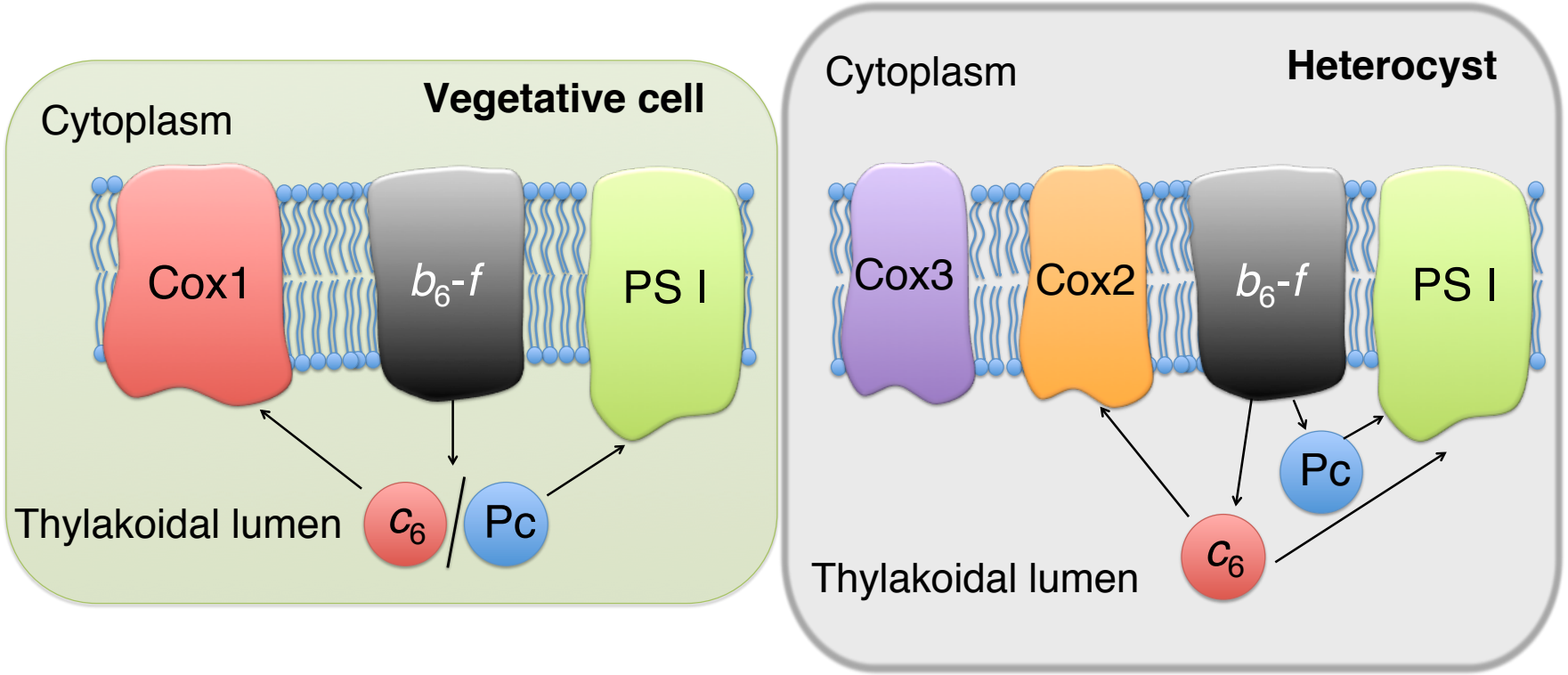
Cytoplasm
Vegetative cell
Cox1
b_6-f
PS I
Thylakoidal lumen
c_6
Pc
Cytoplasm
Heterocyst
Cox3
Cox2
b_6-f
PS I
Pc
c_6
Thylakoidal lumen

Cytochrome c_6 is the main respiratory and photosynthetic soluble electron donor in heterocysts of the cyanobacterium *Anabaena* sp. PCC 7120

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Abstract

Cytochrome c_6 is a soluble electron carrier, present in all known cyanobacteria, that has been replaced by plastocyanin in plants. Despite their high structural differences, both proteins have been reported to be isofunctional in cyanobacteria and green algae, acting as alternative electron carriers from the cytochrome b_6-*f* complex to photosystem I or terminal oxidases. We have investigated the subcellular localization of both cytochrome c_6 and plastocyanin in the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120 grown in the presence of combined nitrogen and under diazotrophic conditions. Our studies conclude that cytochrome c_6 is expressed at significant levels in heterocysts, even in the presence of copper, condition in which it is strongly repressed in vegetative cells. However, the copper-dependent regulation of plastocyanin is not altered in heterocysts. In addition, in heterocysts, cytochrome c_6 has shown to be the main soluble electron carrier to cytochrome *c* oxidase-2 in respiration. A cytochrome c_6 deletion mutant is unable to grow under diazotrophic conditions in the presence of copper, suggesting that cytochrome c_6 plays an essential role in the physiology of heterocysts that cannot be covered by plastocyanin.

Keywords

Cyanobacteria; cytochrome c_6; plastocyanin; cytochrome *c* oxidase, heterocyst; photosystem I.

Abbreviations: Cox, cytochrome *c* oxidase; Cyt, cytochrome; Pc, plastocyanin; PSI and PSII, photosystem I and photosystem II, respectively; sfGFP (superfolder green fluorescence protein).

1. Introduction

Cytochrome (Cyt) c_6 is the ancestral soluble electron donor to photosystem I (PSI) that operates in all known cyanobacteria and most green algae [1]. It is a typical class I *c*-type cytochrome, in which the heme group is covalently attached to a single polypeptide chain (molecular mass, ca. 9.5 kDa) with an α-helix folding pattern [2, 3, 4, 5]. Along evolution, Cyt c_6 has been replaced by plastocyanin (Pc), a classical member of the type I blue copper protein family that contains a single metal atom bound to a relatively small polypeptide chain (molecular mass, ca. 10.5 kDa) [1]. Thus, in plants and in some green algae, Pc acts as the sole soluble electron donor to PSI [6, 7]. However, both proteins are present in a wealth of cyanobacteria and green algae [8], holding up the current paradigm which establishes that both proteins are isofunctional, acting as electron carriers from the Cyt b_6-*f* complex to PSI (in photosynthesis) or Cyt *c* oxidase (Cox) (in respiration) [8, 9]. The convergence of Cyt c_6 and Pc in cyanobacteria clearly confers adaptation to changes in metal homeostasis, being Cyt c_6 highly expressed under copper deficiency and Pc expressed under copper sufficient conditions [8, 10, 11].

In photosynthetic eukaryotes, photosynthesis and respiration are spatially separated in different organelles. Nevertheless, in cyanobacteria both pathways converge in the thylakoids, the major sites of respiratory electron transport as well as photosynthetic light reactions [12, 13, 14, 15]. Thus, cyanobacterial thylakoids are unique due to the convergence of a variety of electron transport routes and the confluence of participating membrane and

soluble elements including plastoquinone, Cyt b_6-*f*, Cox, Cyt c_6 or Pc [15]. In cyanobacterial thylakoid membranes, PSI and photosystem II (PSII) complexes are heterogeneously distributed along the membrane [16], whereas Cyt c_6 and Pc have been located in the thylakoid lumen, participating in both respiratory and photosynthetic electron transport [17]. A second respiratory chain located in the cytoplasmic membrane has been also proposed to occur in *Synechocystis* [15, 18]. This alternative electron pathway might involve dehydrogenase complexes, plastoquinone, Cyt b_6-*f*, Cyt c_6, as well as Cox enzymes [19]. In addition, the presence of a periplasmic Cyt c_6 isoform has been suggested in filamentous cyanobacteria [20, 21], where it could donate electrons (from the periplasmic space) to a Cox in the plasma membrane. However, this alternative localization remains to be confirmed.

Filamentous cyanobacteria from groups IV and V [22] are able to differentiate heterocysts. Heterocysts are cells specialized in nitrogen fixation that differentiate from some vegetative cells in the absence of combined nitrogen [23]. Differentiation is aimed towards the physical separation in distinct cell types of two incompatible processes: oxygenic photosynthesis and nitrogen fixation. The oxygen-sensitive nitrogenase complex requires a microoxic environment that preserves it from the oxygen produced by neighbouring cells. In heterocysts, a fully structural and functional rearrangement of the thylakoid membranes is produced: thylakoids are partitioned in honeycomb and peripheral thylakoids [24], most of the photosynthetic antenna complexes are degraded, oxygen production at PSII is abrogated and photosynthetic CO_2 fixation is blocked [25, 26]. The nitrogenase enzyme requires extensive

amounts of reducing agents and ATP, which are respectively supplied by the catabolism of sugars imported from vegetative cells and by the activity of FoF1-ATP synthase, sustained by the electrochemical gradient created by cyclic electron transport around PSI in the presence of light [27]. Another metabolic trait of heterocysts is a high oxygen consuming activity based on terminal respiratory oxidases, as well as flavodiiron proteins that directly reduce oxygen by the Mehler reaction [28, 29]. In particular, both the heterocyst-specific oxidases Cox2 (a copper-dependent oxidase) and Cox3 (a specific quinol oxidase), highly expressed in heterocysts [29], are required for the elimination of molecular oxygen and to maintain the requirements of the nitrogenase activity [29]. There is no information about the electron donor to Cox2, which is the sole copper oxidase of the heterocyst [29].

Here we have investigated the subcellular localization of both Cyt c_6 and Pc in filaments of *Anabaena* sp. PCC 7120 grown in the presence of combined nitrogen and under diazotrophic conditions. The functional role of these two soluble electron carriers in the respiratory electron transfer chain in heterocysts, has been also studied. Our results indicate that in the presence of copper Cyt c_6 is downregulated in vegetative cells but expressed in heterocysts. In addition, in heterocysts, Cyt c_6 is the main soluble electron donor to Cox2, pointing to a crucial function of this ancestral cytochrome in these specialized cells, not only for the respiratory electron transfer chain, but also for the maintenance of an efficient cyclic photosynthesis.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Anabaena sp. PCC 7120 strain, as well as derived strains, were grown in BG11 (containing $NaNO_3$ or NH_4Cl as N source) or $BG11_0$ (lacking any source of combined nitrogen) medium at 30 ºC under light conditions (25 $\mu E \cdot m^{-2} \cdot s^{-1}$ from led white lamps). BG11 and $BG11_0$ media also contained 0.3 µM $CuSO_4$. For cultures in absence of copper, $CuSO_4$ was not added to the trace metal mix. Cultures were maintained in a shaker when liquid (100 r.p.m.) or in solidified medium with 1% (w/v) Difco agar. The antibiotics Streptomycin and Spectinomycin were used at a concentration of 5 $\mu g \cdot mL^{-1}$. Heterocyst formation was induced in $BG11_0$ medium, for which, cells in BG11 were harvested by centrifugation (3,000×*g*) and washed three times with the appropriate medium.

For analysis of cyanobacterial growth, cultures were inoculated with 0.2 µg $Chl \cdot mL^{-1}$ giving an OD750 nm of about 0.05 (light path, 1 cm) and grew logarithmically until reaching an OD750 nm of about 0.8–0.9. For sampling, the suspensions of filaments were carefully homogenized with a pipette.

Escherichia coli DH5α was used for plasmid constructions, whereas the strains HB101 and ED8654 were used for conjugations with *Anabaena*. *E. coli* cells were grown in LB medium, supplemented with the appropriate antibiotics at standard concentrations [30].

2.2. Strains construction

Labelled strains were obtained by translational fusion of the superfolder green fluorescence protein (sfGFP) to either Cyt c_6 and Pc from *Anabaena* sp. PCC

7120. The entire genomic regions containing the promoter and open reading frame of *petJ* or *petE* genes (encoding respectively for Cyt c_6 and Pc) were amplified by PCR by using the following primer pairs: *petJ*-F (TTAAGCTTCATGCTGTAACTGGC) and *petJ*-R (CCGGGCTAGCGCCGCCGCCGCCCTTCCAATCTGCATC); *petE*-F (CACCCCAGGTCTAAGAGTGCAG) and *petE*-R (AAGCAAGGTCTCACGCCGCCGGCGACAGTGA), respectively. PCR fragments were cloned into the pCSAL34 plasmid (bearing the *sfGFP-mut2* gene [31]). Fusions were then subcloned into the conjugative plasmid pCSV3 [32], producing the plasmids pCAT2 and pCAT6, bearing the *petJ-sfGFP* and *petE-sfGFP* translational fusions. Plasmids were introduced in *E. coli* by transformation and in *Anabaena* by conjugal transfer, as previously described [33]. The genetic structure of selected clones from each mating was tested by PCR with total DNA from the clone as template and using the forward primers of each construction and the reverse primer gfp4 (TCATGTTTGTATAGTTCATCC) to check the presence of the *sfGFP* fusions.

To inactivate the *petJ* gene, flanking regions of the gene were amplified from genomic DNA of *Anabaena* sp. PCC 7120 by using the primers Alr4250-1 (TTTTGGATCCTTACTCTACCTTCCTC) and Alr4251-3 (ACTAAGCTTATGTCGCGTTCTCTCTGCA); and Alr4251-4 (CATAAGCTTTAGTAAATTCCACTTCAAG) and Alr4252-1 (AGAGGATCCTTGTCTGCTGTCACTA), respectively. Fragments were fused by the megaprimer method [34] and cloned in the pSparK I vector (Canvas Biotech), rendering plasmid pVM7. The absence of mutations in the amplified

fragments was corroborated by sequencing. The C.S3 cassette from plasmid pCSE120 (S.K3/L.HEH2 (BamHI)/C.S3 (BamHI); nomenclature as in [33]) was introduced with HindIII into pVM7, rendering the pVM8 plasmid. The entire construct was transferred to the mobilizable plasmid pRL278 through BamHI, rendering plasmid pVM9. Conjugation of *Anabaena* and selection of double recombinants was carried out as previously described [35].

2.3. Western blot analysis

Total cell extracts were supplemented with one-sixth volume of sample buffer containing 375 mM Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 600 mM DTT and 0.012% bromophenol blue, incubated at 95 °C for 5 min, loaded and run in a 15% Laemmli SDS-PAGE system, and transferred to PVDF membrane filters. For detection of Cyt c_6, the procedure described in [36] was used.

2.4. Microscopy

For confocal microscopy, samples were visualized using a 63×1.4 NA oil immersion objective attached to a Leica TCS SP2 or Leica SP5 confocal laser-scanning microscope. sfGFP was excited using 488 nm irradiation from an argon ion laser. Fluorescent emission was monitored by collection across windows of 500-540 nm (sfGFP imaging) and 630-700 nm or 670-720 nm (cyanobacterial autofluorescence). Fluorescence intensity was quantified with the Image J program (version 1.41) and values were processed with the

Microsoft Excel Program (version 14.7).

2.5. Protein expression and purification procedures

Recombinant *Nostoc* sp. PCC 7119 Cyt c_6 and Pc, whose aminoacidic sequences are identical to those from *Anabaena* sp. PCC 7120, were expressed and purified as previously described [37]. Horse-heart respiratory Cyt *c* was purchased from Sigma-Aldrich (c2506). Chlorophyll concentration was determined according to [38]. Heterocysts were isolated from filaments grown in bubbled cultures in media lacking combined nitrogen. Filaments treated with 1 $mg \cdot mL^{-1}$ lysozyme were disrupted by passage three times through a French pressure cell at 3,000 psi, in a buffer containing 50 mM imidazole and 0.5 mM EDTA (pH 8.0). Heterocysts were then collected by successive centrifugation steps at 200 $\times g$ for 10 min at room temperature. Final heterocysts pellets were suspended in 20 mM HEPES, pH 7.5. The purity of heterocyst preparations was examined by microscopy. Membranes enriched in Cox, either from whole filaments or heterocysts, were obtained according to [39].

2.6. Oxygen uptake and nitrogenase activities

Respiration rates were assayed by measuring the rate of O_2 evolution using an Oxygraph O_2 electrode (Hansatech, Cambridge, UK) in a double jacket thermoregulated glass vessel as described in [40], with minor modifications. The temperature was kept constant (25 °C) in the darkness. For Cox activity

determinations, the reaction mixture contained, in a final volume of 1 mL, 5 mM HEPES buffer (pH 7.5), 2.5 mM NaCl, 2 mM sodium ascorbate, an amount of Cox-enriched membranes equivalent to 3–5 $mg \cdot mL^{-1}$ of total protein and 20 μM of *Anabaena* Cyt c_6, *Anabaena* Pc or horse heart Cyt *c*.

Nitrogenase activity was determined as previously described [41] using the acetylene reduction assay in filaments incubated for 48 h in $BG11_0$ medium.

3. Results and discussion

3.1. Subcellular localization of cytochrome c_6 and plastocyanin

In order to ascertain the role of Cyt c_6 and Pc in heterocysts, we have constructed translational fusions of the C-terminal end of each protein to the sfGFP. Since a luminal thylakoid localization is well-established for both proteins, we have used a sfGFP version instead of the standard GFP one. sfGFP is a more stable folding-enhanced GFP, as it contains the 'cycle-3' mutations showing improved tolerance of circular permutation, greater resistance to chemical denaturants and improved folding kinetics [42]. This GFP version has been successfully used to localize proteins in the periplasmic side of the heterocysts membrane, a compartment equivalent to the thylakoid lumen [43]. *Anabaena* strains Cyt c_6-sfGFP and Pc-sfGFP, containing a translational fusion of Cyt c_6 and Pc to the sfGFP, respectively, were generated by a single recombination event, resulting in copies in the genome of both the native gene

and the sfGFP fusion (Suppl. Figure 1). Both strains grew at similar rates as the wild-type strain and did not show any phenotypic defect.

In BG11 medium, containing nitrate or ammonium as nitrogen source, heterocysts development is repressed and all the cells grow in a vegetative state [23]. Under these conditions, it is already known that Cyt c_6 expression is strongly downregulated by copper, whereas Pc expression is strongly induced by the presence of this metal [44, 45, 46]. As expected, we found a clear regulation by copper, exhibited by the expression of Pc and Cyt c_6 in presence and absence of the metallic ion, respectively (Figure 1). sfGFP signals of both translational fusions were detected in the thylakoid lumen, merging with the red fluorescence of photosynthetic pigments. This result validates the use of the sfGFP version to label protein location in the thylakoid lumen.

In $BG11_0$ medium, lacking combined nitrogen, about one of each ten cells in the filament differentiates to heterocysts in a semi-regular pattern [23]. In this condition, both Cyt c_6 and Pc were expressed in vegetative cells according to the copper dependence described above (Figure 2A). However, whereas Pc-sfGFP showed the same copper regulation pattern in both vegetative cells and heterocysts –being expressed in the presence of copper and downregulated in the absence of this metal– (Figure 2B), Cyt c_6-sfGFP was detected in heterocysts in the presence of copper at similar levels than in its absence (Figure 2B). In order to stablish the timing of expression of the Cyt c_6-sfGFP in heterocysts, a time course of induction in $BG11_0$ from ammonium grown cells in the presence of copper was performed (Figure 3). We found detectable GFP signal in heterocysts after 24 h of nitrogen deprivation, reaching

a maximum expression level after 30 h, when the heterocyst is completely mature.

Interestingly, in heterocysts, both Cyt c_6 and Pc locate preferentially in the honeycomb membranes, a thylakoidal domain formed by highly contorted membranes densely packed at the sub-polar regions of the cell. Honeycombs harbour the membrane complexes involved in respiration, like the oxidases Cox2 and Cox3, as well as other components related to the cyclic phosphorylation involving Cyt b_6-f complex and PSI [27, 29], that might interact with Pc and Cyt c_6. According to our results, it is important to point out that in the absence of copper (and therefore in absence of Pc) Cyt c_6 is the only electron carrier that might be involved in these activities, meanwhile in the presence of copper both Cyt c_6 and Pc coexist in the heterocyst at similar levels.

Several possibilities can be considered in order to explain the expression in the heterocyst of Cyt c_6 at significant levels in the presence of copper. Copper is a redox toxic agent, and thus the intracellular concentration of free copper is assumed to be rather low. Although in cyanobacteria copper is transported across the plasma and thylakoidal membranes by the consecutive action of two different P-type ATPases, in the cytoplasm copper is chaperoned by the protein Atx1, whereas Pc acts itself as a copper-quelant protein in the thylakoidal lumen [47, 48]. However, the heterocyst envelope strongly limits diffusion from outside the cell, whereas direct intercellular protein trafficking through septal junctions is limited by the size of the channels [49]. Therefore, the expression of Cyt c_6 in the heterocyst in the presence of copper in the medium would reflect the limitation of this metal to access inside of this

specialized cell and repress Cyt c_6 expression. However, it has to be noted that the existence of still unexplored regulatory mechanisms of the expression of Cyt c_6 in the heterocyst cannot be ruled out.

3.2. Functional analysis of the reaction of cytochrome c_6 and plastocyanin with terminal oxidases

In order to elucidate a possible distinctive function of Cyt c_6 in heterocysts, the reaction of Cyt c_6 and Pc with membranes isolated from heterocysts was studied. First, membranes from vegetative cells grown in the presence of nitrogen, containing the terminal oxidase Cox1 [29], were used as a control (Figure 4). Addition of Cyt c_6 and Pc strongly promoted the O_2 consumption rate in vegetative cells, while the addition of the unspecific electron transfer horse respiratory Cyt *c* did not. Horse respiratory Cyt *c* shows a basal bimolecular rate constant for electron transfer to cyanobacterial terminal oxidases because it does not specifically interact with them [50]. These results are compatible with a specific and efficient reaction of Cyt c_6 and Pc with Cox1.

In heterocysts, expression of Cox1 is annulled, being substituted by the copper oxidase Cox2 and the quinol oxidase Cox3 [29]. In wild-type heterocysts membranes, only Cyt c_6 promoted a high O_2 consumption rate, whereas Pc showed a respiration rate equivalent to the obtained with the unspecific horse Cyt *c* donor (Figure 4). This result points to a selective function of Cyt c_6 instead of Pc in respiration. O_2 consumption rates were also studied in membranes from isolated heterocysts obtained from two deletion mutant strains (ΔCox2 and

ΔCox3) deficient in the heterocyst specific oxidases Cox2 and Cox3, respectively [29]. Heterocyst membranes from the ΔCox2 mutant did not showed any traceable O_2 consumption activity with any of the electron carriers tested (Figure 4), confirming that Cox2 is the main terminal electron acceptor of the soluble protein donors in heterocysts. However, the ΔCox3 mutant showed a behaviour qualitatively similar to the wild-type strain. Thus, a high O_2 consumption respiratory rate was found only in the presence of Cyt c_6, whereas rather low activities were measured when using Pc or respiratory horse Cyt *c*. The activity observed with Cyt c_6 as electron donor was equivalent to that from wild-type heterocysts membranes (Figure 4). This result strongly indicates that Cyt c_6 is the main electron donor of Cox2 in heterocysts, the physiological functional interaction between Cyt c_6 and Cox2 promoting a respiratory route of electrons from the Cyt b_6-*f* complex to Cox2 in heterocyst membranes.

In vegetative cells, it has been shown that Cyt c_6 and Pc are both involved in photosynthetic electron transport, donating electrons to PSI and the respiratory chain [51, 52]. This redundancy might confer plasticity to both processes under environmental changes in metal availability. Nonetheless, our results suggest that in heterocysts Pc mainly acts in photosynthesis, whereas Cyt c_6 efficiently donates electrons both in respiration and photosynthesis. Since respiration is an essential process that maintains low oxygen levels in heterocysts, the expression of Cyt c_6 in heterocysts in the presence of copper in the medium might be crucial for nitrogen fixation and diazotrophic growth.

3.3. Phenotype of the ΔCyt c_6 mutant

In order to ascertain the role of Cyt c_6 in the physiology of heterocysts, a deletion mutant of the *petJ* gene was generated by substituting the open reading frame (ORF) by a C.S3 cassette, conferring resistance to Streptomycin and Spectinomycin, rendering the CVM9 deletion strain (Suppl. Figure 2A).

First, expression of Cyt c_6 was studied both in the wild-type and the ΔCyt c_6 (CVM9) mutant strains by means of western blot. Total protein extracts of both strains grown without copper and with combined nitrogen were prepared, and western blotting with antibodies raised against the Cyt c_6 were performed (Suppl. Figure 2B). In the wild-type strain, a band of ca. 9.7 kDa, corresponding to the molecular weight of Cyt c_6 was obtained in the absence of copper. In the ΔCyt c_6 mutant, the absence of this band corroborated the lack of Cyt c_6.

Growth tests in different media in the presence or absence of copper and combined nitrogen were carried out (Figure 5). As expected, the ΔCyt c_6 mutant was unable to grow in the absence of copper in the medium, independently of the nitrogen source used (Figure 5A). In this condition Pc is repressed and Cyt c_6 is the sole soluble electron donor from the Cyt b_6-*f* complex to PSI in vegetative cells, and to Cox1/Cox2 oxidases in vegetative cells and heterocysts, respectively. Thus, in the deletion mutant both photosynthesis and respiration, essential for a phototrophic growth, are compromised. In presence of copper and combined nitrogen, the growth rate of the ΔCyt c_6 mutant was not statistically different to that of the wild-type strain (Figure 5B). In this condition, Pc is being expressed in vegetative cells, where it replaces Cyt c_6 function both

in photosynthesis and respiration. However, under diazotrophic conditions the growth of the ΔCyt c_6 mutant was severely impaired in the presence of copper (Figure 5B). These results are in clear agreement with the data described above concerning the expression of Cyt c_6 in wild-type heterocysts in the presence of copper and the exclusive role in respiration of the heme protein.

In order to elucidate if the ΔCyt c_6 mutant phenotype is due to inactivation of the nitrogenase enzyme, affecting nitrogen fixation –maybe by the blocking of the oxygen-consuming respiratory process–, nitrogenase activity, measured as the production of ethylene from acetylene, was determined in oxygenic conditions. Nitrogenase activity was not affected in the ΔCyt c_6 mutant (5.36 ± 1.04 nmol·mgChl^{-1}·h^{-1} in the wild-type and 5.84 ± 1.50 nmol·mgChl^{-1}·h^{-1} in ΔCyt c_6 mutant strain; p= 0.729). Similar results have been previously described in ΔCox2 and ΔCox3 single mutants [29]. However, nitrogen fixation is an energy demanding process, with a very high requirement of ATP and reducing equivalents (16 ATP per N fixed) [53] and nitrogenase activity, measured as the production of ethylene from acetylene, might not reflect the energy charge of heterocysts, because this procedure does not require ATP to produce acetylene [53]. It has been suggested that cyclic electron transport around PSI and sucrose supplied from the vegetative cells are the main sources of ATP and reductants, respectively, sustaining nitrogen fixation in heterocysts [54, 55, 56, 57] (Figure 6). Heterocyst thylakoids contain a much larger relative amount of proteins and protein complexes involved in cyclic photosynthetic electron transport and respiration than vegetative cell thylakoids, and thus, in the ΔCyt c_6 mutant strain, even in the presence of copper, the decrease in the levels of

soluble electron carriers in the heterocysts –in particular of Cyt c_6– could explain the lack of growth under diazotrophic conditions (Figure 6). In addition, it has been previously shown that PSI and ATP synthase complexes or terminal oxidases Cox2 and Cox3 are induced in heterocysts [12, 29]. In absence of copper, the electron transport from the Cyt b_6-*f* complex to PSI and Cox2 can be mediated in a satisfactory way by Cyt c_6. However, in presence of copper in the medium, both Pc and Cyt c_6 are expressed in heterocysts. Quantification of the GFP fluorescence shown in Figure 2 clearly shows that the expression of both proteins in the heterocysts is significantly higher than in vegetative cells, as expected from the required high electron-transfer activity of this specialized cell type.

Finally, the fact that the growth in diazotrophic conditions of ΔCyt c_6 mutant was severely impaired in the presence of copper can be globally explained in terms of an important decrease of the energetic charge of heterocysts, due not only to an impaired electron transport to Cox2, but also to a diminished PSI activity. Actually, in vivo experiments have previously shown that in *Anabaena* Cyt c_6 acts more efficiently than Pc as reducing PSI [58]. Both Cyt c_6 and heterocysts can be considered as an ancestral protein and cells, respectively. Cyt c_6 evolved from the bacterial cytochromes in anoxygenic photosynthesis, although Pc was later adopted as its alternative. By this turn, the heterocyst presents metabolic similarities with the primitive anoxygenic photosynthetic bacteria with type-I reaction centres. It can therefore be speculated that Cyt c_6 maintains in the heterocyst specific functions necessary for the functioning of this cell and related to their ancestral origin.

4. Key findings/conclusions

The structure-function relationship of Cyt c_6 and Pc has been intensively studied. These studies have established the dogma that both proteins, despite having different structures, perform the same function acting as alternatively soluble electron transports from the Cyt b_6-f complex to PSI (in the photosynthetic electron transport chain) or to the Cox (in the respiratory electron transport chain), Pc being expressed in presence of copper and Cyt c_6 being expressed under copper deficiency conditions. However, our results suggest that Cyt c_6 plays an essential role in the physiology of heterocyst that cannot be covered by Pc. First, Cyt c_6 is significantly expressed in the heterocyst in the presence of copper, and the lack of the heme protein in these conditions impedes cell growth. Second, Cyt c_6 acts as the sole efficient soluble electron donor to Cox2. These findings might have a great relevance in the regulation of the heterocyst metabolism and in the N_2 fixation by the nitrogenase. In any case, the fact that the ΔCyt c_6 mutant does not growth under diazotrophic conditions, suggests that in heterocysts Cyt c_6 is not only fundamental for the electron transport to Cox2, but also crucial for the electron transfer to PSI and, in general, to maintain the high electron transfer rates need to attain the energetic levels requirements for nitrogen fixation.

Author contribution

F. P. M-H conceived the project. A. T., V. M., C. R-M and F.P. M-H carried out the experiments. J. A. N., V. M. and F.P. M-H interpreted the data and discussed the results. V. M., and F.P. M-H wrote the manuscript (with significant input from J. A. N.), which was corrected, revised and approved by all authors.

Competing financial interest

The authors declare no competing financial interests.

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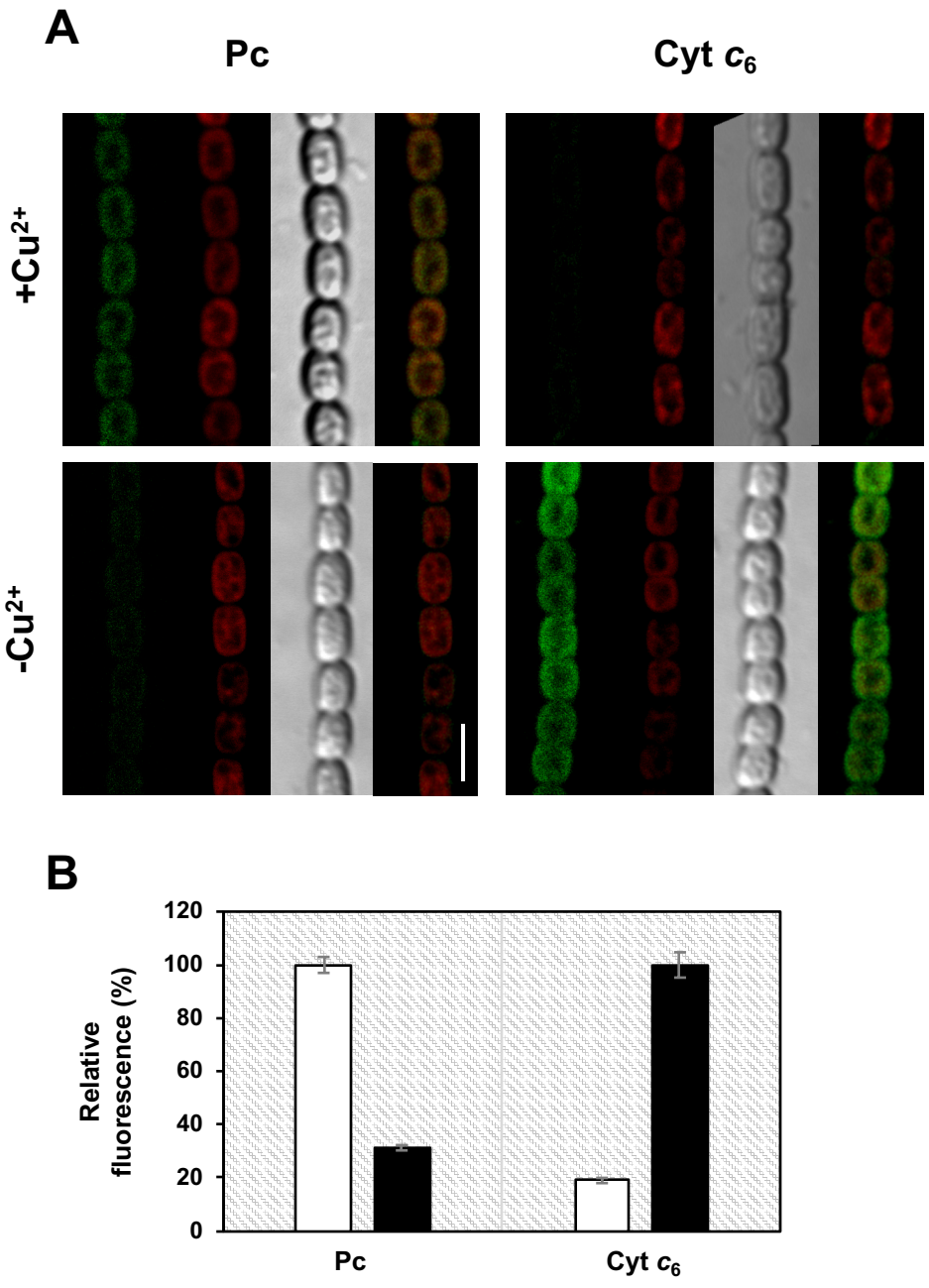


Fig. 1. (A) Micrographs showing the localization of Pc-sfGFP and Cyt c_6-sfGFP by fluorescence microscopy in presence (+Cu^{2+}) or absence (-Cu^{2+}) of copper, with combined nitrogen. In each micrographs panel, from left to right, are shown sfGFP-fluorescence (green), auto-fluorescence (red), bright-field illumination (grayscale), and an overlay (green + red). Brightness and contrast were enhanced to improve visibility. Scale bar, 5 µm. (B) Relative fluorescence of Pc-sfGFP and Cyt c_6-sfGFP in the presence (white) or absence (black) of copper in the medium.

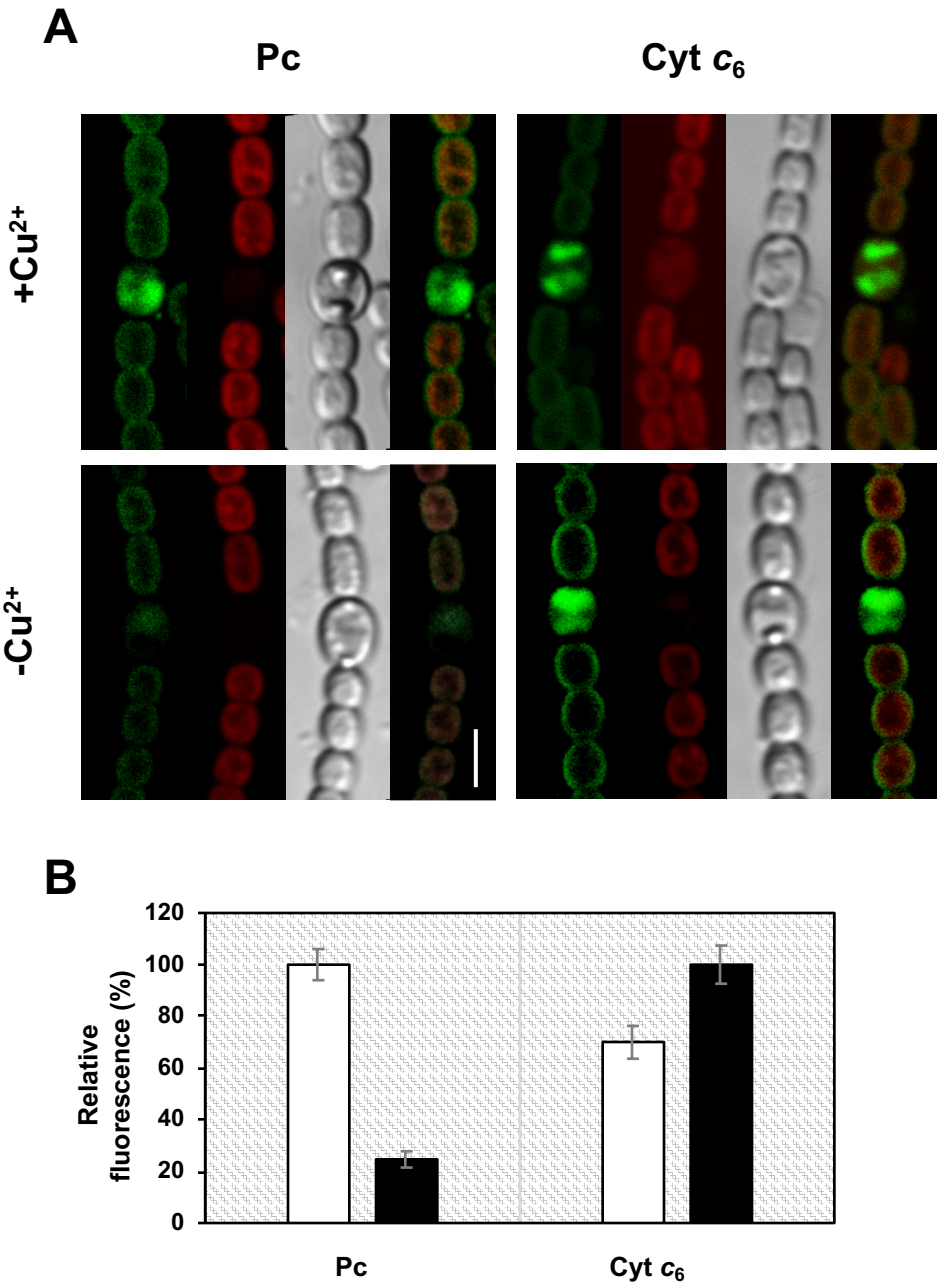


Fig. 2. (A) Micrographs showing the localization of Pc-sfGFP and Cyt c_6-sfGFP by fluorescence microscopy in presence (+Cu^{2+}) or absence (-Cu^{2+}) of copper, without combined nitrogen. In each micrographs panel, from left to right, are shown sfGFP-fluorescence (green), auto-fluorescence (red), bright-field illumination (grayscale), and an overlay (green + red). Brightness and contrast were enhanced to improve visibility. Scale bar, 5 µm. (B) Relative fluorescence of Pc-sfGFP and Cyt c_6-sfGFP in heterocysts, referred to maximum fluorescence of Pc-sfGFP or Cyt c_6-sfGFP, respectively, in the presence (white) or absence (blak) of copper in the medium.

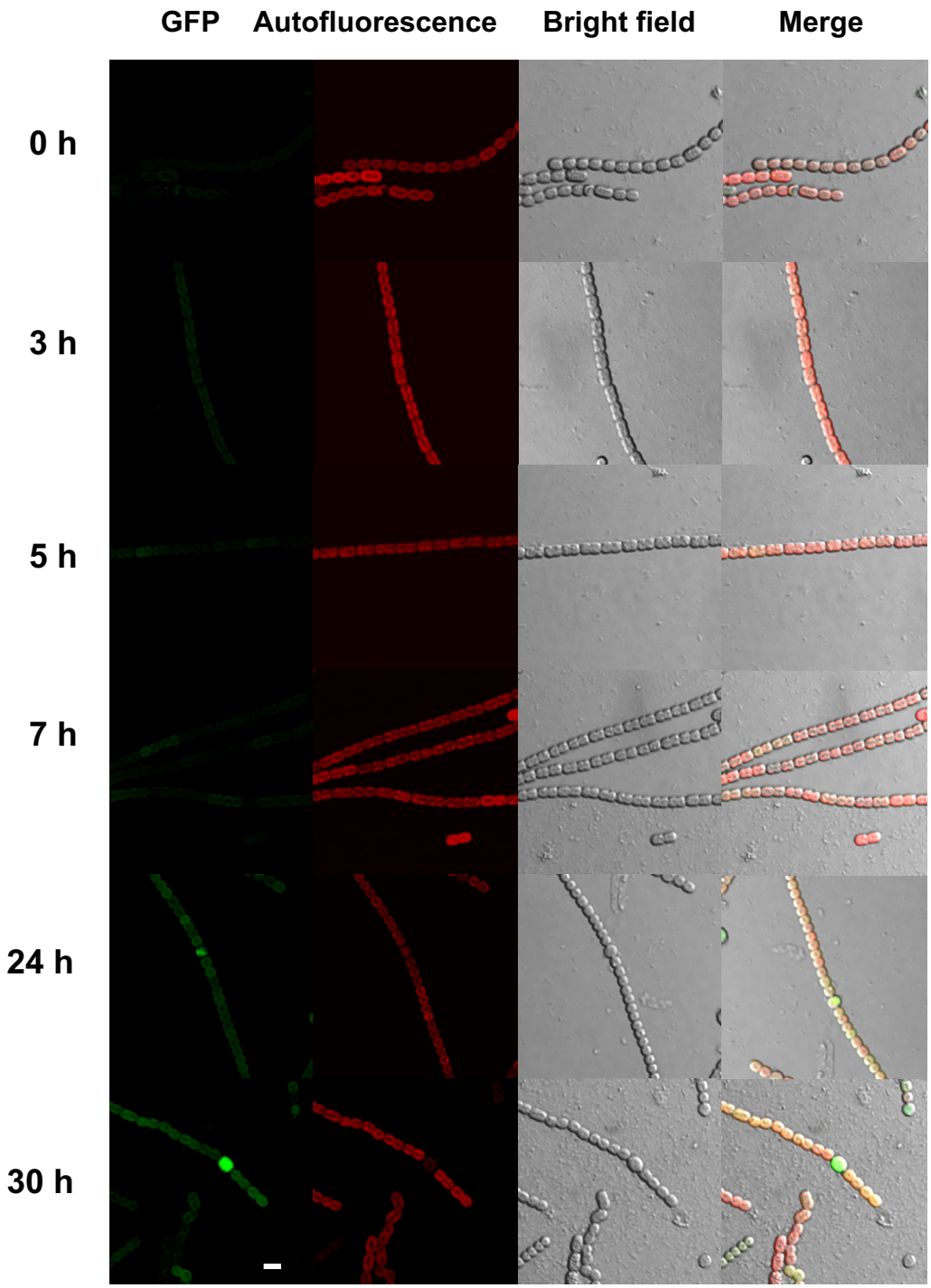


Fig. 3. Time course expression of Cyt c_6-sfGFP in response to nitrogen deprivation in the presence of Cu^{2+} in the culture medium. *Anabaena* were grown in ammonium-containing medium and incubated in the absence of combined nitrogen. The filaments were visualized by confocal microscopy at the times indicated in the figure. In each micrographs panel, from left to right, are shown sfGFP-fluorescence (green), auto-fluorescence (red), bright-field illumination (grayscale), and an overlay (green + red + bright-field illumination). Scale bar, 5 μm.

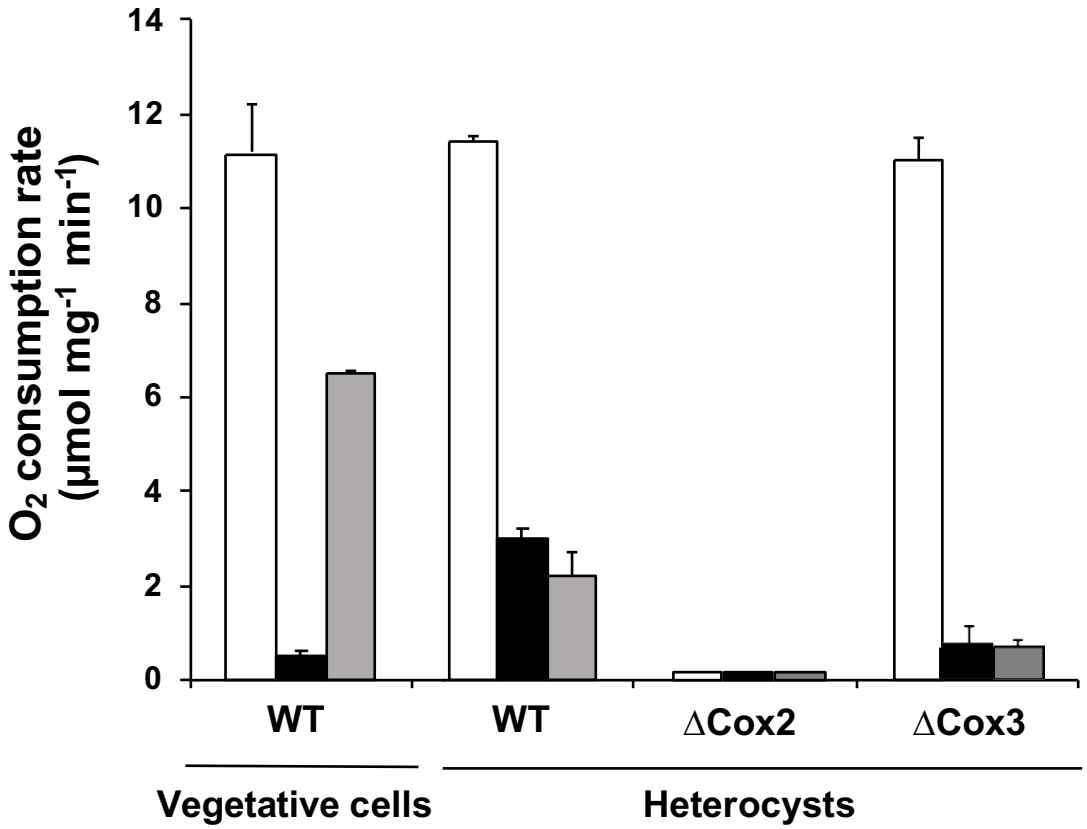


Fig. 4. Reaction of Cyt c_6 and Pc with terminal oxidases in isolated photosynthetic membranes. Rates of oxygen consumption were measured in the presence of Cyt c_6 (white), Pc (grey) or respiratory Cyt *c* from horse (black) in either vegetative or heterocyst cell membranes. Vegetative cells: membranes obtained of vegetative cells from wild-type strain, cultured in continuous light and with combined nitrogen. Heterocysts: membranes obtained of heterocyst cells from wild-type, ΔCox2 and ΔCox3 strains, cultured in continuous light and without combined nitrogen.

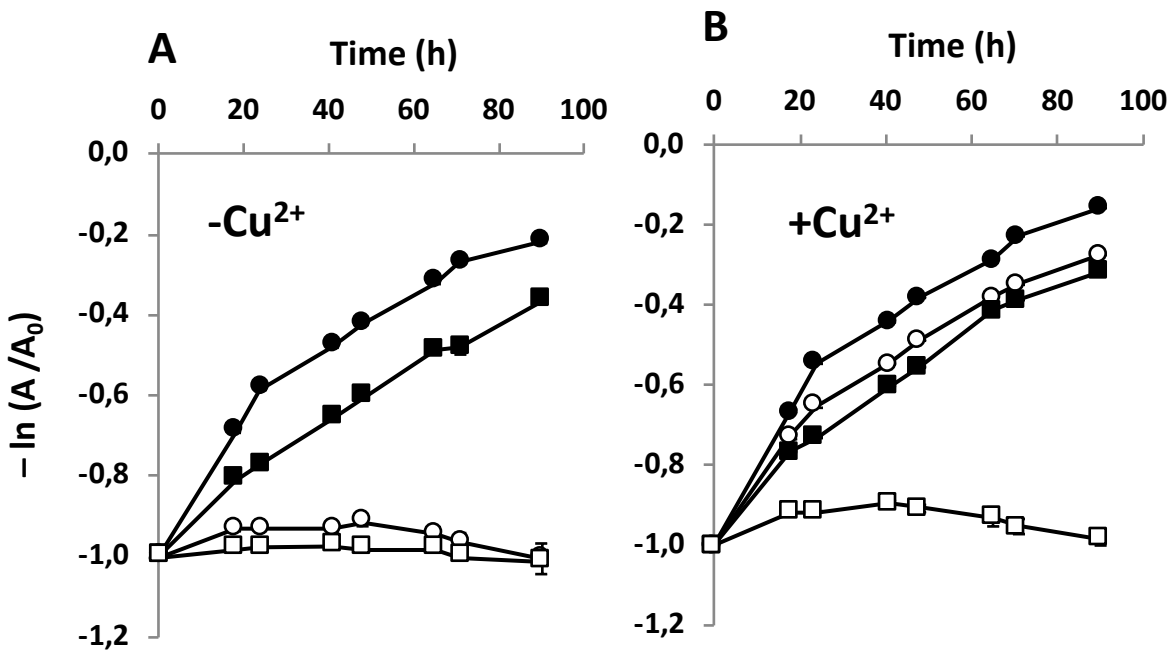


Fig. 5. Growth curve of the *Anabaena* wild-type (filled symbols) and ΔCyt c_6 mutant (empty symbols) strains in presence (circles) or absence (squares) of combined nitrogen, and without (A) or with (B) copper in the culture media.

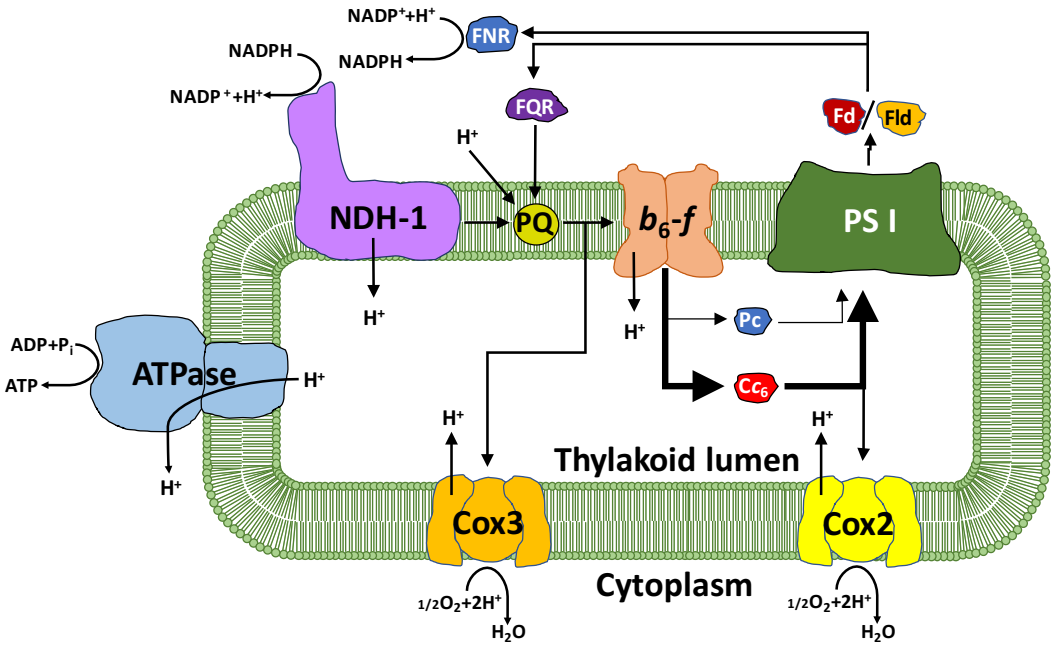
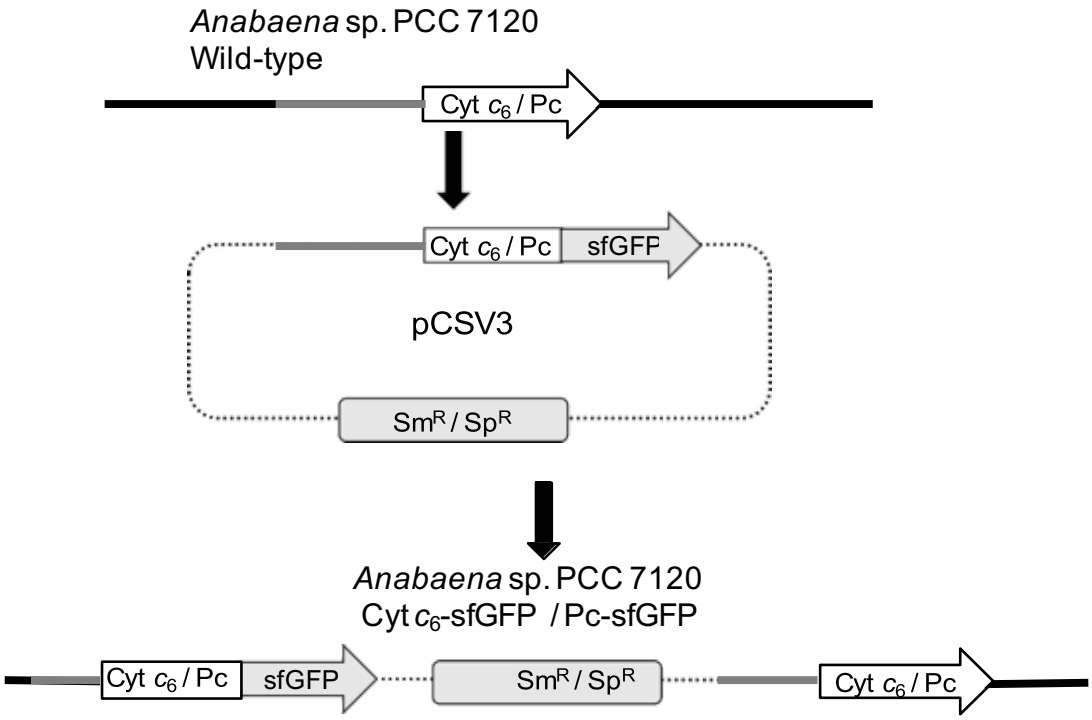
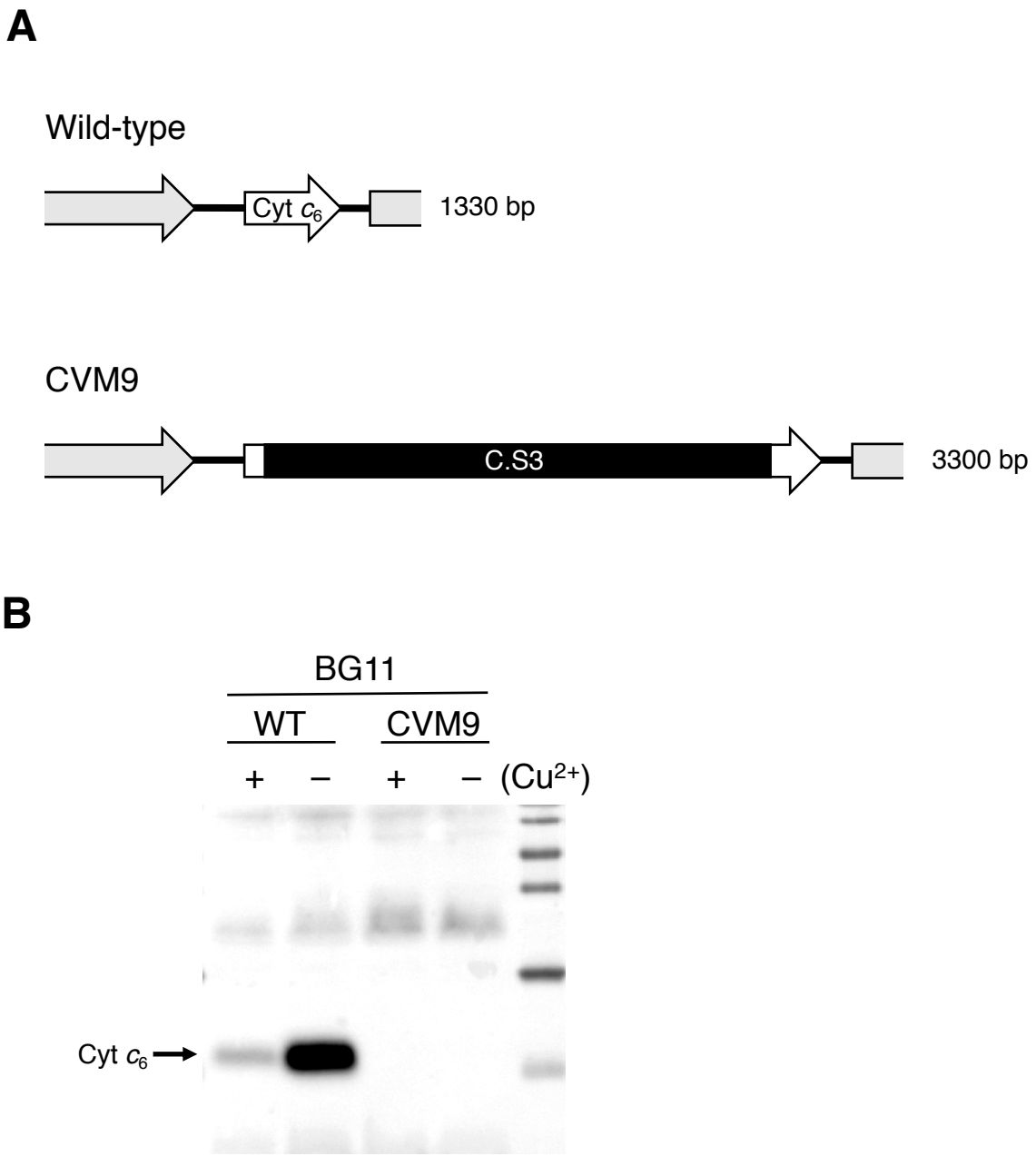


Fig. 6. Schematic diagram of the possible respiratory and photosynthetic electron transfer routes in the thylakoid membrane of heterocysts. NDH-1, NAD(P)H dehydrogenase1; FNR, ferredoxin-NADP+-reductase; FQR, ferredoxin-plastoquinone-reductase; PQ, plastoquinine; b_6-f, cytochrome b_6-f; Pc, plastocyanin; Cc_6, cytochrome c_6; PSI, photosystem I; Fd, ferredoxin; Fld, flavodoxin; Cox2, cytochrome *c* oxidase-2; Cox3, cytochrome *c* oxidase-3; ATPase, ATP synthase.



Suppl. Fig. 1. Scheme of the genetic constructs Cyt c_6_sfGFP and Pc-sfGFP. A DNA fragment containing the promoter region and the ORF of *petJ* or *petE* gene, translationally fussed to *sfGFP* sequence and cloned in plasmid pCSV3 was inserted in the *Anabaena* strain PCC 7120 by single recombination. Exconjugants, harbouring the construct in the chromosome, were selected as Sm^R/Sp^R clones. Ω denotes the transcription and translation stop points that harbour the C.S3 cassette, in order to avoid polar effects.



Suppl. Fig. 2. (A) Scheme of the wild-type *petJ* gene region and the genetic ΔCyt c_6 construct present in plasmid vector pVM9. Double recombinants that had inserted the C.S3 cassette in the chromosome by a double recombination event, and had lost the plasmid were selected as Sm^R/Sp^R and $sucrose^R$ clones respectively. Ω denotes the transcription and translation stop points that harbour the C.S3 cassette, in order to avoid polar effects. (B) Western blot analysis of Cyt c_6 in strains wild-type (WT) and Δ Cyt c_6 (CVM9), grown in BG11 medium in presence (+) or absence (–) of Cu^{2+}. The same amount of total protein from cell extracts was loaded for the two strains, and hybridization with an antibody raised against the Cyt c_6 was performed as described in Materials and Methods section.