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Desiccation-inducible genes are related to N_2-fixing system under desiccation in a terrestrial cyanobacterium ☆

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ABSTRACT

Terrestrial cyanobacteria have various desiccation-tolerant systems, which are controlled by desiccation tolerance-related genes. *Anabaena* (*Nostoc*) sp. strain PCC 7120 is a derivative of the terrestrial cyanobacterium *Nostoc* and is a useful strain for molecular biological analysis. To identify desiccation tolerance-related genes, we selected and disrupted various genes (*all0801*, *all0875*, *alr3090*, *alr3800*, *all4052*, *all4477*, and *alr5182*) and examined their gene expression patterns and predicted their functions. Analyses of gene disruptants showed that viability of the disruptants only decreased under N_2-fixing conditions during desiccation, and the decrease in viability was negatively correlated with the gene expression pattern during desiccation. These data suggest that terrestrial cyanobacteria may acclimate to desiccation stress via N_2 fixation by using desiccation inducible genes, which are not only related to nitrogen fixation or nitrogen metabolism but also to other systems such as metabolism, transcription, and protein repair for protection against desiccation damage under nitrogen-fixing conditions. Further, a photosynthetic gene is required for desiccation tolerance. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

1. Introduction

Cyanobacteria, the photosynthetic, O_2-evolving prokaryotes [1–3], grow in diverse habitats ranging from the tropics to the polar regions [4]. To survive in and acclimate to various conditions, some cyanobacteria have developed desiccation tolerance derived from their archaic origin. These cyanobacteria are subjected to repeated cycles of dehydration, desiccation, and rehydration and can survive under desiccated conditions for long periods. The desiccation-tolerant cyanobacterium *Nostoc commune* is a useful organism for physiological analysis [5–8]. However, the relationship between desiccation tolerance and photosynthesis has not been elucidated.

The genome sequence of the nitrogen-fixing filamentous cyanobacterium *Anabaena* (*Nostoc*) sp. PCC 7120 has been analyzed [9]. *Anabaena* is a useful organism for studying nitrogen fixation using molecular biology [10–13]. Previously, Katoh et al. [14,15] showed that *Anabaena* is a close relative of a terrestrial, desiccation-tolerant cyanobacterium, *Nostoc* sp. HK-01, and analyzed the transcriptome of *Anabaena* under desiccation. Higo et al. [16] analyzed desiccation-inducible gene disruptants and showed that the trehalose metabolism genes are related to desiccation tolerance. Comparing the gene expression patterns of *Anabaena* and *Nostoc* sp. HK-01 in response to desiccation, Yoshimura et al. [17] showed that the group 3 sigma factor gene (*sigJ*) regulates the synthesis of extracellular polysaccharide. Although the *Anabaena* transcriptome has been analyzed under conditions of desiccation [15,16], the functions of only a few desiccation-inducible genes have been elucidated.

Genomic analysis facilitates the investigation of gene function. However, the functions of several genes remain unclear because these genes may be expressed under special conditions, such as nitrogen fixation, heat, cold, osmosis, and salinity, or their functions may be complemented by other gene(s). Our previous study identified the nitrogen fixation-related gene *nifH2* as one of the desiccation-induced genes [15]. Given that *Anabaena* fixes nitrogen, desiccation-induced genes may be related to nitrogen fixation or nitrogen assimilation. However, the relationship between desiccation tolerance, and nitrogen fixation and assimilation has not been investigated.

In this study, the functions of desiccation-induced genes in *Anabaena* sp. PCC 7120 were analyzed using the gene disruption technique and an easy desiccation tolerance test in the presence or absence of a nitrogen source to investigate the relationship between desiccation tolerance and nitrogen metabolism (fixation and assimilation). Typical desiccation-induced genes described in Katoh et al. [15] were selected for analysis, along with a gene related to photosynthesis.

2. Materials and methods

2.1. Organism and culture conditions

Nitrogen-fixing *Anabaena* sp. PCC 7120 was grown at 28 °C in BG11 or BG11o medium (NO_3^--free for N_2-fixing condition) [18] with

Abbreviations: Chl, chlorophyll; PCR, polymerase chain reaction; PS II, photosystem II

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5 mM HEPES-NaOH (pH 7.5) at 20 to 40 μmol photons m^{-2} s^{-1} under continuous fluorescent illumination. To assess desiccation tolerance, cells were grown in 2.5 mL of BG11 or BG11o medium in a 12-well plate placed on a high-speed shaking table. Cell density was estimated by measuring optical density at 750 nm (OD_{750}) using a spectrophotometer (model UV-160A; Shimadzu, Kyoto, Japan; OD_{750} 1 = approximately 5×10^7 cells/mL). The concentration of chlorophyll extracted using methanol was measured at 665 nm (A_{665}) using a spectrophotometer and calculated with A_{665} 1 = 13.42 μg Chl/mL [19]. Wild-type *Anabaena* was maintained on BG11 solid medium supplemented with 1.2% (w/v) agar, 5 mM TES-KOH (pH 8.0), and 0.3% (w/v) sodium thiosulfate. *Anabaena* mutants were maintained on the same solid medium as wild-type cells in the presence of antibiotics (10 μg/mL spectinomycin [Sp] and 2 μg/mL streptomycin [Sm]), but were propagated in the absence of antibiotics for analytical experiments.

2.2. Construction of Anabaena gene disruptants

As described in Katoh et al. [8], the genes *all0801*, *all0875*, *alr3090*, *alr3800*, *all4052*, *all4477*, and *alr5182* were selected for gene-inactivated phenotype analysis. The functions of these genes were determined using BLAST homology search [20] and Pfam search and were compared with a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and families [21]. Gene names were designated using CyanoBase (Kazusa DNA Research Institute, http://bacteria.kazusa.or.jp/cyanobase/Anabaena/index.html). To inactivate the targeted genes, 1.2 to 1.3 kb of DNA was amplified with each set of forward and reverse primers containing an *Xho*I (underlined) site, as shown in Table 1. Each *Xho*I-digested PCR-amplified gene was ligated to the cloning vector pRL271. The SpR/SmR cassette was inserted into a selected restriction site in the middle of the PCR-amplified gene to disrupt the gene [22]. The constructed vector was transferred to *Anabaena* sp. PCC 7120 from *E. coli* using a conjugation method, as described previously [23]. The genes were inactivated by *sucB*-mediated positive selection for double recombination [22].

Gene inactivation was confirmed by PCR using the primers shown in Table 2.

2.3. Viability analysis of desiccated Anabaena

Wild-type and gene-disrupted *Anabaena* cells were grown in BG11 or BG11o medium up to the late logarithmic phase. The cells were harvested by centrifugation, and the pellets were suspended in fresh medium to a final volume corresponding to OD_{750} 10, based on the measured absorbance value before cell harvest. The concentrated cells (10 μL) were then spotted on a "mixed cellulose ester" filter paper (pore size, 0.45 μm; Toyo Roshi Co. Ltd., Tokyo, Japan), and desiccated in a Petri dish (90 × 15 mm) under continuous illumination with white fluorescent lamps (20 to 30 μmol photons m^{-2} s^{-1}) at 28 °C and 30% relative humidity for 0, 4, 8.5, and 24 h. In this experiment, the water content was continuously reduced from 100% to 0% in 8.5 h, as described in Katoh et al. [15] and Higo et al. [16]. The resuspended cell cultures were diluted with a chlorophyll base, and an aliquot of each cell culture was spotted on an agar plate containing growth medium supplemented with 0.6% sodium thiosulfate and 5 mM TES-KOH (pH 8.0). The plates were kept illuminated (20 to 30 μmol photons m^{-2} s^{-1}) at 28 °C for 3 days. Changes in viability were calculated based on microscopic determination of colony forming units, and the viability at 0 h was defined as 100%.

Table 1
Primers used for gene-disruption.

Gene name	Forward primer	Reverse primer
all0801	ACTCGAGGATTCGCAACTATCTC	ACTCGAGTATCTCTCGGTGCAAT
all0875	ACTCGAGATGTAACAGACCCTTA	ACTCGAGGTACATGATAACCAGC
alr3090	ACTCGAGTCTGAGTCTTCCTGGT	ACTCGAGCGTGGGAACGATGATA
alr3800	ACTCGAGATGCGGACGATGTTTA	ACTCGAGCCTACGGTAAGACTTC
all4052	ACTCGAGCGCAACAATTGCGAAT	ACTCGAGAGTACGGTACTATTCC
all4477	ACTCGAGGTATTGCCATTCCTAT	ACTCGAGGACAATTCATTTGCAT
alr5182	ACTCGAGCAAACATTACAACCAC	ACTCGAGCAGCAGTTTCAATTCC

Underline shows *Xho*I site.

Table 2
Primers used for segregation analysis.

Gene name	Forward primer	Reverse primer
all0801	GCAAGTGCAGCAGATTCA	TTTCCCCAAATCCGGGTA
all0875	GAAGACGGTACGTATCAA	CCATCATCACCAGCTTCA
alr3090	GGTGGTATTCTCTCCACA	ATATTCTAGCTGGAGCGT
alr3800	AAGGCAGCGCCTTCTAGA	CTTGCTAGGTACATAGAG
all4052	CTACACCAATGGCATGAA	GGAGATCGCTCATCTGTT
all4477	AGAACGTGCCAAGGTTGA	GCTTGCAGCATCCGAATT
alr5182	GATCTGTCGATCCAAGGT	CACCATAGCCACATTGGT

2.4. Microscopic analysis of Anabaena cells

Wild-type *Anabaena* and disruptants were observed under an optical microscope. To observe chlorophyll fluorescence, excitation light at 435 nm was used.

2.5. Measurement of intracellular trehalose and sucrose

Desiccation was carried out as described in Section 2.4. Modifications were as follows. One hundred microliters of cell suspension was spotted on a filter paper and desiccated. Desiccated cells and filters were soaked in 80% ethanol and treated at 65 °C for 3 h. The supernatant was recovered and evaporated to dryness. Samples were dissolved in a small amount of water and treated with trehalase or invertase to determine the trehalose or sucrose content, respectively. The product glucose was enzymatically measured using the Glucose CII-test (Wako Pure Chemicals, Osaka; [16]) and used for estimating the trehalose and sucrose content.

3. Results

3.1. Selected genes and categories

The selected genes and categories are summarized in Table 3 and Fig. E1. The gene expression categories described in Katoh et al. [15] were rearranged as follows: (i) type U1 included immediate upregulated genes, (ii) type U2 included immediate upregulated genes that were downregulated after the middle phase of desiccation, (iii) type U3 included genes upregulated during the middle phase of desiccation, and (iv) type U4 included genes upregulated after the middle phase of

Table 3
Selected genes and their predicted functions.

Gene name	Predicted function	Gene expression pattern
all0801	Photosystem II-associated peripheral protein	–
all0875	Putative alpha-glucanotransferase with an alpha-amylase catalytic domain	U2
alr3090	Hypothetical protein slightly homologous to manganese catalase	U1
alr3800	SigB2 (*sigB2*) and group 2 sigma 70-type sigma factor E, SigE (*sigE*)	U3
all4052	Transketolase, may participate in sugar metabolism	U1
all4477	DnaK-type molecular chaperone DnaK (*dnaK*)	U4
alr5182	Oxidoreductase, which is also homologous to dehydrogenase	U4