Title:

The two-component response regulator OrrA confers dehydration tolerance by regulating *anaKa* expression in the cyanobacterium *Anabaena* sp. strain PCC 7120

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Running title:

Dehydration tolerance in *Anabaena*

Summary

The aquatic cyanobacterium *Anabaena* sp. strain PCC 7120 exhibits dehydration tolerance. The regulation of gene expression in response to dehydration is crucial for the acquisition of dehydration tolerance, but the molecular mechanisms underlying dehydration responses remain unknown. In this study, the functions of the response regulator OrrA in the regulation of salt and dehydration responses were investigated. Disruption of *orrA* abolished or diminished the induction of hundreds of genes in response to salt stress and dehydration. Thus, OrrA is a principal regulator of both stress responses. In particular, OrrA plays a crucial role in dehydration tolerance because an *orrA* disruptant completely lost the ability to regrow after dehydration. Moreover, in the OrrA regulon, *anaKa* encoding a protein of unknown function was revealed to be indispensable for dehydration tolerance. OrrA and AnaK are conserved among the terrestrial cyanobacteria, suggesting their conserved functions in dehydration tolerance in cyanobacteria.

INTRODUCTION

Cyanobacteria comprise a diverse group of bacteria characterized by oxygen-evolving photosynthesis. Their photosynthetic ability enables them to inhabit almost all illuminated environments. The habitat of cyanobacteria is not limited to aquatic ecosystems, but it extends to terrestrial ecosystems including extremely arid environments such as deserts (Pointing and Belnap, 2012). Some cyanobacterial species belonging to the genera *Nostoc* and *Chroococcidiopsis* are present in dry areas, and they exhibit high tolerance to desiccation (Singh, 2018). For example, *Chroococcidiopsis* strains isolated from deserts could form colonies after 4 years of storage in a dry state, and the terrestrial strain *Nostoc commune* was revived via inoculation into medium after storage in a desiccated state for approximately 90 years (Lipman, 1941; Fagliarone *et al.*, 2017).

Loss of water from cells induces various stresses and damages various cellular components including proteins, membrane lipids, and DNA (Potts, 2001; Singh, 2018).

Desiccation-tolerant cyanobacteria adopt multiple strategies to survive harsh environments during desiccation. *N. commune* cells are embedded in extracellular polysaccharide (EPS), which protects cells from desiccation (Tamaru *et al.*, 2005). In the aquatic cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena*), EPS excretion was increased by overexpression of *sigJ*, which encodes the sigma factor of RNA polymerase, and the mutant displays higher dehydration tolerance than the wild-type strain (WT) (Yoshimura *et al.*, 2007). The accumulation of compatible solutes such as trehalose and sucrose, which stabilize the proteins and cellular membranes of dehydrated cells (Tapia and Koshland, 2014), represents another strategy of desiccation tolerance. *N. commune* and a *Chroococcidiopsis* strain accumulate trehalose and sucrose in response to dehydration, salt and osmotic stress (Hershkovitz *et al.*, 1991; Sakamoto *et al.*, 2009). *Anabaena* also utilizes both sugars as compatible solutes, and disruption of trehalose synthesis genes decreases dehydration tolerance (Higo *et al.*, 2006). Moreover, *Escherichia coli* expressing the sucrose synthesis gene *spsA* from the cyanobacterium *Synechocystis* sp. strain PCC 6803 (*Synechocystis* PCC 6803) accumulates sucrose within cells and exhibits a drastic increase in survival rate after dehydration (Billi *et al.*, 2002).

Oxidative damage caused by reactive oxygen species (ROS) is one of the most deleterious effects of dehydration (França *et al.*, 2007). ROS generation is increased by dehydration, resulting in lipid peroxidation, denaturation of proteins through oxidative modifications, and DNA double-strand breakage. Antioxidant defense systems that suppress ROS generation and scavenge generated ROS protect cellular components from oxidation (Singh, 2018). Because most antioxidant defense systems are proteinous, avoidance of protein oxidation is a key factor for desiccation tolerance (Daly, 2009). Desiccation-resistant bacteria display lower oxidative modification of proteins than desiccation-sensitive bacteria during dehydration (Fredrickson *et al.*, 2008). *Chroococcidiopsis* sp. strain CCMEE 029 is highly tolerant to desiccation, and it does not undergo protein oxidation after 1 year of incubation under dry conditions. Contrarily, *Synechocystis* PCC 6803 exhibits substantial protein oxidation, and it is

incapable of surviving under the same conditions (Fagliarone *et al.*, 2017).

Anabaena is a model organism of bacterial cellular differentiation (Flores *et al.*, 2019). It has been also used to study the molecular mechanisms of responses to stresses including dehydration (Ohmori *et al.*, 2001; Wang *et al.*, 2002). Close relatives of *Anabaena* such as the terrestrial strain *Nostoc* sp. HK-01 exhibit high desiccation tolerance, and *Anabaena* itself displays moderate dehydration tolerance (Katoh *et al.*, 2003; Singh *et al.*, 2013). Because genetic engineering cannot be performed in terrestrial *Nostoc* strains, *Anabaena* serves as a model organism for investigating the molecular mechanisms of desiccation tolerance in the terrestrial strains (Yoshimura *et al.*, 2007; Xu *et al.*, 2020). In *Anabaena*, *orrA*, which encodes a response regulator of the bacterial two-component regulatory system, was identified as a regulator of *lti2* in response to salt and osmotic stresses (Schwartz *et al.*, 1998). We previously indicated that OrrA regulates the expression of *spsA*, *susA,* and *susB*, which participate sucrose synthesis, in response to salt stress (Ehira *et al.*, 2014). Sucrose accumulation under salt stress conditions is lowered by the *orrA* disruption, and intracellular sucrose levels are increased by *orrA* overexpression (Ehira *et al.*, 2014). In this study, we analyzed the global effects of *orrA* disruption on gene expression, observing that OrrA is a master regulator of dehydration and salt stress responses in *Anabaena*. Moreover, among hundreds of genes regulated by OrrA, *anaKa*, which encodes a protein of unknown function, proved essential for dehydration tolerance in *Anabaena*.

RESULTS

OrrA is a major regulator of salt stress response

Sucrose synthesis under salt stress conditions is regulated by OrrA in *Anabaena* (Ehira *et al.*, 2014). The functions of OrrA in global salt stress response regulation were investigated by transcriptome analysis. The gene expression profiles of WT and the *orrA* mutant strain DRorrAS were compared between cells subjected to salt stress (50 mM NaCl) for 3 h and cells before stress treatment. The transcript levels of 298 genes were increased at least twofold by salt stress in WT,

while those of another 1,358 genes were decreased (Table S1). Three genes involved in sucrose metabolism, namely *spsA*, *susA* and *susB*, were upregulated by salt stress as previously reported (Ehira *et al.*, 2014). The upregulated genes included *mth* encoding an enzyme for trehalose synthesis (Higo *et al.*, 2006) and *anaK* genes (all4050, all4051, and all5315), which are predominantly expressed in cyanobacterial resting cells called akinetes in *Anabaena variabilis* (Zhou and Wolk, 2002). Genes encoding proteins that prevent ROS generation, such as *hli/scp* genes (asl0514, asl0873, asr3042, and asr3043) and *dps* genes (all0458 and all1173), and genes that detoxify ROS, such as *katA* and *katB*, were also upregulated (Howe *et al.*, 2018; Tibiletti *et al.*, 2018). The downregulated genes included genes involved in photosynthesis (*psa* and *psb* genes) and carbon fixation (*rbcLXS*, *prk*, *ccmNMLK*, *ecaA*, and *cmpABCD*), and genes encoding components of the phycobilisome (*apc*, *cpc*, and *pec* genes). Moreover, the transcript levels of genes involved in transcription and translation (*rpl* and *rps* genes, *rpoA*, *rpoC1*, and *rpoC2*), and ATP synthesis (*atp* genes) were decreased. The suppression of cell growth under salt stress conditions leads to downregulation of these genes (Ehira *et al.*, 2014).

In DRorrAS, the numbers of genes upregulated and downregulated by salt stress were decreased to 71 and 51, respectively (Table S1). Meanwhile, 37 genes were upregulated in both WT and DRorrAS (Fig. 1), but the degree of upregulation was higher in WT than in DRorrAS. Temporal changes in the expression of salt-induced genes were examined by quantitative reverse transcription (qRT)-PCR (Fig. 2). In WT, *mth* expression was induced within 30 min after NaCl addition, with the *mth* transcript level increasing approximately 800-fold after 60 min, but its induction was completely abolished in DRorrAS (Fig. 2A). The induction patterns of two *dps* genes (all0458 and all1173) differed from each other, but both genes lost salt-responsive induction by *orrA* disruption (Fig. 2B). There are four paralogs of *anaK* (*anaKa*, *anaKb*, *anaKc*, and *anaKd*) in *Anabaena* (Zhou and Wolk, 2002). All *anaK* genes were induced by salt stress, which depended on *orrA* (Fig. 2C). These results indicate that OrrA is a major regulator of salt stress response in *Anabaena*.

127

128 orrA *is necessary for dehydration tolerance*

129 OrrA regulates the expression of most salt-responsive genes, whereas its disruption does not
130 affect growth under salt stress conditions (Ehira *et al.*, 2014). Expression of *orrA* is induced by
131 not only salt stress, but also dehydration (Higo *et al.*, 2006). Moreover, many genes upregulated
132 by salt stress, such as *mth, hli/scp* genes, and *dps* genes, are also upregulated by dehydration
133 (Higo *et al.*, 2006). To investigate the effect of the *orrA* disruption on gene expression in response
134 to dehydration, gene expression profiles were compared between WT and DRorrAS after 3 h of
135 dehydration. In WT, 493 genes were upregulated by dehydration, while 1,307 genes were
136 downregulated (Table S1). Among the upregulated genes in WT, 156 genes were also upregulated
137 in DRorrAS, but the extent of upregulation was significantly lower in DRorrAS than in WT (Fig.
138 1 and Table S1). Salt stress and dehydration increased the expression of the same 239 genes, only
139 23 of which were upregulated by both stresses in DRorrS (Fig. 1). *spsA*, *susA*, and *susB*, which are
140 involved in sucrose metabolism, were also induced by dehydration under the control of OrrA
141 (Table S1). The intracellular sucrose level in WT was increased more than 80-fold after 9 h of
142 dehydration, whereas the level was twofold lower in DRorrAS than in WT (Table 1). Thus, OrrA
143 plays a principal role in regulating gene expression during dehydration in *Anabaena*.

144 To investigate the physiological role of OrrA in dehydration tolerance, growth ability
145 after dehydration was analyzed. *Anabaena* filaments grown in the liquid medium were collected
146 by filtration, and then filaments on the filters were dried in an incubator. After the indicated times,
147 filaments were resuspended in liquid medium and incubated for 3 days. The weight of the filters
148 decreased during the first 8 h of incubation before reaching a plateau, indicating that all water had
149 evaporated (Fig. 3A). In WT, filaments were capable of restarting growth even after 24 h of
150 incubation on the filters (Fig. 3B). DRorrAS filaments were able to regrow after 6 h of incubation
151 on the filters, but their growth was severely impaired after 7 and 8 h of incubation (Fig. 3B).
152 Moreover, DRorrAS completely lost its ability to grow after more than 10 h of incubation on the

153 filters. Thus, *orrA* is necessary for survival under dehydration conditions.

154

155 orrA *is also involved in cold acclimation*

156 OrrA was originally identified as a regulator of the salt and osmolyte responses of *lti2* (Schwartz
157 *et al.*, 1998). In this study, we confirmed the OrrA-regulated *lti2* expression in response to salt and
158 dehydration (Table S1). *lti2* is also known to be upregulated under low temperatures (Ehira *et al.*,
159 2005). In addition, low temperature-responsive genes, such as alr0169, alr0803, alr0804, and
160 alr1819, were under the control of OrrA (Table S1). Hence, we analyzed the effects of *orrA*
161 disruption on cold acclimation. *orrA* expression increased within 30 min after the temperature
162 was decreased from 30℃ to 22℃ (Fig. 4A). Growth of DRorrAS at 30˚C is comparable to that of
163 WT (Ehira *et al.*, 2014), but its growth at 22˚C was slower than that of WT (Fig. 4B). Thus, OrrA
164 also influences cold accilimation.

165

166 anaKa *is a determinant of dehydration tolerance*

167 To further reveal the mechanisms of dehydration tolerance, the involvement of genes regulated by
168 OrrA in dehydration tolerance was investigated. We focused on *anaK* because this gene is highly
169 expressed in akinetes (Zhou and Wolk, 2002), which are cells that are extremely resistant to
170 stresses such as cold and desiccation (Adams and Duggan, 1999). Four *anaK* paralogs, namely
171 *anaKa, anaKb, anaKc*, and *anaKd*, were induced by dehydration ,and this induction depended on
172 OrrA (Table S1). Each *anaK* paralog was inactivated, and the growth of the disruptants after
173 dehydration was determined (Fig. 5). The *anaKa* disruptant DRanaKaK could not grow after
174 incubation on the filters for 24 h, whereas the other *anaK* disruptants retained similar growth
175 ability as WT (Fig. 5A). Time-course experiments illustrated that DRanaKaK lost growth ability
176 at 8 h (Fig. 5B), when water had completely evaporated from filters (Fig. 3A). This indicated that
177 *anaKa* disruption has a fatal effect on survival under dehydration.

178 Disruption of *anaKa* alone resulted in the loss of dehydration tolerance. To examine

the involvement of other genes regulated by OrrA in dehydration tolerance, damage of DRorrAS and DRanaKaK cell after dehydration was evaluated by quantifying phycocyanin excretion from cells (Fig. 6). Phycocyanin is a water-soluble pigment protein and is released extracellularly upon cell lysis (Arii *et al.*, 2015). In WT, phycocyanin excretion was not enhanced by dehydration. In DRorrAS and DRanaKaK, excreted phycocyanin levels were comparable to WT levels before dehydration, but phycocyanin excretion was increased by dehydration. Phycocyanin excretion was more than twofold higher in DRorrAS than DRanaKaK, indicating greater cell membrane damage in DRorrAS. Thus, the *orrA* disruption is more detrimental than the *anaKa* disruption.

DISCUSSION

In this study, we revealed that the two-component response regulator OrrA played principal roles in the regulation of dehydration response and tolerance in *Anabaena*. *orrA* disruption abolished or diminished the induction of approximately 400 genes by dehydration (Fig.1 and Table S1). As previously indicated in response to salt stress (Ehira *et al.*, 2014), OrrA regulated sucrose synthesis under dehydration conditions (Table 1). In addition, the trehalose synthesis genes *mth* and *mts*, which encode maltooligosyl trehalose hydrolase and maltooligosyl trehalose synthase, respectively, were regulated by OrrA (Table S1). Because disruption of *mth* and *mts* results in decreased dehydration tolerance (Higo *et al.*, 2006), the depression of compatible solutes synthesis partly accounts for the susceptibility of the *orrA* disruptant to dehydration. In the OrrA regulon identified this study, *anaKa* was indispensable for dehydration tolerance in *Anabaena* (Fig. 5). *anaKa* encodes a protein of unknown function with a PRC-barrel domain at its N-terminus and a DUF2382 domain at its C-terminus. Although *anaKb*, *anaKc*, and *anaKd* were also induced by dehydration in an OrrA-dependent manner, their disruption did not affect dehydration tolerance. *anaKa* was more strongly induced than the other examined *anaK* genes (Fig. 2, Table S1). Differences in expression levels of AnaK proteins could explain the different phenotypes among the *anaK* mutants. OrrA and AnaK homologs are present in the genome of the

terrestrial cyanobacteria, such as *Nostoc* sp. HK-01, *N. flagelliforme*, and *Chroococcidiopsis thermalis* PCC 7203. OrrA and AnaK homologs are highly expressed in desiccated samples in *N. flagelliforme* (Shang *et al.*, 2019), and an AnaK homolog is induced by dehydration in *Nostoc* sp. HK-01 (Yoshimura *et al.*, 2006), suggesting their conserved functions in desiccation tolerance.

The function of the PRC-barrel domain is unknown. It is found in the H subunit of photosystem reaction center (PRC) of the purple bacteria (Anantharaman and Aravind, 2002). The H subunit of PRC is necessary for functional PRC assembly, and it regulates electron transfer in PRC (Takahashi and Wraight, 1996; Lupo and Ghosh, 2004). The PRC-barrel domain is also found at the C-terminus of RimM, which is involved in RNA processing and ribosome assembly (Anantharaman and Aravind, 2002). The PRC-barrel domain of AnaKa is phylogenetically most closely related to that of the H subunit of PRC (Anantharaman and Aravind, 2002). AnaKa homologs are present in the highly radiation- and desiccation-resistant bacterium *Deinococcus radiodurans* and some species of the genus *Psychrobacter*, which are psychro- and osmo-tolerant bacteria (Kim *et al.*, 2012). In *D. radiodurans*, the expression of the *anaKa* homolog DR1314 is upregulated by heat shock, and its induction is regulated by the extracytoplasmic function sigma factor Sig1 (Schmid *et al.*, 2005). DR1314 is also regulated by the response regulator DrRRA, which is essential for radioresistance in *D. radiodurans* (Wang *et al.*, 2016). The DR1314 disruptant is sensitive to hydrogen peroxide (H_2O_2) exposure and heat shock (Schmid *et al.*, 2005; Wang *et al.*, 2016). Thus, AnaK homologs play important roles in the stress resistance of extremophiles.

It was also indicated that OrrA regulates ROS defense systems (Table 2). ROS, which include singlet oxygen ($^1O_2$), superoxide anion (O_2^-), H_2O_2, and hydroxyl radical (•OH), is the main damaging factor during dehydration (Singh, 2018). O_2^-, which is generated by the reduction of oxygen through photosynthetic electron transport, is detoxified by superoxide dismutase (SOD), which dismutates it into H_2O_2 and O_2 with. In *Anabaena*, *sodA* and *sodB* both encode SOD (Li *et al.*, 2002), but their expression was not upregulated by dehydration. H_2O_2 is removed

by catalases and peroxiredoxin (Prxs), which convert it into water. The catalase genes *katA* and *katB* were induced by dehydration; however, catalase activity is low in *Anabaena*, and Prxs play a more prominent role in H_2O_2 removal (Pascual *et al.*, 2010). Although alr4641 (encoding 2-Cys-Prx), the overexpression of which protects *Anabaena* cells from oxidative stress (Banerjee *et al.*, 2015), is induced by O_2^- and H_2O_2 (Hurtado-Gallego *et al.*, 2019), its expression was altered by dehydration. •OH is formed by the reaction of Fe^{2+} and H_2O_2, and Dps proteins sequestrate iron to prevent •OH formation (Howe *et al.*, 2018). *Anabaena* carries four *dps* genes (all0458, all1173, alr3808, and all4145), and all0458 and all1173 were induced by dehydration. Thus, some H_2O_2-detoxifying enzymes were upregulated during dehydration, but H_2O_2 generation during dehydration is obscure because H_2O_2-responsive genes such as alr4641 and *trxA2* were not induced (Ehira and Ohmori, 2012; Hurtado-Gallego *et al.*, 2019). Conversely, many genes encoding proteins involved in defense against $^1O_2$ were induced by dehydration (Table 2). Excited chlorophylls can transfer excitation energy to oxygen, resulting in $^1O_2$ formation (Latifi *et al.*, 2009). High-light-inducible proteins, which are encoded by the *hli/scp* genes, prevent $^1O_2$ formation by consuming the excess of PSII-generated electrons (Sinha *et al.*, 2012). Orange carotenoid protein (OCP) is an efficient quencher of $^1O_2$ (Sedoud *et al.*, 2014). Recent findings indicated that All3221 and Alr4783 proteins of *Anabaena*, which are homologous to the N-terminal domain (NTD) of OCP, are effective quenchers of $^1O_2$ (López-Igual *et al.*, 2016). Moreover, in *N. flagelliforme*, a remarkable desiccation-tolerant terrestrial cyanobacterium, NTD-OCP proteins accumulated in cells collected from dried fields, suggesting its involvement in desiccation tolerance (Yang *et al.*, 2019). AstaP, another OCP, was originally found in a green alga isolated from the dried surface of heated asphalt in midsummer, and it also displays $^1O_2$ quenching activity (Kawasaki *et al.*, 2013). *Anabaena* carries four genes encoding AstaP homologs (alr1819, all4647, all4894, and all5264), all of wchich were induced by dehydration. It is suggested that *N. commune* deactivates photosynthetic machinery in response to dehydration to protect photosystems from $^1O_2$ generated through the imbalance between chlorophyll excitation

and photosynthetic electron transport (Hirai *et al.*, 2004). Thus, defense systems against $^1O_2$ are especially important in dehydration tolerance of *Anabaena*.

The OrrA regulon defined in this study contains a huge number of genes. A transcriptional network is likely to be involved in the dehydration response, because eight genes encoding transcriptional regulators, including *sigB2* (Ehira *et al.*, 2014), were upregulated by dehydration under the control of OrrA (Table S1). In addition, the expression of more than 10 genes encoding signal transduction proteins, such as sensor histidine kinase (Hik) and Ser/The kinases, increased in an OrrA-dependent manner. These regulators constitute a regulatory network of the dehydration response of *Anabaena*.

OrrA was identified as a master regulator of the response to salt stress and dehydration and was involved in the response to low temperature (Figs. 1 and 4). OrrA was originally identified as the regulator of *lti2* in response to salt and osmotic response (Schwartz *et al.*, 1998), implying that OrrA also plays an important role in the osmotic stress response. Evaporation of water from the medium surrounding cells increases external solute concentrations, resulting in salt and osmotic stresses. Therefore, a common signaling pathway could activate OrrA in response to dehydration, salt, and osmotic stresses. Because OrrA is a response regulator, its activity is regulated through the phosphorylation by a Hik. Three Hiks are located upstream of *orrA* in the genome of *Anabaena* (Ehira *et al.*, 2014). These Hiks are involved in dehydration response, but none of them is a cognate Hik for OrrA in *Anabaena* (K. Shimonaka & S. Ehira, unpublished data). Identification of a cognate Hik for OrrA would reveal the mechanisms by which dehydration signals are sensed.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions– *Anabaena* sp. strain PCC 7120 and its derivatives were grown at 30˚C under continuous illumination provided by a fluorescent lamp at 30 μE m^{-2} s^{-1} in the nitrogen-free modified Detmer's medium (MDM_0) (Watanabe, 1960). Liquid cultures were

bubbled with air containing 1% (v/v) CO_2. Salt stress conditions were generated by adding 50 mM NaCl to cultures in the late-logarithmic phase (optical density at 750 nm [OD_{750}] = 0.5-0.7). Dehydration stress was imposed on *Anabaena* cells as described previously (Higo *et al.*, 2006). A 25-ml portion of cultures in the late-logarithmic phase was filtered through 47-mm, 0.45-µm pore size mixed cellulose ester filters (Tokyo Roshi Kaisha, Tokyo, Japan) and dried for the indicated times at 30˚C under continuous illumination provided by a fluorescent lamp at 10 µE m^{-2} s^{-1} in a Petri dish.

Mutant construction– All primers used in this study (Table S2) were designed based on genome data from CyanoBase (Fujisawa *et al.*, 2017). DRorrAS was used as an *orrA* disruptant in this study (Ehira *et al.*, 2014). DRanaKaK, DRanaKbK, DRanaKcS, and DRanaKdS, in which *anaKa* (*all4050*), *anaKb* (*all5315*), *anaKc* (*all4051*), and *anaKd* (*alr5332*) , respectively, were disrupted, were constructed by replacing each gene with an antibiotic-resistant gene in the same manner as performed for DRorrAS using primer pairs specific for each gene (Ehira *et al.*, 2014).

DNA microarray analysis– Total RNA was extracted from whole filaments according to Pinto et al. (Pinto *et al.*, 2009) and was treated with DNase I (Takara Bio, Shiga, Japan). Global gene expression was analyzed using the *Anabaena* oligonucleotide microarray as described previously (Ehira and Ohmori, 2006). Microarray analyses were conducted using three sets of RNA samples isolated from independently grown cultures. Two hybridization reactions were performed with different combination of Cy-dyes for each set of RNA samples. Thus, six replicates were available per gene to determine changes in gene expression. Genes with at least twofold differences in transcript levels ($P < 0.05$; Student's *t*-test) were identified. The microarray data were deposited in the KEGG Expression Database (accession numbers ex0001979 to ex0002002).

qRT-PCR– cDNAs were synthesized from 1 µg of total RNA with random hexamer primers using a PrimeScript first strand cDNA synthesis kit (Takara Bio). qRT-PCR was performed using the Thermal Cycler Dice Real Time System II (TP900; Takara Bio) in a 20-µl reaction mixture

containing 10 µl of THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan), 0.2 µM each of gene-specific forward and reverse primers, and cDNA. Relative transcript levels were normalized to the value for 16S rRNA and represented as means of duplicate measurements. Experiments were repeated at least three times.

Dehydration tolerance assay– Cells dehydrated for the indicated times on filter paper were suspended in MDM_0. The suspended cultures were diluted with MDM_0 to OD_{750} of 0.1 and incubated at 30˚C at 10 µE m^{-2} s^{-1}. After 72 h, OD_{750} of the cultures was measured.

Determination of sucrose contents– The low-molecular-mass compounds of cells dehydrated for the indicated time were extracted with 80% ethanol for 3 h at 65˚C (Higo *et al.*, 2006). After centrifugation, supernatants were vacuum-dried, and the residue was dissolved in 0.5 ml of water. The samples were treated with 3 U of invertase (Sigma) in 50 mM sodium acetate (pH 4.5) for 2 h at 37˚C, and the concentration of released glucose was determined using a Glucose Assay Kit (Biovision). The chlorophyll *a* content of cultures was determined as described previously (Mackinney, 1941).

Quantification of excreted phycobiliproteins– Dehydrated cells were resuspended in MDM_0 and statically incubated for 5 min at room temperature. Cells were collected by centrifugation, and the absorbance of the supernatants at 620 nm was measured. A_{620} was normalized by OD_{750} measured prior to centrifugation.

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Author contributions

M. Ohmori and S. Ehira conceived the study. S. Kimura and S. Ehira designed the experiments. S.

Kimura, M. Sato, X. Fan, and S. Ehira conducted the experiments and analyzed the data. S. Ehira wrote the manuscript, and all authors edited and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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488 Table 1. Sucrose contents after dehydration

	Sucrose (μmol/ mg chl*a*)	
Strain	0 h	9 h
WT	0.06 ± 0.06	5.00 ± 0.39
DRorrAS	0.04 ± 0.04	2.26 ± 0.29

489 WT, wild-type *Anabaena* sp. strain PCC 7120; DRorrAS, *orrA* mutant *Anabaena* sp. strain PCC

490 7120; chl*a*, chlorophyll *a*

491

492

493 Table 2. OrrA-regulated genes that are related to ROS defense

ORF No.	Product
Defense against H_2O_2	
alr0998 (*katA*)	Fe catalase
alr3090 (*katB)*	Mn catalase
all0458 (*dps*)	Dps
all1173 (*dps*)	Dps
Defense against $^1O_2$	
asl0514 (*hli/scp*)	High-light-inducible/small CAB-like protein
asl0873 (*hli/scp*)	High-light-inducible/small CAB-like protein
asr3042 (*hli/scp*)	High-light-inducible/small CAB-like protein
asr3043 (*hli/scp*)	High-light-inducible/small CAB-like protein
all3221	Homolog of the N-terminal domain of OCP
alr4783	Homolog of the N-terminal domain of OCP
alr1819	AstaP
all4647	AstaP
all4894	AstaP
all5264	AstaP

494 ROS, reactive oxygen species; ORF, open-reading frame; H_2O_2, hydrogen peroxide; $^1O_2$, singlet

495 oxygen; OCP, orange carotenoid protein

496

497 **Figure legends**

498 **Fig 1.** Venn diagrams of genes upregulated by salt stress and dehydration in wild-type *Anabaena*
499 sp. strain PCC 7120 (WT) and the *orrA* disruptant DRorrAS. The numbers in parenthesis indicate
500 genes upregulated in both WT and DRorrAS.

501

502 **Fig 2.** Temporal changes in the transcript levels of genes upregulated by salt stress. The transcript
503 levels of *mth* (A), *dps* (B), and *anaK* (C) after the addition of 50 mM NaCl were determined by
504 qRT-PCR in WT (black circles) and the *orrA* disruptant (white circles). The transcript levels were
505 determined in duplicated measurements using three independently grown cultures. The transcript
506 level at 0 min in WT was taken as 1. Data are presented as the mean ± SD.

507

508 **Fig 3.** Dehydration tolerance of the *orrA* disruptant.
509 A. Changes in weight of filters during 24h of incubation. Filaments of WT (black circles) and the
510 *orrA* disruptant (white circles) were collected on filters and the filters dried for 24 h. The weight
511 of each filter was measured during the 24-h incubation and presented as a ratio to the control (0 h).
512 B. Growth ability of the *orrA* disruptant after dehydration. Filaments on the filters were
513 resuspended in liquid medium at the indicated time, the optical density at 750 nm (OD_{750}) of the
514 suspension was adjusted to 0.1, and then filaments were incubated for 3 days in liquid medium.
515 OD_{750} after the 3-day incubation was indicated for WT (black circles) and the *orrA* disruptant
516 (white circles). The experiments were repeated using three independently grown cultures. Data
517 are presented as the mean ± SD.

518

519 **Fig 4.** Involvement of OrrA in cold acclimation.
520 A. Changes in the *orrA* transcript levels after a shift to low temperature (22˚C). The transcript
521 level at 0 h was taken as 1.
522 B. Growth of WT (black circles) and the *orrA* disruptant (white circles) at 22˚C. Growth was

523 monitored by measuring OD_{750}. The experiments were repeated with three independently grown
524 cultures. Data are presented as the mean ± SD.
525
526 **Fig 5.** Dehydration tolerance of disruptants of the *anaK* genes. Filaments of disruptants of *anaKa*
527 (ΔKa), *anaKb* (ΔKb), *anaKc* (ΔKc), *anaKd* (ΔKd), and *orrA* (ΔorrA) were subjected to
528 dehydration for 0 (white bars) or 24 h (gray bars), and then their growth ability was evaluated as
529 described in Fig 3 (A). Temporal changes of the growth ability of WT (black circles) and the
530 *anaKa* disruptant (white circles) during 24 h of incubation were determined (B). The experiments
531 were repeated using three independently grown cultures. Data are presented as the mean ± SD.
532
533 **Fig 6.** Excretion of phycocyanin after dehydration. Phycocyanin excreted in the medium from the
534 cells of WT (black bars), the *orrA* disruptant (gray bars), and the *anaKa* disruptant (white bars)
535 that were dehydrated on filters for 0 or 24 h was determined by measuring the absorbance at 620
536 nm.
537

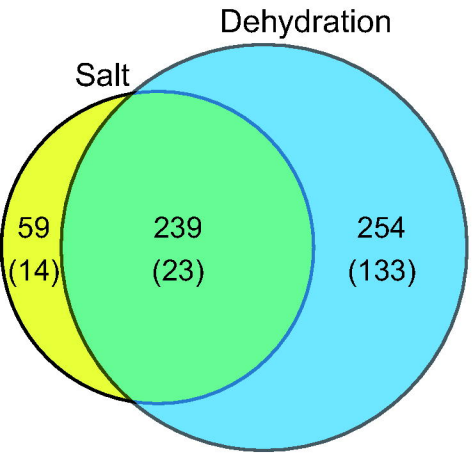

Fig. 1

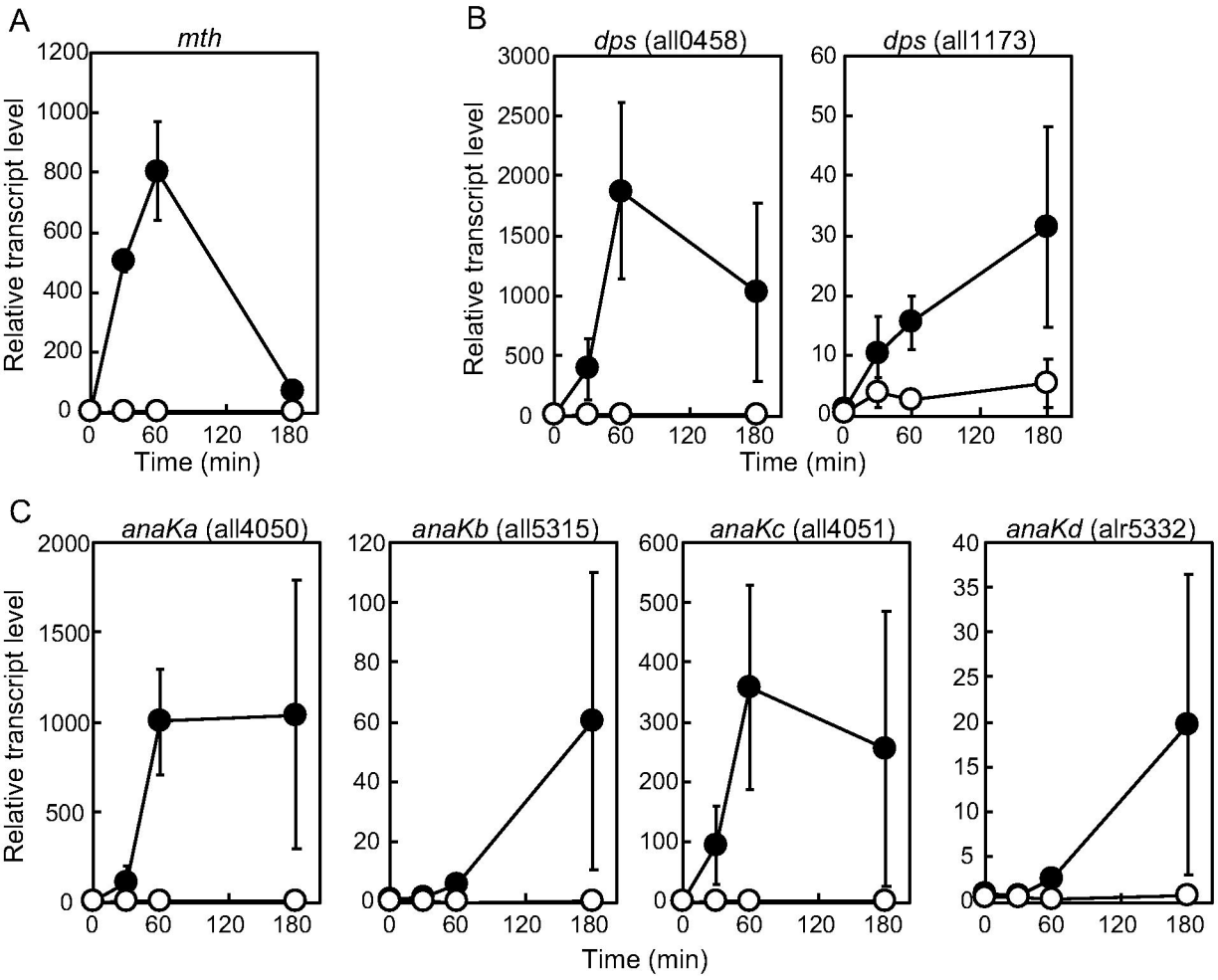
A
mth
Relative transcript level
1200
1000
800
600
400
200
0
0
60
120
180
Time (min)
B
dps (all0458)
3000
2500
2000
1500
1000
500
0
dps (all1173)
60
50
40
30
20
10
0
C
anaKa (all4050)
2000
1500
1000
500
0
anaKb (all5315)
120
100
80
60
40
20
0
anaKc (all4051)
600
500
400
300
200
100
0
anaKd (alr5332)
40
35
30
25
20
15
10
5
0

Fig. 2

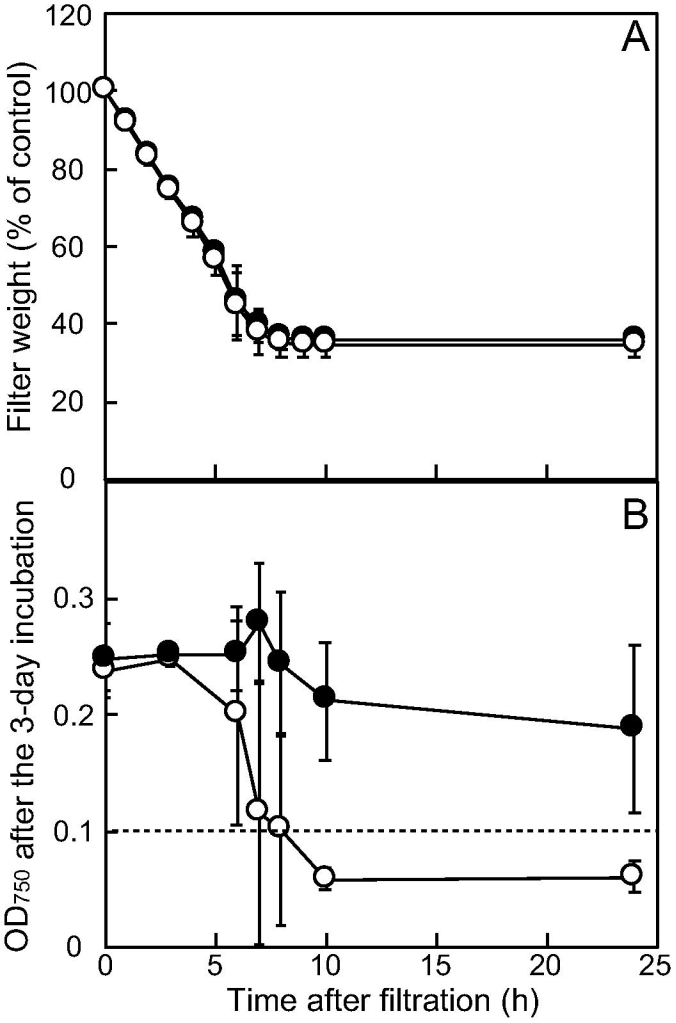
A
B
Filter weight (% of control)
OD750 after the 3-day incubation
Time after filtration (h)
120
100
80
60
40
20
0
0.3
0.2
0.1
0
0
5
10
15
20
25

Fig. 3

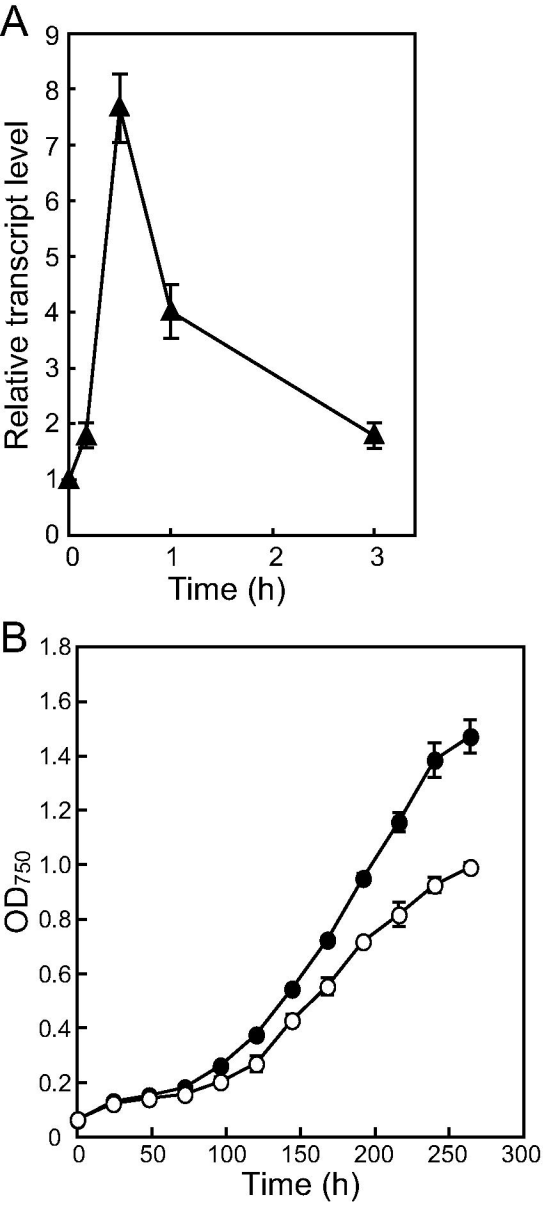
A
Relative transcript level
0
1
2
3
4
5
6
7
8
9
0
1
2
3
Time (h)
B
OD750
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0
50
100
150
200
250
300
Time (h)

Fig. 4

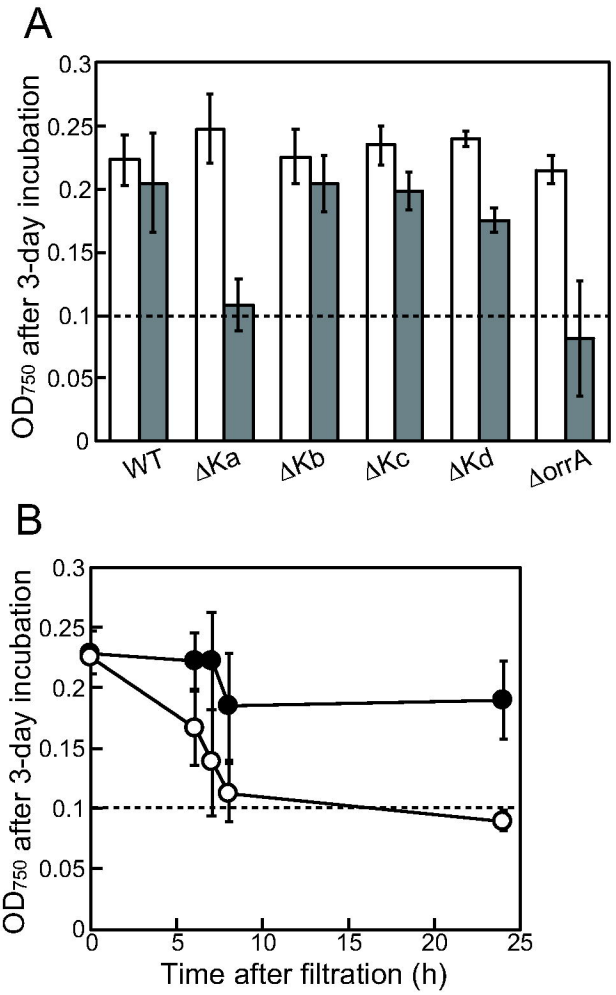
A
0.3
0.25
0.2
0.15
0.1
0.05
0
OD750 after 3-day incubation
WT
ΔKa
ΔKb
ΔKc
ΔKd
ΔorrA
B
0.3
0.25
0.2
0.15
0.1
0.05
0
OD750 after 3-day incubation
0
5
10
15
20
25
Time after filtration (h)

Fig. 5

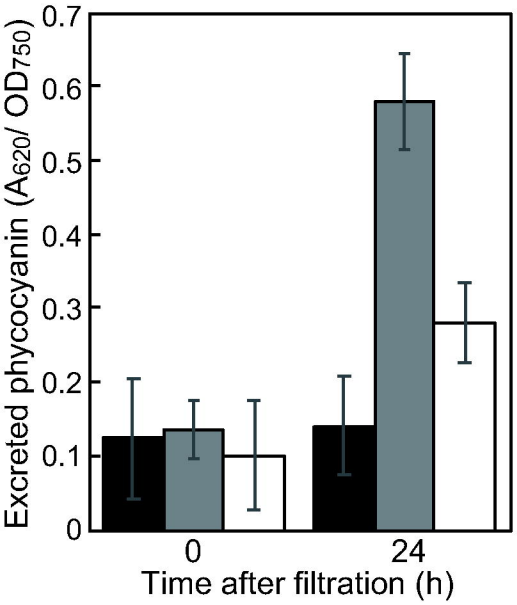
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Excreted phycocyanin (A620/ OD750)
0
24
Time after filtration (h)

Fig. 6