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ORIGINAL ARTICLE

Sucrose synthase is involved in the conversion of sucrose to polysaccharides in filamentous nitrogen-fixing cyanobacteria

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Abstract Higher plants and cyanobacteria metabolize sucrose (Suc) by a similar set of enzymes. Suc synthase (SuS, UDP-glucose: D-fructose 2-α-D-glucosyl transferase, EC 2.4.1.13) catalyses the synthesis and cleavage of Suc, and in higher plants, it plays an important role in polysaccharides biosynthesis and carbon allocation. In this work, we have studied the functional relationship between SuS and the metabolism of polysaccharides in filamentous nitrogen-fixing cyanobacteria. We show that the nitrogen and carbon sources and light regulate the expression of the SuS encoding gene (*susA*), in a similar way that they regulate the accumulation of polysaccharides. Furthermore, glycogen content in an *Anabaena* sp. mutant strain with an insertion inactivation of *susA* was lower than in the wild type strain under diazotrophic conditions, while both glycogen and polysaccharides levels were higher in a mutant strain constitutively overexpressing *susA*. We also show that there are soluble and membrane-bound forms of SuS in *Anabaena*. Taken together, these results strongly suggest that SuS is involved in the Suc to polysaccharides conversion according to nutritional and environmental signals in filamentous nitrogen-fixing cyanobacteria.

Keywords *Anabaena* · Heterocyst-forming cyanobacteria · Glycogen · Sucrose cleavage

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Abbreviations

ADP-Glc	ADP-glucose
AGPase	ADP-glucose pyrophosphorylase
Av-*susA*	*Anabaena variabilis* sucrose syntase encoding gene
Av-SuS	*Anabaena variabilis* sucrose synthase
Fru	Fructose
Suc	Sucrose
SPS	Sucrose-phosphate synthase
SuS	Sucrose synthase
susA	*Anabaena* sp. PCC 7119 sucrose synthase encoding gene

Introduction

Sucrose (Suc) is synthesized by plants, unicellular algae and cyanobacteria as part of the carbon dioxide assimilation pathway (Salerno and Curatti 2003), a critical piece of the global biogeochemical cycle of carbon on the Earth (Falkowski 1997; Ting et al. 2002). While Suc metabolism is an essential pathway in plants, its significance in oxygenic photosynthetic bacteria is still not fully understood. Higher plants and cyanobacteria metabolize Suc by a similar set of enzymes (Salerno and Curatti 2003; Vargas et al. 2003). Particularly, Suc breakdown can take place by the action of invertases (Inv), which catalyzes the irreversible hydrolysis of Suc into hexoses, and by Suc synthase (SuS, UDP-glucose: D-fructose 2-α-D-glucosyl transferase, EC 2.4.1.13), which catalyzes a readily reversible reaction of Suc synthesis and cleavage. SuS is widespread in the plant kingdom and in filamentous heterocyst-forming cyanobacteria (Salerno and Curatti 2003; Koch 2004). In higher plants, SuS has critical functions in long-distance carbon allocation, stress responses, symbiosis with other organisms, and in Suc to polysaccharides inter-

conversion (Avigad 1982; Winter and Huber 2000; Koch 2004). The first genetic evidence demonstrating the importance of SuS in starch production was obtained from studies with *sh1* starch deficient maize mutants (Chourey and Nelson 1976). More recent data from genetically engineered potato and carrot plants supported those findings, since a decreased SuS activity was paralleled by reduced starch content in tubers and roots (Zrenner et al. 1995; Tang and Sturm 1999). Additionally, the existence of a membrane-bound form of SuS also supports a direct function of the enzyme in the channeling of UDP-Glc towards the synthesis of structural polysaccharides (Haigler et al. 2001; Koch 2004).

Knowledge of the relationship between Suc and glycogen metabolism, and particularly, the function and regulation of SuS in filamentous nitrogen-fixing cyanobacteria, remains still at present fragmentary (Salerno and Curatti 2003). These cyanobacterial strains can differentiate a photosynthetic vegetative cell into a heterocyst as part of a strategy that allows simultaneous oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation by spatial separation of both processes. Thus, an exchange of carbon assimilation and nitrogen fixation products occurs between both cell types (Wolk 2000). Glycogen synthesis and mobilization is involved in assimilated carbon feeding to heterocysts during nitrogen fixation. During the light phase, most studied cyanobacteria accumulate glycogen, which is then mobilized to provide reductants and ATP during the night, in either the vegetative cells or the heterocysts. Nitrogen fixation can, therefore, also take place at night, even if at a much lower rate (Fay 1976; Lockau et al. 1978; Ernst and Böger 1985).

The interconnection between glycogen and Suc metabolism in *Anabaena* N_2-fixing filaments was more recently studied. It has been shown that in *Anabaena* sp. PCC 7119 SuS is encoded by the *susA* gene that is also present in *Anabaena* sp. PCC 7120 (Curatti et al. 2000; Salerno and Curatti 2003) and that the enzyme activity is modulated by the nitrogen source at the transcriptional level (Curatti et al. 2002, 2006). Different lines of evidence point to a key role of Suc cleavage by SuS, located in the photosynthetic vegetative cells, in the control of carbon flux in the nitrogen-fixing filaments of heterocystous cyanobacteria (Porchia et al. 1999; Curatti et al. 2000, 2002, 2006). Also, the participation of SuS in a Suc synthesis/cleavage cycling, as reported in higher plants, and a potential contribution of SuS to the synthesis/cycling of sugar nucleotides, has been recently proposed. These data suggest that Suc to glycogen interconversions might take place in the *Anabaena* nitrogen-fixing filaments (Cumino et al. 2007).

The synthesis of storage polysaccharides (glycogen and starch, in bacteria and plants, respectively) utilizes ADP-Glc as the glucosyl donor. Moreover, ADP-Glc synthesis, catalyzed by ADP-Glc pyrophosphorylase (AGPase, EC 2.7.7.27), is the main regulatory step in the accumulation of these reserve compounds. AGPase activity was shown to be allosterically regulated by 3-phosphoglycerate (activator) and inorganic phosphate (inhibitor) in both cyanobacteria and higher plants (Ballicora et al. 2003). In contrast to plants, cyanobacteria are capable of utilizing not only UDP-Glc but also ADP-Glc as substrates for Suc synthesis by the sequential action of Suc-phosphate synthase (SPS, EC 2.4.1.14) and Suc phosphate-phosphatase (EC 3.1.3.24) (Porchia and Salerno 1996; Curatti et al. 1998; Cumino et al. 2002). In addition, as was demonstrated in the mutant strain of *Synechocystis* sp. PCC 6803 lacking AGPase (Miao et al. 2003), the carbon flow may shift toward UDP-Glc formation allowing the biosynthesis of Suc. In cyanobacteria and plants, storage polysaccharide accumulation responds to light/dark cycles at the level of allosteric regulation of AGPase by the 3-PGA/Pi ratio (Ballicora et al. 2003). In *Anabaena variabilis*, glycogen accumulation is also enhanced by sugar feeding to the cell culture medium (Wolk and Shaffer 1976; Ernst and Böger 1985; Lang et al. 1987; Jensen 1990; Fernández-Valiente et al. 1992; Reddy et al. 1996). This cyanobacterium can be cultivated under heterotrophic conditions in the dark, in contrast to the related strains *Anabaena* sp. PCC 7119 and PCC 7120 where Suc metabolism has been more extensively characterized.

In the present work, we show that regulation of the expression of *susA* in *Anabaena* either by environmental or nutritional inputs or when altered by mutagenesis, is related to polysaccharide levels. These findings support a role of SuS in the cycling of sugar-nucleotides and in the regulation of the flux of carbon between Suc and polysaccharides.

Materials and methods

Cyanobacterial strains and growth

Anabaena variabilis strain ATCC 29413, *Anabaena* (*Nostoc)* sp. PCC 7119 and 7120, and derivative mutant strains $SusA^-$, formerly named LC30 with an insertion inactivation of *susA,* and $SusA^+$, a constitutively overexpressing *susA* mutant, were cultured in the presence of different nitrogen sources as previously described (Curatti et al. 2002). Fru was added to the growth medium at a final concentration of 5 mM. *Escherichia coli* DH5α and BL21(DE3): pLysS (Novagen) strains were grown in Luria–Bertani medium supplemented with 50 μg ml^{-1} carbenicillin (Sambrook and Russell 2001).

Cell fractionation

Membrane and periplasm proteins were purified from 2 l of cyanobacterial cultures at logarithmic phase of growth (Norling et al. 1998; Zhu et al. 1999). Malate dehydroge-

nase activity, used as a cytoplasmic marker, was assayed in the direction of oxalacelate reduction (Reng et al. 1993). Quantification of proteins was done by Bradford's dye-binding assay (Bradford 1976).

Protein extraction, enzyme assays and immunoblot analysis

Total protein extracts from *Anabaena* cells were prepared as previously reported, and desalted through Sephadex G-50 columns (Porchia et al. 1999). Proteins were separated by SDS-PAGE using 9% polyacrylamide gels. Polypeptides were visualized with Coomassie blue or electroblotted onto nitrocellulose membranes (HyBond C, Amersham), which were probed with anti-*Anabaena* sp. PCC 7119 SuS polyclonal antibodies (Curatti et al. 2002).

AGPase and SuS activity were determined according to previous reports (Sowokinos et al. 1993; Porchia et al. 1999).

Carbohydrate and chlorophyll determination

Soluble sugars were extracted from 50 ml cell cultures at mid-log phase of growth with alkaline water. Suc was determined after invertase hydrolysis using a coupled-enzyme method (Puebla et al. 1997). For glycogen extraction, the cell pellet was washed with 80% ethanol, resuspended in 400 µl H_20 and autoclaved at 120°C for 1 h. Glycogen was determined in the supernatant after treatment with α-amyloglucosidase (Keppler and Decker 1981). Insoluble polysaccharides were extracted as follows. After glycogen extraction, the remaining cell pellet was washed with boiling water, resuspended in 1.8 N HCl (500 µl) and incubated at 100°C for 1 h. The reaction was neutralized with NaOH. Reducing sugars were determined by the Somogyi and Nelson reagent as described (Porchia et al. 1999). Chlorophyll content was quantified in methanolic cell extracts (MacKinney 1941).

Isolation, manipulation and analysis of nucleic acids

Plasmids were isolated and modified according to standard protocols (Sambrook and Russell 2001). Isolation of high-molecular-mass genomic DNA from *A. variabilis* was performed as previously described (Cai and Wolk 1990) and analysed by electrophoresis in 1% agarose gels. Total RNA was extracted from *A. variabilis* cells harvested after a 48-h treatment in different carbon and nitrogen sources, and light/dark conditions by the Trizol procedure (Gibco-BRL).

Molecular cloning of *Anabaena variabilis susA* gene and its expression in *Escherichia coli*

Genomic DNA of *A. variabilis* (50 ng) was used in 25-µl PCR reactions containing 20 pmol of each of the oligonucleotides 5′-CGGGATCCATGTCAGAATTGATGCAAGGC-3′ (*Bam*HI site underlined), 5′-TGATGTCGACGCTTACCGATATTTATACTGTTCTAATAGTTGTTGCG-3′ (*Sal*I site underlined) and Taq DNA Polymerase (Invitrogene) as recommended by the manufacturer. The PCR conditions were 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 1 min, and a single step of 72°C for 3 min, using a PTC-100 thermal cycler (Model-96 V, MJ Research, Inc. Mass. USA). The PCR product was cloned in the pGEM-T easy (Promega) and named pAVA. The insert was then sequenced. The DNA sequences were compiled and analysed using the BLASTp protocol (Altschul et al. 1990) and the Clustal X method running the Megalign program of the DNAStar package with the PAM 250 residue weight table. Comparisons were performed with data obtained from GenBank and EMBL databases. The sequence of *A. variabilis susA* gene (Av-*susA*) was deposited in the EMBL data base under the accession number AJ292758.

The 2,148-bp *Bam*HI-*Eco*RI DNA fragment comprising the open reading frame of Av-*susA* was excised from pAVA and transferred to pRSET-A (Novagen). The resulting construct was introduced into *E. coli* strain BL21(λDE3):pLysS (Novagen) to produce His6::SusA recombinant protein. His6-tagged fusion protein was purified by Co^{2+}-affinity chromatography (TALON resin, Clontech) as described earlier (Cumino et al. 2002).

Northern blot analysis

Anabaena variabilis RNA samples (20 µg) from each growing condition were separated in a 1% agarose-formaldehyde denaturing gel, and immobilized in positively charged nylon membranes (0.45 µm, Nytran-Schleicher and Schuell), by alkaline passive transference. A probe containing the first 1,187 nucleotides of Av-*susA* encoding sequence was amplified from *A. variabilis* total DNA by PCR and labelled with [α-^{32}P]dCTP by the random primer extension system (NEN). Prehybridization and hybridization of the membrane was carried out at 65°C and the autoradiography was performed as previously described (Torres and Salerno 2007).

Results

Anabaena variabilis susA gene cloning and characterization of its gene product

To study the expression of SuS in *A. variabilis*, we first identified its encoding gene (Av-*susA*) and amplified it by the PCR methodology and reported its sequence to the EMBL database (accession number AJ292758). Av-*susA* encodes an 806-amino acid polypeptide 96% identical to the *Anabaena* sp. PCC 7119 *susA* gene product (Curatti

et al. 2000). Most of the amino acid substitutions are clustered in the first one hundred amino-acid residues at the N-terminal end of the predicted protein. For functional characterization of Av-*susA*, the full length DNA encoding region was expressed in *E. coli* as a fusion protein with a His6 oligopeptide, Av-SuS::His6, which was further purified by affinity chromatography using a Co^{2+} resin (Fig. 1). The recombinant protein presented similar immnunological reactivity (Fig. 1 lane 5) and identical biochemical properties with regard to substrate specificity and kinetic parameters as the previously characterized SuS protein (not shown) from *Anabaena* sp. PCC 7119 (Porchia et al. 1999; Curatti et al. 2000). No significant effect of the allosteric regulators of AGPase activity (3-phosphoglycerate or inorganic phosphate) was observed on *A. variabilis* SuS activity, as assayed in vitro (not shown).

Effect of Fru, light and ammonium on Av-SuS expression

As shown in Fig. 2a, b, the addition of ammonium to *A. variabilis* cells diazotrofically cultivated in the light produced an increase in SuS activity and in the accumulation of Av-*susA* mRNA, in agreement with previous findings in *Anabaena* sp. PCC 7119, a related strain which is an obligate phototroph (Curatti et al. 2002, 2006). Then, we analyzed the effect of continuous light and Fru feeding, both reported as triggering factors for glycogen accumulation (Ernst and Böger 1985; Lang et al. 1987; Jensen 1990; Fernández-Valiente et al. 1992), on Av-SuS expression.

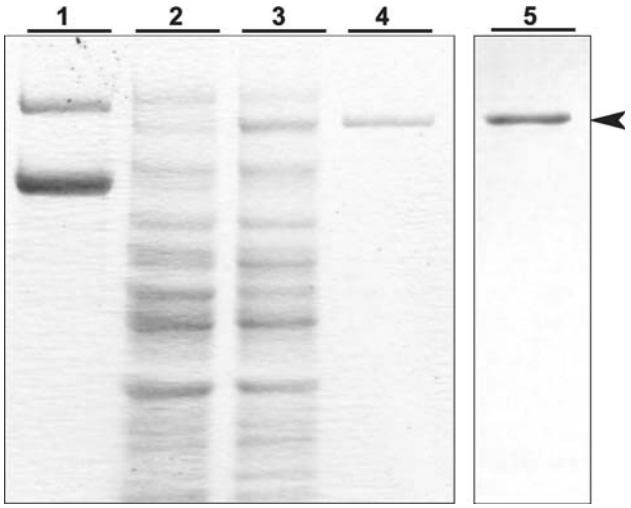

Fig. 1 Functional characterization of Av-*susA* gene product. SDS-PAGE analysis (*lanes 1–4*) of His-tagged Av-SuS (His6::Av-SuS) expressed in *E. coli*. *Lane 1*, molecular-mass standard proteins (116 and 66 kDa); *lanes 2* and *3*, total proteins of non-induced and IPTG-induced *E. coli* strain BL21(λDE3) [pLysS p28H], respectively; *lane 4*, affinity purified His6::Av-SuS; *lane 5*, immnoblot analysis using anti-SuS polyclonal antibodies from *Anabaena* sp. PCC 7119 SuS. The *arrow* indicates the position of the His6::Av-SuS polypeptide

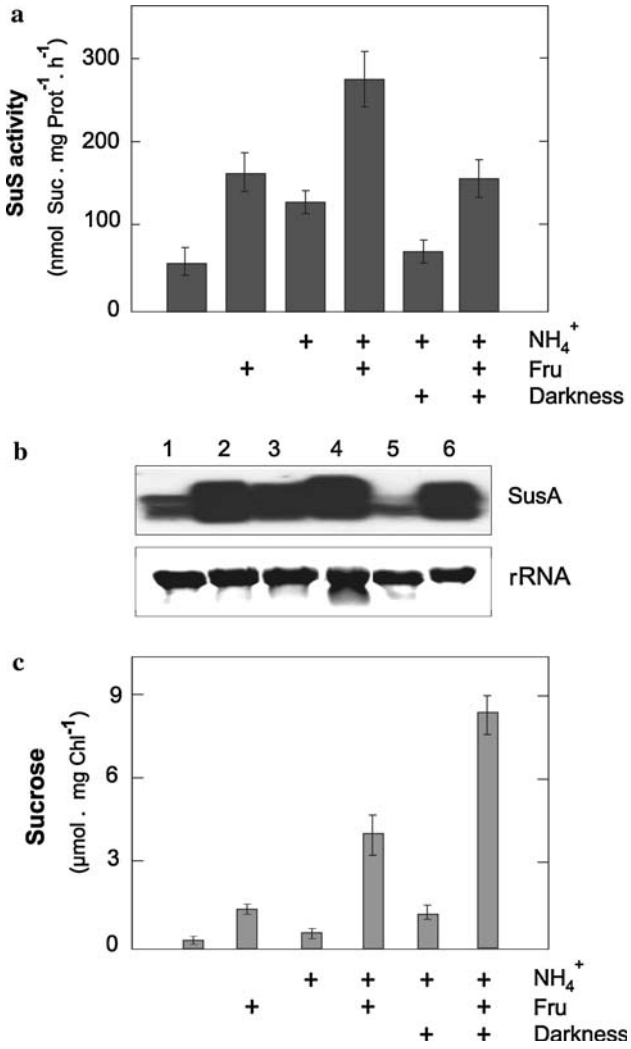

Fig. 2 Effect of the carbon and nitrogen sources, and light on Av-*susA* expression and Suc level. *Anabaena variabilis* cells cultivated diazotrophically in the light were shifted to the indicated conditions for 48 h. **a** SuS activity, assayed in the direction of Suc synthesis in cell-free extracts. **b** Northern blot analysis. Total RNA was extracted from *A. variabilis* treated cells as indicated in **a**. A 1,187-bp DNA fragment from Av-*susA* was used as probe. **c** Suc level determined in *A. variabilis* treated cells as indicated in (**a**). Data represents the mean and standard deviation of triplicates from at least two independent experiments

The addition of 5 mM Fru also produced and increase in SuS activity and Av-*susA* mRNA levels. Two different transcripts were observed in *A. variabilis* RNA samples, suggesting that transcription might take place from different promoters as previously shown in PCC 7119 and 7120 (Curatti et al. 2006). Ammonium and Fru, amended together, exerted an additive effect on SuS activity and Av-*susA* expression in the light. In the darkness, Fru (but not ammonium) was able to induce Av-*susA* mRNA accumulation (Fig. 2b, lanes 5 and 6). No additive effect on SuS activity was observed when both ammonium and Fru were amended in combination in the dark. This result was

consistent with the analysis of the effect of light and Fru on the levels of Av-SuS polylpeptide in ammonium-grown cells (Fig. 3, lanes 3 and 4). The increase in the accumulation of Av-SuS polypetide by the addition of Fru was observed to the greatest extent when cells were transferred from the darkness to the light (Fig. 3, lanes 6 and 8).

Similar results on the effect of the nitrogen source and light were obtained with the obligate phototrophic strain *Anabaena* sp. PCC 7120, cultivated under a diel photoperiod regime (Cumino et al. 2007 and not shown).

Effect of ammonium, Fru and light on Suc levels

Suc contents were determined to aid in the understanding of the partitioning of intracellular carbon according to the availability of ammonium, Fru and light, and the possible participation of SuS in the process. The ratios of Suc to chlorophyll levels could be calculated since chlorophyll content of *A. variabilis* cells slightly decreases by the presence of Fru (Haury and Spiller 1981; Fernández-Valiente et al. 1992). Also darkness does not significantly change the chlorophyll content of *A. variabilis* heterotrophic cells (Mannan and Pakrasi 1993).

Whereas under continuous light the presence of ammonium caused a two-fold accumulation of Suc in relation to diazotrophically-cultivated cells (Fig. 2c), the addition of Fru produced an eight-fold increase in Suc levels. The simultaneous addition of ammonium and Fru enhanced Suc levels by 47-fold. When ammonium-grown cells were transferred to the darkness, Suc levels increased by 3-fold and when Fru was also present, Suc levels raised to 87-fold in comparison with phototrophically- and diazotrophically-cultivated cells.

Effect of genetic manipulation of *susA* expression on polysaccharide levels

To further investigate whether the regulation of SuS activity and Suc content by light and Fru could be related to the accumulation of polysaccharides, we used a set of mutant strains with genetic manipulation of *susA* expression (Curatti et al. 2002, 2006). The cyanobacterial cells were cultivated photoautotrophically under a 12 h/12 h light/dark photoperiod and cells were collected at the middle of the light period. It was observed that in diazotrophically-grown cells, the glycogen level was four-fold lower in the mutant strain SusA$^-$ that bears an insertion inactivation of the *susA* gene than in the *Anabaena* sp. PCC 7119 parental strain (Table 1). The AGPase activity was 0.90 ± 0.1 nmol h^{-1} mg prot^{-1} in protein extracts of diazotrophically-grown SusA$^-$ cells, two-fold higher than in PCC 7119 cells. On the contrary, when cells were grown in the presence of ammonium, glycogen was not detected either in the wild-type cells or in the derivative SusA$^-$ mutant strain, but was accumulated in the mutant strain SusA$^+$ that constitutively over-expresses *susA*. Also a nearly two-fold increase in the accumulation of a fraction of polysaccharides that remained insoluble after extensive soluble sugars and glycogen extraction was observed as a result of the overexpression of *susA* in the SusA$^+$ mutant strain (Table 1).

Subcellular localization of SuS activity

In higher plants, either soluble or membrane-bound isoforms of SuS have been characterized. Whereas soluble SuS isoforms are involved in the cleavage of Suc for respiration and anabolic pathways, a membrane-associated form has been implicated in the synthesis of structural polysaccharides as cellulose and callose (Amor et al. 1995; Carlson and Chourey 1996; Winter et al. 1997; Sturm et al. 1999; Winter and Huber 2000; Haigler et al. 2001; Komina et al. 2002; Koch 2004) and a tonoplast-associated form was presumably implicated in sucrose mobilization from the vacuole (Etxeberria and Gonzalez 2003). Given the observations on the expression pattern of SuS and susA mutational experiments, which strongly suggested that SuS is involved in glycogen and possibly in structural polysaccharides

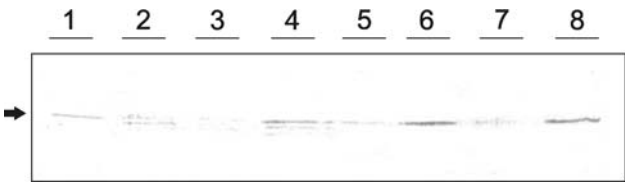

Fig. 3 Effect of light and exogenous Fru on Av-SuS polypeptide content. Western blot analysis using anti-SuS polyclonal antibodies prepared from *Anabaena* sp. PCC 7119 SuS. *Anabaena variabilis* cells were cultivated during 48 h in the presence of NH_4^+ in the light (*lanes 1–4*) or in the dark (*lanes 5–8*), and 5 mM Fru was added to a sub-set of cultures (*lanes 2, 4, 6, and 8*). Cultures were shifted to the opposite light condition for 12 h (*lanes 1, 2, 5, and 6*), or for 48 h (*lanes 3, 4, 7, and 8*). Cell-free extracts (50 µg protein) were loaded in each lane. The *arrow* indicates the migration of Av-SuS polypeptide

Table 1 Effect of *Anabaena susA* expression on polysaccharide contents

Anabaena strain	Glycogen(µmol Glc. mg chorophyll^{-1})		Polysaccharides(µmol Glc. mg chlorophyll^{-1})
	NH_4^+	N_2	NH_4^+
PCC 7119	ND[b]	0.40 ± 0.05	1.6 ± 0.3
SusA$^-$[a]	ND[b]	0.10 ± 0.05	1.6 ± 0.2
SusA$^+$[a]	0.46 ± 0.05	–[b]	2.8 ± 0.2

ND not detected

[a] SusA$^-$ and SusA$^+$ mutants were formerly named LC30 and LC60, respectively (Curatti et al. 2002)

[b] SusA$^+$ does not grow in this condition

accumulation, we investigated the subcellular localization of *Anabaena* SuS. As shown in Fig. 4, SuS activity and polypeptide were detected in both soluble (cytoplasmic) and insoluble (membrane-bound) fractions.

Discussion

Previous studies in *Anabaena* sp. PCC 7119 and PCC 7120 showed a close relationship between Suc and nitrogen metabolism, coordinated at the transcriptional level (Curatti et al. 2002, 2006; Cumino et al. 2007). After the isolation and characterization of Av-*susA*, the SuS enconding gene of a mixo/heterotrophic cyanobacterium strain, we show a broader response of SuS expression in *Anabaena* strains regarding the nitrogen source and the up-regulation of *susA* by light and Fru. Importantly, we present evidence for the participation of SuS in polysaccharides accumulation in *Anabaena* cells.

The similarity between the functionally characterized *Anabaena* SuS encoding sequences (accession number AJ010639 and AJ292758), and of the biochemical properties of Av-SuS in comparison with *Anabaena* sp. PCC 7119 SuS indicate an analogous function of the enzyme in *Anabaena* spp. strains (Porchia et al. 1999; Curatti et al. 2000, 2002, 2006; Cumino et al. 2007). Moreover, after our first experimental results, the sequence of the *A. variabilis*

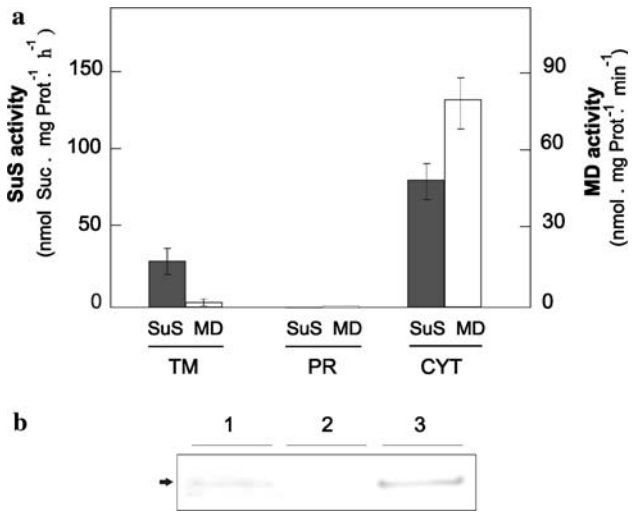

Fig. 4 Subcellular localization of SuS activity in *Anabaena* sp. cells. **a** SuS activity was assayed in total membranes (*TM*), periplasmic (*PR*) and cytoplasmic (*CYT*) protein fractions extracted from *Anabaena* sp. cells. Malate-dehydrogenase activity (*MD*) was used as cytoplasmic marker. Data are the mean ± SE of five independent experiments. **b** Immunoblotting of protein extracts. *Lanes 1–3*, *TM*, *PR* and *CYT*, respectively. Polypeptides were revealed using anti-SuS polyclonal antibodies prepared from *Anabaena* sp. PCC 7119 SuS. Approximately 100 μg of protein were loaded on each lane. *Arrows* indicate the positions of Av-SuS polypeptide

genome became public (http://genome.jgi-psf.org/finished_microbes/anava/anava.home.html), uncovering a very similar repertoire of genes putatively encoding the enzymes for Suc metabolism that have been studied in *Anabaena* sp. PCC 7120 (Salerno and Curatti 2003; Vargas et al. 2003).

We show that the expression of Av-*susA* is modulated by ammonium as previously demonstrated for *Anabaena* sp. PCC 7119. Thus, it can be inferred that SuS may be controlled by a developmental program during the differentiation of heterocysts (Curatti et al. 2002, 2006). The expression of Av-*susA* is also regulated at the transcriptional level by light and the exogenous addition of Fru. The presence of at least two mRNA species in Northern blot analyses (Fig. 2b) may indicate a possible transcription from different promoters as shown in *Anabaena* sp. PCC 7120 (Curatti et al. 2006). It is likely that *Anabaena* SuS-encoding gene expression has a very complex regulation, and that several signal transduction cascades for developmental, nutritional, and environmental signals may converge in the modulation of SuS activity, thus playing a key role in the control of the carbon flux in the nitrogen-fixing filaments.

A recent analysis that models the Suc metabolic network in nitrogen-fixing *Anabaena* filaments found that AGPase flux alone, as calculated by metabolic simulation, is insufficient to supply the ADP-Glc needed for glycogen and Suc production. Consequently, it was proposed that a concomitant production of ADP-Glc might also be ascribed to Suc cleavage by SuS in the vegetative cells (Cumino et al. 2007). The correlation observed in the present study between glycogen and insoluble polysaccharide levels, and *susA* expression (Table 1) supports the role of Suc cleavage by SuS as a contributor to the sugar nucleotide pools for the biosynthesis of polysaccharides in *Anabaena* sp. Moreover, this function of SuS is further confirmed by experiments in SusA$^-$, the mutant strain lacking SuS, where glycogen level was significantly reduced although AGPase activity is twice as high as in the wild-type strain. Also, the presence of a membrane-bound form of SuS resembles the cellular localization of SuS in higher plants (Haigler et al. 2001), and may be in agreement with a role of SuS in the biosynthesis of structural polysaccharide. The proposal that SuS has a direct function in the biosynthesis of polysaccharides in filamentous cyanobacteria is consistent with recent findings in plant leaves were the characterization of both SuS-overexpressing and SuS-antisense-mRNA-expressing transgenic lines indicated that (a) ADP-Glc produced by SuS may be linked to starch biosynthesis; (b) SuS exerts a strong control on the starch biosynthetic process (Muñoz et al. 2005).

In *A. variabilis*, where Fru enhanced the accumulation of Av-*susA* mRNA in the dark, the Av-SuS polypeptide level and SuS activity did not increase proportionally. Regarding

the regulation of the expression of Av-*susA*, since Fru somehow mimicked the effect of light on Av-*susA* mRNA accumulation, it can be speculated that a photosynthetic product(s) related to the carbon status of the cell might be acting as a signal rather than a direct effect of light.

It has been recently shown that in *Anabaena* sp. the expression of *susA* and *rbcLS* (ribulose-1,5-biphosphate carboxylase/oxygenase encoding gene) is coordinated at the transcriptional level in a network that integrates signals from nitrogen metabolism and development (Curatti et al. 2006). This network also appears to comprise the biosynthesis of reserve and structural polysaccharides and highlights the function of SuS in metabolic homeostasis.

Taken together, our work strongly suggests that in filamentous nitrogen-fixing cyanobacteria, at least part of the carbon fixed during the light period, is channeled towards the accumulation of reserve and structural polysaccharides in a pathway that involves Suc synthesis and cleavage. The regulation of the expression of the SuS encoding gene by light, nitrogen, and carbon source is one of the regulatory steps of this pathway.

A diagram of carbon cycling in vegetative cells of *Anabaena variabilis* based on this work and previous reports is shown in Fig. 5. Suc and glycogen synthesis are especially active in nitrogen-fixing filaments, where SuS contributes to the glycogen synthesis yielding ADP-Glc from the cleavage of Suc (Cumino et al. 2007; this work). Although *susA* expression is up regulated by ammonium, in the absence of this nitrogen source, it is high enough to allow Suc channeling towards the biosynthesis of glycogen, increasing its pool size. Thus, the function of SuS in the control of the carbon flux from vegetative cells to heterocysts might be part of an extended network of carbon cycling in these cells. The alkaline/neutral invertase activity that is upregulated during nitrogen fixation (Vargas et al. 2003) is thought to decrease Suc pool size while keeping a high Suc turnover rate. When ammonium is available, its assimilation becomes the main sink of carbon skeletons in vegetative cells and the stimulation of both Suc and glycogen breakdown redirects carbon towards ammonium assimilation. Conversely to the vegetative cells of the diazotrophic filaments, Suc synthesis is attenuated by down-regulation of Suc-synthesis involved genes (Cumino et al. 2007) and Suc breakdown is increased by upregulation of SuS, what might result in relatively smaller Suc pool size and slower Suc turn over. This interpretation is consistent with what has been shown in eukaryotic algae regarding enhanced starch and Suc breakdown (Miyachi and Miyachi 1985) and a decrease of the flow of recently assimilated carbon to Suc and starch (Kanazawa et al. 1970) during ammonium assimilation. A similar regulation of glycogen metabolism by ammonium was observed in the unicellular, non-nitrogen fixing cyanobacterium *Anacystis nidulands* (García-González et al. 1992; Lara and Guerrero 1997). In *A. variabilis*, exogenously supplied Fru stimulates both Suc synthesis through SPS (Cumino et al. 2007) and Suc cleavage by SuS in such a way that Suc turnover is accelerated at the same time Suc pool size builds up. Finally, light exerts a more profound effect on SuS than on SPS activity, and might ensure a faster turnover of Suc that redirects carbon flux towards polysaccharides biosynthesis and significantly controls Suc pool size.

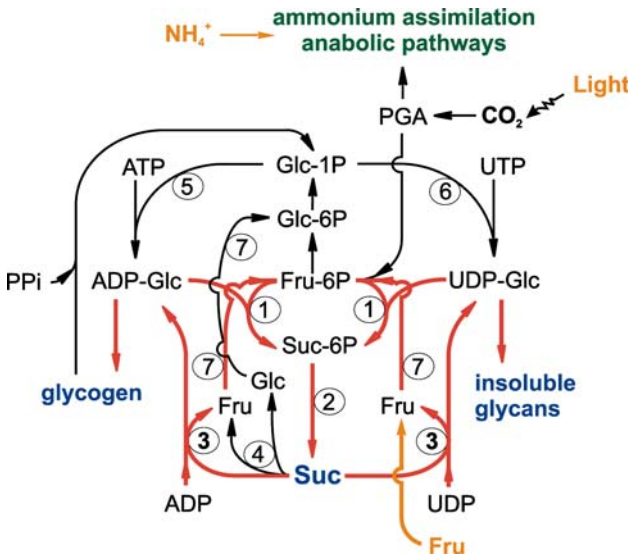

Fig. 5 Diagram of carbon cycling in *Anabaena variabilis* vegetative cells. Photosynthetic carbon fixation through the Calvin Cycle and/or organic carbon assimilation occurs, which lead to Suc and polysaccharides biosynthesis. SuS activity is regulated at the transciptional level by the carbon and nitrogen sources and light, which represent one of the instances where carbon flux is controlled in filamentous cyanobacteria. See "Discussion" for a more detailed explanation of the model. Enzymes are indicated with *circles*: (*1*), SPS; (*2*), sucrose-phosphate phosphatase; (*3*), SuS; (*4*), alkaline/neutral invertase; (*5*), AGPase; (*6*), UDP-Glc pyrophosphorylase, and (*7*), hexokinase

This study contributes to an emerging concept that shows that not only the enzymology of Suc and polysaccharides metabolism has been conserved during evolution from cyanobacteria to higher plants, but also that some basic regulatory aspects of carbon assimilation have been conserved as well. Additionally, as *A. variabilis* is one of the organisms of choice for the nitrogenase-mediated phosynthetic production of hydrogen as a nonpolluting fuel (Dutta et al. 2005; Sakurai and Masukawa 2007; Ghirardi et al. 2007), and Fru feeding to this cyanobacterium significantly stimulates growth, heterocyst development, nitrogenase activity and hydrogen evolution (Haury and Spiller 1981; Lang et al. 1987; Reddy et al. 1996), a better understanding of the mechanism and regulation of this system at the genetic and biochemical levels can be useful for designing genetically modified strains for energy purposes in the near future.

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