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Nitrate Assimilatory Genes and Their Transcriptional Regulation in a Unicellular Red Alga *Cyanidioschyzon merolae*: Genetic Evidence for Nitrite Reduction by a Sulfite Reductase-Like Enzyme

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***Cyanidioschyzon merolae* is a unicellular red alga living in acid hot springs, which is able to grow on ammonium, as well as nitrate as sole nitrogen source. Based on the complete genome sequence, proteins for nitrate utilization, nitrate transporter (NRT) and nitrate reductase (NR), were predicted to be encoded by the neighboring nuclear genes *CMG018C* and *CMG019C*, respectively, but no typical nitrite reductase (NiR) gene was found by similarity searches. On the other hand, two candidate genes for sulfite reductase (SiR) were found, one of which (*CMG021C*) is located next to the above-noted nitrate-related genes. Given that transcripts of *CMG018C*, *CMG019C* and *CMG021C* accumulate in nitrate-containing media, but are repressed by ammonium, and that SiR and NiR are structurally related enzymes, we hypothesized that the *CMG021C* gene product functions as an NiR in *C. merolae*. To test this hypothesis, we developed a method for targeted gene disruption in *C. merolae*. In support of our hypothesis, we found that a *CMG021G* null mutant in comparison with the parental strain showed decreased cell growth in nitrate-containing but not in ammonium-containing media. Furthermore, expression of *CMG021C* in the *nirA* mutant of a cyanobacterium, *Leptolyngbya boryana* (formerly *Plectonema boryanum*), could genetically complement the NiR defect. Immunofluorescent analysis indicated the localization of CMG021C in chloroplasts, and hence we propose an overall scheme for nitrate assimilation in *C. merolae*.**

Keywords: *Cyanidioschyzon merolae* • Nitrate assimilation • Nitrite reductase • Nitrogen regulation • Red alga • Sulfite reductase.

Abbreviations: GOGAT, glutamine:2-oxoglutarate amido-transferase; GS, glutamine synthase; HA, hemagglutinin; MA medium, modified Allen's medium; NCR, nitrogen catabolite repression; NiR, nitrite reductase; NR, nitrate reductase; NRT, nitrate/nitrite transporter; ORF, open reading frame; PEG, polyethylene glycol; SiR, sulfite reductase.

Introduction

Cyanidioschyzon merolae is a unicellular red alga living in acid hot springs (pH 1–3, 40–50°C), with each cell containing only one mitochondrion, one chloroplast and one nucleus. Recently, the complete genome sequences of these three organelles were determined (Ohta et al. 1998, Ohta et al. 2003, Matsuzaki et al. 2004, Nozaki et al. 2007), and the extremely simple and minimally redundant gene content was uncovered. Taking advantage of these biological characteristics, we have been developing various tools for biological analysis in *C. merolae* (Minoda et al. 2004, Imamura et al. 2008, Ohnuma et al. 2008,

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Imamura et al. 2009, Ohnuma et al. 2009, Kobayashi et al. 2009). These studies have helped to make this organism a model plant cell for studying fundamental cellular processes, such as metabolic regulation and interaction among organelles.

Nitrogen is an essential and major component of every cell, and autotrophic organisms such as plants usually utilize ammonium or nitrate as the nitrogen source. Since the nitrogen assimilation is predominantly performed by the glutamine synthetase (GS)–glutamate synthase (GOGAT) cycle, ammonium ions are the obvious substrates for the assimilatory reaction (Suzuki and Knaff 2005). However, environmental ammonium is oxidized to nitrate by nitrifying bacteria and other abiotic processes, and thus plants need to reduce nitrate back to ammonium prior to the GS reaction. In higher plant and green algal cells, nitrate anions are imported into the cell by specific transporters (Galvan and Fernández 2001), and reduced to nitrite by NAD(P)H-dependent nitrate reductase (NR) in the cytosol. Nitrite is subsequently reduced to ammonium by ferredoxin-dependent nitrite reductase (NiR) in plastids. GS is usually found in the cytosol (GS1) as well as in plastids (GS2), and the cytosolic enzyme is composed of isozymes encoded by multiple genes (Crawford 1995, Lam et al. 1996). As an intriguing exception, localization of GS in mitochondria was recently found in Arabidopsis (Taira et al. 2004).

While, compared with other nutrients, little information is available on the regulatory mechanisms controlling the expression of genes involved in plant nitrogen metabolism, we recently identified a transcription factor, R2R3-type MYB transcription factor CmMYB1, which functions as a central nitrogen assimilation regulator in *C. merolae* (Imamura et al. 2009). Under nitrogen deprivation, *CmMYB1* transcripts increase first, and subsequently CmMYB1 protein accumulates in the nucleus and binds promoter regions of key nitrogen assimilation genes, such as *NRT*, *NR* and *GS*. Consequently, expression of these nitrogen-responsive genes is induced to acclimate to nitrogen-limited environments. In contrast, these nitrogen-responsive genes are repressed in media containing glutamine or ammonium but not glutamate or nitrate. Therefore, it is suggested that CmMYB1 mediates the nitrogen catabolite repression (NCR)-sensitive transcription of nitrogen assimilation genes and *C. merolae* cells sense glutamine or ammonium as the nitrogen signal.

In a series of studies dealing with the nitrogen assimilation process in *C. merolae*, we found a unique nuclear gene (*CMI233C*, gene number in http://merolae.biol.s.u-tokyo.ac.jp/) that encodes GS and localized the gene product to the cytosol (Terashita et al. 2006). Thus, ammonium ions imported from the environment should be assimilated into glutamine in the cytosol. However, the assimilatory pathway of nitrate is not well understood in *C. merolae*, although it can grow in media containing nitrate as the sole nitrogen source. Recently, in vitro biochemical assays using a recombinant protein suggested that a putative sulfite reductase [*CmNiR* (*CmSiRB*) see Results] has potentially a nitrite-reducing activity; however, its function in the nitrate assimilatory pathway in the cells has not been demonstrated (Sekine et al. 2009). In this study, we characterized the nitrate assimilatory pathway and regulation of the related genes using a newly developed gene disruption method for *C. merolae*. Based on our in vivo results, we propose a nitrogen assimilation scheme for *C. merolae*. In addition, the full method for the *C. merolae* gene disruption is first described herein.

Results

Nitrate assimilatory genes in the C. merolae genome

In a related unicellular red alga, *Galdieria sulphuraria* (formerly *Cyanidium caldarium*), the characteristics of nitrate reduction and NR have been described (Rigano and Violante 1973, Di Martino Rigano et al. 1984). However, specific reports of this process for *C. merolae* are lacking. Since *C. merolae* can grow in media containing nitrate as the sole nitrogen source (data not shown), it should have a complete nitrate-reducing and assimilatory pathway. To understand the nitrate assimilation pathway in *C. merolae*, we searched for nitrate/nitrite-related genes based on the completed genome sequence. We found genes for the nitrate (and/or nitrite) transporter (NRT; *CMG018C*) and NR (*CMG019C*) as adjacent genes, each showing high similarity to orthologs of other organisms (**Supplementary Fig. S1A, B**). Thus, we named these genes (gene products), *CmNRT* (CmNRT) and *CmNR* (CmNR), respectively. CmNRT is composed of 568 amino acids (61.66 kDa), and belongs to the MFS (major facilitator superfamily) transporters. CmNR is composed of 951 amino acids (105.82 kDa), and shows the highest similarity to green plant NRs containing a typical domain structure of MoCo, heme and FAD domains, in this order from the N-terminus (Crawford 1995). However, in initial searches, we could not find any candidates for an NiR-encoding gene. Since this was unexpected, we searched for similar proteins and noticed that there are two candidate genes putatively encoding sulfite reductases (SiRs). Both SiR homologs (CMG021C and CMJ117C) made a tight phylogenetic cluster with SiR genes in other species (Sekine et al. 2009), and one of the coding genes, *CMG021C*, is located next to the *CmNR* gene (**Fig. 1A**). It is well known that SiR is an enzyme structurally related to NiR (Nakayama et al. 2000). Furthermore, it was recently reported that the specificity of SiR could be converted to NiR by introducing a specific mutation (Nakayama et al. 2000), and conversely, an NiR-type enzyme was shown to function as an SiR in *Mycobacterium tuberculosis* (Schnell et al. 2005). Thus, we hypothesized that the *CMG021C* gene product could function as an NiR in *C. merolae*, and genetically tested the hypothesis in this study.

Expression analysis of nitrate-related genes in C. merolae

The expression of *CmNRT*, *CmNR* and the two SiR-related genes (*CMG021C* and *CMJ117C*) was analyzed by RNA gel blot hybridization (**Fig. 1B**). *Cyanidioschyzon merolae* cells were cultivated

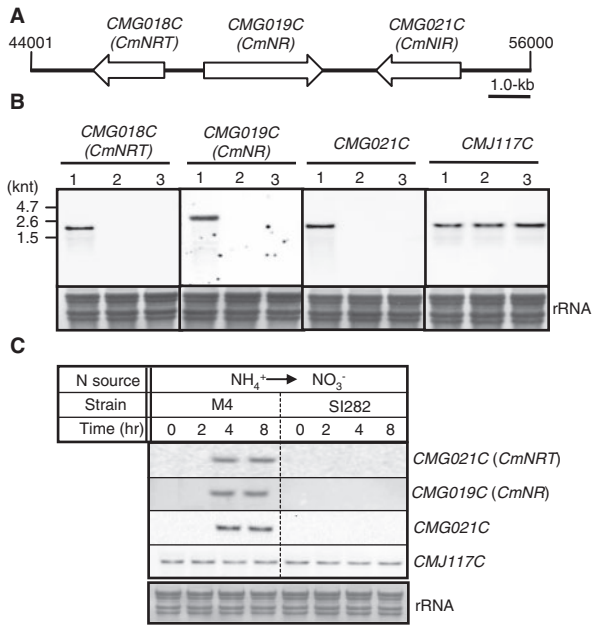

Fig. 1 Structure and expression of the nitrate assimilatory genes. (A) Structure of the nitrate gene cluster in *C. merolae*. The line represents a region of chromosome 7, position 44,001–56,000, in the *Cyanidioschyzon* genome database (http://merolae.biol.s.u-tokyo.ac.jp/). The bar indicates 1.0 kb. (B) Expression analysis of *CmNRT* (*CMG018C*), *CmNR* (*CMG019C*) and two SiR-related genes (*CMG021C* and *CMJ117C*). Total RNA was prepared from *C. merolae* cells grown in medium containing either NO_3^- (lane 1), NH_4^+ (lane 2) or NO_3^- and NH_4^+ (lane 3) as the nitrogen source, and expression of the genes was examined by RNA gel blot hybridization analysis with specific probes. A 7 µg aliquot of total RNA was loaded in each lane. The positions of the molecular weight markers are shown on the left. Electrophoretic patterns of rRNAs stained with methylene blue are also shown below as loading controls. (C) Transcript levels of *CmNRT* (*CMG018C*), *CmNR* (*CMG019C*) and two SiR-related genes (*CMG021C* and *CMJ117C*) under nitrate conditions in M4 and SI282 cells. The *C. merolae* cells were harvested at the indicated time after nitrogen source change, from ammonium (NH_4^+) to nitrate (NO_3^-), and total RNAs were prepared from the cells. Total RNA (4 µg) was then subjected to RNA gel blot hybridization analysis with specific probes for the indicated genes. Other conditions are the same as in (B).

in media containing ammonium, nitrate or both ammonium and nitrate as the nitrogen source, and total RNAs were prepared from cells of mid-logarithmic phase (OD ~0.6). As shown in **Fig. 1B**, *CmNRT* and *CmNR* transcripts were detectable in nitrate-grown cells (lane 1) but not in ammonium-grown cells (lane 2). These transcripts of nitrate-grown cells were absent in the presence of ammonium (lane 3), indicating a possible NCR mechanism (ter Schure et al. 2000). The expression pattern of *CMG021C* is basically the same as those of *CmNR* and *CmNRT*. On the other hand, *CMJ117C* was constitutively expressed irrespective of the nitrogen source (**Fig. 1B**), as expected from its role as an SiR. Since we have shown previously that a *CmMYB1* null mutant strain, SI282 (Imamura et al. 2009), could not grow on nitrate as the sole nitrogen source, we next investigated the possibility that CmMYB1 regulates gene expression of *CmNRT*, *CmNR* and *CMG021C* in nitrate-containing medium, using SI282 and its parental strain, M4 (Minoda et al. 2004). We found that nitrate-induced accumulation of those transcripts was not detectable in SI282 (**Fig. 1C**). On the other hand, the level of transcripts of *CMJ117C* was not changed in either strain, irrespective of the nitrogen condition. These results indicate that CmMYB1 is indispensable for nitrate-responsive gene expression and the transcriptional systems for the clustered genes, *CmNRT*, *CmNR* and *CMG021C*, but not for *CMJ117C*. Thus, the expression analyses supported our hypothesis that CMG021C works as an NiR in *C. merolae*.

Construction of the CMG021C null mutant and its growth curve in nitrate-containing media

To examine genetically whether CMG021C is involved in the nitrate assimilation pathway, we developed a method for gene disruption in *C. merolae* and used this method to knock-out *CMG021*. For the gene targeting, it is essential to induce homologous recombination in the targeted gene region. However, in a previous study we showed that an introduced *URA5.3* (*CMK046C*) gene of *C. merolae* was efficiently used to repair the mutated *URA5.3* allele in the M4 mutant (Minoda et al. 2004). Thus, we constructed a modified marker gene to prevent homologous recombination at the *URA5.3* locus, in which the *URA3* portion of the *URA5.3* gene was substituted by the orthologous cDNA portion of *G. sulphuraria*, a related unicellular red alga (**Supplementary Fig. S2**). After constructing the DNA for gene targeting, introduction into the M4 mutant and selection of the uracil autotrophic cells, we obtained a mutant strain lacking the *CMG021C* gene and named it SI021. Subsequently, we conducted PCR and immunoblot analyses to verify the expected recombination in SI021. These results are shown in **Fig. 2B**. When a set of primers, F1 and R1 (**Fig. 2A**), was used, we detected 2.0 and 2.3 kb bands with M4 and SI021 genomic DNA as a template DNA, respectively, as was predicted (**Fig. 2B**, left). When a set of primers, F2 and R2, was used, we only detected a band of 1.8 kb with SI021 genomic DNA, as was predicted (**Fig. 2B**, middle). Furthermore, CMG021C protein detected in M4 grown in nitrate medium was not detectable in SI021 (**Fig. 2B**, right). These results clearly showed that the expected recombination by a double cross-over reaction successfully disrupted the *CMG021C* gene in SI021. It is of note that the protein levels of CMG021 and CMJ117C were well correlated with those of the transcripts (**Figs. 1B, 2B**). Secondly, we investigated growth of SI021 in media containing nitrate or ammonium. As shown in **Fig. 2C**, the knock-out of the *CMG021C* gene was not lethal for the cells, but the cell growth was significantly reduced in nitrate-containing medium as compared with the parental strain. However, in ammonium-containing medium, the cell growth of both strains was almost identical. Thus, these results again suggested that CMG021C functions in the nitrate assimilation pathway as an NiR. The cell growth of SI021 under the nitrate condition might be compensated by CMJ117C, and this point will be discussed below.

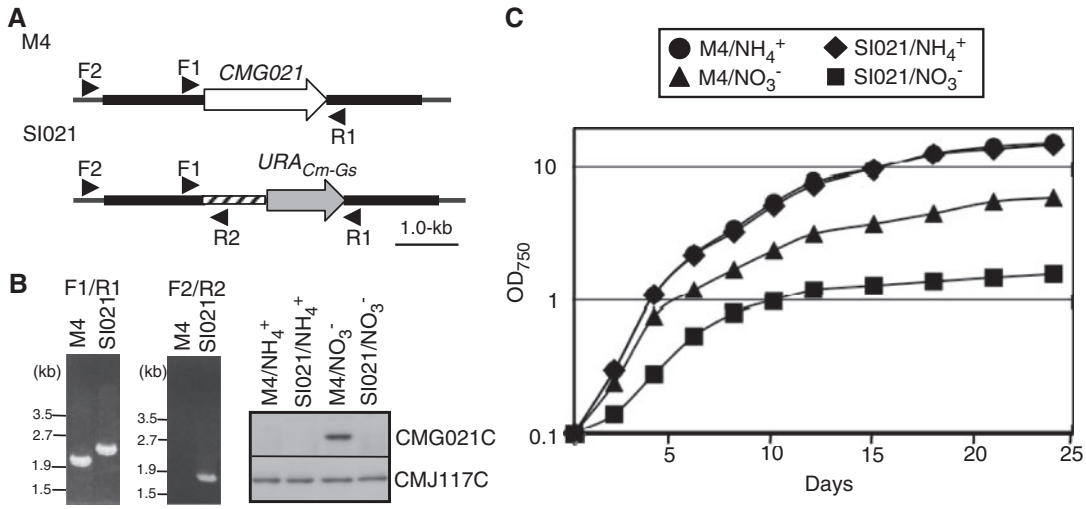

Fig. 2 Construction of the *CMG021C* null mutant and its growth curve when grown in nitrate medium. (A) Schemas for the loci of *CMG021C* in M4 and SI021 strains. White and gray arrows indicate *CMG021C* and the artificial UMP synthase gene ($URA_{Cm\text{-}Gs}$), respectively. Thick black and hatched lines indicate the upstream or downstream region of the *CMG021C* gene and the upstream region of *URA5.3* gene of *C. merolae*, respectively. The positions of primers for PCR are shown with arrowheads. The bar indicates 1.0 kb. (B) Confirmation of the *CMG021C* knock-out. The genomic DNA was analyzed by PCR with the set of primers F1/R1 (left) or F2/R2 (middle). Total proteins (10 μg for each lane) prepared from the indicated strains and growth conditions in the log phase were subjected to immunoblot analysis with antibodies against CMG021C or CMJ117C (right). (C) Growth curve of M4 and SI021 cells. M4 and SI021 cells were grown in an ammonium medium to mid-log phase, and collected by centrifugation. After washing with the nitrate or ammonium medium twice, cells were inoculated in the nitrate or ammonium medium at an OD_{750} of 0.1, and their growth was periodically monitored.

Functional complementation of a cyanobacterial nitrite reductase mutant by CMG021C

To examine further the possibility that CMG021C functions as an NiR, we expressed the CMG021C protein in an NiR-deficient mutant, PN1 [an insertional mutant of *nirA* (Suzuki et al. 1995)], of a cyanobacterium, *Leptolyngbya boryana* (formerly *Plectonema boryanum*), and checked for growth on nitrate. To this end, a shuttle plasmid that can replicate in both *Escherichia coli* and *L. boryana* containing the *CMG021C* gene was constructed and introduced into PN1. After confirming the expression of the *CMG021C* transcript by RNA gel blot hybridization analysis (**Fig. 3A**), the growth of the complemented strain PN1 (pPBHNIR) on nitrate was compared with that of the parental *L. boryana* and PN1 (**Fig. 3B**). After shifting from an ammonium medium to a nitrate medium, PN1 stopped growing within several days, while PN1 (pPBHNIR) continued to grow, although the growth rate was slightly lower than that of the wild type. Thus, genetic complementation of the nitrate growth phenotype strongly indicated a nitrite-reducing activity of the CMG021C gene product in the cyanobacterial cells. Based on these observations, we conclude that the *CMG021C* gene physiologically functions as an NiR and so named it *CmNiR*, whereas *CMJ117C* was named *CmSiR*, for the presumed SiR-coding gene. Since the CmSiR expression plasmid could not be constructed presumably because of the toxic effect for *E. coli* cells, it was impossible to estimate the nitrite-reducing activity of CmSiR using the cyanobacterial system.

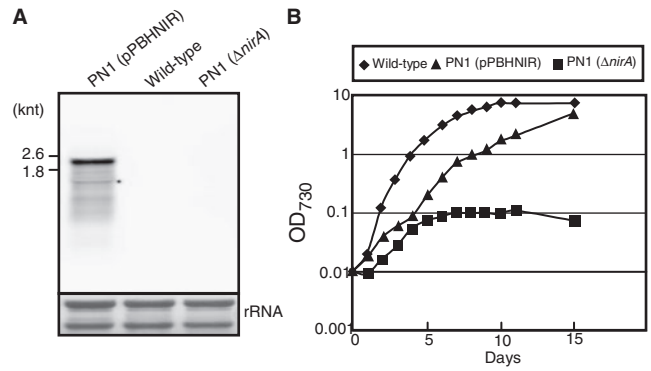

Fig. 3 Complementation of the nitrite reductase mutant PN1 of the cyanobacterium *L. boryana* by *CMG021C* (*CmNiR*). The *CMG021C* gene was cloned onto a *Leptolyngbya–E. coli* shuttle plasmid to make pPBNIR, and introduced into the *Leptolyngbya nirA* mutant PN1 to examine the complementation of the mutation. (A) Expression of *CMG021C* in *Leptolyngbya*. Total RNA was prepared from *Leptolyngbya* wild-type strain, PN1, and PN1 was introduced with pPBHNIR grown in ammonium medium. The expression of *CMG021C* was examined by RNA gel blot hybridization analysis with a specific probe. A 3 μg aliquot of total RNA was loaded in each lane. The positions of the size markers and the electrophoretic pattern of rRNAs are also shown. (B) Growth of *Leptolyngbya* strains in nitrate medium. After pre-cultivation in ammonium medium, *Leptolyngbya* wild-type strain, PN1, and PN1 introduced with pPBHNIR were inoculated in nitrate medium and growth was monitored periodically by measuring the OD_{730}.

Localization analysis of CmNiR and CmSiR in C. merolae

To clarify further the nitrate-assimilating pathway in *C. merolae*, it is important to determine the intracellular localization of each assimilatory enzyme. To this end, we constructed expression plasmids from which C-terminal 3× hemagglutinin (HA) epitope-tagged CmNiR or CmSiR proteins were expressed from the respective putative promoter regions (upstream ~1.5 kb), and used these for transient expression in *C. merolae* cells. After introducing the expression plasmids, cells were cultivated for 6 h in either nitrate or ammonium medium, and immunoblot analysis of total *C. merolae* proteins was performed, using an anti-HA mouse monoclonal antibody (**Fig. 4A**). The anti-HA antibody did not significantly react with any native *C. merolae* protein. Although a faint signal of about 50 kDa was always detected from native *C. merolae* protein, we ignored this since no background signal was detected in the immunolocalization analysis. Introduction of a CmNiR-HA expression plasmid resulted in a signal of 83 kDa only from proteins of the nitrate-grown cells, indicating that the adjacent upstream promoter region of 1.5 kb included sufficient information for NCR. In contrast, a 92 kDa protein was detected, irrespective of the nitrogen source in the medium, when the CmSiR-HA expression plasmid was introduced. The intensity of the CmNiR-HA signal was weaker than that of the CmSiR-HA signal (**Fig. 4A**). However, we could not quantify the protein expression levels from these results, since the signal intensity might reflect the transformation efficiency rather than the protein amounts in each cell. Results of the immunolocalization analysis by fluorescent microscopy are shown in **Fig. 4B**. The CmSiR-HA signal (yellow-green) was detected in the chloroplast and overlapped with the chlorophyll fluorescence (red) irrespective of the cultivation medium. On the other hand, while the CmNiR-HA signal was also detected in the chloroplast, the signal was only detected in nitrate-containing media, consistent with the expression analyses (**Figs. 1, 2**). These results indicated that both CmSiR and CmNiR are localized in chloroplasts.

Discussion

In the present study, we characterized nitrate assimilatory genes in an acidophilic unicellular red alga, *C. merolae*. Whereas unique genes for the NRT and NR were identified, no typical NiR-coding gene was found in the complete genome sequence

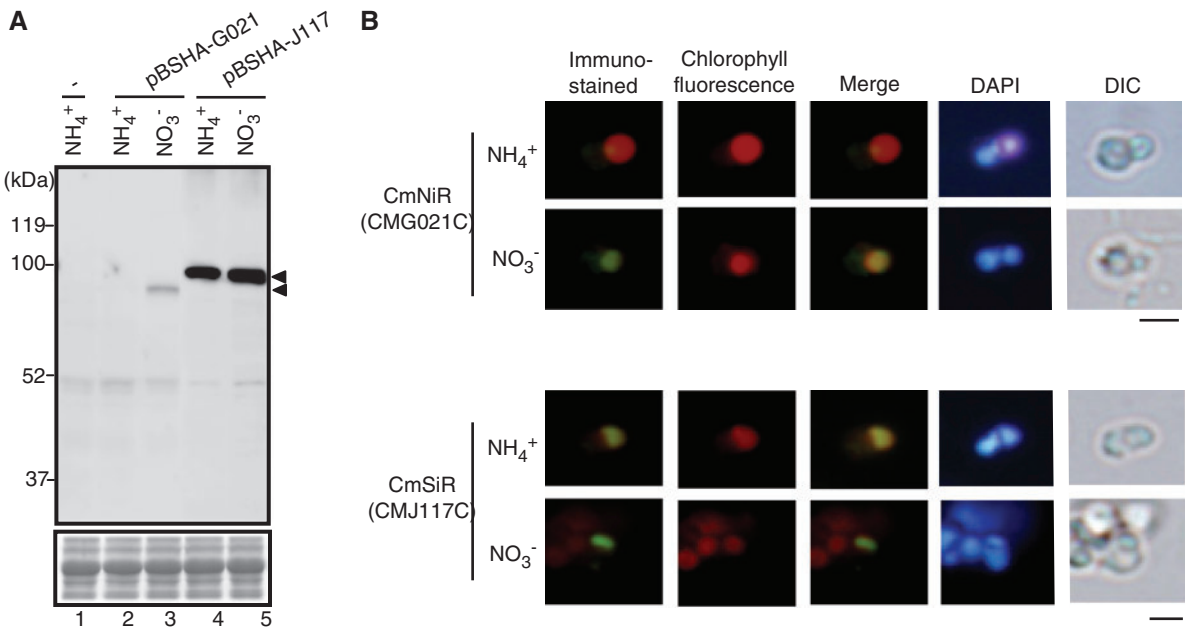

Fig. 4 Subcellular localization of HA-tagged CmNiR and CmSiR. (A) Transiently expressed HA-tagged CmNiR and CmSiR in *C. merolae*. Aliquots of total proteins (10 µg) from wild-type (–) cells or cells of each transformant (with pBSHA-G021 or -J117) grown under NH_4^+ or NO_3^- condition were subjected to immunoblot analysis. HA-tagged proteins were first reacted with Alexa Fluor 488-conjugated mouse monoclonal anti-HA antibody (Invitrogen, Carlsbad, CA, USA) and subsequently reacted with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Promega, Madison, WI, USA), and then detected with an ECL-Plus Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK). Arrowheads indicate the positions of HA-tagged CmNiR or CmSiR. Total protein (~55 kDa) stained with Coomassie Brilliant Blue is shown as a loading control (lower panel). (B) Intracellular localization of CmNiR and CmSiR. Localization of HA-tagged CmNiR and CmSiR under NH_4^+ or NO_3^- conditions was examined with Alexa Fluor 488-conjugated mouse monoclonal anti-HA antibody by immunofluorescent microscopy (yellow-green signal, immunostained). Intrinsic chlorophyll fluorescence (red signal, chlorophyll fluorescence), a merged image of immunostaining and intrinsic chlorophyll fluorescence (merge), 4′, 6-diamidino-2-phenylindole staining of cells (DAPI) and a differential interference contrast image (DIC) are shown. Bars correspond to 2 µm.

(Nozaki et al. 2007). Our analysis presents several genetic lines of evidence that one of two SiR-related proteins encoded by the *C. merolae* genome indeed functions as an NiR (CmNiR). Although NiR is related to SiR at the amino acid sequence level (**Supplementary Fig. S1C**), to our knowledge this is the first case of an SiR-type protein functioning as an NiR in vivo. Unicellular red algae of Cyanidiales live in acidic hot springs, and their predominant nitrogen sources would be those retrieved from hot water emerging from reductive underground sources. Oxidized nitrogen compounds, such as nitrate and nitrite, are usually generated by microbial activities in oxidizing environments. Thus, nitrate assimilation might be essential in only very limited situations in these organisms. After some irreversible mutations, such as loss of NiR, requirements to re-establish nitrate assimilation activity could have led to the evolution of an unusual nitrate assimilation system as in *C. merolae*. Similar observations of an unusual nitrate assimilatory system were recently made in *G. sulphuraria*, a related unicellular red alga, whose genome structure was recently determined (A. Weber, unpublished). In this case, although this alga can grow on nitrate as the sole nitrogen source, the typical NR-coding gene was not identified from the genome, while an NiR gene was present. Thus, the nitrate-reducing activity should be compensated for by other enzymatic systems. The opportunity to use nitrate as the sole nitrogen source could be relatively rare, and this may be a common characteristic of the living habitat for these algae.

Structurally, NiR and SiR are related enzymes sharing high sequence similarity (**Supplementary Fig. S1C**). However, the canonical SiR itself appears to have very weak NiR activity, if any (Nakayama et al. 2000, Hirasawa et al. 2004). Thus CmNiR would have experienced some structural modification to accomplish the change in specificity. It has been reported that a single amino acid change, Arg193 to glutamate, of maize SiR changed the substrate specificity from sulfite to nitrite (Nakayama et al. 2000). However, in the case of both the SiR-related proteins of *C. merolae* (CmNiR and CmSiR), the corresponding amino acid residues are arginine, and the relevant change could not explain the novel specificity observed with CmNiR. Recently, Sekine et al. (2009) characterized CmNiR (CmSiRB) enzymatic properties through in vitro assays using a recombinant CmNiR protein. The nitrite-reducing activity of CmNiR is about seven times higher and about four times lower than that of *Zea mays* SiR and *Synechocystis* sp. PCC 6803 NiR, respectively. With respect to the K_m value, CmNiR has a two orders of magnitude higher affinity for sulfite than for nitrite. In contrast, the k_{cat} value of CmNiR for sulfite was two orders of magnitude lower than that for nitrite. These in vitro assays demonstrated that CmNiR has a relatively high nitrite-reducing activity and a low sulfite-reducing activity, and also is consistent with our results and conclusion that the *CMG021C* gene encodes NiR. However, the strain lacking the *CmNiR* gene, SI021, could grow even under the nitrate growth condition. Because it was difficult to express CmSiR as a holo form in *E. coli* cells, the enzymatic properties of CmSiR have not been characterized so far, while it was shown that *Z. mays* SiR has about five times lower activity for nitrite than for sulfite (Sekine et al. 2009). This raises the possibility that the growth of SI021 under the nitrate condition is compensated by CmSiR. Further experimental analyses, including biochemical characterization of these enzymes, as well as the structural analysis, e.g. X-ray crystallography, would be required to resolve the structural basis of the modified specificity.

Based on the present and previous expression analyses, it is now clear that there is nitrogen-responsive transcriptional regulation in *C. merolae*. It should also be noted that while the gene expression is activated in the nitrate medium, it is repressed by the presence of ammonium, a process that is most probably related to the NCR mechanism found in other organisms (ter Schure et al. 2000, Cooper 2002). In a previous study, we identified a R2R3-type MYB transcription factor, CmMYB1, which is the central nitrogen assimilation regulator under nitrogen-depleted conditions in *C. merolae* (Imamura et al. 2009). We have here made clear that CmMYB1 is also involved in the nitrate-responsive gene expression of *CmNRT*, *CmNR* and *CmNiR* (**Fig. 1C**). Our previous studies also indicated that CmMYB1 mediates the NCR-sensitive transcription of the nitrogen assimilation genes in response to nitrogen depletion, and *C. merolae* cells sense glutamine or ammonium as the nitrogen signal, but nitrate does not appear to be specifically sensed. In other plants, it has been reported that *Chlamydomonas* Nit2, which is the transcription factor possessing GAF, glutamine-rich, leucine zipper and RWP-RK domains, is the central regulator required for nitrate signaling on the NR (*NIA1*) gene promoter and that intracellular nitrate is needed for NIT2 function to modulate *NIA1* transcript levels (Camargo et al. 2007). *NIT2* expression is up-regulated under nitrogen deprivation and nitrate growth conditions, but is repressed under ammonium growth conditions, implying that the NCR mechanism is involved in *NIT2* expression. In Arabidopsis, transcripts of nitrate assimilation genes, *NRT1/CHL1*, *NIA1*, *NIA2* and *NiR* encoding nitrate transporter, nitrate reductase 1, nitrate reductase 2 and nitrite reductase, respectively, are induced after change to nitrate from ammonium growth conditions; however, those genes are transcribed even in the presence of ammonium in the medium (Wang et al. 2000, Jonassen et al. 2009). Recently, Jonassen et al. reported that bZIP-type transcription factors, HY5 and HYH, are activators and inhibitors of light-dependent expression of *NIA2* and *NRT1/CHL1* genes, respectively (Jonassen et al. 2008, Jonassen et al. 2009). However, the nitrate-responsive gene expression of *NRT1/CHL1*, *NIA1*, *NIA2* and *NiR* did not depend on the two transcription factors. This raises the possibility that other types of transcription factor(s) contribute to the expression. One candidate would be a MYB-type transcription factor (AT1G01060), as only the gene expression of this factor was significantly up-regulated in response to the nitrate condition of all the transcription factors in the microarray analysis (Wang et al. 2000). Thus, the mechanisms of nitrate-responsive transcription seem to be different among plant lineages.

As for the nitrate assimilation pathway, intracellular localization of CmNR is predicted to be cytosolic by the TargetP program [http://www.cbs.dtu.dk/services/TargetP (Emanuelsson et al. 2000)]. On the other hand, localization of CmNiR has been elusive; thus we performed immunolocalization experiments in *C. merolae* using the recently developed transient expression technique (Ohnuma et al. 2008). CmNiR was localized to the plastid and, together with earlier cytoplasmic localization results for CmGS (Terashita et al. 2006), an overall nitrogen assimilation scheme was predicted, as shown in **Fig. 5**. Nitrate is imported into cells by CmNRT, and reduced to nitrite by CmNR in the cytosol. Nitrite is then imported into the plastid through an unknown transporter, where it is further reduced to ammonium by CmNiR. However, as mentioned above, there is a possibility that CmSiR also has NiR activity. Although still awaiting experimental confirmation, we posit that the electron donor for the CmNR reactions would be NAD(P)H, analogously to other plant systems (Crawford 1995). The electron donor for CmNiR was shown to be ferredoxin (Sekine et al. 2009), which would be reduced by PSI in the plastid. The reduced ammonium would be transported back to the cytosol where it is assimilated into glutamine by cytosolic CmGS (Terashita et al. 2006). Extracellular ammonium can also be imported by a specific transporter and assimilated by CmGS in the cytosol. The produced glutamine is then imported into the plastid and used as the substrate for the GOGAT reaction. GOGAT should be localized in the plastid as the unique GOGAT gene (*gltB; CMV060C*) is encoded by the plastid genome.

Materials and Methods

Cells and growth

Cyanidioschyzon merolae 10D cells were routinely grown in modified Allen's (MA) medium as described by Minoda et al. (2004). In the case of nitrogen-controlled media, 20 mM $(NH_4)_2SO_4$ was substituted with 5 mM $NaNO_3$ and 20 mM Na_2SO_4, 2.5 mM $(NH_4)_2SO_4$ and 17.5 mM Na_2SO_4, or 5 mM $NaNO_3$, 2.5 mM $(NH_4)_2SO_4$ and 17.5 mM Na_2SO_4 for NO_3^-, NH_4^+ and NO_3^-/NH_4^+ medium, respectively. *Leptolyngbya boryana* cells were grown as described by Fujita et al. (1992) and Yamazaki et al. (2006) in BG11 medium containing either 3.75 mM $(NH_4)_2SO_4$ (for ammonium medium) or 7.5 mM $NaNO_3$ (for nitrate medium) as a nitrogen source. Chloramphenicol at 25 μg ml^{-1} was added to the medium when required.

RNA manipulations

Cyanidioschyzon merolae and *L. boryana* cells were collected by centrifugation at 3,000×*g* for 5 min and stored at −80°C until use. The frozen cells were thawed on ice and suspended in RNA extraction buffer [50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.5% (w/v) SDS] and total RNA was extracted by acid phenol. The extracted total RNA was purified with phenol:chloroform:isoamylalcohol (25:24:1) followed by ethanol precipitation. Isolated total RNA was separated by gel electrophoresis (1.2% agarose/3% formaldehyde) and transferred to a Biodyne

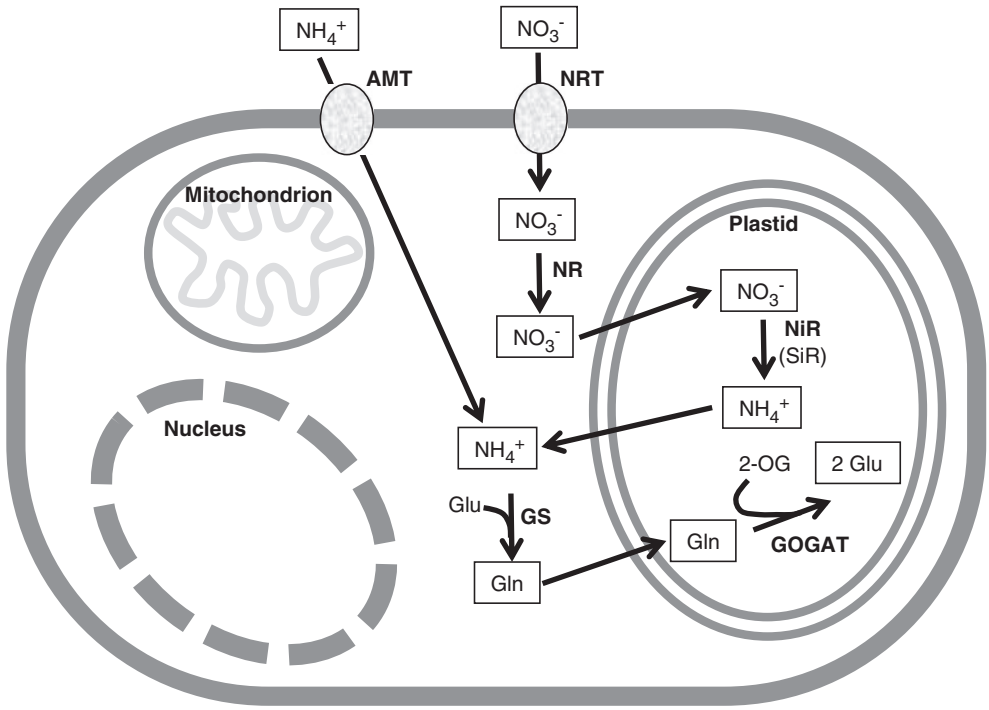

Fig. 5 Scheme of the nitrogen assimilation pathway in *C. merolae*. Nitrate and ammonium are assimilated through complex intracellular traffic in *C. merolae* cells. AMT, ammonium transporter; 2-OG, 2-oxoglutarate; GOGAT; glutamine:2-OG amidotransferase. See text for details.

Plus nylon membrane (Pall Corporation, Ann Arbor, MI, USA) by a standard capillary transfer method. An RNA gel blot hybridization analysis was performed as described previously (Kanamaru et al. 2001). Gene-specific probes were constructed by PCR with specific primers: 5′-GGTGCAGCAATACCCAC TTT-3′ and 5′-TCCAATGGTCGTCACTTTCA-3′ for *CMG018C* (*CmNRT*), 5′-TATTGGAGACACGGTGCAGGTCAAG-3′ and 5′-TGCACGCATCATCCTGAAATCCCGC-3′ for *CMG019C* (*CmNR*), 5′-ATCCGTTGACCGAGGTACTG-3′ and 5′-TGCAG TCATCGGAGATGAAG-3′ for *CMG021C* (*CmNiR*), and 5′-CG CTCTTACATGACCGACAA-3′ and 5′-GTTGCTCTGGCATGA TGTTG-3′ for *CMJ117C* (*CmSiR*) (Kanamaru et al. 2001). The signals were detected with a chemiluminescent image analyzer LAS3000 (Fujifilm, Tokyo, Japan).

Construction of a selectable URA marker gene for transformation

Using a pair of oligonucleotide primers, KpnI-URA5.3 (5′-GG GGTACCGAACTGAGGGGCGAACGC-3′, underlined sequence denotes the *Kpn*I site) and URA5.3-SpeI (Minoda et al. 2004), and the *C. merolae* genome as a template, PCR was carried out for 25 cycles (98°C for 10 s, 55°C for 5 s and 72°C for 3 min) with PrimeSTAR® HS DNA Polymerase (TAKARA BIO INC., Otsu, Japan). The amplified fragment was digested with *Kpn*I and *Bam*HI and inserted into the *Kpn*I–*Bam*HI site of pKF18k [GenBank accession No. D63846 (TAKARA BIO INC.)] to make pKFURAB3. pKFURAB3 has a 2,582 bp DNA fragment, which contains the *URA5.3* (*CMK046C*) gene and 903 bp of upstream and 288 bp of downstream region sequence.

Isolation of total RNA of *G. sulphuraria* was performed as previously described (Linka et al. 2008). The *G. sulphuraria* cDNA was synthesized using 1 μg of total RNA and an oligo(dT) primer. Reverse transcription was carried out at 65°C for 5 min, at 42°C for 50 min and at 70°C for 15 min with SuperScript™ II RT (Invitrogen, Carlsbad, CA, USA). The reaction mixture was incubated at 37°C for 30 min with RNase H. The coding sequence of *URA5.3* cDNA was amplified from *G. sulphuraria* cDNA by PCR using a pair of oligonucleotide primers, GsURA5.3-F (5′-ATGTCTTCACTGGACGCATTG-3′) and GsURA5.3-R (5′-TC ATTCAACGTATTCTTCAAGTCG-3′), and a cDNA synthesis reaction mixture as a template. PCR was carried out for 35 cycles (98°C for 30 s, 53°C for 30 s and 68°C for 2 min) with Expand High Fidelity polymerase (Roche, Basel, Switzerland). PCR products (1,422 bp) were subcloned into the pGEM-T Easy vector system (Promega, Madison, WI, USA) to make pGSUra5.3, and sequenced.

To obtain the upstream region of *URA5.3* (903 bp) and the *URA5* region (512 bp) of *C. merolae* ($URA5_{Cm}$, 1,415 bp), using a pair of oligonucleotide primers, URACm-GS_Nter_F (5′-AA TTCGAGCTCGGTACCGAACTGAG-3′, underlined sequence denotes the *Kpn*I site) and URACm-GS_R (5′-GATAACCGACC TGAGTGGCGGAGTGTTCCGACAAGGTCGCTGATGC GGAA-3′), and pKFURAB3 as a template, PCR was carried out for 30 cycles (98°C for 10 s, 40°C for 5 s and 72°C for 2 min) with PrimeSTAR® HS DNA Polymerase. The *URA3* region of *G. sulphuraria* ($URA3_{Gs}$, 904 bp) was obtained in the same way using the primer pair URACm-GS_F (5′-TTCCGCATCAGCGAC CTTGTCGGAACACTCCGCCACTCAGGTCGGTTATC-3′) and URACm-GS_Cter_R1 (5′-AGTAAGCTTTCATTCAACG TATTCTTCAAGTCGTTGTAAATAAGCATCCC-3′, underlined sequence denotes the *Hin*dIII site), and pGSUra5.3 as a template. Amplified fragments of $URA5_{Cm}$ with the upstream region of *URA5.3* of *C. merolae* and $URA3_{Gs}$ were electrophoresed and purified using a Wizard® SV Gel and PCR Clean-Up System (Promega). PCR (98°C for 10 s, 60°C for 5 s and 72°C for 3 min) was performed with fragments $URA5_{Cm}$ with the upstream region and $URA3_{Gs}$ without template for 10 cycles. Then the amplified fragment as a template, and primers URACm-GS_Nter_F and URACm-GS_Cter_R2 (5′-ACGACGGCCAGT AAGCTTTCATTCAACGTA-3′, underlined sequence denotes the *Hin*dIII site) were added to the mixture and PCR was carried out for 20 cycles (98°C for 10 s, 40°C for 5 s and 72°C for 3 min) to amplify the DNA fragment $URA_{Cm\text{-}Gs}$ with the upstream region (2,310 bp) (**Supplementary Fig. S2**). The DNA fragment was named URAPCG marker and was digested with *Kpn*I and *Hin*dIII, and inserted the *Kpn*I–*Hin*dIII site of pKF18k to make pKFURACm-Gs.

Preparation of DNA for construction of the CMG021C null mutant

The DNA fragment was produced by a two-step PCR method. For the first PCR, fragment 1 (1.5 kb of the upstream region of the *CMG021C* gene, −1,500 to −1, where +1 is the initiation codon) was amplified by PCR with the primer set G021_1500up_F (5′-CGGAATGCAGAAGAGCAGCGGCTTG-3′) and G021_R_ 5′URACm-Gs (5′-CCTCAGTTCGGTACCCAGAGTATACCGCA CGCGCAACTAT-3′, underlining indicates the adaptor sequence), and *C. merolae* genomic DNA as a template; fragment 2 (1.5 kb of the downstream region of the *CMG021C* gene, +2,038 to +3,537) was amplified by PCR with the primer set G021_F_5′URACm-Gs (5′-GTTGAATGAAAGCTTTAGCTG CTGCTGGCGAATCCATGAA-3′, underlining indicates the adaptor sequence) and G021_1500down_R (5′-TTCCAGGAA CTCGAAGCATGGCG-3′), and *C. merolae* genomic DNA as a template; fragment 3 (2.3 kb of a selectable URAPCG marker) (**Supplementary Fig. S2**) was amplified by PCR with the primer set 5′URACm-Gs_F_G021 (5′-CGTGCGGTATACTCTG GGTACCGAACTGAGGGGCGAAC-3′, underlining indicates the adaptor sequence) and 5′URACm-Gs_R_G021 (5′-TCGCC AGCAGCAGCTAAAGCTTTCATTCAACGTATTCTT CAAGTCG-3′, underlining indicates the adaptor sequence), and pKFURACm-Gs as a template DNA. The second PCR was performed with a set of primers, G021_1400up_F (5′-TGTATCGAAACGAGTGCCGGCA-3′) and G021_1400up_R (5′-CTGTCGTTGGCCACGTGAATC-3′), using fragments 1, 2 and 3 as the template DNAs. The resultant 5.1 kb DNA fragment (the *CMG021C* upstream region + the URAPCG marker cassette + the *CMG021C* downstream region from the 5′end to the 3′ end in this order) (**Fig. 2A**) was used for the transformation.

Construction of the chromosomal deletion mutant and confirmation of the CMG021C null mutant strain

Cyanidioschyzon merolae M4 (Minoda et al. 2004) was used for gene disruption. Cells were grown in MA2 medium (Ohnuma et al. 2008) containing uracil (0.5 mg ml^{-1}) in a glass vessel under continuous white light (50 µE m^{-2} s^{-1}) at 40°C with 2% CO_2, unless otherwise noted. MA2 medium was solidified with 0.5% (w/v) gelrite. H_2SO_4 (500 µl l^{-1}) was added to correct a pH shift by gelrite. Transformation was performed as previously described (Ohnuma et al. 2008) with modification. A 5 µg aliquot of the PCR fragment was used for transformation. After overnight incubation, cells were collected by centrifugation (2,000×*g* for 5 min at 40°C), suspended with 200 µl of MA2 medium and spotted on MA2 plates as below. Plating was performed as previously described (Shimogawara et al. 1998) with modifications. Cornstarch was washed sequentially with distilled water and ethanol. The washed starch was stored at 4°C in 75% ethanol until use. Before each experiment, ethanol-washed starch was washed once with 10× MA-I [200 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 2% (v/v) trace elements (stock trace elements: 2.85 g^{-1} H_3BO_3, 1.8 g l^{-1} $MnCl_2{\cdot}4H_2O$, 0.105 g l^{-1} $ZnCl_2$, 0.39 g l^{-1} $Na_2MoO_4{\cdot}2H_2O$, 0.04 g l^{-1} $CoCl_2{\cdot}6H_2O$, 0.043 g l^{-1} $CuCl_2$) and 3 ml l^{-1} H_2SO_4] by centrifugation and resuspension. Subsequently, cornstarch was washed twice with MA medium (Minoda et al. 2004). The starch was finally resuspended to 20% slurry in MA medium, and polyethylene glycol (PEG) 4000 was added to 0.4% (w/v). A 15 µl aliquot of the starch suspension was dropped on the top of solid medium at regular intervals in a 9 cm diameter Petri plate to make 20–30 spots. Then 10 µl of the cells was dropped on to the cornstarch spots after extra moisture of the spot of cornstarch was dried up. Wild-type cells were spotted at appropriate intervals as nurse cells. Cells were incubated under continuous white light at 40°C with 5% CO_2 until colony formation.

To verify the expected recombination in SI021 we performed PCR with the following primers: G021_TF_Check_F1 (F1 in **Fig. 2A**, 5′-TGCGCGTGCGGTATACTCTG-3′), G021_TF_Check_R1 (R1, 5′-GGATTCGCCAGCAGCAGCTA-3′), G021_TF_Check_F2 (F2, 5′-TGATTCCGGCATGCGATACCTC-3′) and URA_UP_R2 (R2, 5′-CGCGCAATCATCCTCCCACTAGAAG AT-3′) and carried out immunoblot analyses with the antibody against CMG021C (Sekine et al. 2009) as described previously (Imamura et al. 2009).

Complementation analysis

The PN1 mutant of *L. boryana*, which was constructed by insertionally inactivating the NiR structural gene *nirA* (and hence it cannot utilize nitrate and nitrite as a nitrogen source) (Suzuki et al. 1995), was used as a host for heterologous expression of the CMG021C open reading frame (ORF). The plasmid used for expression of CMG021C in *L. boryana* was a derivative of pPBHLI18, a plasmid constructed for expression of the *chlL* gene under the control of the T5 promoter in the cyanobacterium (H Takagi, T Hase and Y Fujita, unpublished data). The ORF of *CMG021C* was amplified with primers 5′-ACATGCATGCGAAT GATGTTCGTCACGTACGC-3′ (underlined sequence denotes the *Sph*I site) and 5′-TGATCACGATACGGGCGCTGCCGGCA-3′ (underlined sequence denotes the *Bcl*I site) from *C. merolae* total DNA. PCR was carried out for 25 cycles (96°C for 20 s, 57°C for 45 s and 68°C for 2.5 min) with KOD-Plus DNA polymerase (TOYOBO, Tokyo, Japan). After digestion with *Sph*I and *Bcl*I, a CMG021C expression plasmid, pPBHNIR, was constructed by replacing the *chlL*-containing *Sph*I–*Bam*HI fragment of pPBHLI18 with the *CMG021C*-containing *Sph*I–*Bcl*I fragment. Thus, CMG021C is expected to be constitutively expressed under control of the T5 promoter in cyanobacterial cells with pPBHNIR. Transformation of *L. boryana* by electroporation was conducted as described by Fujita et al. (1992). Cells were grown in an ammonium medium to mid-log phase (OD_{730} ~1.0), and collected by centrifugation. After washing with nitrate medium three times, cells were inoculated in nitrate medium at OD_{730} 0.01 and their growth monitored periodically under standard growth conditions.

Immunofluorescence microscopy

For construction of plasmids for HA epitope-tagged CmNiR (CMG021C) and CmSiR (CMJ117C) expression, the ORFs of *CmNiR* and *CmSiR* were amplified together with each 5′-flanking region (1.5 kb) from *C. merolae* total DNA with the sets of primers 5′-GTACTAGTCGGAATGCAGAAGAGCAGCG-3′ and 5′-GT ACTAGTCGATACGGGCGCTGCCGGCA-3′for *CmNiR*, and 5′-GTACTAGTGAAAAGAAGCCCACCGAACAAGAATG-3′ and 5′-GTACTAGTGTCCATCGCAGTATTCCCATCTGT-3′ for *CmSiR* (each underlined sequence denotes the *Spe*I site). PCR was carried out for 30 cycles [94°C for 15 s, 56°C (*CmNiR*) or 45°C (*CmSiR*) for 30 s, and 68°C for 4.5 min] with KOD-Plus DNA polymerase. Each fragment was digested with *Spe*I, and cloned into the *Spe*I site of pBSHAb-T3′ (Ohnuma et al. 2008) to construct pBSHA-G021 and pBSHA-J117. Both genes were inserted in the same orientation as the HA epitope-tag. Plasmids were transiently transformed into *C. merolae* as described elsewhere (Ohnuma et al. 2008) with some modifications: after PEG treatment, cells were incubated overnight in MA2 medium, collected by centrifugation (2,000×*g* for 5 min) and gently resuspended in either NH_4^+ or NO_3^- media. After cultivation for 6 h, cells were subjected to immunoblot and subcellular localization analyses as described previously (Ohnuma et al. 2008).

Supplementary data

Supplementary data are available at PCP online.

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