environmental stresses have been investigated in detail in cyanobacteria and have been summarized in a recent review (24). In brief, PSII is inevitably damaged during photosynthesis, while environmental stresses do not affect PSII photodamage, or affect it only slightly, but inhibit PSII repair (25, 26). ATP is required for most of the steps of PSII repair (10, 24), such as the degradation of damaged D1, transcription of *psbA* genes, synthesis and maturation of D1, and reassembly of the PSII complex. Also, overproduction of the D1:2 protein has been proven to make *Synechococcus* cells more tolerant to photoinhibition of PSII under stress conditions (27). Based on our observations and these previous reports, we hypothesize that the increased F_oF_1 ATP synthase activity in *Synechococcus* caused by the C252Y mutation first improves the intracellular energy supply, accelerates the ATP-dependent repair of the D1 protein, changes the proportion of the D1:2 protein, and recovers inhibited photosynthetic activity; then photosynthesis powers more energy-dependent processes, including NPQ, as well as carbon fixation pathways, to further protect cells from stresses and accelerate cell growth. Our findings demonstrate the roles of AtpA in photosynthesis and stress tolerance in cyanobacteria and indicate that AtpA might be a promising candidate for improving stress tolerance in other photosynthetic systems, such as other cyanobacteria, microalgae, and even plants.

Through homology modeling analysis, it is clear that cysteine 252 is located neither in the active center nor at the interaction surface between the α and β subunits but rather on the surface of the whole F_1 complex (see Fig. S8 in the supplemental material). Thus, the C252Y mutation seems to have no effects on neighboring residues but instead affects protein activity or protein-protein interactions. This mutation could disrupt or induce protein-protein interactions between AtpA and some chaperones, such as Atp23 (28), Atp11/12 (29), and FMC1 (30) in yeast and HSP90 (31) in all eukaryotes, which might stabilize and affect the activity of F_oF_1 ATP synthase.

The fact that most cyanobacterial species have a conserved tyrosine at the 252nd amino acid position raised another question: why some cyanobacterial species harboring Y252 in their AtpA proteins (for instance, *Synechocystis* sp. strain PCC 6803) do not show stress tolerances as high as that of Sye2973. This could be explained through the different genetic backgrounds of Sye2973 and these other cyanobacterial species.

According to a previous report (7), Sye2973 is a high-temperature-tolerant mutant of *S. elongatus* UTEX 625; the latter showed good tolerance to 38°C in the beginning but

lost this ability over 6 decades of culturing in labs. Similarly, RpoS is rarely inactivated in natural *Escherichia coli* isolates within their mammalian hosts but is frequently disrupted during stab storage in labs. Considering these reports, we hypothesize that a similar microevolution event took place in Sye7942. Sye7942 AtpA might have had Y252, just like most of its homologs, when this strain (also known as *Anacystis nidulans* R2) was isolated from wild environments. Then the Y252C mutation in AtpA might have appeared during serial subculturing for decades under lab culture conditions, which routinely use 30°C and weak illumination. And Sye7942 is speculated to have finally lost its original high stress tolerance without facing stress pressures for decades due to the point mutation of AtpA.

How did cyanobacteria, a group of ancient microorganisms, survive the huge environmental changes over the past 3.5 billion years of evolutionary history? The effective stress acclimation strategies observed in this study and previous work provide part of the explanation. In fact, the dynamic nature of cyanobacterial genomes is also an important reason. Besides spontaneous mutations (32), most cyanobacteria are naturally competent (33–35) or able to be transformed by conjugation (36). Thus, they can take up DNA molecules from the living environment and easily integrate them into their own genomes by homologous recombination. In addition, the presence of multiple copies of chromosomes (37) provides cyanobacteria with the ability to tolerate certain harmful mutations. Mutations benefiting the cyanobacterial host are selected and fixed in the genome of the host under selective pressures (32). The transformation, site-saturation mutagenesis, and screening experiments that we performed in this study have illustrated how cyanobacteria might have evolved via a single amino acid point mutation in the face of environmental selection pressures.

Our work here highlights the importance of ATP synthase in the environmental stress tolerance of cyanobacteria, and it demonstrates the plasticity of genetic mutations for stress acclimation. Generally, our findings will better our understanding of stress acclimation mechanisms and be valuable for the improvement of the stress tolerance of photosynthetic organisms, including cyanobacteria and higher plants.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* strain DH5 α (TaKaRa, Dalian, China) was used for cloning. Sye7942 was from Xudong Xu's lab at the Institute of Hydrobiology, Chinese Academy of Sciences. Sye2973 was purchased from the Culture Collection of Algae at The University of Texas at Austin (UTEX). The strains constructed and used in this study are listed in Table 1. Sye7942 and Sye2973 were cultured in BG11 medium (38) at 30°C under 3% CO₂, under continuous illumination (50 $\mu\text{E m}^{-2} \text{s}^{-1}$); these are defined as the normal culture conditions in this work. The growth of each cyanobacterial strain was monitored by measuring the optical density at 730 nm (OD₇₃₀).

For evaluating the stress tolerance of *Synechococcus* strains, *Synechococcus* cultures were inoculated into column photobioreactors (39) containing 200 ml liquid BG11 medium with an initial OD₇₃₀ of 0.05 and were then grown under normal conditions (30°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) or high-light (30°C, 500 $\mu\text{E m}^{-2} \text{s}^{-1}$), high-temperature (41°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$), or high-light and high-temperature (41°C, 500 $\mu\text{E m}^{-2} \text{s}^{-1}$) stress conditions. All cultures were bubbled with 3% CO₂.

For the determination of AtpA protein levels, F₀F₁, ATP synthase activities, and gene transcript levels, *Synechococcus* cultures were inoculated at an initial OD₇₃₀ of 0.2, grown under normal conditions (30°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$, 3% CO₂) for 48 h, and then incubated at 45°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination, and 3% CO₂ aeration for 2 or 4 h.

For the determination of intracellular glycogen contents, *Synechococcus* strains were inoculated into column photobioreactors (39) containing 200 ml liquid BG11 medium with an initial OD₇₃₀ of 0.05 and were then grown under normal (30°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) and high-temperature stress (41°C, 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) conditions. All cultures were bubbled with 3% CO₂.

Plasmid and strain construction. For the identification of genes responsible for stress tolerance, 21 protein-encoding genes containing SNPs between Sye7942 and Sye2973 were cloned from the chromosome of Sye2973 via PCR using the primers listed in Table 2. PCR products were purified using the E.Z.N.A. Cycle Pure kit (Omega Bio-tek, Norcross, GA, USA) and were ligated into the pEASY-Blunt vector (TransGen Biotech, Beijing, China). The resulting plasmids were then verified by Sanger gene sequencing and are listed in Table 2. For *in vivo* site-saturation mutagenesis of AtpA, pSK038 was used as the template for the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The primers are listed in Table 2, and the resulting plasmids were confirmed by Sanger DNA sequencing and are listed in Table 2.

Transformation of Sye7942. Sye7942 was transformed with a recombinant plasmid according to a previously reported method (35) with some modifications. Briefly, 500 μl wild-type Sye7942 with an OD₇₃₀ of 0.5 to 1.0 was centrifuged at 6,000 $\times g$ for 2 min and was then resuspended in 200 μl of fresh

BG11 medium. Next, 200 ng of each plasmid was added, and the mixture was incubated in the dark at 30°C overnight. For two-step plate screening (Table S3 in the supplemental material), DNA-*Synechococcus* mixtures were spread on solid BG11 plates with 1.5% agar after transformation and were incubated at 43°C and 220- $\mu\text{E m}^{-2} \text{ s}^{-1}$ illumination for 3 days. Then transformants were picked, streaked on BG11 plates, and incubated at 43°C and 500- $\mu\text{E m}^{-2} \text{ s}^{-1}$ illumination for 3 days. Wild-type Sye7942 and Sye2973 were used as negative and positive controls, respectively. Transformants that survived after the two-step screening were considered true high-light- and high-temperature-tolerant colonies for subsequent genotyping experiments. The SNP-containing DNA fragments were amplified from the genomic DNA of these transformants for Sanger sequencing. The AtpA point mutants of Sye7942 were rescreened at 43°C and 500- $\mu\text{E m}^{-2} \text{ s}^{-1}$ illumination for 3 days until the sequencing results indicated that all copies of the gene were mutated. Only homozygous mutants were used in the subsequent physiological and metabolic analyses.

Sequence analysis. For sequence alignment of the AtpA protein, the amino acid sequences of AtpA were retrieved by using blastP against the nonredundant protein database with the AtpA sequence from Sye7942, and sequences with >90% coverage and >49% identity were selected for the subsequent multiple-sequence alignment, performed with BioEdit (40). Logos were generated using the WebLogo server (41) with the multiple-sequence alignment mentioned above as an input. For phylogenetic analysis, an unrooted neighbor-joining tree was created in MEGA6 (42) using the default parameters. Bootstrap analysis was performed using 1,000 replicates.

For whole-genome resequencing of Sye7942-C252Y, genomic DNA was isolated, fragmented, blunted, modified with a 3'-A overhang, ligated to Illumina's standard sequencing adapters, and amplified by PCR. The library was sequenced as paired-end reads using an Illumina HiSeq 2500 sequencer. Library construction and sequencing were performed by Allwegene Tech (Beijing, China). The data were deposited in the NCBI Sequence Read Archive (SRA) under accession number [SRR6246213](https://www.ncbi.nlm.nih.gov/sra/SRR6246213).

The genome and plasmid sequences of Sye7942 were obtained from GenBank (accession numbers [NC_007595.1](https://www.ncbi.nlm.nih.gov/nuccore/NC_007595.1), [NC_004073.2](https://www.ncbi.nlm.nih.gov/nuccore/NC_004073.2), and [NC_004990.1](https://www.ncbi.nlm.nih.gov/nuccore/NC_004990.1)) and were used as reference sequences for read mapping. BWA (43) was used for mapping reads to reference sequences, and SAMtools (44) was used to identify SNPs between reference sequences and our data. The SNP-containing DNA fragments detected were amplified from the genomic DNA of both Sye7942 and Sye7942-C252Y and were verified by Sanger sequencing. Only the nucleotide mutation appearing in Sye7942-C252Y and not in Sye7942 was considered the true SNP.

Preparation of membrane vesicles. Cells were grown from an initial OD_{730} of 0.2 to 1.2 under normal conditions and were then exposed to 45°C for 2 or 4 h. The method used has been described in a previous report (45). The cells were collected at $6,000 \times g$ for 10 min at 4°C and were suspended in precooled buffer A (1.0 M betaine, 0.4 M D-sorbitol, 20 mM Tris-HCl [pH 7.0], 15 mM CaCl_2 , 15 mM MgCl_2 , 1 mM 6-amino-*n*-caproic acid, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Then the cells were lysed by two passages through a French pressure cell (Constant Systems Limited, Daventry, UK) at a pressure of 2×10^7 Pa. The homogenate was centrifuged at $4,000 \times g$ for 20 min at 4°C to remove the unbroken cells and cell debris. The thylakoid membrane was collected by centrifugation at $20,000 \times g$ for 1 h at 4°C and was then resuspended in buffer A. The thylakoid membrane preparations were frozen in liquid nitrogen and were stored at -80°C.

Determination of the activity of F_0F_1 ATP synthase. The acid/base transition method was used to determine the activity of F_0F_1 synthase at 25°C, as described in a previous report (46). Thylakoid membrane vesicles were incubated in buffer B (acid medium, containing 23 mM succinic acid, 6.7 mM MgCl_2 , 2 mM KCl, 50 mM NaCl, 10 mM Tris-HCl [pH 6.0], 5 mM NaH_2PO_4 , 1.4 pM valinomycin, 10 pM diuron, and 3.75 mM dithiothreitol) for 1 min at a concentration of 20 μg chlorophyll/ml. Then the suspension was diluted 1:1 with buffer C (6.7 mM MgCl_2 , 2 mM KCl, 50 mM NaCl, 200 mM Tris-HCl, 5 mM dithiothreitol, and 2 mM ADP) and was incubated for 30 s. The reaction in the 200- μl reaction mixture was stopped by boiling for 5 min. The ATP concentration was analyzed by a luciferase luminescence assay (Beyotime, Haimen, China). The activity of F_0F_1 ATPase synthase was calculated according to the ATP concentration, the reaction time, and the chlorophyll content.

Determination of intracellular ATP contents. Intracellular concentrations of ATP were determined and calculated with an ADP/ATP ratio bioluminescence assay kit (K255-200; BioVision) according to the instructions.

Immunoblot analysis. An anti-AtpA serum was generated by immunizing rabbits with the synthetic oligopeptide CLKTSKPEFIEKVQS. The total protein extracted from Sye7942, Sye7942-C252Y, and Sye2973 was quantified using the Bradford method (47), and equal amounts (5 μg) of protein were resolved by SDS-PAGE with a 12% sodium dodecyl sulfate-polyacrylamide gel (see Fig. S9 in the supplemental material). Proteins were then blotted onto nitrocellulose membranes and were detected with the anti-AtpA antibody.

Photosynthetic parameter determination. Rapid light response curves and fluorescence induction measurements were determined using a Water-PAM (water pulse-amplitude-modulated) chlorophyll fluorometer (Walz GmbH, Effeltrich, Germany) after 15 min of dark adaptation. An actinic light intensity of 61 to 1,017 $\mu\text{E m}^{-2} \text{ s}^{-1}$ was used in the rapid light response curves. The fluorescence yields F' and F_m' were determined before or after the last saturating light pulse (800 ms, 3,000 $\mu\text{E m}^{-2} \text{ s}^{-1}$), respectively, at the end of each actinic illumination period. The ETR was calculated using the formula $\text{PAR} \times (F_m' - F')/F_m'$, where PAR (photosynthetically active radiation) is the actinic light intensity (48, 49). The fluorescence induction measurements were performed under 61- $\mu\text{E m}^{-2} \text{ s}^{-1}$ continuous actinic light. At the same time, saturating flashes (800 ms, 3,000 $\mu\text{E m}^{-2} \text{ s}^{-1}$) were provided every minute until the maximal fluorescence yield (F_m' , for the light-adapted samples) was stable. After the measurements,

TABLE 4 Primers for real-time PCR analysis

Gene	Primer designation	Primer sequence
<i>rbcl</i>	Synpcc7942_1426-1	AATCCACAAATCGCAAGCA
	Synpcc7942_1426-2	TGAAACCAGCCGTCAAGAA
<i>rbcS</i>	Synpcc7942_1427-1	GGAAGAGTTCTACTGGACGAT
	Synpcc7942_1427-2	TTGGCACTGCTTGATGTT
<i>glgC</i>	Synpcc7942_0603-1	ACTCTGCCTCGCTCAACC
	Synpcc7942_0603-2	CTCATCCACATCCCCTCTT
<i>psbA1</i>	psbA1-1	GCCCTTTACAACCTCAAGAT
	psbA1-2	ATCATCAGCACGCCGAAC
<i>psbA2</i>	psbA2-1	ATTAAGTCTCGTAAATAGTTCAACT
	psbA2-2	TGGTTACCCACTCGCAGAA
<i>psbA3</i>	psbA3-1	GGCATTACGCCTTAGACATAC
	psbA3-2	CGGTGCTGGTTACCCACT
<i>rnpB</i>	rnpB-1	AGCAAGGTGGAGGGACAA
	rnpB-2	CGAAGACAGAGGGCAGTTAT

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to the samples at a final concentration of 20 μM . Then the maximal fluorescence yield ($F_{m, \text{DCMU}}$) was measured under the actinic light and saturating flash (800 ms, 3,000 $\mu\text{E m}^{-2} \text{s}^{-1}$). The NPQ was calculated as $(F_{m, \text{DCMU}} - F_m')/F_m'$.

The photosynthetic oxygen evolution rate was measured by a Clark-type oxygen electrode (DW1; Hansatech, Norfolk, UK). The temperature was controlled by a thermostatic water bath at 30°C and 45°C, and the light intensity was 625 $\mu\text{E m}^{-2} \text{s}^{-1}$. The cells were collected by centrifugation at $4,000 \times g$ for 5 min, resuspended in fresh BG11 medium supplemented with 1 mM KHCO_3 , and incubated under environmental conditions for 20 min. The photosynthetic oxygen evolution rate was calculated according to the chlorophyll content, which was determined as described in previous studies (50).

Low-temperature fluorescence (77 K fluorescence emission spectra) was measured with a Hitachi F-4500 fluorescence spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan) as described in a previous report (51). Samples were prepared at a concentration of 4 μg chlorophyll ml^{-1} and were immediately frozen in liquid nitrogen. The excitation wavelength was 430 nm, and the emission spectra were recorded from 674.2 to 750.2 nm, with bandwidths of 10 nm and 5 nm, respectively. The intensity of fluorescence emission at 695 nm (F_{695}), arising from PSII, was divided by the intensity of emission at 715 nm (F_{715}), arising from PSI. F_{695}/F_{715} was used as a relative indicator of the distribution of excitation energy between PSII and PSI (PSII/PSI ratio) (11).

P700 reduction kinetics were determined as described previously (52) using a Joliot-type spectrophotometer (JTS-10; Bio-Logic, Grenoble, France). DCMU was added to the samples at a final concentration of 20 μM . The excitation wavelength was 630 nm (320 $\mu\text{E m}^{-2} \text{s}^{-1}$), and the determination wavelengths were 705 nm and 750 nm. The turnover rate of PSI, in electrons per second (K), was calculated according to the formula $K = 0.693/\text{half-time of P700 reduction (in seconds)}$.

qRT-PCR. Both Sye7942 and Sye7942-C252Y were grown under normal conditions to the exponential phase and were shocked at 45°C for 2 h. Cultures with or without heat shock were collected and quickly frozen in liquid nitrogen. RNA extraction and quantitative real-time reverse transcription-PCR (qRT-PCR) were performed as described previously (53, 54). The primers are listed in Table 4.

Glycogen content determination. For the determination of cellular glycogen contents, *Synechococcus* cells were collected at $12,000 \times g$ for 10 min at 4°C, washed twice with sterile water, suspended in 30% (wt/vol) KOH, and incubated at 95°C for 2 h. Ethanol was added to the suspension at a final concentration of 75% (vol/vol), with subsequent incubation at -20°C for 2 h to precipitate glycogen. Glycogen was collected by centrifugation ($12,000 \times g$, 10 min, 4°C), washed with 70% and 98% ethanol in turn, and dried in a SpeedVac concentrator (Eppendorf, Germany). The dried glycogen was resuspended in 100 mM sodium acetate (pH 4.5) and was enzymatically hydrolyzed to glucose by amylo-glucosidase (Novozymes, Denmark) at 60°C for 2 h. The glycogen content was determined by monitoring the glucose concentration using a biosensor (SBA-40C; Shandong Academy of Sciences, China).

Statistical analysis. The values shown in graphs are expressed as means $\pm$ standard deviations. The significance of differences between groups was determined by t tests using IBM SPSS Statistics, version 19 (SPSS, Chicago, IL, USA). A P value of <0.05 was considered to indicate significance.

Accession number(s). All whole-genome sequencing data that support the findings of this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) with accession number [SRR6246213](https://doi.org/10.1128/AEM.01222-18).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01222-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.**ACKNOWLEDGMENTS**

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X.L. and X.T. conceived and supervised the study. W.L., X.T., K.S., S.Z., and C.L. performed the experiments and analyzed the data. X.T., W.L., K.S., G.L., and X.L. wrote the report.

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