Thioredoxin peroxidase in the Cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract The amino acid sequence deduced from the open reading frame designated sll0755 in *Synechocystis* sp. PCC 6803 is similar to the amino acid sequences of thioredoxin peroxidases from other organisms. In the present study, we found that a recombinant SLL0755 protein that was expressed in *Escherichia coli* was able to reduce H_2O_2 and tertiary butyl hydroperoxide with thioredoxin from *E. coli* as the electron donor. Targeted disruption of open reading frame sll0755 in *Synechocystis* sp. PCC 6803 cells completely eliminated the H_2O_2-dependent and tertiary butyl hydroperoxide-dependent photosynthetic evolution of oxygen and the electron flow in photosystem II. These results indicate that the product of open reading frame sll0755 is a thioredoxin peroxidase whose activities are coupled to the photosynthetic electron transport system in *Synechocystis* sp. PCC 6803.

Key words: Chlorophyll fluorescence; Cyanobacterium; Hydrogen peroxide; Thioredoxin peroxidase; *Synechocystis* sp. PCC 6803

1. Introduction

Active oxygen species (AOS), such as O_2^-, $^{\bullet}OH$ and H_2O_2, are generated as a result of the incomplete reduction of O_2 during respiration and photosynthesis. Organisms living in oxygenic environments have an absolute requirement for mechanisms that detoxify AOS. It is suggested that AOS are extremely reactive and can cause severe damage to cell components by, for example, inactivating proteins, cleaving DNA and in the peroxidation of unsaturated fatty acids in cell membranes [1]. In plant chloroplasts, O_2^-, the primary product of AOS, is immediately dismutated to H_2O_2 and O_2 by superoxide dismutase and H_2O_2 is reduced to H_2O by ascorbate peroxidase, which uses ascorbate as the electron donor. The univalently and divalently oxidized products of ascorbate, monodehydroascorbate and dehydroascorbate, respectively, are then re-reduced to ascorbate via the Halliwell-Asada pathway [2].

In Cyanobacteria, H_2O_2 is scavenged by peroxidases and/or catalases [3]. The peroxidases use electrons generated during the photosynthetic electron transport and such a peroxidase activity is not observed in the presence of DCMU or in the dark. The peroxidases are referred to as 'photoreductant peroxidases', but the activities of the peroxidases and the donors of electrons have not been fully characterized [4].

Several years ago, Kim et al. isolated a novel antioxidant enzyme, thioredoxin peroxidase (TPX), from yeast [5]. The enzyme catalyses not only the reduction of H_2O_2 to H_2O but also the reduction of alkyl hydroperoxides to the corresponding alcohols and H_2O, with thioredoxin as the electron donor [6,7]. Baier and Dietz reported that some plants have a gene for a homolog of TPX, *bas1*, and that the product of this gene is localized in the chloroplast stroma [8,9]. Furthermore, the genome of the Cyanobacterium *Synechocystis* sp. PCC 6803 includes an open reading frame (ORF) designated sll0755 that encodes a putative homolog of TPX [9,10]. However, the functions of TPX in photosynthetic organisms remain unclear.

In this study, we analyzed the enzymological properties of recombinant SLL0755 protein that was synthesized in *Escherichia coli*. The function in vivo of the gene product was also examined by disrupting the ORF in intact *Synechocystis* sp. PCC 6803 cells.

2. Materials and methods

2.1. Organisms and culture conditions

The wild-type strain of *Synechocystis* sp. PCC 6803 was originally provided by Dr J.G.K. Williams (Dupont de Nemours, Wilmington, DE, USA). *Synechocystis* sp. PCC 6803 was grown photoautotrophically at 34°C in BG-11 medium supplemented with 4 mM HEPES-KOH (pH 7.5) under illumination, provided by fluorescent lamps at 30 μmol photons m^{-2} s^{-1}. The culture was agitated on a reciprocal shaker (NR-3, TAITEC, Saitama, Japan) at 100 strokes/min.

2.2. Overexpression of the product of ORF sll0755 in E. coli

Chromosomal DNA from the *Synechocystis* cells was purified with ISOPLANT (Nippon Gene, Osaka, Japan) and used as a template for the polymerase chain reaction (PCR). A 0.6 kbp fragment of DNA that contained ORF sll0755 was amplified by PCR with the following primers: 5′-AGGTACCCCATATGCGCATGCCAGAGGTATTA-AGGGTAGGA-3′ and 5′-ATGAGCTCCTAAGGTTCCGCCAC-TGT-3′. The first primer was designed to introduce a *Nde*I site with an ATG codon for the initiation of translation and the newly introduced site is underlined. The reaction mixture after PCR was frac-

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Abbreviations: AOS, active oxygen species; DCMU, 3-(3,4 dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol; IPTG, isopropyl-D-thiogalactopyranoside; MeSH, 2-mercaptoethanol; ORF, open reading frame; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *t*-BuOOH, tertiary butyl hydroperoxide; TPX, thioredoxin peroxidase

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tionated by electrophoresis and the amplified fragment was extracted from an agarose gel with Geneclean II (BIO 101, La Jolla, CA, USA) and cloned into the TA cloning vector pT7Blue-T (Novagen, Madison, WI, USA). The resultant plasmid was used for transformation of *E. coli* JM109. Both strands of the cloned fragment were sequenced with an automated DNA sequencer (ABI377A, Perkin Elmer, Foster City, CA, USA) to confirm the identity of the product of PCR. The plasmid was digested with *Nde*I and *Bam*HI and the resultant 0.6 kbp DNA fragment was cloned into the vector pET16b (Novagen) for the subsequent expression of a His-tagged fusion protein, after pET16b had been digested with *Nde*I and *Bam*HI. The product was designated pET-TPX and used for the transformation of *E. coli* BL21(DE3)-pLysS. Transformed cells were grown at 37°C in 2-YT medium supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. When the absorbance at 600 nm of the culture reached 0.6, IPTG was added to a final concentration of 0.4 mM and the culture was incubated for an additional 6 h at 30°C.

All procedures of purification of the fusion protein were performed at 0–4°C. The *E. coli* cells were sonicated (10 kHz) with an ultrasonic processor (VP-60, TAITEC) for 10 min, with four 2 min interruptions, on ice in 50 mM HEPES-KOH buffer (pH 7.8) that contained 1 mM ethylenediaminetetraacetic acid (disodium salt), 1 mM phenylmethylsulfonyl fluoride and 10 μM leupeptin. The lysate was centrifuged at $41\,000\times g$ for 30 min and the supernatant was brought to 60% saturation with ammonium sulfate. The resultant precipitate was collected by centrifugation at $10\,000\times g$ for 15 min. After the pelleted proteins had been dissolved in a small volume of the above described buffer, the fusion protein was purified with His Bind Resin (Novagen) according to the manufacturer's instruction. It was further purified by ion exchange chromatography on a Mono-Q column (Pharmacia, Uppsala, Sweden) with a gradient of 0.0–0.5 M NaCl and subsequent gel filtration chromatography on a Superdex 200HR (Pharmacia).

The concentration of protein was determined as described by Bradford [11] with bovine serum albumin as the standard.

2.3. SDS-PAGE

Fractionation by SDS-PAGE was performed on a 12.5% (w/v) polyacrylamide gel as described by Laemmli [12]. For SDS-PAGE under non-reducing conditions, 2-mercaptoethanol (MeSH) was omitted from the loading buffer that was used to denature the purified SLL0755 fusion protein. Proteins on gels were stained with Coomassie brilliant blue R-250.

2.4. Assays of thioredoxin-dependent H_2O_2-reducing and tertiary butyl hydroperoxide-reducing activities

Thioredoxin and NADPH:thioredoxin oxidoreductase from *E. coli* and NADPH were used as the coupled electron-donor system, as described elsewhere [7]. The *trxA* and *trxB* genes for thioredoxin and NADPH:thioredoxin oxidoreductase, respectively, from *E. coli* were amplified by PCR and cloned into pET16b. The resultant plasmids were used to transform *E. coli* BL21(DE3)pLysS and His-tagged thioredoxin and His-tagged NADPH:thioredoxin oxidoreductase were expressed in *E. coli* in the same manner as described above for the His-tagged SLL0755 protein. These proteins were purified with His Bind Resin according to the manufacturer's instructions. The reaction mixture for assays (1 ml) consisted of 20 mM potassium phosphate buffer (pH 7.0) that contained 0.1 mM NADPH, 0.2 mg/ml bovine serum albumin, 60 μg thioredoxin, 65 μg NADPH:thioredoxin oxidoreductase, 2.5 μg TPX and H_2O_2 or tertiary butyl hydroperoxide (*t*-BuOOH) at various concentrations. The activity of TPX was determined spectrophotometrically by monitoring the oxidation of NADPH at 340 nm.

2.5. Targeted disruption of the ORF sll0755 in Synechocystis cells

A 2.1 kbp DNA fragment that contained the encoding region of ORF sll0755 was amplified by PCR with chromosomal DNA from *Synechocystis* sp. PCC 6803 as a template and 5′-AAGATGACCGGGTGGGCACA-3′ and 5′-CACACAGGTTTCGCCGTGGC-3′ as primers. The amplified fragment of DNA was cloned into pT7Blue-T and its nucleotide sequence was confirmed as described above. The spectinomycin/streptomycin resistance gene cassette (Sp^r/Sm^r, 2 kbp) was excized from pRL453 [13] by digestion with *Bam*HI and inserted into the *Bgl*II site of ORF sll0755 in pT7Blue-T. The resultant plasmid was designated pTPX-Sp^r/Sm^r. Then, *Synechocystis* cells were transformed with this plasmid to generate *tpx*$^-$ cells as described by Williams [14]. Transformed cells were selected on agar-solidified BG-11 medium supplemented with 30 μg/ml spectinomycin and complete segregation of ORF sll0755 in *tpx*$^-$ cells was confirmed by PCR with the appropriate primers. *tpx*$^-$ cells were cultured photoautotrophically in the same way as wild-type cells.

2.6. Analysis of the role of TPX in Synechocystis cells in vivo

The role of TPX in vivo was analyzed with wild-type and *tpx*$^-$ mutant *Synechocystis* cells that had been suspended in BG-11. The photosynthetic evolution of oxygen by the cells was monitored with

Fig. 1. Alignment of the deduced amino acid sequences of SLL0755 from *Synechocystis* and TPXs from various organisms. The completely conserved residues are boxed and residues in TPXs that are identical to residues in SLL0755 are indicated by dots. The two Cys residues involved in the reduction of peroxides and the dimerization of TPXs are underlined. The identity scores and positive similarity score for the sequence of mature proteins compared to the sequence of SLL0755 were calculated with the FASTA program [26]. The abbreviations and accession numbers in the SWISS-PROT database of the sequences are as follows: *Synechocystis*, SLL0755 of *Synechocystis* sp. PCC 6803, Q55624; *Porphyra*, YCF42 of *Porphyra purpurea*, P51272; spinach, BAS1 of *Spinacia oleracea*, O24364; *Arabidopsis*, BAS1 of *Arabidopsis thaliana*, Q96291; human, TSA of *Homo sapiens*, P32119; yeast, TSA of *Saccharomyces cerevisiae*, P34760.

an oxygen electrode (Hansatech, Norfolk, UK) at 25°C under illumination at 1000 μmol photons/m^2/s. Iodoacetamide was added at 10 mM to the suspension of cells to inhibit the fixation of CO_2 via the Calvin cycle [15]. After the evolution of oxygen had stopped in the light, *t*-BuOOH was added to the suspension of cells at a final concentration of 70 μM.

The yield of chlorophyll fluorescence was measured with a chlorophyll fluorometer (PAM, WALZ, Effeltrich, Germany) as described previously [3,16].

3. Results and discussion

3.1. Homology between SLL0755 of Synechocystis sp. PCC 6803 and TPXs of other organisms

The ORF designated sll0755 encodes a protein of 200 amino acid residues with a calculated molecular mass of 22 509 Da. The deduced amino acid sequence is very similar to those of proteins reported as TPX, thiol-specific antioxidant protein or 2-Cys peroxiredoxin, from other organisms (Fig. 1). The similarities among these proteins extend throughout their entire sequences. In particular, the deduced amino acid sequence of SLL0755 includes two conserved cysteine residues, Cys^{50} and Cys^{172}, in the amino- and carboxy-terminal regions, respectively. These residues form the catalytic center and allow the formation of inter-subunit disulfide bonds in TPX [17]. The amino acid motifs around the two cysteine residues, ^{46}FTFVCPTEI^{54} and ^{170}EVCP^{173}, are also strongly conserved, the former motif is known as an F-motif [8]. The deduced amino acid sequence of SLL0755 is very similar to those of TPXs from a red alga and from higher plants but lacks the amino-terminal extension that constitutes the signal for targeting to plastids [9].

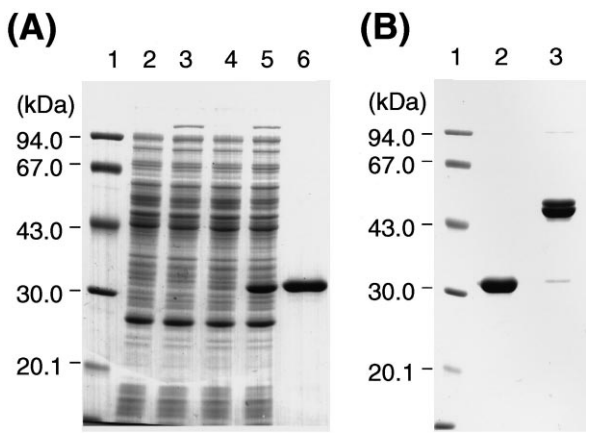

Fig. 2. Analysis by SDS-PAGE of the recombinant *Synechocystis* SLL0755 protein expressed in *E. coli*. (A) Crude extracts and the purified recombinant SLL0755 protein: lane 1, molecular mass markers; lane 2 and 3, crude lysates of *E. coli* cells that harbored pET16b and were incubated without and with 0.4 mM IPTG for 2 h at 37°C, respectively; lane 4 and 5, crude lysates of *E. coli* cells that harbored pET-TPX and were incubated without and with 0.4 mM IPTG for 2 h at 37°C, respectively; lane 6, recombinant SLL0755 protein after purification by affinity chromatography. 10 μg of protein was loaded in each lane. (B) Analysis by SDS-PAGE under non-reducing conditions of the purified recombinant SLL0755 protein. SDS-PAGE was performed in the presence of MeSH and in its absence. Lane 1, molecular mass markers; lane 2 and 3, recombinant SLL0755 protein that was purified and treated with 1 mM H_2O_2 for 30 min at 25°C with and without 5 mM MeSH, respectively. 10 μg of protein was loaded in each lane.

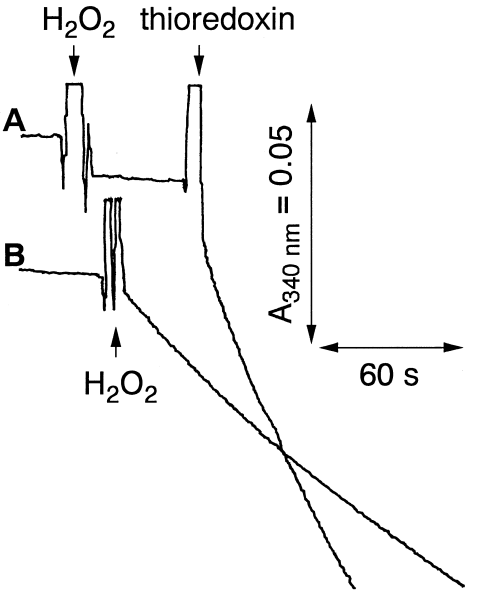

Fig. 3. The thioredoxin-dependent reduction of H_2O_2 by the recombinant SLL0755 protein. (A) The reaction mixture (1 ml) contained 0.1 mM NADPH, 5.0 μg SLLO755 and 65 μg NADPH: thioredoxin oxidoreductase. After the addition of 50 μM H_2O_2, the reaction was started by the addition of 60 μg thioredoxin. The oxidation of NADPH was monitored at 340 nm. (B) The reaction was started by the addition of 50 μM H_2O_2 to a reaction mixture (1 ml) that contained 0.1 mM NADPH, 2.5 μg SLLO755, 60 μg thioredoxin and 65 μg NADPH:thioredoxin oxidoreductase.

3.2. Characterization of recombinant SLL0755 expressed in E. coli

ORF sll0755 was cloned and its encoded product was expressed as a fusion protein, with a histidine tag at the amino-terminus, under control of the T7 promoter in *E. coli*. The fusion protein was purified by affinity chromatography (Fig. 2A). The molecular mass of the fusion protein was estimated to be about 28 kDa by SDS-PAGE. This value was about 5 kDa higher than that deduced from the putative amino acid sequence. Delayed migration of TPX during SDS-PAGE was also reported for TPXs from yeast and plant sources [8,18]. The absorption spectrum of the purified SLL0755 fusion protein did not show any characteristics typical of the conventional redox cofactors of catalases and peroxidases.

The TPXs from yeast and the human brain can be dissociated into subunits in the presence of SDS under reducing conditions but they exist as dimers under oxidizing conditions [6,17,19]. The formation of an inter-subunit disulfide bond is involved in the catalytic activity of these proteins. To confirm that this phenomenon is also a property of the TPX from *Synechocystis*, we incubated the fusion protein with 1 mM H_2O_2 in the presence and in the absence of 5 mM MeSH and then subjected it to SDS-PAGE under non-reducing conditions. The fusion protein was detected in a dimeric form, with a molecular mass of 46 kDa, in the absence of MeSH. In the presence of MeSH, the protein migrated as a monomer (Fig. 2B). In the absence of MeSH, the fusion protein existed in two different dimeric forms with different mobilities during non-reducing SDS-PAGE. The difference might have been due to different inter-subunit disulfide linkages, with resultant

differences in packing and migration of dimers, as reported for TPX from yeast [17]. These results indicate that the protein from *Synechocystis* has catalytic cysteine residues that are oxidized by H_2O_2 to generate inter-subunit dimers.

In TPX of yeast, the Cys^{47}-SH residue of one subunit of a homodimeric molecule is oxidized to cysteine sulphonic acid, Cys^{47}-SOH, by H_2O_2. A water molecule (H_2O) is removed from Cys^{47}-SOH and from Cys^{170}-SH in the other subunit to generate an inter-subunit disulfide bond. It has been proposed that the pK_a of the thiol group of Cys^{47} in the yeast enzyme is lowered by the positively charged side chain of Arg^{124}. This arginine residue is conserved in the amino acid sequences of all TPXs reported to date, as it is in SLL0755. Thus, Cys^{47}, which corresponds to Cys^{50} in SLL0755, might function as the nucleophile for the reduction of H_2O_2 to water [20].

In the present study, the TPX activity of the SLL0755 fusion protein was assayed with the thioredoxin-NADPH:thioredoxin oxidoreductase coupling system from *E. coli*, with NADPH as the electron donor. The SLL0755 fusion protein catalyzed the decomposition of H_2O_2 and the oxidation of NADPH depended on the presence of H_2O_2, thioredoxin and NADPH:thioredoxin oxidoreductase (Fig. 3). The rate of the reaction was proportional to the amount of SLL0755 protein included in the assay. The TPX activity of SLL0755 was completely insensitive to KCN. These results indicated that SLL0755 was TPX and, therefore, ORF sll0755 was designated *tpx*. We determined the K_m for H_2O_2 and the maximal velocity of the reduction of H_2O_2 by the recombinant TPX from *Synechocystis* to be 0.18 ± 0.12 μM and 3.2 μmol NADPH oxidized/mg protein/min, respectively. The reaction specificity for H_2O_2 of *Synechocystis* TPX, k_{cat}/K_m, was calculated to be 1.4×10^7 s^{-1} M^{-1}. This value is slightly higher than the analogous values for catalase and ascorbate perox-

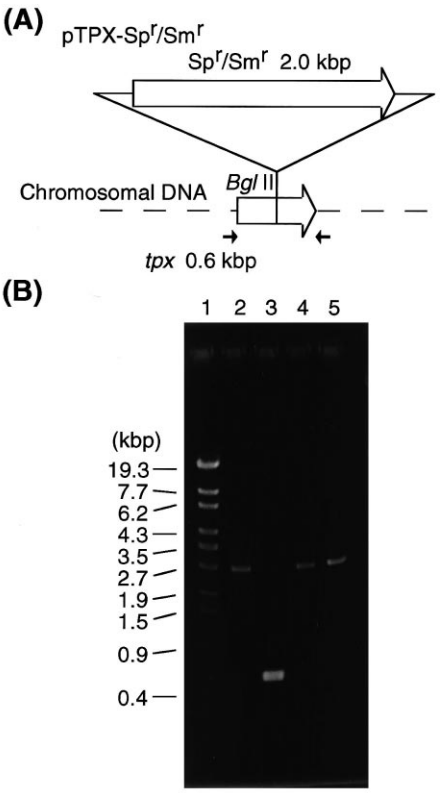

Fig. 4. The targeted disruption of the *tpx* gene in *Synechocystis* sp. PCC 6803. (A) The construction of plasmid pTPX-Sp^r/Sm^r that was used for the disruption of the gene in *Synechocystis* sp. PCC 6803. Small arrows indicate the primers used for the analysis by PCR that was designed to evaluate the extent to which the native gene for TPX was replaced by the mutated gene. (B) Analysis by PCR of the native *tpx* gene and the mutated *tpx* gene in *tpx*$^-$ cells with the following templates: lane 1, DNA fragments of bacteriophage digested with *Eco*T14I (molecular size markers); lane 2, the disruption vector pTPX-Sp^r/Sm^r; lane 3, chromosomal DNA from wild-type cells; lanes 4 and 5, chromosomal DNA from *tpx*$^-$ cell lines designated number 1 and 2, respectively.

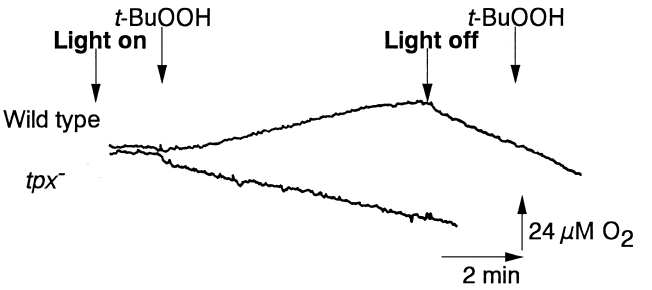

Fig. 5. The *t*-BuOOH-dependent evolution of oxygen by wild-type *Synechocystis* sp. PCC 6803 and by *tpx*$^-$ cells. Light (1000 μmol photons m^{-2} s^{-1}) was turned on and off and *t*-BuOOH was added at a final concentration of 70 μM at the times indicated by appropriately labelled arrows.

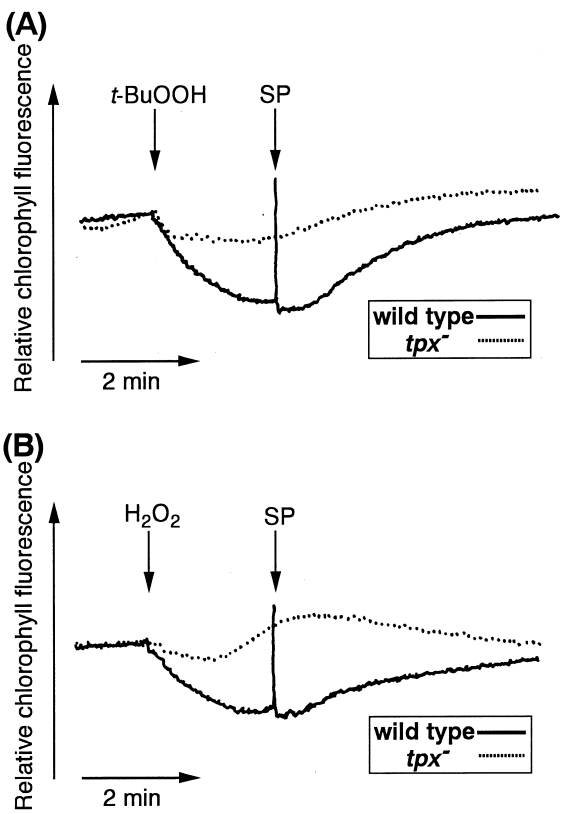

Fig. 6. The *t*-BuOOH-dependent (A) and H_2O_2-dependent (B) quenching of chlorophyll fluorescence in wild-type and *tpx*$^-$ cells of *Synechocystis* sp. PCC 6803. 70 μM *t*-BuOOH and 100 μM H_2O_2 were added, as indicated, and a saturating light pulse (SP) was applied at times indicated by arrows.

idase from *Synechococcus* sp. PCC 7942 and spinach, respectively [21].

Furthermore, the recombinant TPX catalyzed the decomposition of *t*-BuOOH at a rate similar to the rate of decomposition of H_2O_2. Glutathione peroxidase is known as a scavenger of alkyl hydroperoxides in land plants and green algae but a similar alkyl hydroperoxide-scavenging activity has not previously been detected in *Synechocystis* sp. PCC 6803 [22,23].

3.3. Targeted disruption of the gene for TPX in Synechocystis

To examine the function of TPX in vivo in *Synechocystis*, we disrupted the *tpx* gene by insertion of an Sp^r/Sm^r gene cassette at the *Bgl*II site in the *tpx* gene (Fig. 4A). We performed PCR with chromosomal DNA from wild-type and *tpx*$^-$ mutant cells and with the vector pTPX-Sp^r/Sm^r, which had been constructed for the transformation, as templates and an appropriate set of primers (indicated schematically in Fig. 4A). A PCR with chromosomal DNA from wild-type cells as a template amplified a 0.6 kbp DNA fragment, while a PCR with DNA from cells with a disrupted gene yielded a fragment of 2.6 kbp. Our results indicated that the *tpx* gene in all *tpx*$^-$ mutant cells had been disrupted by insertion of the gene cassette. The mutant cells were able to grow under low intensity light (30 μmol photons m^{-2} s^{-1}), an observation that indicates that TPX is not essential for the growth of *Synechocystis* under non-oxidative conditions, as observed similarly in the case of *tpx* null mutants of yeast and *E. coli* [24,25].

The H_2O_2-dependent evolution of oxygen by *Synechocystis* sp. PCC 6803 occurred only in the light [3]. Therefore it is likely that the electrons required for the reduction of H_2O_2 to H_2O are supplied from the photosynthetic electron transport system. If the TPX of *Synechocystis* scavenges peroxides using thioredoxin as the electron donor, the evolution of oxygen from photosystem II should occur in wild-type cells but not in *tpx*$^-$ mutant cells in the presence of *t*-BuOOH in the light. In wild-type cells, we did in fact observe the *t*-BuOOH-dependent evolution of oxygen in the light but the evolution of oxygen stopped upon turning off the light. Further addition of *t*-BuOOH did not cause the evolution of oxygen in the dark. In contrast to wild-type cells, *tpx*$^-$ cells did not show any evidence of the *t*-BuOOH-dependent evolution of oxygen in the light (Fig. 5). These results indicate that TPX reduces *t*-BuOOH using electrons from photosystem I.

The steady state fluorescence of chlorophyll was transiently quenched by H_2O_2 and by *t*-BuOOH (Fig. 6). The peroxide-dependent quenching of chlorophyll fluorescence was not influenced by 1 mM KCN, a result that suggests that peroxidases such as ascorbate peroxidase do not function in *Synechocystis*. In the *tpx*$^-$ cells, the peroxide-dependent quenching of chlorophyll fluorescence was not observed (Fig. 6). The slight quenching observed just after the addition of peroxide was also observed after the addition of an equal volume of distilled water. These observations, together with the other results reported herein, indicate that TPX is the only enzyme that scavenges H_2O_2 and alkyl hydroperoxides, with thioredoxin as the electron donor, in *Synechocystis* sp. PCC 6803.

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