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Functional characterization of *Synechococcus* amylosucrase and fructokinase encoding genes discovers two novel actors on the stage of cyanobacterial sucrose metabolism☆

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ABSTRACT

Plants and most cyanobacteria metabolize sucrose (Suc) with a similar set of enzymes. In *Synechococcus* sp. PCC 7002, a marine cyanobacterium strain, genes involved in Suc synthesis (*spsA* and *sppA*) have been characterized; however, its breakdown was still unknown. Indeed, neither invertase nor sucrose synthase genes, usually found in plants and cyanobacteria, were found in that *Synechococcus* genome. In the present study, we functionally characterized the *amsA* gene that codes for an amylosucrase (AMS), a glycoside-hydrolase family 13 enzyme described in bacteria, which may catabolyze Suc in *Synechococcus*. Additionally, we identified and characterized the *frkA* gene that codes for a fructokinase (FRK), enzyme that yields fructose-6P, one of the substrates for Suc synthesis. Interestingly, we demonstrate that *spsA*, *sppA*, *frkA* and *amsA* are grouped in a transcriptional unit that were named Suc cluster, whose expression is increased in response to a salt treatment. This is the first report on the characterization of an AMS and FRK in an oxygenic photosynthetic microorganism, which could be associated with Suc metabolism.

Introduction

In the biosphere, sucrose (Suc) is mainly synthesized by plants, unicellular algae, and cyanobacteria as part of the carbon dioxide assimilation pathway [1,2]. In plant life, Suc is a key molecule transported from leaves to heterotrophic tissues, playing a crucial role in growth and development, in stress responses and in signal transduction pathways [3]. Suc metabolism has been described in plants, some unicellular algae and cyanobacteria, most of them using a similar set of enzymes [4,5]. Thus, Suc biosynthesis in cyanobacteria has been reported either in filamentous heterocyst-forming (*Anabaena* sp. PCC 7119, PCC 7120), or in unicellular strains (*Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002) [4,6,7]. It is achieved by a two-step pathway, involving the sequential action of sucrose-phosphate synthase (SPS) and sucrose-phosphate-phosphatase (SPP) [4,8,9]. On the other hand, Suc breakdown was shown to be carried out by the action of alkaline/neutral invertases (A/N-Inv), non-glycosylated proteins (in contrast to plant acid invertases) grouped in the glycoside hydrolase family 100 [10–12], which catalyze the irreversible hydrolysis of Suc into hexoses, and/or by Suc synthase (SuS) [13–15]. This enzyme has been reported in heterocyst-forming strains providing sugar nucleotide for glycogen synthesis and seems to be related to nitrogen fixation [16,17]. Recently, SuS was also described in three unicellular strains (*Microcystis aeruginosa* PCC 7896, *Gloebacter violaceus* PCC 7421, and *Thermosynechococcus elongatus* BP-1) [18,19].

Synechococcus sp. PCC 7002 is a euryhaline cyanobacterium considered as a model marine strain. Because it is considered as a potential platform for biotechnological applications, global transcription profiles were recently performed [20,21]. This strain is capable of growing in conditions ranging from freshwater up to over 1 M salt concentration, and tolerates high-light intensity. In response to salinity, cells synthesize glucosylglycerol, as the main compatible solute, Suc, and glucosylglycerate to a minor extent [22,23]. The functional characterization of Suc biosynthesis encoding genes (*spsA* and *sppA*), the analysis of their contiguous genome location and their co-transcription were recently reported [7].

Abbreviations: AMS, amylosucrase; FRK, fructokinase; GH, glycoside hydrolase; SPS, sucrose-phosphate synthase; SPP, sucrose-phosphate phosphatase; Suc, sucrose; SuS, sucrose synthase; A/N-Invertase, alkaline neutral invertase; *Syn* 7002, *Synechococcus* sp. PCC 7002.

☆ Sequences reported in this paper have been deposited in the EMBL-EBI database (accession nos. HG798542 and HG798543, *Synechococcus* sp. PCC 7002 *amsA* and *frkA* gene for amylosucrase and fructokinase, respectively).

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In this study we investigated Suc breakdown in the marine cyanobacterium *Synechococcus* sp. PCC 7002 and functionally characterized the *amsA* gene that codes for an amylosucrase (AMS), which is able to catabolyze Suc. Additionally, we characterized the *frkA* gene that codes for a fructokinase (FRK), enzyme that yields fructose-6P, one of the substrates for Suc synthesis. Interestingly, the coding genes for SPS, SPP, AMS and FRK are grouped in a four-genes transcriptional unit, whose expression is increased in response to salt. This is the first report on the characterization of an AMS in an oxygenic photosynthetic organism and on a FRK protein in cyanobacteria. Remarkably, both enzymes could be involved in Suc metabolism.

Materials and methods

Bacterial strains and growth

Synechococcus sp. strain PCC 7002 (*Syn* 7002) cells were routinely cultivated in ASNIII – BG11 medium supplemented with B12 vitamin. *Escherichia coli* DH5α and BL21(DE3):pLysS (Novagen) strains were grown in Luria Bertani medium supplemented with 50 μg/ml carbenicillin. To study the effect of salt, cells previously cultured at standard conditions up to exponential phase, were collected and transferred to BG11 medium for 5 days. At this time, the cultures were supplemented with NaCl (684 mM final concentration). To determine Suc hydrolysis activity, *Syn*-7002 cells were permeabilized as described previously [24].

Protein extraction and partial Suc hydrolysis activity purification

Homogenates were prepared from cells previously powered in liquid nitrogen in the presence of glass beads with mortar as described [25]. Extracts were centrifuged at 18,000 × *g* for 30 min. The clarified supernatant was loaded onto a Deae-Sephadex column (1 × 18 cm) equilibrated with 20 mM Hepes–NaOH (pH 6.5), 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.5 mM phenyl-methylsulfonylfluoride and 20% glycerol. The column was washed with 100 ml of the equilibration buffer and eluted with a linear NaCl gradient (0–0.5 M) in the same buffer [25]. Fractions with Suc breakdown activity were pooled and concentrated. Protein concentrations were determined according to Bradford [26] using bovine serum albumin as standard.

Cloning and expression of FRK and AMS genes

Sequences of two *orf*s (named 876-*orf* and 1986-*orf*) were retrieved from the *Syn*-7002 genome (http://www.ncbi.nlm.nih.gov) and deposited in the EMBL-EBI database after functional characterization as fructokinase (FRK) and amylosucrase (AMS) encoding genes, respectively. Two DNA fragments of 910 and 2015 bp were PCR-amplified using the primer pairs *frkA* −15 + 4 F/*frkA* +863 + 879 R and *amsA* −3 + 12 F/*amsA* +1980 + 1997 R (Table S1), respectively. Amplification products were ligated into the pRSET-A vector (Invitrogen, Carlsbad, CA) between the restriction sites *Bam*HI and *Hind*III for *frkA*, or *Bam*HI and *Eco*RI for *amsA*, obtaining the recombinant plasmids pR-*frkA* and pR-*amsA*, respectively. The identity of each construct was confirmed by DNA sequencing. *E. coli* BL21(DE3)pLyS cells were transformed with pR-*frkA* or pR-*amsA*, to produce the recombinant proteins His_6::7002-FRK and His_6::7002-AMS, respectively [27]. The expression of the recombinant proteins His_6::7002-FRK and His_6::7002-AMS was induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG) when the culture reached an A_{600nm} of about 0.5. After 3 h induction at 30 °C, cells were harvested and each fusion protein (His_6::7002-FRK or His_6::7002-AMS) was purified throughout Co^{2+} affinity chromatography (TALON® resin, Clontech) and concentrated for further studies. Cell extracts from IPTG-treated or non-induced cells and purified His_6::7002-FRK and His_6::7002-AMS were analyzed by SDS-PAGE [7].

Supplementary Table S1 can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.04.003.

Isolation of RNA and RT-PCR assays

RNA from *Syn*-7002 cells was isolated using the TRIZOL reagent (Gibco-BRL/Invitrogen). For RT-PCR analysis, total RNA (2.5 μg) treated with DNase (RQ1 RNase-free DNase, Promega) was reverse-transcribed using MMLV (Moloney murine leukemia virus) reverse transcriptase (Promega) and the specific primer *amsA* +1980 + 1997 R (Table S1). Subsequently, the cDNA was amplified using combinations of the primers: (a) RT *spsA–sppA* F/*sppA* +173 + 193 R, (b) RT *sppA–frkA* F/*frkA* +139 + 158 R, and (c) RT *frkA–amsA* F/*amsA* +230 + 250 R (Table S1). As a control, cDNA of 16S rRNA was amplified using the primers SYN172 16S F/OXY1313 16S R. PCR reactions were run on a Mastercycler® epgradient cycler (Eppendorf) for 20 cycles of 94 °C (1 min), 58 °C (1 min), and 72 °C (1 min), and a single step at 72 °C (5 min).

Assay for AMS activity

In the standard procedure, aliquots of cell extracts or purified enzymes were incubated at 30 °C for 30 min in Na-phosphate buffer (pH 7.5) supplemented with 100 mM Suc. Reducing sugars were quantified by coupling hexokinase, phosphoglucose isomerase (PGI) plus glucose 6-phosphate dehydrogenase, in the presence of NADP. The amounts of glucose and fructose were distinguished by adding or omitting PGI, and following spectrophotometrically the appearance of NADPH [28]. The production of fructose corresponds to total Suc consumed, and therefore, determination of fructose was considered as total activity. Suc hydrolysis activity was measured by the amount of glucose produced. Transglycosylation activity was calculated by subtracting the amount of glucose from the amount of fructose released. The effect of pH on enzymatic activity was investigated within a range of pH 5.0–10.0 (0.1 M sodium citrate buffer for pH 5.0–7.0, 0.1 M Tris–HCl for pH 7.0–9.0, and 0.1 M glycine–NaOH for pH 9.0 and 10.0) at 30 °C. The effect of temperature on the activity was studied between 30 and 50 °C at pH 7.5.

Assay for FRK activity

FRK activity was measured at 30 °C using the coupling enzymes PGI and G6PDH [28]. The reaction mixture contained 100 mM Hepes–NaOH (pH 7.5), 10 mM ATP, 20 mM $MgCl_2$, 10 mM fructose, 1.2 mM NADP, 0.25 U of PGI, and 2 U of G6PDH. The reaction was started by addition of fructose. The reduction of NADP was monitored on a Shimadzu UV1700 spectrophotometer (Japan) at 340 nm. To measure FRK substrate specificity, the production of ADP was quantified as the rate of NADH oxidation after incubation at 30 °C with coupled enzymes. The reaction mixture contained 50 mM Tris–HCl (pH 9.0), 5 mM ATP, 10 mM $MgCl_2$, 0.5 mM NADH, 5 mM phosphoenolpyruvate, 1 U of pyruvate kinase, and 1 U of lactate dehydrogenase, in 1-ml final volume. The rate of NADH oxidation was evaluated at 340 nm. Different saccharides were tested at 5 mM final concentration.

Phylogenetic analyses

Amino-acid sequences deduced from nucleotide sequences homologous to *Syn*-7002 *asmA* or *frkA* genes were first compared with those present in public databases using the

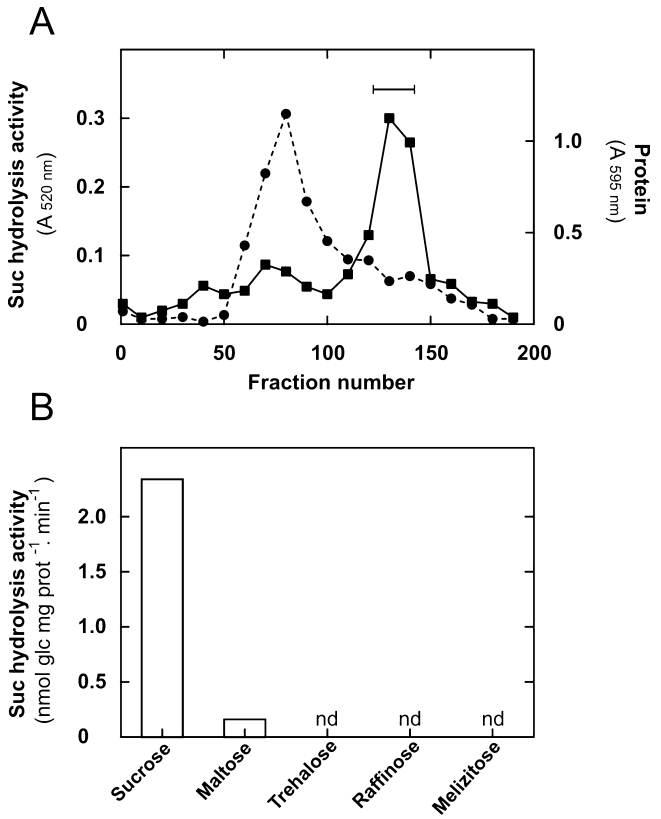

Fig. 1. Detection of Suc hydrolysis activity in *Synechococcus* sp. PCC 7002 cells. (A) DEAE-Sephacel chromatography of crude extracts from *Syn*-7002 cells. Suc hydrolysis activity (■) and protein (●– – –●). (B) Hydrolysis specificity. Activity was assayed incubating an aliquot of the concentrated pooled fractions (120–145) from the Deae-Sephacel chromatography, with different sugars (10 mM final concentration), at pH 7.5 during 1 h at 30 °C. Reductant monosaccharides were determined by the Somogyi–Nelson method. Nd: not detected.

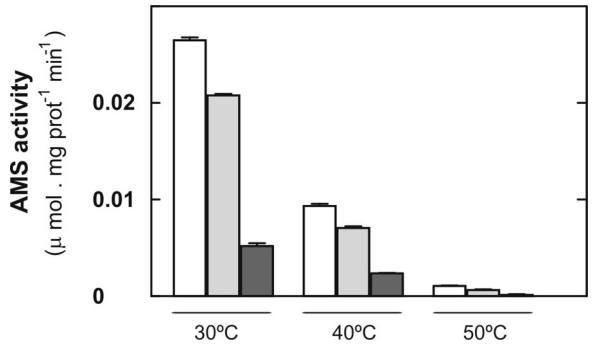

Fig. 2. Enzyme activity of the recombinant His_6::7002-AMS at different temperatures. The reaction was performed in a mixture containing an aliquot of the purified protein, 10 mM Suc, and 100 mM sodium-phosphate buffer, pH 7.5. Production of fructose was considered a quantification of total activity (white bars). Suc hydrolysis activity was estimated from the amount of glucose produced (light grey bars). Transglycosylation activity was calculated by subtracting the amount of glucose to that of fructose produced (dark grey bars).

basic local alignment search tool BLAST network service (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed with the neighbor-joining and maximum parsimony methods, and bootstrap analyses (1000 replicates) were done with *MEGA* version 5. The Integrated Microbial Genomes (IMG, http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) system was used to derive the comparison of gene cluster organization.

Results

Search for Suc degrading proteins in Synechococcus sp. PCC 7002

In preliminary experiments looking for Suc hydrolysis activity in *Synechococcus* sp. PCC 7002 (hereinafter *Syn* 7002), we measured Suc breakdown into reductant monosaccharides in toluene-permeabilized cells (Fig. S1) and in crude extracts. To partially purify this activity, crude extracts were chromatographed onto an ion exchange column (Fig. 1A). Suc hydrolysis activity was specific for Suc when it was assayed using the concentrated partially purified enzyme (Fig. 1B).

Supplementary Fig. S1 can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.04.003.

A comprehensive analysis of *Syn*-7002 genome revealed that there is no homologous sequence to those encoding invertases and/or SuSs, Suc catabolizing proteins reported in cyanobacteria and plants [4,5]. Consequently, we investigated the presence in the genome of other sequences related to Suc breakdown. A careful survey of the open reading frames (*orfs*) surrounding the recently described *Syn*-7002 *spsA–sppA* genes [7] disclosed two *orfs* (SYNPCC7002_A0885 and SYNPCC7002_A0886) annotated as putative α-amylase (AMS) subunit A and as putative fructokinase (FRK), member of the family of carbohydrate kinases, respectively. AMS enzymes had been reported to exhibit Suc hydrolysis activity, among others [29].

Functional identification of Syn-7002 amylosucrase gene

The deduced amino-acid sequence of the putative AMS *orf* (SYNPCC7002_A0885, named 1986-*orf*) was 39.7%, 37.8%, 40.6%, 42.2%, and 42% identical to those of functionally characterized AMSs from *Neisseria polysaccharea* [30], *Deinococcus radiodurans* [31], *D. geothermalis* [32], *Alteromonas macleodii* [33], and *Arthrobacter chlorophenolicus* A6 [34], respectively. The calculated Mr of the deduced amino-acid peptide is 76,499 Da. Sequence alignments show a high conservation of amino-acid residues among different microorganisms (Fig. S2). For functional characterization, the 1986-*orf* was PCR-amplified, cloned, and expressed in *E. coli*.

Supplementary Fig. S2 can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.04.003.

It is known that AMS exhibits multiple catalytic activities. Primarily, it can hydrolyze Suc to glucose and fructose or transfer glucose from Suc hydrolysis to another glucose or acceptor molecule. As a side reaction, it is also able to catalyze the isomerization of Suc to turanose or trehalulose [29,33,35,36]. When *E. coli* cells expressing the 1986-*orf* sequence were incubated with Suc, they exhibited hydrolysis and transglucosylation activity. Both activities were highest at 45 °C for the *A. macleodii* AMS [33], but this was not the case for the recombinant *E. coli* protein (His_6::7002-AMS) (Fig. 2). The purified recombinant protein showed an apparent molecular mass as predicted (Fig. 3A) and maximum activities at pH between 6.5 and 7.0 (Fig. 3B). Thus, the 1986-*orf* was characterized as an AMS encoding gene (*amsA*).

We retrieved homologs with high similarity to the *amsA* sequence from a few among all cyanobacterial genomes sequenced to date, most belonging to unicellular strains and to the *Synechococcus* genus (e.g. PCC 73109, 8807, 7117 and 7003, four strains closely related to Syn 7002). These *Synechococcus* sequences grouped with SPS and SPP putative genes, similarly to the functionally characterized *Syn*-7002 *asmA* (Fig. S3A).

Supplementary Fig. S3 can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.04.003.

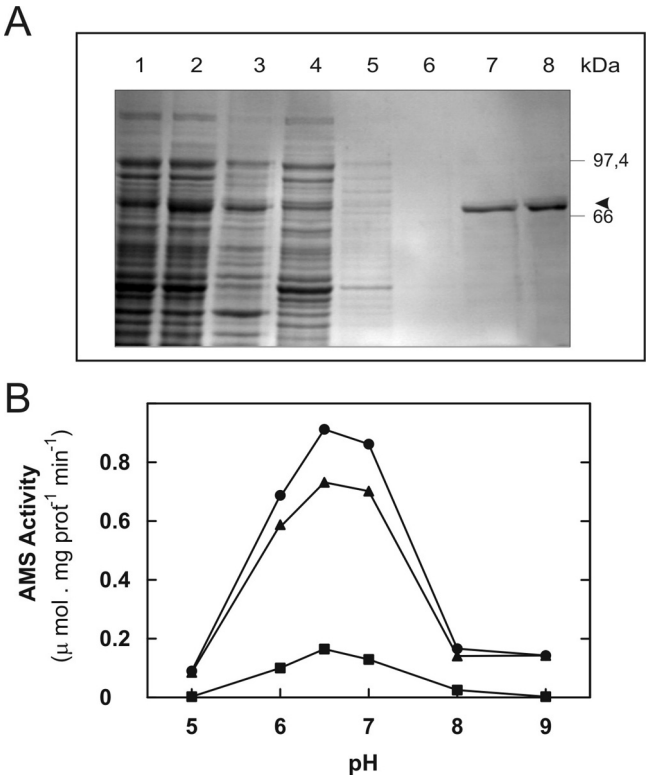

Fig. 3. Characterization of the *Synechococcus* sp. PCC 7002 AMS encoding gene (*asmA*) by heterologous expression in *E. coli*. (A) Characterization of the protein product of 1986-*orf* expression in *E. coli*. Polypeptides were separated by SDS-PAGE in a 12% (w/v) gel and Coomassie blue stained. Lanes 1 and 2, total soluble extract from *E. coli* (pR-*amsA*) non-induced or 1-M IPTG induced cells, respectively; lane 3, insoluble proteins of crude extract; lanes 4 and 5, proteins eluted from the Ni-column after a first and second washing with 5 mM imidazole (pH 7.5); lanes 6 and 7, proteins eluted with 20 and 150 mM imidazole (pH 7.5), respectively; lane 8, concentrated purified recombinant His_6::7002-AMS. The arrowhead indicates the position of His_6::7002-AMS. (B) Effect of pH on total activity (●–●), Suc hydrolysis (■--■), and transglycosylation activity (▲–▲), using recombinant purified His_6::7002-AMS.

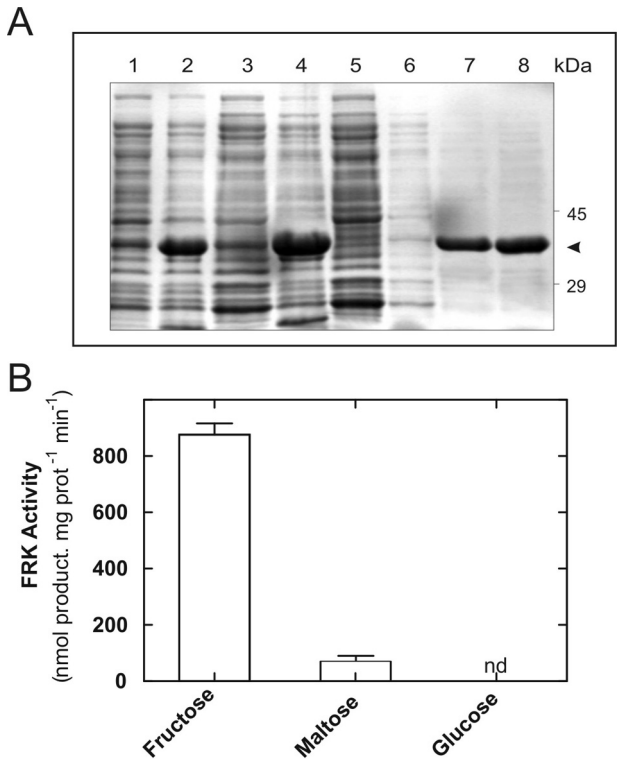

Fig. 4. Characterization of the *Synechococcus* sp. PCC 7002 FRK encoding gene (*frkA*) by heterologous expression in *E. coli*. (A) Characterization of the protein product of 876-*orf* expression in *E. coli*. Polypeptides were separated by SDS-PAGE in a 12% (w/v) gel and stained with Coomassie blue. Lanes 1 and 2, total extract from *E. coli* (pR-*frkA*) non-induced or 1-M IPTG induced cells, respectively; lanes 3 and 4, soluble and insoluble fraction of IPTG-induced *E. coli* cells, respectively; lanes 5 and 6, proteins eluted from the Ni-column after 5 and 20 mM imidazole (pH 7.5) washing, respectively; lane 7, proteins eluted with 150 mM imidazole (pH 7.5); lane 8, concentrated purified recombinant His_6::7002-FRK. The arrowhead indicates the position of His_6::7002-FRK. (B) Substrate specificity. Different sugars at 5 mM final concentration were incubated in the presence of His_6::7002-FRK, Hepes-NaOH pH 7.5, and 5 mM Mg^{2+}-ATP, for 30 min at 30 °C. Values are the mean ± S.D.; $n = 3$.

Functional identification of Syn-7002 FRK gene

The 876-*orf* neighbor to *spsA–sppA* genes encodes a putative 291-amino-acid protein with an apparent molecular mass of 32,398 Da, homologous to characterized FRKs. The deduced amino-acid sequence shares 24% and 47% identity with the characterized and crystallized *Halothermothrix orenii* FRK and with *Methylomicrobium alcaliphilum* FRK, respectively [37,38]. The putative *Syn*-7002 FRK contains a typical ATP binding motif and putative substrate recognition sequences present in the *H. orenii* FRK (Fig. S4). The functional characterization of the 876-*orf* was performed by heterologous expression in *E. coli* (Fig. 4). The apparent molecular mass of the His-tagged recombinant protein was about 33 kDa calculated from SDS-PAGE, which is in agreement with the predicted deduced amino-acid sequence. The purified His_6::7002-FRK catalyzed the highly specific ATP-dependent phosphorylation of fructose into fructose-6-phosphate, one of the substrates for Suc synthesis. FRK activity was dependent on Mg^{2+} with maximum pH around 7.5 (not shown). These results indicate that the 876-*orf* encodes an authentic FRK protein and was named *frkA*.

Supplementary Fig. S4 can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.04.003.

Homologs to *frkA* sequence could be retrieved from bacterial, a few cyanobacterial and plant genomes. *Syn*-7002 *frkA* sequence also grouped with Suc-biosynthesis related sequences (*spsA* and *sppA*), pattern that was found in other four *Synechococcus* strains and in *Cyanobium gracile* (also known as *Synechococcus* sp. PCC 6307) genomes (Fig. S3A).

Syn-7002 Suc cluster

The occurrence of a cluster containing *spsA*, *sppA*, *frkA and amsA* genes suggested that *frkA* and *amsA* may be related with Suc metabolism in *Syn* 7002. Thus, this arrangement was named Suc cluster. The *frkA* gene is separated by 14 and 110 bp from *sppA* and *amsA*, respectively (Fig. 5A). In silica analysis using VIMSS Predicted Operon software supported that hypothesis. Total RNA isolated from *Syn*-7002 cells treated with cloramphenicol and salt [7] was transcribed into cDNA by using the external primer *amsA* +1980 + 1997 (Table S1, Fig. 5A) in a reverse transcriptase reaction. From the cDNA product, intergenic regions (*spsA–sppA*, *sppA–frkA*, and *frkA–amsA*) were PCR amplified (Fig. 5B) using the corresponding primer pairs (a, b, and c, Table S1), indicating the existence of a single transcript. Additionally, the effect of a salt treatment on the expression of Suc-cluster individual genes was investigated by RT-PCR. Levels of *spsA*, *sppA*, *frkA* and *amsA* transcripts increased after 2 h of salt treatment (Fig. 6).

Discussion

Although SPS and SPP, involved in Suc synthesis, have been fully characterized in the marine cyanobacterium *Synechococcus* sp. PCC 7002 [7], proteins responsible for Suc breakdown were

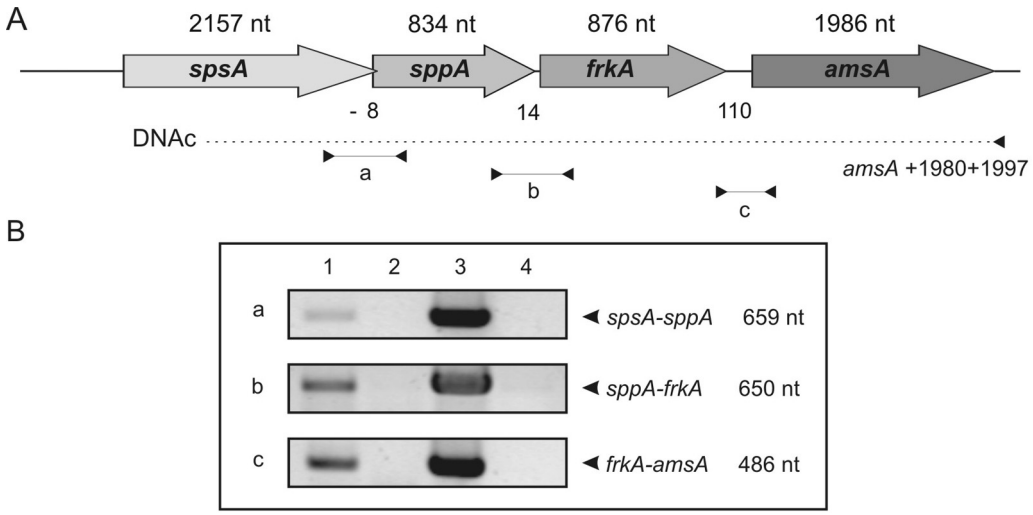

Fig. 5. Genomic organization and expression of the Suc cluster. (A) Schematic representation of the contiguous location of the genes *spsA*, *sppA*, *frkA* and *amsA* in the *Synechococcus* sp. 7002 genome. Distances between genes or overlapping are indicated. Location of the primers used to amplify intergenic regions (a, *spsA–sppA*; b, *sppA–frkA*; and c, *frkA–amsA*) (Table S1) are indicated. (B) Gel electrophoresis of the RT-PCR products from the amplification of total RNA prepared from *Syn*-7002 cells treated with 684 mM NaCl and 30 μg/ml choramphenicol [7]. (a–c) Amplification products including intergenic spaces of contiguous genes (*spsA–sppA*, *sppA–frkA*, and *frkA–amsA*, respectively). Lane 1, PCR-amplification products; lanes 2 and 4, negative controls (without reverse transcriptase or without template, respectively); lane 3, positive control, using genomic DNA as template.

still unknown. Since we determined Suc hydrolytic activity in *Syn*-7002 cells (Figs. 1 and S1), the lack of sequences homologous to those encoding known Suc-breakdown proteins in oxygenic-photosynthetic organisms (alkaline/neutral invertases and/or SuS) prompted the present study. Exploring the neighboring sequences to *spsA-sppA* [7] brought to light the existence of sequences encoding putative proteins that could be related to Suc metabolism (Figs. S3A and 5A). The functional characterization of those sequences confirmed that they code for AMS and FRK enzymes (Figs. 3 and 4).

Although *Syn* 7002 is a model strain for marine cyanobacteria, it represents a particular case regarding Suc metabolism. In most reported cyanobacterial genomes, SPS and SPP genes are located separately, in different regions of the chromosome; however, in *Syn* 7002 this does not happen. Not only was it shown that 7002-*spsA* and 7002-*sppA* genes are co-transcribed [7], but also that they are

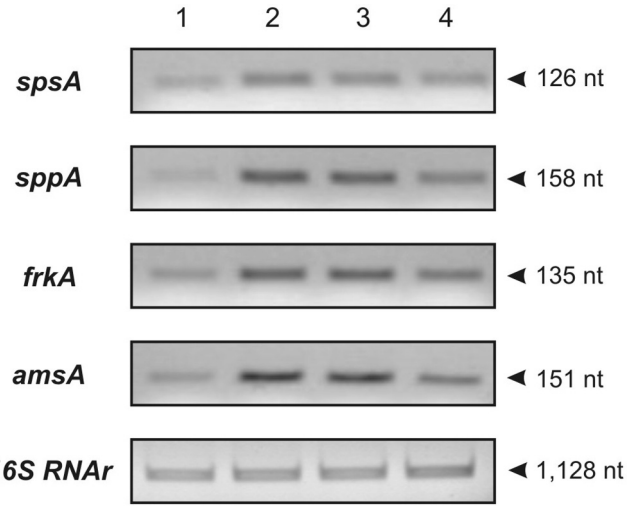

Fig. 6. Expression analysis by RT-PCR of *spsA*, *sppA*, *frkA* and *amsA* from total RNA from salt treated *Synechococcus* sp. 7002 cells. Amplification products were separated by eletrophoresis in 2% agarose and stained. Lane 1, control (no salt addition); lanes 2–4, salt (684 mM) treated cells for 2, 6 and 24 h, respectively. Amplification of *Syn*-7002 RNAr 16S was used as control.

grouped with two other genes (Figs. S3A and 5A) coding for proteins that could be related to Suc metabolism. Thus, *amsA* encodes a protein that can hydrolyze Suc (7002-AMS), and *frkA* encodes a fructose specific kinase (7002-FRK), which can yield one of the substrates for Suc synthesis. Therefore, the *sps–spp–frk–ams* genomic organization was called Suc cluster. A similar four-gene organization was found in few cyanobacterial genomes (Fig. S3A) and in several alpha proteobacteria. Recently, its occurrence was reported in the methanotroph strain *Methylomicrobium alcaliphilum* 20Z [38,39], suggesting a possible lateral gene transfer from bacteria as the origin of the *Syn*-7002 Suc cluster. Additional phylogenetic analyses performed with sequences of 7002-AMS and 7002-FRK homologs from cyanobacteria, plants and bacteria support a bacterial origin for both proteins (Figs. S5 and S6). While AMS is likely to be restricted to microorganisms, FRK proteins could be found in cyanobacteria and plants.

Supplementary Figs. 5 and 6 can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.04.003.

Suc hydrolysis by an AMS (EC 2.4.1.4) has not been reported for oxygenic photosynthetic organisms, however, it was for bacteria, where these proteins have been extensively studied [31–33,40]. AMS that belongs to glycoside hydrolase (GH) family 13 (the α-amylase family), is organized in five domains, namely A, B, C, N, and B′. The first three domains are characteristic of the GH family, and B′ and N domains are only found in AMS [31]. The amino-acid residues required for catalytic activities are conserved in the 7002-AMS and in the other cyanobacterial genomes. Remarkably, Suc catabolism by AMS allows *Syn*-7002 cells not only to hydrolyze Suc to monosaccharides supplying carbon and energy, but also to synthesize amylose-like polymers, without the requirement of an expensive nucleotide activated sugar as donor.

FRK (EC 2.7.1.4) enzymes have been described in numerous bacteria, where their encoding genes are commonly found in an operon involved in Suc utilization, including genes for Suc transport [41–43], and in various plant species (*Solanum lycopersicum*, *Zea mays*, *Oryza sativa*, *S. tuberosum*, *Beta vulgaris*, among others) [44–48]. FRK proteins belong to the ribokinase-like superfamily of sugar kinases. By comparison with the crystal structure of the *H. orenii* protein [37], the residues Thr243 and Gly274 (but not the Asn299) are conserved in 7002-FRK (Fig. S4), similarly to other

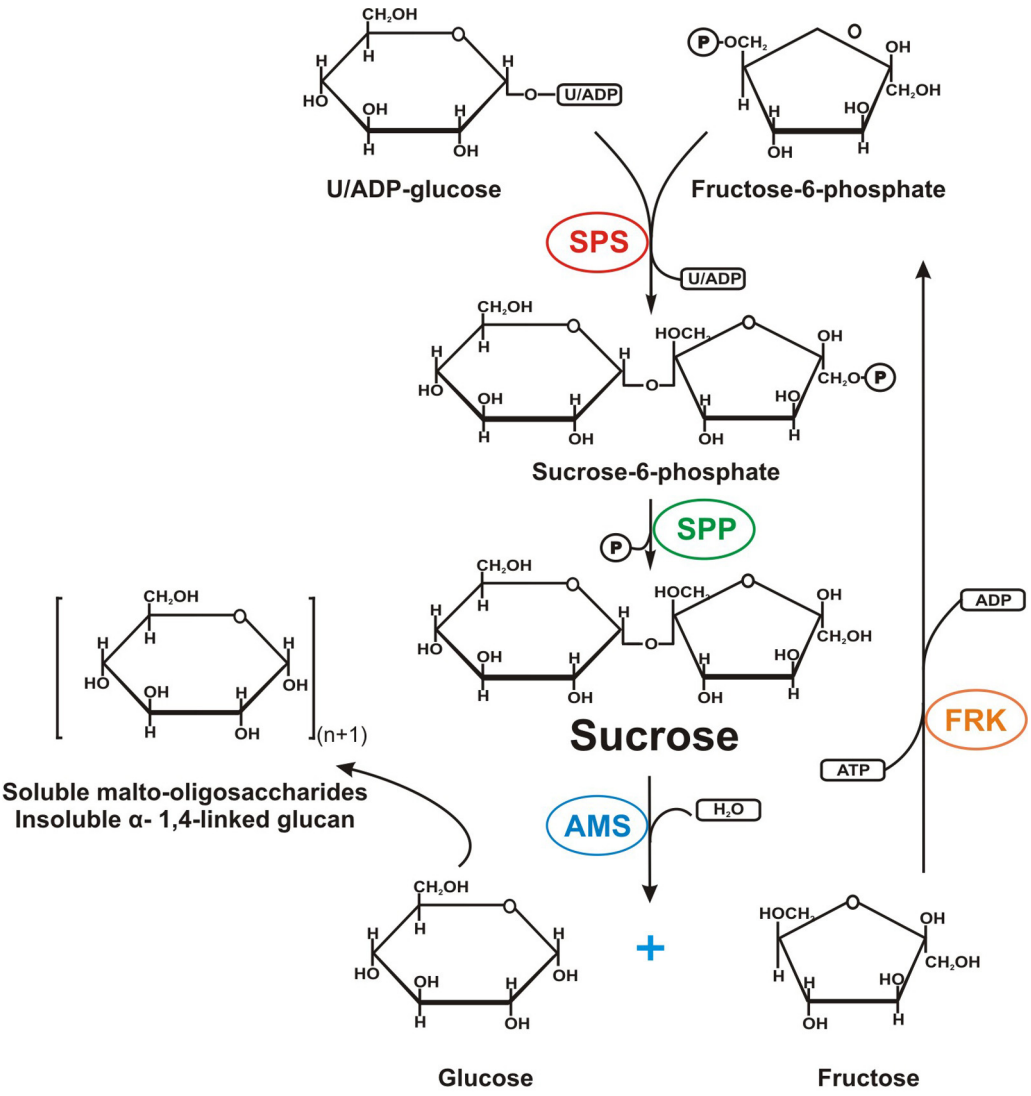

Fig. 7. Schematic representation of Suc metabolism reactions in *Synechococcus* sp. 7002. Suc biosynthesis pathway involves the sequential action of SPS and SPP [7]. yielding Suc and inorganic phosphate (Pi). In the present study we demonstrate that AMS is able to catalyze Suc hydrolysis to glucose and fructose, and/or transferring the glucose moiety to a maltooligosaccharide or α 1,4-glucan, and that FRK is able to activate the released fructose by phosphorylation in the presence of ATP. The resulting fructose-6P can re-enter the route of Suc biosynthesis.

characterized FRK from bacteria [38]. It has been reported that in plants and bacteria, FRK regulates the rate and localization of carbon usage by channeling fructose into a metabolically active state for glycolysis [49]. In plants, fructose phosphorylation is particularly important in sink tissues, where the action of SuS produces fructose that must be phosphorylated to maintain the carbon flux to starch or respiration. In contrast, to date there is no report on the characterization of a cyanobacterial FRK. We functionally characterized the *frkA* gene from *Syn* 7002 (a strain that lacks SuS activity) located in the Suc cluster (Fig. 5), suggesting that it may play a role in regenerating the substrate for Suc synthesis (Fig. 7).

The four genes grouped in the Suc cluster suggest an operon typology, which is also supported by in silica prediction and experimental evidence (Fig. 5). Most operons are under the control of a single transcriptional promoter located upstream of the first gene. We have previously determined the RNA 5′-ends upstream of the *spsA* gene, which corresponds to transcription start points (*tsps*) from RNA total obtained from salt-treated *Syn*-7002 cells, mapped by primer extension methodology. Two RNA sizes (RNA_I and RNA_{II}) were observed, starting at the −62 (putative transcription starting point I, tsp_I) and −94 (putative tsp_{II}) nucleotides upstream of the translation initiation site [7]. Interestingly, it was found a (GGAtC-N19-CGTT) motif similar to the consensus sequence GGAAC(N16/17)CGTT found in the binding site of an alternative sigma factor involved in responses to saline and osmotic stress in some eubacteria [50]. Since the expression of the four genes of the Suc cluster increased with a 2-h salt treatment, it could be suggested that the co-transcription could take place from one or both putative *tsps*. However, it cannot be discounted that additional *tsps* may regulate individually the transcription of *amsA* or *frkA* since it has also been reported more complex transcriptional regulation with multiple promoters and transcriptional terminators in a single operon [51].

Our results suggest that a Suc cycling mechanism might be operating in *Syn*-7002 cells under certain conditions such as salt treatment, allowing cell metabolism to shift easily from Suc production to degradation. Such mechanism has been reported in plant tissues and in the cyanobacterium *Anabaena* sp. PCC 7120 [15, Salerno, personal communication], but its function remain poorly understood. It has been suggested that Suc cycles allow the pathway's net flux a high degree of sensitivity to respond to factors modulating rates of synthesis and degradation, controlling

respiration, maintaining osmotic potential, controlling sugar accumulation, and promoting sugar signaling [52,53].

Finally, the gene structure *spsA–sppA–frk–ams* has not been reported in any other oxygenic photosynthetic organism and opens new insights in Suc metabolism regulation in relation to carbon assimilation and response to stress. Taking together our results we propose a picture of Suc metabolism reactions in *Syn* 7002 (Fig. 7).

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