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ORIGINAL ARTICLE

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Sucrose synthase and RuBisCo expression is similarly regulated by the nitrogen source in the nitrogen-fixing cyanobacterium *Anabaena* sp.

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Abstract In higher plants and cyanobacteria, sucrose (Suc) metabolism is carried out by a similar set of enzymes. The function and regulation of Suc metabolism in cyanobacteria has begun to be elucidated. In strains of *Anabaena* sp., filamentous nitrogen-fixing cyanobacteria, Suc synthase (SuS, EC 2.4.1.13) controls Suc cell level through the cleavage of the disaccharide. The present work shows that there are two *sus* genes in *Anabaena* (*Nostoc*) sp. that are co-regulated regarding the nitrogen source; however, only *susA* accounts for the extractable SuS activity and for the control of the Suc level. Primer extension analysis has uncovered the sequence of the *Anabaena susA* and *susB* ammonium-activated putative promoters, which share a high sequence similarity with that of *rbcLS* encoding ribulose bisphosphate carboxylase/oxygenase (EC 4.1.1.39) and other ammonium up-regulated genes. Moreover, *susA* and *rbcLS* expression is developmentally co-localized to the vegetative cells of the nitrogen-fixing cyanobacterial filaments. Our results strongly suggest the existence of a regulatory network that would coordinate the expression of key genes for Suc and nitrogen metabolism, carbon fixation, and development in *Anabaena* sp.

Keywords *Anabaena* sp. · Ammonium regulation · Cyanobacteria · Ribulose bisphosphate carboxylase/oxygenase · Sucrose metabolism · Sucrose synthase

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Abbreviations Chl: Chlorophyll · *gfp*: Green fluorescent protein gene · *rbcLS*: Ribulose bisphosphate carboxylase/oxygenase gene · RuBisCO: Ribulose bisphosphate carboxylase/oxygenase · *sus*: Sucrose synthase gene · Suc: Sucrose · SusB: Gene product of *susB* · SuS: Sucrose synthase · tsp: Transcription start point

Introduction

In higher plants, sucrose (Suc) metabolism is not only essential for the allocation of carbon resources but also participates in a regulatory network that coordinates metabolism and development. The intricate mechanism underlying this regulation is beginning to be uncovered (Smeekens 2000; Winter and Huber 2000; Coruzzi and Zhou 2001; Koch 2004; Rolland and Sheen 2005). In plants and cyanobacteria, Suc is synthesized from the sequential action of Suc-phosphate synthase (EC 2.4.1.14) and Suc-phosphate phosphatase (EC 3.1.3.24), and it can be cleaved by Suc synthase (SuS, EC 2.4.1.13) or irreversibly hydrolyzed by invertases (Inv, EC 3.2.1.26) when there is a high demand of hexoses (Winter and Huber 2000; Salerno and Curatti 2003). The role of SuS in higher plants is well documented. It has the dual capacity to direct carbon toward both polysaccharide biosynthesis (starch, cellulose, and callose) and respiration, it is crucial for carbohydrate import by heterotrophic tissues, and it is also related to environmental stress tolerance and symbiosis with other organisms (Koch 2004). SuS is encoded by a gene family (six genes in the case of *Arabidopsis thaliana*) with a complex expression pattern depending on tissue specificity and environmental and developmental signals (Baud et al. 2004).

Recent studies on cyanobacteria have shed some light on the physiological role of Suc metabolism and, particularly, on the function and regulation of SuS in oxygenic–photosynthetic prokaryotes (Salerno et al. 1995;

Salerno and Curatti 2003). When cells of *Anabaena* sp. or related strains are subjected to combined-nitrogen step-down, some of the vegetative cells of the filaments differentiate into heterocysts, which gain the capacity of nitrogen fixation but lose the function of CO_2 fixation. Thus, an active exchange of assimilated carbon and nitrogen occurs between both cell types (Wolk et al. 1994). In these cyanobacteria, both global nitrogen control and heterocyst differentiation is mainly exerted by *Nit*rogen *c*ontrol *A* (NtcA), a transcriptional activator of the CAP family, which binds to the consensus sequence $GTN_{10}AC$ (Herrero et al. 2004). NtcA activates the expression of genes involved in the utilization of alternative sources of nitrogen in the absence of ammonium, and also initiates a cascade of gene expression that triggers and maintains heterocyst differentiation (Golden and Yoon 2003; Herrero et al. 2004). Like other members of the CAP family, NtcA can act both as an activator or repressor of the target genes, depending on the relative location of the consensus sequence in the promoter. When ammonium is not available, NtcA binds to the σ^{70}–like −10 or −35 elements of the target gene promoters, preventing their expression, while ammonium relieves that inhibition. The gene encoding ribulose−1,5–bisphosphate carboxylase/oxygenase (*rbcLS*) is expressed only in vegetative cells (Elhai and Wolk 1990) and it has been proposed that it is repressed by NtcA in heterocysts (Ramasubramanian et al. 1994; Jiang et al. 1997; García-Domínguez et al. 2000; Herrero et al. 2001; Herrero et al. 2004). On the other hand, factor 2, an additional DNA-binding protein, is only present in vegetative cells and binds the *rbcLS* gene promoter, suggesting that it may act as an activator of *rbcLS* in those cells (Ramasubramanian et al. 1994).

Based on the determination of Suc related enzyme activities in *A. variabilis* cell extracts enriched in vegetative cell or heterocystic proteins, it had been proposed that SuS activity was mainly concentrated in the vegetative cells of the nitrogen-fixing filaments and was responsible for Suc synthesis (Schilling and Ehrnsperger 1985). However, the characterization of the *susA* gene in the *Anabaena* (*Nostoc*) sp. strain PCC 7119, coding for a SuS protein (SusA), and further studies showed that not only did SusA catalyze the cleavage of Suc in vivo, but also revealed that it is down-regulated after combined-nitrogen starvation (Curatti et al. 2002). It was also shown that Suc levels increase up to tenfold or decrease more than 50-fold in *Anabaena* mutant cells where *susA* was interrupted or over-expressed, respectively. Moreover, *susA* over-expression was detrimental only for diazotrophic growth. This evidence, together with the demonstration of an Inv activity localized to the *A. variabilis* heterocysts (Schilling and Ehrnsperger 1985), suggests the involvement of Suc in diazotrophic metabolism and that SusA may participate in the control of the carbon flux, from the vegetative cells to the heterocysts, in the nitrogen-fixing filaments (Curatti et al. 2002). A *susA* homologous gene, named *susB*, has also been identified in the *Anabaena* (*Nostoc*) sp. PCC 7120 genome (Curatti et al. 2002; Salerno and Curatti 2003).

In this study, we have examined the effect of ammonium on the expression of *susA* and *susB* genes and have used a mutational approach to clarify the contribution of SusB in the control of the Suc level in *Anabaena* sp. cells. We have also determined the sequences of the nitrogen source-regulated *sus*-genes putative promoters and shown that a developmental down-regulation of *susA* expression occurs in the heterocysts.

Materials and methods

Bacterial strains and growth conditions

Anabaena (*Nostoc*) sp. strain PCC 7119 and the derivative mutant strains LC30 (*susA::sp*), LC80–82 and LC90–92 (*susB::kan*), LC120–122 (*susA::sp*/*susB::kan*), LC150, LC160 and LC170 (green fluorescent protein (GFP) transcriptional fusion with *susA* promoter in sense, antisense, and without promoter, respectively) and AMC486 (GFP transcriptional fusion with *rbcLS* promoter in sense orientation) were cultured in a BG11 medium in the presence of different nitrogen sources, as previously described (Curatti et al. 2002). When required, antibiotics were added: neomycin (Nm) at 300 $\mu g\ ml^{-1}$ or 50 $\mu g\ ml^{-1}$ for solid or liquid medium, respectively; spectinomycin (Sp) and streptomycin at 1.5 $\mu g\ ml^{-1}$ or 5 $\mu g\ ml^{-1}$ each for solid or liquid medium, respectively. *Escherichia coli* DH5α, HB101, and ED8654 cells were grown in a Luria–Bertani medium and supplemented with different antibiotics at 37°C for cloning and conjugation (Elhai and Wolk 1988; Sambrook and Russell 2001).

Isolation, manipulation, and analysis of nucleic acids

Plasmids were isolated and modified according to standard protocols (Sambrook and Russell 2001). The isolation of genomic DNA from cyanobacteria was performed as previously described (Cai and Wolk 1990). RNA was isolated using the TRIZOL reagent (Invitrogen). RNA quality and polymerase chain reaction (PCR) products were analyzed by electrophoresis in 1% agarose gels.

Expression in *Escherichia coli* and purification of SusB recombinant protein

The BLAST analysis of the *Anabaena* (*Nostoc*) sp. strain PCC 7120 genome (http//:www.kazusa.or.jp/cyano/) uncovered an open reading frame (*orf all1059*) of 2,437 bp, 58% identical to SusA. The DNA fragment corresponding to that *orf* (tentatively called *susB*) was PCR amplified from *Anabaena* sp. PCC 7119 genomic DNA with the primers SS11B (CGGGATCCATGC

ACGAACTATTTCACCCTATTTTTGCTAATGGT) and SS12E (CGGAATTCGCTAATGACAACTCATG TGCTTCGCTAAAATTTG), containing a *Bam*HI or an *Eco*RI site, respectively. The amplicon was ligated into the corresponding restriction sites of the expression vector pRSET-A (Invitrogen) and the resulting plasmid was named pSSB1. *Anabaena* sp. PCC 7119 *susB* was completely sequenced and it was found to be 100% identical to the homologous sequence of the strain PCC 7120. The plasmid pSSB1was introduced into the *E. coli* strain BL21(λDE3) pLysS (Novagen). The induction of the expression and purification of the recombinant histidine-tagged SusB protein by Co^{2+} affinity chromatography (TALON resin, Clontech) was carried out as described previously (Cumino et al. 2002).

Reverse transcription polymerase chain reaction

First strand cDNA synthesis was carried out using total RNA (2.5 μg) and specific reverse primers and incubated in the presence of M-MLV reverse transcriptase (Curatti et al. 2002), following the manufacturer's instructions (Promega). For the synthesis of *susA* cDNA, two different oligonucleotides were used: SS4 (AGCGGTCGA CAATTCTCTTTCTGAGTCAATGGC) or SS6 (GGG ATGCGAGAGATAAAAGCTTCCAGGG) that anneal, down-stream or up-stream, the insertional mutation of *susA* in the strain LC30, respectively. For the synthesis of *susB*, *rbcLS* (encoding RuBisCO) and *rnpB* (encoding the constitutively expressed ribonuclease P, Vioque 1997) cDNAs, the reverse primers SS15 (CTGGAATTGGGCTAGTAGTTGTTTTTCGGC), 20-lwRL (TCAGAGGGTATCCATTGCCTCAAACT CGAA) or 18-lwrpn (AAAAGAGGAGGGTGGT GGTAAGCCGGGTTCTGT) were used, respectively. The cDNAs were PCR amplified using the oligonucleotides SS3 (CGGGATCCATGTCAGAATTGATGC AAGCG) and SS4, or SS3 and SS6, for *susA*, SS11B and SS15 for *susB*, 20-lwRL and 19-upRL (TACGCTC AAACGAAGACTCAGACAAAATCT) for *rbcLS*, and 18-lwrpn and 17-uprpn (TAGGGAGAGAGT AGGCGTTGGCGGTTGCAG) for *rnpB*, according to the following protocol: 94°C for 1 min, 23 or 18 cycles (for *susA* and *susB* or *rbcLS* and *rnpB*, respectively) at 94°C for 30 s, 55°C for 45 s, and 72°C for 1.5 min, and a single step at 72°C for 3 min, using a PTC−100 thermal cycler (Model-96 V, MJ Research, Inc., MA, USA). Control reactions were incubated at 30°C for 10 min in the presence of RNase A to ascertain RNA dependence of the RT-PCR signals.

Primer extension analysis

The oligonucleotides used for the primer extension analyses of *susA* were: SS1 (GCAGGTAATTTT TATCTTGCTGACGC, which corresponds to positions +71 to +96 from the translation start point), SS2 (GGGTAACGTAGGCTAACAACC, positions −65 to −47) or SS7 (ATCATGTTTTTCTTCACTATCTA, positions +25 to +48). The plasmid pSS7 (Curatti et al. 2000) was used to generate the dideoxy-sequencing ladders with the same primers. For *susB*, the oligonucleotides used were SS21 (TAACGTTTACCTGAGGCAT CTAATG, positions +70 to +95) or SS23 (AAT TTGGCTATTACTGATCAAGACC, positions −170 to −144). The plasmid pSS4.0 was generated by ligating a PCR product, obtained from the amplification of *Anabaena* sp. PCC 7119 genomic DNA with the oligonucleotides SS13 (CTTCCATCACATCCCCTTCACC CTCGCTCT) and SS14 (ATTCGCCGTGAGTAAA GTTGTGTAGT), to the pGEM T-Easy vector (Promega). The sequences of the putative promoter regions of *susA* and *susB*, generated in the primer extension analyses, indicated that they were identical in the *Anabaena* sp. PCC 7119 and PCC 7120 genomes. The oligonucleotides were end-labeled with T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P]ATP (Amersham) as previously described (Muro-Pastor et al. 1999) and mixed with 25–40 μg of total RNA in the presence of 10 mM Tris–HCl (pH 8.0), 150 mM KCl and 1 mM EDTA. The mixtures were incubated first at 85°C for 10 min and then at 50°C for 1 h. The extension reactions were carried out at 47°C for 1 h in a final volume of 45 μl containing the whole reaction mixture: 0.25 mM of each deoxynucleoside triphosphate, 200 U of reverse transcriptase (Superscript II; Invitrogen) and the buffer recommended by the manufacturer. The reaction mixtures were then treated with RNase A (DNase free, Sigma) and, after precipitation, the DNA was resuspended in formamide loading dye and loaded onto a 6% polyacrylamide 7 M urea sequencing gel, next to the corresponding sequencing ladder. Images of the radioactive gels were obtained by autoradiography (Sambrook and Russell 2001).

Insertional inactivation of *sus* genes

For *susB* interruption, the Km/Nm resistance determinant encoded by the *kan* gene was introduced into an *Xba*I site within the *susB orf* of pSSB1. Then a *Spe*I DNA fragment, containing the *susB*::*kan* mutated allele, was ligated into the *Spe*I site of the conjugative vector pRL277 (Cai and Wolk 1990) to generate pLC80 or pLC90, bearing *kan* in the same or in the opposite transcriptional direction as *susB*, respectively. pLC80 and pLC90 were transferred to *Anabaena* sp. PCC 7119 by conjugation (Elhai and Wolk 1988) and double recombinants were identified by their Suc/Nm-resistance phenotype and named LC80–82 or LC90–92, according to the relative orientation of the *kan* gene. *susA::sp*/*susB::kan* double-mutant strains (LC120–122) were generated by inactivating *susA* with plasmid pLC30 (Curatti et al 2002), which bears the *susA::sp* mutant allele of *susA* (*sp* states for the Sp resistance determinant) in the mutant strain LC90. Homozygosity was

tested by the PCR analysis of the *susA* or *susB* genomic locus using oligonucleotides SS3 and SS4 or SS11B and SS15, for *susA* or *susB*, respectively.

Cellular localization of *susA* expression

To determine the cellular localization of *susA* expression, a 700 bp DNA fragment bearing *gfp*-mut2 (encoding a modified version of the GFP, Cormack et al. 1996) was ligated into the shuttle vector pAM505 (kindly provided by Dr. James Golden), between the *Eco*RI and proximal *Pst*I sites, to generate pAM505::GFP. A 0.93-kbp DNA fragment, containing the up-stream region of *susA*, was generated by the PCR amplification, using *Anabaena* sp. PCC 7119 genomic DNA and the oligonucleotides SS25 (AGAT AACTGCATTCAACTGACACC) and SS7, and ligated into the plasmid vector pGEM-T Easy (Promega), generating $pP_{susA}1.0$. The 0.93-kbp *Eco*RI DNA fragment was ligated into the *Eco*RI site of the shuttle vector pAM505::GFP to generate pLC150 or pLC160 (P_{susA} in the same or the opposite transcriptional direction of *gfp*-mut2, respectively). The shuttle vectors pLC150, pLC160, and pAM505::GFP were transferred to *Anabaena* sp. PCC 7119, by conjugation, to generate the strains LC150 (bearing a P_{susA}-GFP transcriptional fusion) and LC160 and LC170 (as controls), respectively. The cell localization of *susA* expression was analyzed in a fluorescence microscope (Nikon, model ECLIPSE E600).

Sucrose and sucrose synthase activity determinations

Sucrose content and SuS activity determinations were carried out as previously described (Porchia et al. 1999).

Results

Effect of the nitrogen source and *susA* inactivation on the expression of *sus* genes in *Anabaena* sp.

A comparative expression analysis of *Anabaena susA* and *susB* genes was performed by multiplex RT-PCR, adding reverse primers for *susA*, *susB*, *rbcLS*, and *rnpB* together to the RNA samples for the synthesis of cDNAs, which, in turn, were PCR amplified in separate reactions. Similar to what had been demonstrated for *susA* (Curatti et al. 2002), *susB* and *rbcLS* expression was higher in ammonium- than in nitrate- or dinitrogen-grown cells. The levels of *rnpB* mRNA remained unchangeable in the three nitrogen source conditions (Fig. 1a). The expression of *susB* was higher in the *Anabaena* sp. mutant strain LC30, which lacks *susA* and accumulates higher amounts of Suc, than in the wild-type cells (Curatti et al. 2002), for the three nitrogen sources analyzed (Fig. 1b). On the other hand, the expression of *susA* remained unaltered in the LC30 genetic background (Fig. 1b). The expression of *susB* was remarkably lower in the nitrate-grown cells (Fig. 1).

Mutational analysis of the function of *susB* in *Anabaena* sp.

To investigate the catalytic function of SusB, its encoding gene was over-expressed in *E. coli*, as a fusion protein with His_{6X}, and the resulting recombinant protein (His_{6X}::SusB) was affinity purified (not shown). His_{6X}::SusB did not catalyze the synthesis or the cleavage of Suc, using uridine diphosphate (UDP)-Glc or adenosine diphosphate (ADP)-Glc as substrates in the

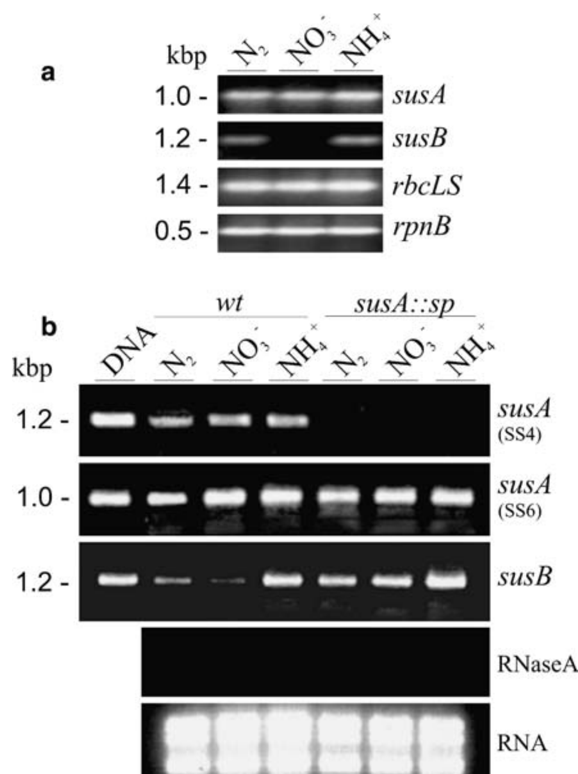

Fig. 1 RT-PCR analysis of the effect of the nitrogen source (**a, b**) and the mutation of *susA* (**b**) on *Anabaena* sp. *sus* genes expression. Aliquots of total RNA (2.5 μg) from *Anabaena* sp. cells cultivated in the presence of the indicated nitrogen sources were used in each RT-PCR reaction. Oligonucleotides SS4 or SS6, which anneal down-stream or up-stream the insertional mutation in the mutant strain LC30 (*susA::sp*), respectively, were used for *susA* cDNA synthesis. SS15 was used for *susB* cDNA synthesis and also for the negative control (RNA samples treated with RNaseA). The oligonucleotides 20-lwRL or 18-lwrpn were used for *rbcLS* or *rpnB* cDNA synthesis, respectively. The oligonucleotides were added together (**a**) or separately (**b**) for multiple or unique cDNA-synthesis reactions, respectively. Polymerase chain reaction amplification using genomic DNA of *Anabaena* sp. PCC 7119 was used as positive control (**b**). Also RNA samples (5 μg) stained with ethidium bromide are shown (**b**)

presence of fructose or UDP or ADP, in addition to Suc, respectively.

To further investigate the metabolic and physiological function of SusB, homozygous insertional-mutant strains, with an Nm-resistance cartridge orientated in the same (LC80 and LC82) or the opposite (LC90–92) transcriptional direction as *susB*, were generated (Fig. 2). Homozygous double-mutant strains (LC120–122) were obtained by insertion inactivation of *susA* in the mutant strain LC90 (Fig. 2). SuS activity was not modified in any cell-free extract from each *susB* mutant strain cultivated in the presence of ammonium as nitrogen source (not shown). The Suc levels were not modified in LC80 or LC90 but increased to about five-fold in LC120 (not shown), as reported for the single *susA* mutation in the strain LC30 (Curatti et al. 2002). The inactivation of *susB* in *Anabaena* sp. PCC 7119 or in LC30 did not modify the growth performance of the resultant mutants (LC90 and LC120 strains, respectively), using ammonium, nitrate, or dinitrogen as nitrogen sources, in comparison with the original strains (not shown).

Primer extension analysis of the expression of *Anabaena* sp. *sus* genes

To study the regulation of the expression of the *sus* genes, the oligonucleotides SS1, SS2, and SS7 and the total RNA, isolated from cells grown in the presence of different nitrogen sources, were used to determine the tsps of *susA*. With oligonucleotide SS1, three major RNA 5′-ends were detected that corresponded to putative tsps situated at positions −39, −83, and −101 from the translation start point, which were named tspI, tspII, and tspIII, respectively (Fig. 3a). These putative tsps were confirmed by conducting similar experiments with the oligonucleotide SS7. Besides, tspI was further confirmed with oligonucleotide SS2. The three mRNA species were more abundant for ammonium-grown cells. Although at lower levels than for ammonium-grown cells, the mRNAs that originated from tspII and tspIII were also present in the RNA samples from cells cultivated in the presence of nitrate or dinitrogen. However, the mRNA associated to tspI, the most abundant in ammonium-grown cells, could not be detected when cells were cultivated in the presence of the alternative nitrogen sources. Similar experiments were performed to determine the tsps of *susB*. Using either oligonucleotide SS21 or SS23, a unique putative tsp located at position −244 could be determined in the RNA samples from *Anabaena* cells grown only in the presence of ammonium, but not from diazotrophically grown cells (Fig. 3b).

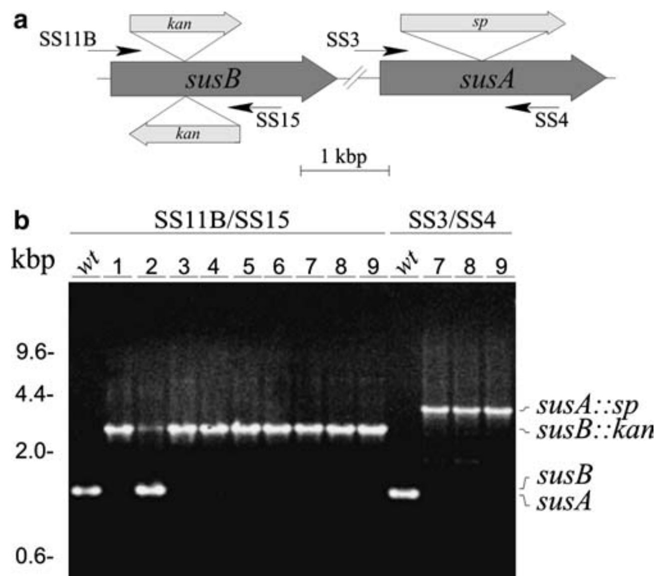

Fig. 2 Insertional inactivation of *susA* and *susB* in *Anabaena* sp. **a** Integration sites of the *sp*- or *kan*-resistance cartridges introduced into the *Anabaena* sp. chromosome to inactivate *susA* or *susB*, respectively. **b** Polymerase chain reaction analysis of the *susA* or *susB* loci using genomic DNA of *Anabaena* sp. strains: PCC 7119 (wt), LC80–82 (lanes 1–3), LC90–92 (lanes 4–6) or LC120–122 (lanes 7–9), and the indicated pair of oligonucleotides

The putative promoters of the *sus* genes share sequence similarity with regulatory elements of the *rbcLS* promoter in *Anabaena* sp.

The putative −10 and −35 boxes of *susA* (particularly those associated with *tsp*I and *tsp*II) and *susB* are similar to those of *E. coli* σ^{70} promoters (Fig. 4a). Interestingly, the *sus* gene putative promoters share high sequence similarity with that of the *rbcLS* gene (Nierzwicki-Bauer et al. 1984). It has been shown that the *Anabaena* sp. *rbcLS* promoter binds the transcriptional factor NtcA at positions centered at the nucleotides +2 and −52 and factor 2, from −12 to –77, regarding the tsp, respectively (Ramasubramanian et al. 1994). The putative *susB* promoter is similar to the putative promoter I of *susA*, which presented the highest response to ammonium. These putative promoters match a region in the *Anabaena rbcLS* promoter, around the tsp at position +2 (Fig. 4a, c). The region up-stream to the −35 box of the *susA* putative promoter III is also highly similar to the region around nucleotide −52 of the *rbcLS* promoter (Fig. 4a, b). Besides, the spacer distance between *susA* tspI and tspIII, and the *rbcLS* regulatory elements centered at positions +2 and −52, is similar (62 or 54 nucleotides, respectively).

Additionally, *Anabaena susA* and *susB* putative promoters share sequence similarity with other cyanobacterial ammonium-activated promoters (Fig. 4d), like those of *gorA*, encoding gluthatione reductase, and *gifA*, and *gifB*, which code for gluthamine synthase inhibitors in *Synechocystis* sp. PCC 6803 (Jiang et al. 1997; García-Domínguez et al. 2000).

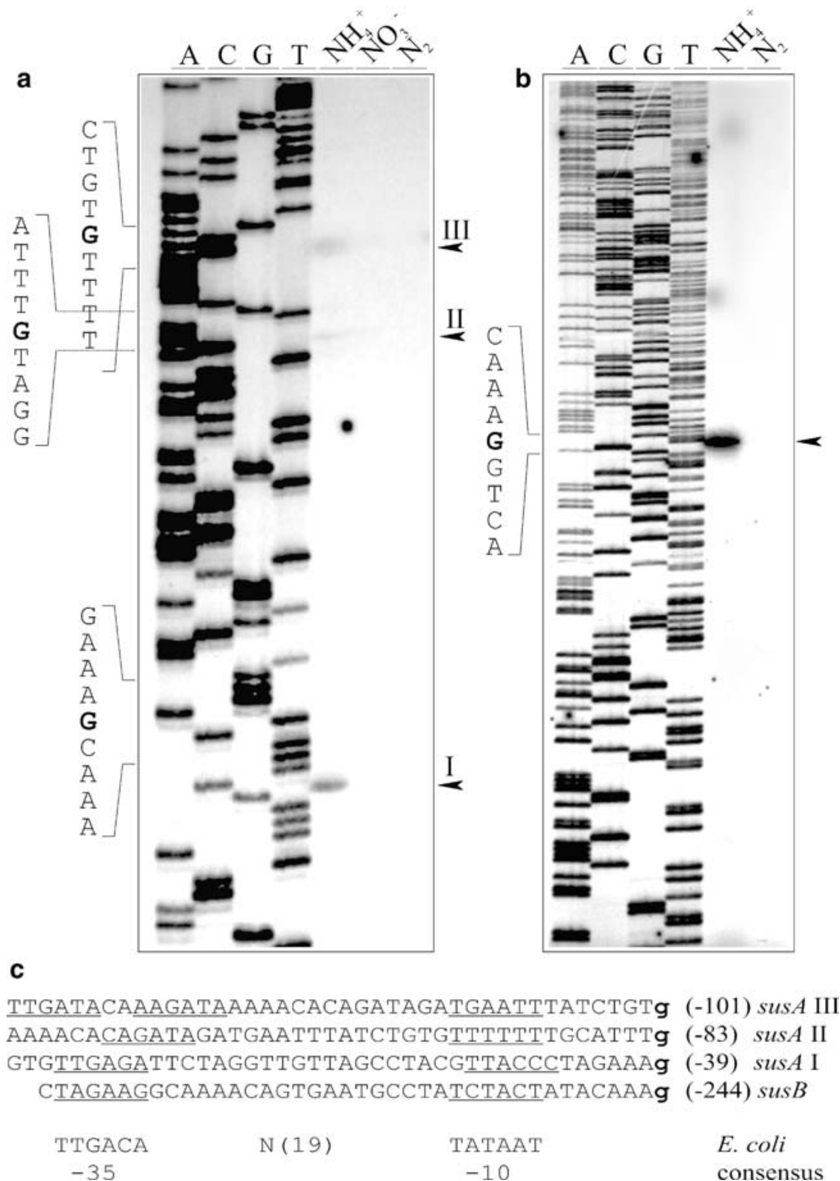

Fig. 3 Mapping of the transcription start sites (tsps) of the *Anabaena* sp. *susA* and *susB* genes. Primer extension analysis was carried out using total *Anabaena* RNA (40 μg) of cells cultivated in the presence of different nitrogen sources, and the oligonucleotides SS1 or SS21 for of *susA* (**a**) or *susB* (**b**) mRNA extension, respectively. The sequencing ladders were generated using the vector pSS7 and the primer SS1 for *susA* (**a**), or pSS4.0 and SS21 for *susB* (**b**). **c** Upstream sequences from the *susA* and *susB* tsps, which are indicated in bold lower case and their relative positions from the translation start are indicated between *parentheses*. The putative −10 and −35 boxes are underlined and, as a reference, the consensus boxes of *E. coli* genes are also shown

The expression of *susA* is localized to the vegetative cells of *Anabaena* sp. nitrogen-fixing filaments

It has been demonstrated that *rbcLS* is not expressed in mature heterocysts of *Anabaena* sp. (Elhai and Wolk 1990). Unlike NtcA that is present in both vegetative cells and heterocysts, factor 2 is specific of the vegetative cells of *Anabaena* sp. (Ramasubramanian et al. 1994). The sequence similarity between the promoter III of *susA* and that of the *rbcLS* factor 2-binding element (Fig. 4b) led us to determine the cellular expression of the *susA* promoter, using a transcriptional fusion with *gfp-mut2*. A 930-bp DNA fragment up-stream from the translation start site directed the expression of *gfp* in *Anabaena* sp. cells (strain LC150), while similar constructs, bearing the 930-bp DNA fragment orientated in the opposite direction or without the promoter, did not show expression of the reporter gene (not shown). Figure 5 shows that in diazotrophically grown LC150 cells, *susA* expression is localized to vegetative cells, similar to *rbcLS*.

Discussion

In plants, carbon and nitrogen metabolism intermediates act as signals that regulate the transcription of genes encoding enzymes involved in many essential processes, including photosynthesis, carbon and nitrogen metabolism, and resource allocation (Coruzzi and Zhou 2001; Koch 2004; Palenchar et al. 2004; Rolland and Sheen 2005). The mechanism underlying this regulation seems to be very complex and is poorly understood. The cyanobacteria model has been very useful in order to

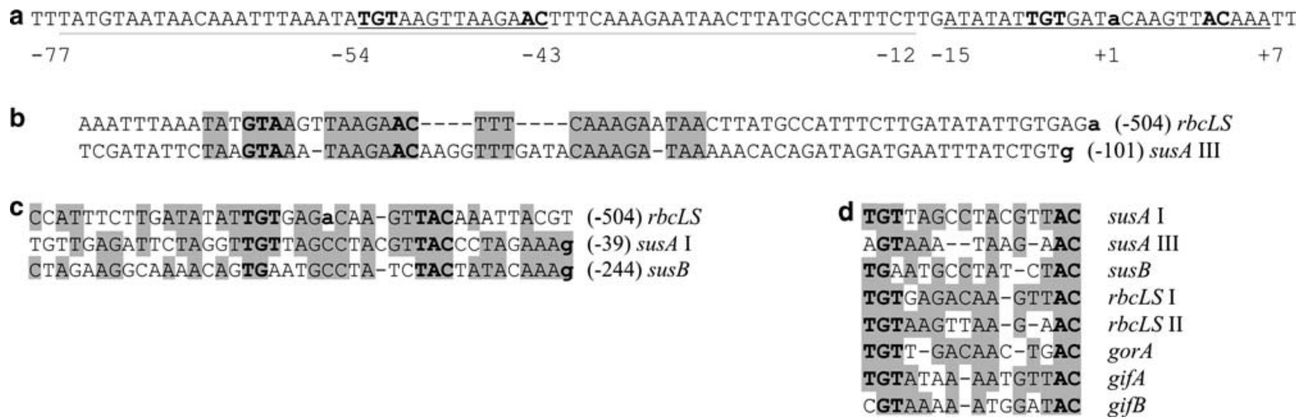

Fig. 4 Sequence alignment of *Anabaena* sp. *susA* and *susB* gene promoters. **a** *Anabaena* sp. *rbcLS* promoter and regulatory elements (Ramasubramanian et al. 1994). The two putative NtcA binding regions were *thin black underlined* while factor−2 binding element is *thick gray underlined*. *Numbers* indicate relative positions from the tsp (+1). **b** Alignment of the *susA* III and *rbcLS* promoters. **c** Alignment of the *susA* promoter I, and *susB*, and *rbcLS* promoters. **d** Alignment of the *sus* gene promoters with those of other cyanobacterial genes known to be up-regulated by ammonium at the NtcA binding region. In (**b**–**d**), gaps (*dashes*) were introduced to optimize the alignment and identical nucleotides were *shaded*. In (**b**) and (**c**) *numbers* indicate the position of the *tsps* relative to the translation start. The tsps and the consensus for NtcA binding sites are indicated in *bold lowercase* and *bold uppercases*, respectively

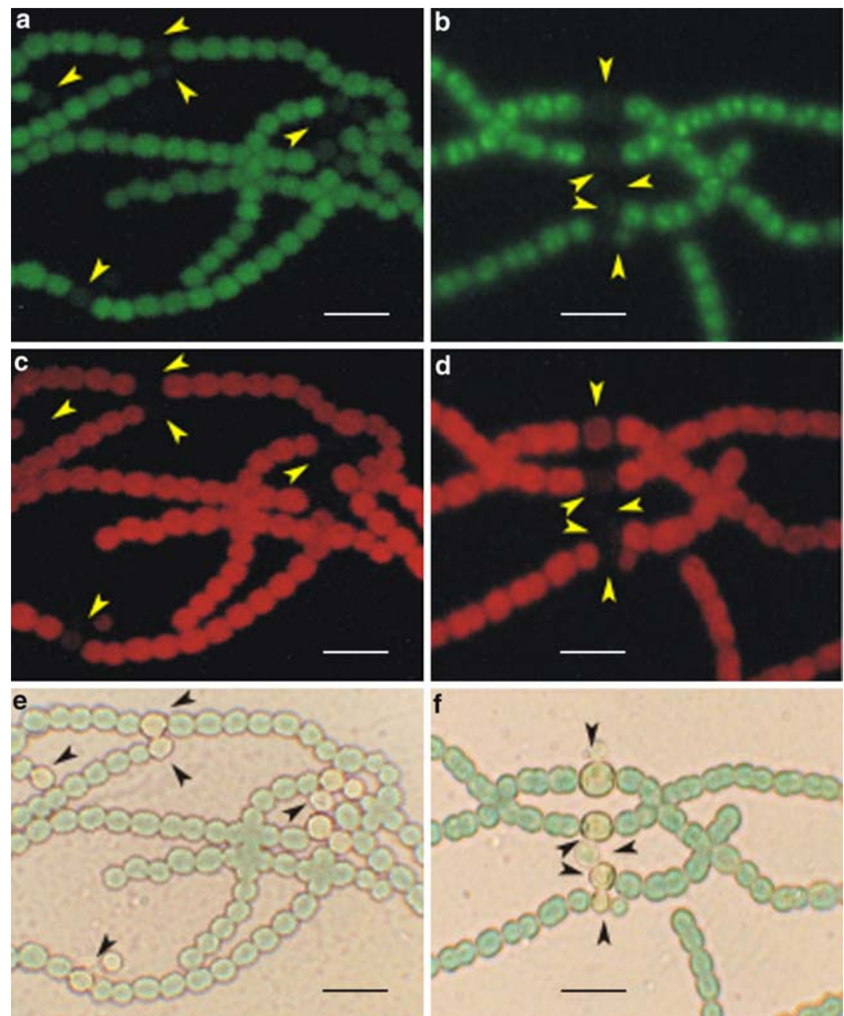

Fig. 5 Cellular localization of *susA* expression. Diazotrophically grown cells of *Anabaena* sp. strains LC150 (**a, c, e**) or AMC486 (**b, d, f**) expressing *gfp* from the *susA* or *rbcLS* promoters, respectively. The fluorescence images (**a**–**d**) were obtained with blue light excitation and with (**a, b**) or without (**c, d**) the red fluorescence (mainly from chlorophyll) blocked. **e, f** These are bright-field images of the strains. *Yellow arrows* indicate the position of the heterocysts and the *bar* corresponds to 10 μm

understand several aspects of chloroplasts evolution and function, photosystem damage and repair, and redox signaling (Melis 1999; Leister 2003; Pfannschmidt 2003). Recent studies have shown a close phylogenetic relationship between the Suc metabolizing enzymes of cyanobacteria and higher plants and have suggested that the cyanobacterial model might also be useful to address complex questions such as metabolic signaling in plant cells (Salerno and Curatti 2003; Desplats et al. 2005). The findings presented in this study are important not only for the understanding of Suc metabolism in oxygen-evolving photosynthetic prokaryotes, but also because they indicate that some regulatory networks, comprising Suc and nitrogen metabolism, carbon fixation and development, are also operative in filamentous cyanobacteria.

We have previously reported that SusA catalyzes the cleavage of Suc in vivo in *Anabaena* sp. and that *susA* expression is down-regulated after combined nitrogen starvation. These results led us to suggest that SusA may be playing a role in the control of the carbon flux in nitrogen fixing filaments (Curatti et al. 2002). The presence of two homologous *sus* genes (about 60% identical) in most filamentous cyanobacteria (Salerno and Curatti 2003) raised the question of their biochemical and physiological functions and the regulation of their expression. Our attempts to clarify the role of SusB have had limited success, mainly because the mutational analysis did not reveal any mutant phenotype that could be associated to the lack of the *susB* gene. This result indicates that the contribution of SusB to SuS activity and the control of Suc levels are likely to be negligible. Although the evidence presented herein cannot rule out the possibility that SusB's in vitro activity requirements could be distinct from those of other SuSs, it clearly indicates that SusB is the closest relative to the SuS proteins, but with different biochemical function(s) and/or properties. On the other hand, the fact that *susA* and *susB* are co-expressed, according to the presence of ammonium as the nitrogen source (sharing very similar putative promoters), and that *susB* expression is induced in a mutant strain lacking *susA*, indicates that the function of both *sus* genes is coordinated at the transcriptional level. The study of *susB*'s functions, other than that related to Suc and nitrogen metabolisms, was beyond the scope of this work.

Primer extension analyses suggest that the similar putative promoter-regions, inferred from the putative tsps I for *susA* and that of *susB*, account for most of the ammonium activation of the *sus* genes expression. It is possible that the putative promoter-regions II and III of *susA* are responsible for *susA* expression using nitrate or dinitrogen as alternative nitrogen sources. However, the abundance of these mRNA species, and also that of susB in dinitrogen-grown cells, determined by primer extension analyses, does not quantitatively fit the total mRNA accumulation, determined by RT-PCR. This raises the question whether there are ''still unseen'' *sus*-genes tsps for the alternative nitrogen sources.

It has been proposed that NtcA, which activates the expression of genes involved in the utilization of alternative sources of nitrogen in the absence of ammonium in cyanobacteria, also acts as a repressor of genes that are activated by ammonium. When ammonium is not available, NtcA binds to the σ^{70}-like -10 or -35 elements of those genes preventing their expression, while ammonium relieves that inhibition (Ramasubramanian et al. 1994; Jiang et al. 1997; García-Domínguez et al. 2000; Herrero et al. 2001; Flores and Herrero 2005). The striking similarities between the *sus* genes promoters and those of other ammonium-activated cyanobacterial genes (mainly of *rbcLS*) suggest that *Anabaena* sp. *sus* genes expression may be regulated by a similar mechanism.

The localization of the expression of *susA*, which accounts for most (if not all) SuS activity in the *Anabaena* sp. vegetative cells, supports the hypothesis that SuS may contribute to controlling the carbon flux from the vegetative cells of the nitrogen-fixing filaments (Curatti et al. 2002). Since the ratio of heterocysts to vegetative cells is 1 to 10–15 (Herrero et al. 2004), heterocyst differentiation accounts for a minimal decrease of *susA* expression in the diazotrophic filaments in comparison with those cultivated in the presence of ammonium. Thus, *susA* and *rbcLS* expression appears to be regulated by the nitrogen source at two different levels: a strong down-regulation by a nitrogen-source-dependent developmental program in the heterocysts and a weak modulation by the nitrogen source in the vegetative cells.

The presence of the *rbcLS* factor 2-binding-like element in the putative *susA* promoter strongly suggests that *susA* may be the target of a factor 2-dependent developmental program to differentiate gene expression in vegetative cells and heterocysts. There is a direct repeat AGA, separated by eight nucleotides in the *rbcLS* factor 2-binding element that is separated by 16 nucleotides in the homologous region of the *susA* putative promoter III, which would yield promoter structures differentiated by almost one complete turn of the DNA helix. It may be speculated that these differences in promoter structure may account for the variation in the expression levels of *susA* and *rbcLS*. It has been shown that factor 2 also binds, in vitro, the promoters of *xisA* and *nifH*, two genes that encode proteins with functions in the heterocyst, but with comparatively less affinity than that of the *rbcLS* promoter. The putative *sus*A promoter region is more similar to that of the *rbcLS* factor 2-binding element than to the corresponding region on the *xisA* promoter (Ramasubramanian et al. 1994). Thus, the available information suggests that *susA* may be the target of a factor 2- (or a related DNA-binding protein-) dependent developmental program that plays a role in differentiating gene expression in vegetative and heterocyst cells. According to current evidence, the question that remains to be answered is whether factor 2 may act as an activator or a repressor of developmentally regulated genes that have to be expressed in vegetative cells or heterocysts, respectively.

The similarities between the putative *sus* gene promoters and those of other ammonium-activated cyanobacterial genes (like *rbcLS*) suggest that *Anabaena* sp. *sus* gene expression may be modulated by a similar NtcA-dependent mechanism. Many examples have been reported on NtcA-regulated genes, which have promoter elements that do not match perfectly the consensus $GTN_{10}AC$. It has been proposed that this, together with the cellular levels of active NtcA, may determine a hierarchy of gene expression regulation during the developmental program for the differentiation of heterocysts (Herrero et al. 2004). The *sus*-gene putative nitrogen source-regulated promoters may better fit that subset of genes.

The co-regulation of the expression of *rbcLS* and *susA* strongly suggests that in cyanobacteria, as in plants, CO_2 fixation and Suc utilization are coordinated at the transcriptional level in a network that also integrates signals from nitrogen metabolism. Although the regulatory scenarios seem to be rather typical of eukaryotes, in the case of higher plants, or prokaryotes, in the case of *Anabaena* sp., the evolutionary convergence towards the coordination of Suc metabolism together with carbon fixation and nitrogen assimilation could represent a functional constrain and promises new insights into both plant and cyanobacterial research. The results presented in this work provide the basis for further studies directed to uncover the mechanism for the coordination of the expression of genes for Suc metabolism, with other key genes of nitrogen and carbon metabolisms and development at the molecular level in oxygenic photosynthetic organisms.

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