β -1,4-Glucanase-like protein from the cyanobacterium *Synechocystis* PCC6803 is a β -1,3-1,4-glucanase and functions in salt stress tolerance

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In the present study, we characterized the gene (Cyanobase accession number slr0897) designated *Ssglc* encoding a β -1,4-glucanase-like protein (SsGlc) from *Synechocystis* PCC6803. The deduced amino acid sequence for *Ssglc* showed a high degree of similarity to sequences of GH (glycoside hydrolase) family 9 β -1,4-glucanases (cellulases) from various sources. Surprisingly, the recombinant protein obtained from the *Escherichia coli* expression system was able to hydrolyse barley β -glucan and lichenan (β -1,3-1,4-glucan), but not cellulose (β -1,4-glucan), curdlan (β -1,3-glucan), or laminarin (β -1,3-1,6-glucan). A ¹H-NMR analysis of the enzymatic products revealed that the enzyme hydrolyses the β -1,4-glycosidic linkage of barley β -glucan through an inverting mechanism. The data indicated that SsGlc was a novel type of GH9 glucanase which could specifically hydrolyse the β -1,3-1,4-linkage of glucan. The growth of mutant *Synechocystis* cells in which the *Ssglc* gene was disrupted by a kanamycin-resistance

cartridge gene was almost the same as that of the wild-type cells under continuous light (40 μ mol of photons/m² per s), a 12 h light (40 μ mol of photons/m² per s)/12 h dark cycle, cold stress (4 °C), and high light stress (200 μ mol of photons/m² per s). However, under salt stress (300–450 mM NaCl), growth of the *Ssglc*-disrupted mutant cells was significantly inhibited as compared with that of the wild-type cells. The *Ssglc*-disrupted mutant cells showed a decreased rate of O₂ consumption and NaHCO₃-dependent O₂ evolution as compared with the wild-type cells under salt stress. Under osmotic stress (100–400 mM sorbitol), there was no difference in growth between the wild-type and the *Ssglc*-disrupted mutant cells. These results suggest that SsGlc functions in salt stress tolerance in *Synechocystis* PCC6803.

Key words: cyanobacterium, glucanase, lichenase, glycoside hydrolase, salt stress, *Synechocystis* PCC6803.

INTRODUCTION

Photosynthetic organisms possess many kinds of glycoside hydrolases (GHs). The genes encoding these enzymes have been isolated from a number of plant species, and grouped into GH families on the basis of sequence homology and the hydrophobic clustering of deduced amino acid sequences [1–7]. A structural classification is a very efficient way to estimate the substrate and reaction specificities of enzymes from their deduced amino acid sequences, and is widely used in research on carbohydrate-active enzymes from plants. Starch, a storage material in plants, is hydrolysed by enzymes such as α - and β -amylases and α -glucosidases, which belong to the families GH13, GH14, GH31 and GH77 [8,9]. Cellulose- and xylan-related GHs belonging to the families GH5, GH9 and GH10 are very important for cell wall modifications, and hence might function during growth, expansion and abscission [9]. Plants have several defence strategies against pathogenic attack. Chitinases belonging to the GH18 and GH19 families and some GH17 glucanases are expressed in response to an attack and act directly on the pathogen [10–12]. The enzymes can also be induced to express by a variety of defence-related signal molecules [13]. Family GH1 β -glucosidases are now implicated in the control of a variety of signalling molecules, such as hormones, coniferin and flavonol glucosides, because of their glycosylation or deglycosylation activity [14]. However, it should be noted that a similarity in sequence does not always mean a similarity in substrate specificity or physiological function. It is still essential to characterize a recombinant enzyme produced in some heterologous expression system.

There is little information on physiological function and enzymatic property of GHs in photosynthetic prokaryotes. These enzymes might differ in structure and function from those found in plants. The photosynthetic prokaryote cyanobacterium *Synechocystis* PCC6803 possesses a gene (Cyanobase accession number slr0897) whose product is homologous with the endo- β -1,4-glucanases from various organisms. As in the case of plant species, the gene product from slr0897 might have an important role in modifying cell walls or defending against fungal pathogens. In the present study, we isolated and characterized the slr0897 gene, which encodes a glucanase-like protein (SsGlc) and is designated *Ssglc*, and constructed a gene expression system in *Escherichia coli*. The recombinant proteins produced by the expression system were then examined with respect to their molecular and enzymatic properties. Furthermore, we also examined the physiological function of SsGlc in cyanobacteria using mutant cells in which the *Ssglc* gene was disrupted by a kanamycin-resistance cartridge gene.

EXPERIMENTAL

Materials

Barley β -glucan and lichenan were from Sigma Chemical Co. Cellulose, CM-cellulose, laminarin, xylan and curdlan were from Nachalai Tesque. Reagents and enzymes were purchased from TaKaRa. All other chemicals were of analytical grade and were purchased from commercial sources.

Abbreviations used: GH, glycoside hydrolase; SsGlc, *Synechocystis* PCC6803 glucanase-like protein.

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The nucleotide sequence data for *Ssglc* reported in this paper have been deposited in the DDBJ, EMBL, GenBank® and GSDB nucleotide sequence databases under accession number AB266254.

Organism and culture

The wild-type strain of *Synechocystis* PCC6803 and its mutant cells were grown photoautotrophically at 27 °C in Allen's medium at 40 μmol of photons/ m^2 per s under fluorescent lamps. Exponential-phase cells of *Synechocystis* PCC6803 ($D_{730} = 0.6\text{--}1.0$) were diluted with fresh medium and subjected to stress. *E. coli* cells, strains DH5a and BL21(DE3)pLysS, were cultured at 37 °C in LB (Luria–Bertani) broth.

Expression of SsGlc in *E. coli*

Chromosomal DNA was isolated from *Synechocystis* PCC6803 by the method of Williams [15]. A DNA fragment containing the slr0897 ORF (open reading frame) encoding SsGlc was amplified by PCR with 5'-CGCTCCTCCATATGAATAGC-3' (*Ssglc*-f) and 5'-GCATTCTGAGCTTGTTC-3' (*Ssglc*-r). The forward primer was designed to introduce an NdeI site with an ATG codon for the initiation of translation (bold sequence). Amplified DNA fragments were cloned into a pT7Blue-T vector (Novagen) and sequenced with an automated DNA sequencer (ABI310A, Applied Biosystems). For the construction of the plasmids to express SsGlc, the plasmids were digested with NdeI and cloned into a pET3a vector (Novagen) digested with the same restriction enzyme. The resulting constructs, designated pETSsGlc, were introduced into the *E. coli* strain BL21(DE3)pLysS, and the recombinant enzymes were produced in *E. coli* by a method described previously [16]. SDS/PAGE was performed on 10% (w/v) polyacrylamide slab gels as described previously [16].

Enzyme assays and purification of recombinant SsGlc

The recombinant *E. coli* cells or the *Synechocystis* PCC6803 cells were harvested by centrifugation at 5000 *g* for 10 min, suspended in 100 mM potassium phosphate buffer (pH 7.0), and sonicated at 10 kHz for a total of 80 s with four intervals of 20 s each. These lysates were centrifuged at 12000 *g* for 15 min, and the supernatants (crude enzymes) were used for enzyme assays. The activity of the glucanase was measured by determining the rate of production of reducing sugar from the hydrolysis of substrates. The reaction mixture (1 ml) consisted of 0.25% (w/v) substrate and the enzyme in 100 mM sodium phosphate buffer (pH 6.0). After incubation at 37 °C for 10 min, the reaction was terminated by the addition of 2 ml of a dinitrosalicylic acid solution consisting of 30% (w/v) potassium sodium (+)-tartrate tetrahydrate, 1% 3,5-dinitrosalicylic acid and 1.6% NaOH. The amount of reducing sugar was measured and quantified by the methods of Miller [17] using glucose as a standard. In order to analyse the enzymatic properties, recombinant SsGlc was purified with a cellulose column (1.5 cm $\times$ 20 cm) equilibrated with 100 mM potassium phosphate buffer (pH 7.0). The column was washed with 200 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 1 M NaCl, and then eluted with 50 ml of distilled water. The active fractions were combined and concentrated to a final volume of 1 ml by ultra-filtration (Amicon Ultra-4). Protein concentrations were determined by the method of Bradford [18] with BSA as a standard. Enzymatic properties were analysed using the purified SsGlc. The substrate specificity of SsGlc was determined by incubating the purified enzyme at 37 °C for 10 min with various types of β -glucan (0.25%) from different sources. Optimum pH was determined at 37 °C in 100 mM citrate phosphate buffer (pH 3.0–6.5) and 100 mM potassium phosphate buffer (pH 5.5–8.0) under the same conditions as described above. Kinetic analyses were performed over a substrate concentration range 0.2–20 mg/ml.

The kinetic parameters K_m and k_{cat} were derived from Lineweaver–Burk plots.

Analysis of enzymatic products

Barley β -glucan (5 mg) was dissolved with 99.9% $^2\text{H}_2\text{O}$ (0.6 ml), and the substrate solution was mixed with 0.4 nmol of the purified recombinant SsGlc in an NMR tube. After mixing with the enzyme, the NMR tube was immediately set as an NMR probe, and ^1H -NMR spectra were recorded at 270 MHz and 25 °C. For determining the anomeric form of the reaction products, data were collected for the first 4 h of the reaction.

To determine the linkage hydrolysed, the polysaccharide substrate was hydrolysed intensively by repeating the application of the enzyme (0.4 nmol for each) at an interval of 24 h, and ^1H -NMR spectra were obtained at 270 MHz and 70 °C every 24 h.

Generation of the *Ssglc*-disrupted mutants (*SsΔglc*)

Synechocystis PCC6803 cells were transformed with the plasmid pETSsGlc/*kan*^r, which was derived from pETSsGlc by disrupting the *Ssglc* gene. After pETSsGlc was digested with NotI and blunted with Mung bean nuclease, the *Ssglc* gene was interrupted with a 1.2 kb HincII *kan*^r from the plasmid pUC4K (GE Healthcare). The resultant plasmid (pETSsGlc/*kan*^r) was introduced into *Synechocystis* PCC6803 cells by the method of Golden et al. [19]. Transformed cells were selected on agar-solidified Allen's medium supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin, and the complete segregation of *Ssglc*-interrupted cells was confirmed by genomic PCR with appropriate primers.

Measurements of O₂ consumption and NaHCO₃-dependent O₂ evolution

The rates of O₂ consumption and NaHCO₃-dependent O₂ evolution were determined by measuring the CO₂-dependent oxygen evolution as described previously [20]. Cultures were washed and resuspended to a D_{750} of 0.3 in fresh medium. A sample (1.0 ml) of these samples and 1 mM NaHCO₃ were placed in a DW1 liquid-phase oxygen electrode chamber (Hansatech) and stirred gently at 27 °C with 100 μmol of photons/ m^2 per s. The rates of O₂ consumption and NaHCO₃-dependent O₂ evolution were calculated in terms of μmol of O₂ evolved/h per mg of chlorophyll. The chlorophyll content of *Synechocystis* PCC6803 cells was measured by the method of Lichtenthaler [21].

RESULTS

Isolation and characterization of the *Ssglc* gene

The *Ssglc* gene consisted of 3210 bp encoding 1070 amino acids with a calculated molecular mass of 112 129 Da. The deduced amino acid sequence for *Ssglc* showed 30–50% identity with that of β -1,4-glucanases from plants, eukaryotic algae, yeast and mammals, and less than 30% identity with that of β -1,3-glucanases, β -1,3-1,4-glucanases and β -1,3-1,6-glucanases from various sources. A phylogenetic tree constructed using deduced amino acid sequences of glucanases from various sources showed that SsGlc can be classified into the β -1,4-glucanase group (Figure 1A). SsGlc has a modular structure as shown in Figure 1(B). The N-terminal region is composed of two cellulose-binding domains belonging to the family CBM-2 (8–105 and 435–532) and an intervening region of unknown function (106–434). The second half of SsGlc consists of a catalytic domain (589–1043) belonging to family GH9.

Figure 1 Phylogenetic tree for the amino acid sequences of glucanases from various sources (A) and schematic structure of SsGlc (B)

Clustal X (<http://www.ebi.ac.uk/clustalw>) software and TreeView X (<http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/index.html>) software were used for analysis and drawing respectively. Black boxes indicate the cellulose-binding domain (CBM-2). Shaded boxes indicate the catalytic domain belonging to family GH9. The white box indicates an unknown region which does not show any homology with various amino acid sequences reported previously. *A. kawachii*, *Aspergillus kawachii*; *A. quisqualis*, *Ampelomyces quisqualis*; *A. thaliana*, *Arabidopsis thaliana*; *A. thaliana* Landsberg erecta, *Arabidopsis thaliana* Landsberg erecta; *B. brevis*, *Bacillus brevis*; *B. circulans*, *Bacillus circulans*; *B. germanica*, *Blattella germanica*; *B. licheniformis*, *Bacillus licheniformis*; *C. fimi*, *Cellulomonas fimi*; *C. thermocellum*, *Clostridium thermocellum*; *F. x ananassa*, *Fragaria x ananassa*; *H. jecorina*, *Hypocrea jecorina*; *H. virens*, *Hypocrea virens*; *N. crassa*, *Neurospora crassa*; *N. koshunensis*, *Neotermes koshunensis*; *N. tabacum*, *Nicotiana tabacum*; *O. sativa*, *Oryza sativa*; *P. furiosus*, *Pyrococcus furiosus*; *P. sachalinensis*, *Prunus sachalinensis*; *S. rostrata*, *Sesbania rostrata*; *V. fungicola*, *Verticillium fungicola*; *Z. mays*, *Zea mays*.

Figure 2 SDS/PAGE analysis of recombinant SsGlc

Lane 1, crude extract of *E. coli* BL21(DE3)pLysS transformed with pETSsglc; lane 2, crude extract of *E. coli* BL21(DE3)pLysS transformed with pET3a; lane 3, purified recombinant SsGlc. Molecular masses are indicated in kDa.

Expression of recombinant SsGlc in *E. coli* and its purification

As shown in Figure 2, a protein band corresponding to 112 kDa, the calculated molecular mass of SsGlc, was clearly overexpressed using pETSsglc as compared with the band obtained with pET3a (lanes 1 and 2). The recombinant SsGlc protein accounted for nearly 20% of all the soluble protein in *E. coli* cells, and was used for the purification. The recombinant SsGlc was successfully purified by successive separations using cellulose column chromatography as described in the experimental section. SDS/PAGE of the purified protein produced a single band with a molecular mass of approx. 112 kDa (Figure 2, lane 3).

Substrate specificity of recombinant SsGlc

In the crude enzymes from the recombinant *E. coli* transformed with pETSsglc, we could not detect any activity directed toward CM-cellulose as a substrate. Then, we measured the hydrolytic activity of the recombinant SsGlc towards various β -glucans which differ in linkage types and the ratio of linkage types. Activity by the recombinant SsGlc was detected when barley β -glucan or lichenan was used as the substrate. The specificity of the activity of the purified recombinant SsGlc towards various β -glucans was determined in percentage terms relative to the activity toward barley β -glucan (Table 1). The purified recombinant SsGlc could hydrolyse β -1,3,4-glucans (barley β -glucan and lichenan). However, the recombinant SsGlc did not show any activity directed towards CM-cellulose (β -1,4-glucans), curdian (β -1,3-glucans) and laminarin (β -1,3,6-glucans).

Anomeric form of the enzymatic products

$^1\text{H-NMR}$ spectra showing the enzymatic hydrolysis of barley β -glucan are shown in Figure 3(A). In the early stage of the enzymatic reaction (~ 30 min), only the α -anomeric signal of the reducing end produced was significantly detected at 5.14 p.p.m. The appearance of the β -anomer was much delayed (30 min) when compared with that of the α -anomer. The time course of the anomers' formation was obtained from the signal areas of the spectra, and is shown in Figure 3(B). The result indicates that the enzyme hydrolyses the glycosidic linkage through an inverting mechanism.

Table 1 Substrate specificity and kinetic parameters of the recombinant SsGlc

nd, not determined.

Substrate (origin)	Major linkage type	Specific activity (μmol of glucose/min per mg of protein)	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (ml/s · mg)
Barley β -glucan	1,4; 1,3- β - (2.3–2.7:1)	3.2 ± 0.1 (100%)	2.0 ± 0.5	8.4 ± 0.6	4.3
Lichenan (<i>Cetraria islandica</i>)	1,4; 1,3- β - (2:1)	2.2 ± 0.1 (69%)	4.2 ± 1.5	3.7 ± 0.6	0.88
Curdian (<i>Alcaligenes faecalis</i>)	1,3- β -	0	nd	nd	nd
Laminarin (<i>Laminaria digitata</i>)	1,3; 1,6- β - (7:1)	0	nd	nd	nd
CM-cellulose	1,4- β -	0	nd	nd	nd
Xylan	1,4- β -	0	nd	nd	nd

Figure 3 ^1H -NMR spectroscopic analysis of products from SsGlc

(A) Time-dependent ^1H -NMR spectra showing hydrolysis of barley β -glucan by purified recombinant SsGlc. (B) Time course of the anomers' formation from barley β -glucan catalysed by purified recombinant SsGlc. Red., reducing end.

Glycosidic linkage cleaved by the enzyme

With intensive hydrolysis of barley β -glucan, the intensity of the anomeric ^1H -signal assigned to the β -1,4-linkage (4.95 p.p.m.) was significantly reduced, whereas the intensity of the β -1,3-signal did not change (Figure 4). This indicates that the enzyme cleaves the β -1,4-glycosidic linkage of barley β -glucan.

Kinetic analysis of the enzymatic reaction

A kinetic analysis of the enzymatic reaction was performed using the two different substrates, barley β -glucan and lichenan. Initial velocities determined from the increase in reducing sugars were plotted against the substrate concentrations, and the substrate-velocity curves for both substrates exhibited a shape typical of Michaelis-Menten kinetics (results not shown). The kinetic parameter values calculated from the curves are listed in Table 1. The optimum pH and temperature for the activity of SsGlc were examined using lichenan as a substrate and were found to be 7.0 and 42°C respectively.

Targeted disruption of the *Ssglc* gene in *Synechocystis* PCC6803

We performed a PCR analysis with DNA of wild-type and Ss Δglc mutant cells that had been transformed with the vector

pET*Ssglc/kan^r*. PCR with chromosomal DNA of wild-type cells as a template amplified a 0.5 kb DNA fragment for *Ssglc*, while PCR with DNA from Ss Δglc mutant cells yielded a fragment of 1.7 kb (results not shown). These results indicated that the *Ssglc* gene in Ss Δglc mutant cells had been disrupted by the insertion of the *kan^r* gene.

Glucanase activity toward barley β -glucan was also detected in the crude extract prepared from *Synechocystis* PCC6803 wild-type cells: the specific activity against barley β -glucan was 730.3 ± 40.7 nmol of glucose/min per mg of chlorophyll. On the other hand, no glucanase activity was detected in Ss Δglc mutant cells.

Effects of various forms of stress on the growth of Ss Δglc and wild-type cells

To elucidate the physiological role of SsGlc in *Synechocystis* PCC6803, we analysed the growth ability of Ss Δglc and wild-type cells under normal and stressful conditions. The growth rate of the Ss Δglc mutant was almost the same as that of the wild-type under continuous light ($40 \mu\text{mol}$ of photons/ m^2 per s, 27°C) (Figure 5A), a 12 h light ($40 \mu\text{mol}$ of photons/ m^2 per s)/12 h dark cycle (27°C) (Figure 5B), high light ($200 \mu\text{mol}$ of photons/ m^2 per s,

Figure 4 ^1H -NMR spectroscopic analysis of the hydrolysis of barley β -glucan by SsGlc

(A) Time-dependent ^1H -NMR spectra showing the hydrolysis of barley β -glucan by the purified recombinant SsGlc. (B) Time course of the degradation of β -1,4- and β -1,3-linkages in barley β -glucan catalysed by the purified recombinant SsGlc. Arrows indicate the points at which the enzyme was added.

Figure 5 Growth curves of wild-type and Ss Δ glc mutant *Synechocystis* PCC6803 cells under various light and temperature conditions

Wild-type ($\circ$) and Ss Δ glc mutant ($\bullet$) *Synechocystis* PCC6803 cells were cultured in 1 litre of Allen's medium under continuous light ($40 \mu\text{mol}$ of photons/ m^2 per s, 27°C) (A), a 12 h light ($40 \mu\text{mol}$ of photons/ m^2 per s)/12 h dark cycle (27°C) (B), high light ($200 \mu\text{mol}$ of photons/ m^2 per s, 27°C) (C) and low temperature conditions ($40 \mu\text{mol}$ of photons/ m^2 per s, 4°C) (D) with bubbling of sterile air at 1 litre/min. Each point represents the mean for five assays (coefficient of variation $< 5\%$). OD 730 = D_{730} .

27°C) (Figure 5C) and low temperature ($40 \mu\text{mol}$ of photons/ m^2 per s, 4°C) (Figure 5D).

As shown in Figure 6, the growth of the Ss Δ glc mutant cells was significantly inhibited until the stationary phase in the medium

Figure 6 Effect of salt stress on the growth of wild-type and Ss Δ glc mutant *Synechocystis* PCC6803 cells

Wild-type ($\circ$) and Ss Δ glc mutant ($\bullet$) *Synechocystis* PCC6803 cells were cultured in 1 litre of Allen's medium under continuous light ($40 \mu\text{mol}$ of photons/ m^2 per s, 27°C) with various concentrations of NaCl [100 (A), 200 (B), 300 (C), 450 (D) mM] and bubbling with sterile air at 1 litre/min. Each point represents the mean for five assays (coefficient of variation $< 5\%$). OD 730 = D_{730} .

containing 300 mM NaCl. Furthermore, the Ss Δ glc mutant cells could not grow at all in the medium containing 450 mM NaCl. By contrast, the growth of the wild-type *Synechocystis* PCC6803 cells was not altered under these conditions.

Figure 7 Effect of osmotic stress on the growth of wild-type and SsΔglc mutant *Synechocystis* PCC6803 cells

Wild-type (○) and SsΔglc mutant (●) *Synechocystis* PCC6803 cells were cultured in 1 litre of Allen's medium under continuous light ($40 \mu\text{mol}$ of photons/ m^2 per s, 27°C) with various concentrations of sorbitol [100 (A), 200 (B), 300 (C), 400 (D) mM] and bubbling with sterile air at 1 litre/min. Each point represents the mean for five assays (coefficient of variation < 5%). OD 730 = D_{730} .

Figure 7 shows the effect of the osmotic stress caused by sorbitol on the growth of the wild-type and SsΔglc mutant *Synechocystis* PCC6803 cells. There was no significant difference in the growth of either when cultured in medium containing 100–400 mM sorbitol.

Effect of salt stress on photosynthesis and O_2 evolution in SsΔglc and wild-type cells

Figure 8 shows the effect of 0.8 M NaCl on the rates of NaHCO_3 -dependent O_2 evolution and O_2 consumption in the *Synechocystis* PCC6803 wild-type and SsΔglc mutant cells. The efficiency of NaHCO_3 -dependent O_2 evolution and O_2 consumption in the wild-type cells was retained for 72 h. However, in the mutant cells, the rates were immediately suppressed in the early stage of the NaCl treatment.

DISCUSSION

A gene (slr0897) possessing significant sequence similarity to glucanases from various sources is present in the *Synechocystis* PCC6803 genome. The deduced amino acid sequence of SsGlc indicated that the catalytic domain (589–1043) belongs to family GH9, resembling that of β -1,4-glucanases (cellulase). On the basis of a phylogenetic tree of glucanases from various sources, SsGlc can be classified into the β -1,4-glucanase group (Figure 1A). Interestingly, the N-terminal region other than the catalytic domain contains two cellulose-binding domains (CBM-2: 8–105 and 435–532) separated by a sequence of unknown function (Figure 1B). SsGlc appears to have a unique modular structure unlike any other β -1,4-glucanase reported previously [22–25].

Figure 8 Changes in NaHCO_3 -dependent O_2 evolution and O_2 consumption of the wild-type and SsΔglc mutant *Synechocystis* PCC6803 cells under salt stress

Wild-type and SsΔglc mutant *Synechocystis* PCC6803 cells were cultured in 1 litre of Allen's medium under continuous light ($40 \mu\text{mol}$ of photons/ m^2 per s, 27°C) with 800 mM NaCl and bubbling with sterile air at 1 litre/min. Changes in the rate of oxygen evolution and consumption in wild-type (○) and SsΔglc mutant (●) *Synechocystis* PCC6803 cells during incubation in the presence of 1 mM NaHCO_3 under $100 \mu\text{mol}$ of photons/ m^2 per s and $0 \mu\text{mol}$ of photons/ m^2 per s were measured using an oxygen electrode chamber as described in the Experimental section. Each point represents the mean for five assays (coefficient of variation < 5%).

We studied the substrate specificity of SsGlc (Table 1). Unlike typical β -1,4-glucanases, SsGlc did not exhibit any activity towards CM-cellulose (β -1,4-glucan). After the testing of various β -glucans differing in linkage type and ratio, SsGlc was found to hydrolyse only barley β -glucan and lichenan. The substrate specificity of SsGlc was basically similar to that of the β -1,3-1,4-glucanases reported thus far [23,26–28]; that is, the substrate must have both β -1,3- and β -1,4-linkages in its polysaccharide chain, but only β -1,4-linkages are hydrolysed (Table 1 and Figure 4). SsGlc can hydrolyse neither β -1,3-glucan nor β -1,4-glucan. Thus SsGlc should be classified as a β -1,3-1,4-glucanase on the basis of substrate specificity. This is the first time a β -1,3-1,4-glucanase has been classified in the family GH9. Like the other family 9 GHs, SsGlc was found to be an inverting enzyme (Figure 3). However, most of the β -1,3-1,4-glucanases reported to date belong to families GH16 and GH17, of which all members are retaining enzymes [22]. To our knowledge, SsGlc is the first example of an inverting β -1,3-1,4-glucanase.

The optimum pH for SsGlc was 7.0, higher than that for any other glucanase from a plant or micro-organism. Generally, the optimum pH for glucanases from plants and other organisms is around 4 [26,27,29,30]. In plants, β -glucanases are located in the vacuole or cell wall, where the pH is lower than in the cytosol and chloroplast [31]. In cyanobacterial cells, the pH is around 7.5, indicating that SsGlc can function under physiological conditions in these cells.

The kinetic analysis of SsGlc produced values for two different substrates, barley β -glucan and lichenan, in which the ratio of

1,4-linkages to 1,3-linkages is 2.3–2.7 and 2.0 respectively. The K_m value for the former substrate (2.0 mg/ml) is lower than that for the latter (4.2 mg/ml). The difference seems to influence directly the overall catalytic activity (k_{cat}/K_m), the values of which are 4.3 and 0.88 respectively. SsGlc is likely to prefer a substrate with a higher ratio of 1,4-linkages. This is consistent with the result of an experiment involving the intensive digestion of barley β -glucan (Figure 4), which indicates that the β -1,4-linkage of β -1,3,1,4-glucan is specifically hydrolysed by this enzyme. An earlier study on the bacterial β -1,3,1,4-glucanase [22] found that the enzyme hydrolyses β -1,4-linkages in 3-O-substituted glucopyranose units. SsGlc might have similar cleavage specificity. However, intensive treatment of barley β -glucan with SsGlc brought about only a 16% loss of β -1,4-linkages (Figure 6). The result suggests that SsGlc strictly recognizes the extended region of a polysaccharide chain lowering the specific activity towards polysaccharide substrates. In fact, the k_{cat}/K_m value for *Bacillus licheniformis* β -1,3,1,4-glucanase toward barley β -glucan was reported to be approx. 2400 [32], which is much larger than that reported for SsGlc here. The unusual substrate recognition of SsGlc might be related to its physiological function described below.

In order to clarify the physiological functions of SsGlc in *Synechocystis* PCC6803 cells, we created and characterized *Ssglc*-disrupted (*SsΔglc*) mutant cells. The growth of mutant cells in which the *Ssglc* gene was disrupted by a kanamycin-resistance cartridge gene was almost the same as that of the wild-type cells under continuous light (40 μ mol of photons/m² per s, 27 °C), the 12 h light (40 μ mol of photons/m² per s)/12 h dark cycle (27 °C), cold stress (40 μ mol photons/m² per s, 4 °C) and high light stress (200 μ mol photons/m² per s, 27 °C) (Figure 5). These results suggest that SsGlc was not involved in either cell division or the remodelling of the cell wall. However, under salt stress, the growth of *SsΔglc* mutant cells was significantly inhibited compared with that of the wild-type cells (Figure 6). No structural changes or significant differences in the composition of the insoluble sugars of cell walls between the *SsΔglc* mutant and the wild-type cells were observed by photomicroscopy and solid-state ¹³C-CP/MAS (cross-polarization/magic angle spinning) NMR spectroscopy (M. Tamoi, H. Kurotaki and T. Fukamizo, unpublished work), indicating that the increased sensitivity to NaCl did not result from the cell wall architecture of *SsΔglc* mutant cells. The efficiency of photosynthesis and O₂ consumption were immediately suppressed under salt stress (Figure 8). Accordingly, the suppression of photosynthesis and O₂ consumption may result in the growth inhibition of the *SsΔglc* mutant cells under salt stress.

The ¹H- and ¹³C-NMR analyses of the cell extracts showed that levels of some soluble sugars were obviously reduced in *SsΔglc* mutant cells compared with the wild-type cells (M. Tamoi, H. Kurotaki and T. Fukamizo, unpublished work). The lack of SsGlc appears to reduce the soluble sugar content in *Synechocystis* PCC6803 cells, and the reduction might enhance the sensitivity of *SsΔglc* mutant cells to salt stress. It was reported that *Synechocystis* PCC6803 is able to tolerate up to 1.2 M of NaCl [33,34]. In *Synechocystis* PCC6803 cells, glycosylglycerol was recognized to function as a major osmoprotectant factor. Furthermore, it was recently reported that sucrose is accumulated during salt stress and functions as a secondary osmolyte in cyanobacterial cells [35]. Under sorbitol stress, the growth of *SsΔglc* mutant cells was almost the same as that of the wild-type cells (Figure 7), indicating that SsGlc functions in salt stress tolerance, and not in osmotic stress tolerance in *Synechocystis* PCC6803 cells. The SsGlc protein itself or the enzymatic products might protect the photosynthetic machinery from salt stress, but it is unclear which is a major determinant of salt stress tolerance.

Anyway, it is important that the novel β -1,3-1,4-glucanase studied here functions in salt stress tolerance in photosynthetic organisms.

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REFERENCES

- Henrissat, B. (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280**, 309–316
- Henrissat, B. and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781–788
- Davis, G. and Henrissat, B. (1995) Structures and mechanisms of glycosyl hydrolases. *Structure* **3**, 853–859
- Henrissat, B. and Bairoch, A. (1996) Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* **316**, 695–696
- Campbell, J. A., Davies, G. J., Bulone, V. and Henrissat, B. (1997) A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.* **326**, 929–939
- Henrissat, B. and Davies, G. (1997) Structure and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* **7**, 637–644
- Coutinho, P. and Henrissat, B. (1999) Carbohydrate-active enzymes: an integrated database approach. In *Recent advances in carbohydrate bioengineering* (Gilbert, H., Davies, G., Henrissat, B. and Svensson, B., eds), pp. 3–12, Royal Society of Chemistry, Cambridge
- Myers, A. M., Morell, M. K., James, M. G. and Ball, S. G. (2000) Recent progress toward understanding biosynthesis of the amylopectin crystal. *Plant Physiol.* **122**, 989–998
- Henrissat, B., Coutinho, P. M. and Davies, G. (2001) A census of carbohydrate-active enzymes in the genome of *Arabidopsis thaliana*. *Plant Mol. Biol.* **47**, 55–72
- Schlumberg, A., Mauch, F., Vogeli, U. and Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**, 365–367
- Brogie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J. and Brogie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254**, 1194–1197
- Collinge, D. B., Kragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmussen, U. and Vad, K. (1993) Plant chitinases. *Plant J.* **3**, 31–40
- Reymond, P. and Farmer, E. E. (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* **1**, 404–411
- Mita, S., Murano, N., Akaike, M. and Nakamura, K. (1997) Mutants of *Arabidopsis thaliana* with pleiotropic effects on the expression of the gene for β -amylase and on the accumulation of anthocyanin that are inducible by sugars. *Plant J.* **11**, 841–851
- Williams, J. G. K. (1988) Construction of specific mutants in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Methods Enzymol.* **167**, 766–778
- Tamoi, M., Ishikawa, T., Takeda, T. and Shigeoka, S. (1996) Enzymic and molecular characterization of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Synechococcus* PCC 7942: resistance of the enzyme to hydrogen peroxide. *Biochem. J.* **316**, 685–690
- Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**, 426–428
- Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- Golden, S. S., Brusslan, J. and Haselkorn, R. (1987) Genetic engineering of the cyanobacterial chromosome. *Methods Enzymol.* **153**, 215–231
- Tamoi, M., Takeda, T. and Shigeoka, S. (1999) Functional analysis of fructose-1,6-bisphosphatase isozymes (*fbp-I* and *fbp-II* gene products) in cyanobacteria. *Plant Cell Physiol.* **40**, 257–261
- Lichtenthaler, H. K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* **148**, 350–382
- Planas, A. (2000) Bacterial 1,3-1,4-glucanases: structure, function and protein engineering. *Biochim. Biophys. Acta* **1543**, 361–382
- Eckert, K., Zielinski, F., Leggio, L. L. and Schneider, E. (2002) Gene cloning, sequencing, and characterization of a family 9 endoglucanase (CelA) with an unusual pattern of activity from the thermoacidophile *Alicyclobacillus acidocaldarius* ATCC27009. *Appl. Microbiol. Biotechnol.* **60**, 428–436
- Keitel, T., Simon, O., Borris, R. and Heinemann, U. (1993) Molecular and active-site structure of a *Bacillus* 1,3-1,4- β -glucanase. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5287–5291

- 25 Zverlov, V. V., Volkov, I. Y., Velikodvorskaya, T. V. and Schwarz, W. (1997) Highly thermostable endo-1,3- β -glucanase (laminarinase) LamA from *Thermotoga neapolitana*: nucleotide sequence of the gene and characterization of the recombinant product. *Microbiology* **143**, 1701–1708
- 26 Akiyama, T., Kaku, H. and Shibuya, N. (1996) Purification and properties of a basic endo-1,3- β -glucanase from rice (*Oryza sativa* L.). *Plant Cell Physiol.* **37**, 702–705
- 27 Akiyama, T., Kaku, H. and Shibuya, N. (1998) Purification, characterization and NH₂-terminal sequence of an endo-(1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucanase from rice (*Oryza sativa* L.) bran. *Plant Sci.* **134**, 3–10
- 28 Sun, L., Gurnon, J. R., Adams, B. J., Graves, M. V. and Etten, J. V. (2000) Characterization of a β -1,3-glucanase encoded by chlorella virus PBCV-1. *Virology* **276**, 27–36
- 29 Kotake, T., Nakagawa, N., Takeda, K. and Sakurai, N. (1997) Purification and characterization of wall-bound exo-1,3- β -D-glucanase from barley (*Hordeum vulgare* L.) seedlings. *Plant Cell Physiol.* **38**, 194–200
- 30 Woodward, J. R. and Fincher, G. B. (1982) Purification and chemical properties of two 1,3;1,4- β -glucan endohydrolases from germinating barley. *Eur. J. Chem.* **121**, 663–669
- 31 Staehelin, L. A. and Newcomb, E. H. (2000) Membrane structure and membranous organelles. In *Biochemistry and Molecular Biology of Plants* (Buchanan, B. B., Gruissem, W. and Jones, R. L., eds), pp. 2–50, American Society of Plant Physiologists, Rockville
- 32 Planas, A., Juncosa, M., Cayetano, A. and Querol, E. (1992) Studies on *Bacillus licheniformis* endo- β -1,3-1,4-D-glucanase: characterization and kinetic analysis. *Appl. Microbiol. Biotechnol.* **37**, 583–589
- 33 Hagemann, M. and Erdmann, N. (1997) Environmental stresses. In *Cyanobacterial Nitrogen Metabolism and Environmental Biotechnology* (Rai, A. K., ed.), pp. 155–221, Springer Verlag, Berlin
- 34 Reed, R. H. and Stewart, W. D. P. (1985) Osmotic adjustment and organic solute accumulation in unicellular cyanobacteria from freshwater and marine habitats. *Mar. Biol.* **88**, 1–9
- 35 Desplats, P., Folco, E. and Salerno, G. L. (2005) Sucrose may play an additional role to that of an osmolyte in *Synechocystis* sp. PCC6803 salt-shocked cells. *Plant Physiol. Biochem.* **43**, 133–138

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