Differential transcriptional regulation of orthologous *dps* genes from two closely related heterocyst-forming cyanobacteria

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Abbreviations: 5′ RACE - 5′ Rapid Amplification of cDNA Ends; 5′ UTR - 5′untranslated region; DNA-binding proteins from starved cells; GFP - Green Fluorescent Protein; ORF - open reading frame; ROS - reactive oxygen species; TSS - Transcriptional start site.

Abstract

In cyanobacteria _DNA-binding proteins from starved cells (Dps) play an important role in the cellular response to oxidative and nutritional stresses. In this study we have characterized the cell-type specificity and the promoter regions of two orthologous _dps _genes, _Npun_R5799 _in _Nostoc punctiforme _and _alr3808 _in _Anabaena _sp. PCC 7120. A transcriptional start site (TSS), identical in location to the previously identified proximal TSS of _alr3808_, was identified for _Npun_R5799 _under both combined nitrogen supplemented and N$_2$-fixing growth conditions. However, only _alr3808 _was also transcribed from a second distal TSS. Sequence homologies suggest that the promoter region containing the distal TSS is not conserved upstream of orthologous genes among heterocyst-forming cyanobacteria. The analysis of promoter GFP-reporter strains showed a different role in governing cell-type specificity between the proximal and distal promoter of _alr3808_. We here confirmed the heterocyst specificity of the distal promoter of _alr3808_ and describe a very early induction of its expression during pro-heterocyst differentiation. In contrast, the complete promoters of both genes were active in all cells. Even
though *Npun_R5799* and *alr3808* are orthologs, the regulation of their respective expression differs, indicating distinctions in the function of these cyanobacterial Dps proteins depending on the strain and cell-type.

**Introduction**

Cyanobacteria are a diverse group of prokaryotes capable of oxygenic photosynthesis. Some genera also have the capacity to fix atmospheric N\textsubscript{2} into ammonia. These two processes are normally incompatible but cyanobacteria have evolved a range of protective strategies (Bergman *et al.*, 1997; Thiel, 2004). One such strategy, used by a group of filamentous cyanobacteria, is to, in times of combined nitrogen limitation, differentiate a new cell-type, the heterocyst, at semi-regular intervals along the filaments (Wolk *et al.*, 1994; Kumar *et al.*, 2010). During heterocyst differentiation the cell undergoes a range of modifications to its morphology, physiology and genome. The end result is a cell-type with a severely reduced internal oxygen concentration suitable for nitrogen fixation. During growth on N\textsubscript{2} fixed carbon is supplied by the neighboring vegetative cells (Wolk, 1968; Cumino *et al.*, 2007) while heterocysts, which are incapable of oxygenic photosynthesis and carbon fixation, supply the vegetative cells with fixed nitrogen (Wolk *et al.*, 1974). Under laboratory conditions, differentiation of a vegetative cell into a mature N\textsubscript{2}-fixing heterocyst takes approximately 20-24 hours depending on the growth rate (Adams, 2000). The first detectable step is the formation of pro-heterocysts that require further maturation before allowing the expression of nitrogenase, the enzyme complex responsible for nitrogen fixation. The formation of pro-heterocysts can be microscopically detected by the gradual thickening of the heterocyst envelope and changes in autofluorescence due to the degradation of
phycobilisome proteins associated with the inactivation of oxygenic photosynthesis (Maldener & Muro-Pastor, 2010). The nutritional stress (nitrogen limitation) and the metabolic changes that take place during the heterocyst differentiation process temporarily result in a cellular metabolic imbalance (Herrero et al., 2001), which might lead to an accumulation of reactive oxygen species (ROS). Although not fully understood at the cell-type specific level mechanisms aimed at protecting the cells against oxidative stress must be present in both vegetative cells and developing heterocysts. In heterocyst-forming cyanobacteria a wide range of enzymes involved in the defense against ROS, such as peroxiredoxins, catalases and DNA-binding proteins from starved cells (Dps) have been identified (Banerjee et al., 2013). It has also been shown that in cyanobacterial cells oxidative stress is closely connected to iron homeostasis (Latifi et al., 2009), which is interesting since N2-fixing cyanobacteria are known to contain higher levels of cellular iron (Kustka et al., 2003). The regulation of the iron metabolism is in turn linked to the regulation of the nitrogen metabolism through crosstalk between the two transcriptional regulators NtcA and FurA (Cheng et al., 2006; López-Gomollón et al., 2007).

Dps proteins, which belong to the ferritin superfamily, are involved in both iron homeostasis and the cellular defense against oxidative stress (Andrews, 2010; Chiancone & Ceci, 2010; Haikarainen & Papageorgiou, 2010). Two of the main functions of Dps proteins are to detoxify hydrogen peroxide and to serve as iron storage complexes. In addition, some members have also been shown to bind DNA and physically protect it from degradation. Multiple paralogous dps genes exist in heterocyst-forming cyanobacteria (Sato et al., 2012; Ekman et al., 2014), several of which are regulated by the availability of combined nitrogen both at the transcript (Ehira & Ohmori, 2006; Flaherty et al., 2011; Mitschke et al., 2011) and protein level (Stensjö et al., 2007,
Ow et al., 2008; 2009). Higher transcript or protein levels in heterocyst as compared to vegetative cells have been shown for several of the paralogs, indicating different physiological functions in the two cell-types (Ow et al., 2008; 2009; Ekman et al., 2014). The mechanisms that confer cell specificity and stress specific induction of the Dps proteins in multicellular cyanobacteria are however largely unknown. We present the transcriptional regulation and cell-type specificity of one uncharacterized dps gene in *Nostoc punctiforme*, Npun_R5799, and its ortholog alr3808 from *Anabaena* sp. PCC 7120 (from now on *Anabaena*). While both strains are heterocyst-forming they show different physiological adaptations, e.g. differences in genome size, ability to differentiate multiple cell-types and symbiotic competence (Meeks et al., 2001). With this work we aim at getting a deeper understanding of the regulation of Dps proteins in heterocyst-forming cyanobacteria.
Materials and Methods

Strains and growth conditions

*Nostoc punctiforme* ATCC 29133-S (also known as UCD 153; Campbell *et al.*, 2007), and mutant strains (promoter-GFP reporter strains *N. punctiforme* ATCC 29133-S UX1-UX5) were grown at 30 °C under continuous illumination of 40 μmol photons m\(^{-2}\) s\(^{-1}\) with gentle agitation.

The cells were either grown in BG11\(_0\), i.e. BG11 (Rippka *et al.*, 1979) without nitrate, or in BG11\(_0\) medium supplemented with 2.5 mM NH\(_4\)Cl and 5 mM HEPES-NaOH (pH 7.5). For nitrogen step down the cultures were normalized to 2 μg chlorophyll a mL\(^{-1}\) and cultivated in media with combined nitrogen for seven days. The cells were harvested, washed three times with BG11\(_0\) and re-suspended in BG11\(_0\). Samples were taken at different time points after nitrogen deprivation. Experiments involving *Anabaena*, the *ntcA* mutant strain CSE2 (Frías *et al.*, 1994) and the *hetR* mutant strain 216 (Buikema & Haselkorn, 1991) were carried out as previously described (Mischke *et al.*, 2011).
In silico analysis of promoter regions

Sequence alignment of 400 nt regions located upstream of the translational start sites of several orthologous dps genes from sequenced genomes of heterocyst-forming cyanobacteria were performed by using ClustalW2 (Larkin et al., 2007).

Primer extension analysis to identify 5'ends

Primer extension analysis was carried out as previously described (Muro-Pastor et al., 1999). RNA was isolated using hot phenol (Mohamed & Jansson, 1989). Oligonucleotides for primer extension analysis are listed in Table 1. The oligonucleotides were labeled using polynucleotide kinase (Fermentas) and γ-32P dATP. Primer extension was carried out with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. 10-20 µg RNA sample was used in each of the primer extension assays.

Promoter-gfp constructs

All replicative plasmids constructed are derivatives of pSUN119 (Argueta et al., 2004). Genomic DNA was isolated as previously described (Tamagnini et al., 1997). Different truncation fragments of the upstream regions of Npun_R5799 and alr3808 were amplified from genomic DNA of N. punctiforme and Anabaena sp. PCC 7120 respectively, using defined primer pairs (Table 1). The obtained PCR products were cloned into the pSUN119 vector (Argueta et al., 2004) using restriction digestion (PstI & KpnI), generating the plasmids pUX1, pUX2, pUX3, pUX4 and pUX5, respectively, bearing the distal, proximal and complete promoter regions of Npun_R5799, as well as the distal and complete promoter regions of alr3808. The constructs were transferred into N. punctiforme by electroporation (Holmqvist et al., 2009).
Fluorescence microscopy

Green fluorescent protein (GFP) fluorescence was analyzed by laser confocal microscopy as described previously (Ekman et al., 2014).

Results

The two orthologous dps genes Npun_R5799 and alr3808 are differentially regulated

A recent study in Anabaena identified two true transcriptional start sites (TSSs) upstream of alr3808 by using terminator exonuclease treatment (Mitschke et al., 2011), one of which showed a transcriptional dependence on the heterocyst regulatory transcription factor HetR (Kim et al., 2011). Transcript analyses were performed in order to examine the regulation of Npun_R5799 in comparison to alr3808 and its putative importance for the heterocyst-specific metabolism. Primer extension analysis of RNA isolated from Anabaena cultures confirmed the presence of two transcriptional start sites upstream of alr3808 (Figure 1A). The proximal TSS was located 44 nucleotides (nt) upstream of the translation initiation site (ATG) and the distal TSS was located 317 nt upstream of the ATG. In the case of N. punctiforme in both ammonium supplemented and N2-fixing cultures only a single putative TSS, identical in position to the proximal TSS in Anabaena, was identified (Figure 1A; Supplementary Figure 1). In Anabaena, transcription from both the distal and proximal promoters of alr3808 was enhanced by the removal of combined nitrogen (Figure 1). Furthermore, consistent with previous RNASeq data (Mitschke et al., 2011), primer extension analysis using RNA from the Anabaena mutant strains 216 (hetR) and CSE2 (ntcA) show that transcription from the distal promoter is HetR dependent and does not require
NtcA (Figure 1B). However, in RNA isolated from the Anabaena mutant strains CSE2 (ntcA) and 216 (hetR) the transcript originating from the proximal promoter (TSS2) was not severely affected by removal of either the main nitrogen regulator NtcA or the main heterocyst differentiation regulator HetR (Figure 1B). This indicates that expression from the proximal promoter of alr3808 does not require NtcA or HetR and is therefore most likely active in all cells of the filaments.

The promoter regions of alr3808, Npun_R5799 and orthologous genes among heterocyst-forming cyanobacteria are only partially conserved

The genomes of Anabaena and N. punctiforme both encode five Dps-like proteins, each forming a separate phylogenetic cluster together with different subsets of Dps proteins from other cyanobacterial species (Ekman et al., 2014). The corresponding genes from heterocyst-forming cyanobacteria within the cluster containing Npun_R5799 show a high sequence identity (82-90%) (Supplementary Table 1). We hypothesize that the strong conservation of the genes could indicate an important physiological function and similar regulation among these Dps proteins. To test this, we aligned the upstream regions (400 nt upstream of the ATG) of a subset of genes orthologous to Npun_R5799 from heterocyst-forming cyanobacteria (Supplementary Table 1; data not shown). In general, a low sequence conservation of the sequences aligning with the region surrounding the distal TSS (TSS1) in Anabaena was observed. However, the alignment of the distal promoter region of alr3808 (-400 to -300 nt upstream of the ATG) and its closest homolog in Anabaena variabilis ATCC 29413 showed a high sequence conservation, including sequences matching the DIF motif (TCCGGA) associated with heterocyst-specific expression (Mitschke et al., 2011), centered around -35 nt from the distal TSS of alr3808 (Figure 2A). The
low sequence conservation in *N. punctiforme* is in accordance with the lack of a distal TSS, as indicated in the previous section. In contrast, the aligned regions surrounding the proximal TSS (TSS2) were highly conserved for all the orthologous genes (Figure 2B). This region includes a conserved putative extended -10 box region, TGNTAN3T (Mitchell *et al.*, 2003), as well as a conserved position of the TSS in two previously investigated species, *Anabaena*, *Nodularia spumigena* CCY9414 (Mitschke *et al.*, 2011; Voß *et al.*, 2013) and the detected 5'-end in *N. punctiforme* (Figure 2B).

**Different cell-type dependent promoter activities of the upstream promoter regions of alr3808 and Npun_R5799**

To investigate the cell-type specificity of the different promoter regions of *Npun_R5799* and *alr3808*, GFP fluorescence emission was examined from strains bearing different promoter-GFP reporter fusions in a WT *N. punctiforme* background (Figure 3). The strains containing the complete promoter regions, including both the distal and proximal promoters, of *alr3808* and *Npun_R5799* showed GFP fluorescence emission in all cell-types along the filaments of *N. punctiforme*. This was true for filaments from combined nitrogen-supplemented (0 h), as well as in N2-fixing cultures (24 h) (Figure 3). The GFP fluorescence originating from expression by the HetR-dependent distal promoter of *alr3808* (fragment -490 to -215 nt with respect to the ATG), including the DIF motif (Mitschke *et al.*, 2011), increased following the removal of combined nitrogen (Figure 3). Twenty four hours after the removal of combined nitrogen the fluorescence was completely specific to the mature heterocysts, recognized by their morphology, pattern along the filaments, and loss of auto-fluorescence (Figure 3). In contrast, the construct bearing the corresponding region from *Npun_R5799* (-460 to -139 nt with respect to the ATG) did not show
any GFP fluorescence emission under any of the tested conditions (Figure 3). Expression from the proximal promoter of Npun_R5799 mimicked the random distribution of fluorescence produced by the complete promoter region (Figure 3), confirming that only the proximal part of the investigated promoter region is active in N. punctiforme. In contrast, the heterocyst-specific expression from the distal TSS of alr3808 is clearly observed above the background expression of the proximal TSS in the strain containing the complete promoter region of alr3808 (Figure 3).

In order to further analyze the initiation of expression of the alr3808 distal promoter following nitrogen step down, changes in GFP-fluorescence was analyzed at different time points after the removal of combined nitrogen (Figure 4). The GFP accumulation from the alr3808 distal promoter was induced early during the formation of the pro-heterocysts, within 5.5 hours after the removal of combined nitrogen (Figure 4). These early pro-heterocysts lack morphologically distinct features but were localized at semi-regular intervals along the filaments in a similar fashion to the later appearance of distinguishable pro-heterocysts and mature heterocysts.

Discussion

Dps proteins are widespread among cyanobacteria and heterocyst-forming filamentous cyanobacteria typically have 2-6 isoforms (Ekman et al., 2014). The phylogenetic separation of the different isoforms into different clades implies evolutionary as well as functional differences both in activity and regulation (Alaleona et al., 2010). The work presented here is part of a larger exploration of the importance of ferritin-like proteins, including the Dps proteins, in the robustness and variable lifestyle of cyanobacteria, with specific focus on the multicellular cyanobacterium N. punctiforme. In addition to the role of Dps proteins in iron homeostasis,
binding and protection of DNA against oxidative damage, Dps proteins have shown differential regulation in response to changes in the availability of combined nitrogen as well as in different cyanobacterial cell-types (Ow et al., 2009; Mitschke et al., 2011; Ekman et al., 2014). In the present study we show that a distal TSS of the complex promoter of alr3808 is active specifically in the pro-heterocysts and heterocysts and is induced early in the differentiation process. Additionally, we show that both TSSs upstream of alr3808, which were previously identified in the promoter region of alr3808 using RNAseq (Mitschke et al., 2011), produce transcripts that reach the open reading frame (ORF). However, only the sequence upstream of one of these two TSSs is conserved among the majority of heterocyst-forming cyanobacteria (Figure 2). Although Npun_R5799 and alr3808 show high sequence similarity, their promoters seem to be regulated differently, and therefore these Dps proteins may have different roles in the physiology of heterocyst-forming cyanobacteria. The alignments of sequences from a broad selection of sequenced heterocyst-forming cyanobacteria were made possible by the recent contribution from the Cyano-GEBA initiative (Shih et al., 2013), e.g. allowing the identification of conserved features in promoters.

Our promoter activity studies indicate a similar cellular distribution of transcripts, from the proximal promoters of both Npun_R5799 and alr3808, along the filaments of both N2-fixing and ammonium grown cultures of N. punctiforme. The detected transcripts in the ntcA mutant strain of Anabaena (Figure 1B) also indicate that NtcA is not required for basal expression of alr3808. In addition to being regulated in response to nitrogen deficiency, alr3808 is also regulated by different metallo-regulators, such as FurA (the ferric uptake regulator) and Zur (FurB), which are involved in the control of redox homeostasis (Hernández et al., 2007; Sein-Echaluce et al., 2014).
Taken together, these observations indicate a complex regulation of the expression of this gene, suggesting an important physiological function of the encoded protein.

Previous studies have shown that the increased expression from the distal promoter of *alr3808* following removal of combined nitrogen is dependent on HetR (Mitschke *et al*., 2011), indirectly implying heterocyst specificity. Using promoter-GFP reporters we were able to directly show a distinct heterocyst-specific expression of the distal promoter of *alr3808* (Figures 3-4). The GFP signal was localized to pro-heterocysts 5.5 h after combined nitrogen depletion and the cell-type specificity was maintained in mature heterocysts. The early activation of the distal promoter of *alr3808* during pro-heterocyst formation in *Anabaena* is consistent with observations concerning early expression of a minimal DIF motif-containing promoter, that of the small RNA *NsiR1*, which is expressed in pro-heterocysts before any morphological sign of differentiation can be detected (Muro-Pastor, 2014). An early and transient induction of expression of *alr3808* during heterocyst differentiation has also previously been reported in large transcriptomic data sets following the exposure of filaments to nitrogen deprivation (Ehira & Ohmori, 2006; Flaherty *et al*., 2011; Mitschke *et al*., 2011). Increased expression of *alr3808* in heterocysts suggests that the encoded Dps protein may serve a specific role during or after heterocyst differentiation. However, lack of conservation in the distal promoter region in the majority of all heterocyst-forming cyanobacteria indicates a more species-specific function in *Anabaena* sp. PCC 7120 and the closely related strain *A. variabilis* ATCC 29413. It is important to state that this indication is based on sequence similarities and that the possible lack of distal TSS in the majority of heterocyst-forming cyanobacteria needs to be verified experimentally. Because we have shown cell-type specificity of the distal promoter of *alr3808* in *N. punctiforme* we also conclude that the
transcriptional machinery that recognizes the DIF motif and confers heterocyst specificity in *Anabaena* seems to be conserved in *N. punctiforme*.

In earlier phylogenetic studies Npun_R5799 and Alr3808 cluster together with more atypical Dps proteins e.g. DpsA-Te from *Thermosynechococcus elongatus* (Sato *et al.*, 2012; Ekman *et al.*, 2014). DpsA-Te exhibits different physiological properties as compared to the typical Dps proteins. By using Zn$^{2+}$ as an additional cofactor this Dps protein favors O$_2$, instead of H$_2$O$_2$ as the oxidant for Fe$^{2+}$ sequestration (Alaleona *et al*., 2010). Interestingly, Alr3808 from *Anabaena* has earlier been suggested to be involved in iron homeostasis rather than hydrogen peroxide detoxification (Sen *et al*., 2000; Hernández *et al*., 2007). During heterocyst differentiation, the cells experience nitrogen deprivation and at the same time their metabolism undergoes substantial reprogramming, which might result in increased accumulation of ROS and an altered iron homeostasis. Whether the main role of Alr3808 and Npun_5799 is involved in maintaining iron homeostasis, DNA-binding, peroxide tolerance or oxygen scavenging remains to be resolved.

**Conclusion**

Even though *Npun_R5799* and *alr3808* are orthologs, their transcriptional regulation varies, suggesting differences in the function of these Dps proteins between both strains and cell-types. Part of the complex regulation shown for the *alr3808* promoter suggests a specific role for Alr3808 in developing heterocysts. However, identifying the specific function/s of Alr3808 and Npun_R5799 will require further investigation. Together with earlier studies, our results
corroborate the existence of different roles for the conserved multiple paralogous Dps proteins present in heterocyst-forming cyanobacteria.

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References


Campbell EL, Summers ML, Christman H, Martin ME & Meeks JC (2007) Global gene expression patterns of *Nostoc punctiforme* in steady-state dinitrogen-grown heterocyst-
containing cultures and at single time points during the differentiation of akinetes and hormogonia. *J Bacteriol* **189**: 5247-5256.


Tables

Table 1. Oligonucleotides used in the present study. All primers are given in the 5’ to 3’ orientation. Primers named TS# were used to reconstruct the Npun_R5799 promoter regions and primers named ATS# were used for the promoter regions of alr3808.

<table>
<thead>
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<th>Primers</th>
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<th>Restriction site</th>
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<td>pUXS1 and pUXS3</td>
<td>Pst I</td>
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<td>pUXS2 and pUXS3</td>
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<tr>
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<td>Anchor Primer</td>
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Figure 1. Primer extension analysis of the 5'-ends of \textit{Npun}\textunderscore R5799 and \textit{alr3808}. Primer extension was carried out with RNA isolated from filaments of (A) WT \textit{Anabaena} sp. PCC 7120, WT \textit{N. punctiforme}, and (B) WT \textit{Anabaena} sp. PCC 7120 and the mutant strains CSE2 (\textit{ntcA}) and 216 (\textit{hetR}). RNA samples were collected from whole filaments growing in the presence of ammonium (0) or at different time points after combined nitrogen step down. Transcripts corresponding to the proximal TSS (TSS2) are indicated by -44 nt for both \textit{alr3808} and \textit{Npun}\textunderscore R5799. Transcripts corresponding to the distal TSS (TSS1) are indicated by -317 nt.
Figure 2. Sequence alignment of promoter regions of \textit{dps} genes orthologous to \textit{alr3808} and \textit{Npun_R5799} from other heterocyst-forming cyanobacteria. (A) Alignment of the 5'-region (-400 nt to -300 nt with respect to the translation start site) from the two orthologous \textit{dps} genes in \textit{Anabaena} sp. PCC 7120 and \textit{Anabaena variabilis} ATCC 29413. The DIF motif (Mitschke \textit{et al.} 2011) is depicted by a green box centered at -33.5 nt with respect to the distal TSS (TSS1) in \textit{Anabaena} sp. PCC 7120. (B) Alignment of the 100 nt upstream region of \textit{alr3808}, with respect to the translation start site, with the corresponding regions of 24 orthologous \textit{dps} genes. Locus tags for each corresponding gene can be found in Supplementary Table 1. Blue boxes indicate the -10 regions in (A) and the extended -10 regions (Mitchell \textit{et al.}, 2003) in (B) of the corresponding promoters. Red boxes, in both (A) & (B), indicate experimentally verified transcriptional start sites (TSS) in \textit{Anabaena} and \textit{Nodularia} and the detected 5'-end in \textit{N. punctiforme} (Mitschke \textit{et al.}, 2011; Voß \textit{et al.}, 2013; present study). Conserved nucleotides are labeled with “*”.
Figure 3. GFP fluorescence from promoter-gfp fusions in *N. punctiforme*. Filaments harboring the different promoter-gfp constructs were grown with combined nitrogen (0 h) or taken 24 h after the removal of combined nitrogen. The different promoter regions fused to *gfp* are indicated as distal, proximal or complete for the distal, proximal or complete promoter regions of *all3808* and *Npun_R5799*, respectively. The locations of the promoter fragments used, with respect to the translational start site, are indicated. GFP- and auto- fluorescence are presented as a green or red color, respectively. Heterocysts are distinguished by a reduced auto-fluorescence and indicated by ★. Scale bars = 10 µm.
Figure 4. GFP fluorescence from the *alr3808* distal promoter-*gfp* construct in *N. punctiforme* following combined nitrogen deprivation. The images on the top and bottom represent GFP- (green color) and auto- (red color) fluorescence, respectively. Six time points during the early stages of heterocyst differentiation, following combined nitrogen deprivation, are depicted. Scale bars = 10 µm.