Planta (2003) 216: 951–960
DOI 10.1007/s00425-002-0943-x

ORIGINAL ARTICLE

Walter Vargas · Andrea Cumino · Graciela L. Salerno

Cyanobacterial alkaline/neutral invertases. Origin of sucrose hydrolysis in the plant cytosol?

Received: 30 July 2002 / Accepted: 21 October 2002 / Published online: 18 December 2002

Abstract The aim of this work was to investigate the occurrence of invertase (Inv) in cyanobacteria. We describe the first isolation and characterization of prokaryotic alkaline/neutral Inv (A/N-Inv) genes. Two genes (*inv*A and *inv*B) were identified in *Anabaena* sp. PCC 7120, which share about 50–56% identity with plant A/N-Inv and encode proteins of about 53–55 kDa. The identification of these proteins was confirmed by biochemical and immunological studies with recombinant proteins and with the enzymes isolated from *Anabaena* cells. Expression analysis supported the important role of A/N-Inv in nitrogen-fixing growth conditions. Nevertheless, A/N-Inv activities were shown in all filamentous and unicellular cyanobacteria investigated, regardless of their capacity to fix dinitrogen. Searches in complete sequenced genomes showed that A/N-Inv homologues are restricted to cyanobacterial species and plants. In particular, filamentous nitrogen-fixing strains display two A/N-Inv genes and unicellular strains have only one. Phylogenetic analysis leads us to suggest that modern plant A/N-Inv might have originated from an orthologous ancestral gene after the endosymbiotic origin of chloroplasts.

Keywords *Anabaena* sp. · Cyanobacteria · Neutral/alkaline invertases · Nitrogen metabolism · Sucrose metabolism

Abbreviations A-Inv: alkaline invertase · A/N-Inv: alkaline/neutral invertase · An: *Anabaena* · Inv: invertase or invertases · M_r: relative molecular mass · N-Inv: neutral invertase · SPP: sucrose-phosphate phosphatase · SPS sucrose-phosphate synthase · Suc sucrose · SuS sucrose synthase

W. Vargas · A. Cumino · G.L. Salerno (✉)
Centro de Investigaciones Biológicas,
Fundación para Investigaciones Biológicas Aplicadas (FIBA),
7600 C.C. 1348, Mar del Plata, Argentina
E-mail: fibamdq@infovia.com.ar
Fax: +54-223-4757120

Introduction

Sucrose (Suc) occupies a unique position in plant life as it is a major product of photosynthesis with a central role as transport sugar, in growth, development, storage, signal transduction and stress (Levitt 1980; Avigad and Dey 1997; Smeekens 2000). It is also found in cyanobacteria, prokaryotes that perform oxygenic photosynthesis as plants do (Reed et al. 1984; Page-Sharp et al. 1999), and in a few species of purple bacteria (Khmelenina et al. 1999). In prokaryotes, although Suc accumulation is generally associated with environmental stress responses (Reed et al. 1984; Hagemann and Erdmann 1997; Khmelenina et al. 1999; Potts 2000), its function has not been fully elucidated.

Suc metabolism in plants has been extensively studied. Suc is synthesized in the cytoplasm from the sequential actions of sucrose-phosphate synthase (SPS; EC 2.4.1.14) and sucrose-phosphate phosphatase (SPP; EC 3.1.3.24), and it can be reversibly cleaved by sucrose synthase (SuS; EC 2.4.1.13), or irreversibly hydrolyzed by invertases (Inv; EC 3.2.1.26) when there is a high demand for hexoses (Winter and Huber 2000). Inv exist in several isoforms that differ in pH optimum for activity (acidic, neutral and alkaline; Winter and Huber 2000). The specific functions of the different Inv isoforms are not fully elucidated, but they appear to regulate the entry of Suc into different utilization pathways (Sturm 1999). Acidic pH optimum Inv, extensively studied, are cell-wall or vacuolar enzymes and are evolutionarily related to Inv from yeast and bacteria (Sturm and Chrispeels 1990). In contrast, our knowledge about alkaline/neutral Inv (A/N-Inv), which are thought to accumulate in the cytosol (Chen and Black 1992; Van den Ende and Van Laere 1995), is still very limited (Sturm 1999). They have been purified and characterized from soybean hypocotyl (Chen and Black 1992), *Vicia faba* cotyledons (Ross et al. 1996), *Cichorium intybus* roots (Van den Ende and Van Laere

1995), carrot (Lee and Sturm 1996) and sugarcane (Vorster and Botha 1998). Recently, a cDNA from *Lolium temulentum* was characterized as an A/N-Inv by expression in *Escherichia coli* (Gallagher and Pollock 1998), and a carrot cDNA was isolated by using an internal tryptic peptide sequence of the purified neutral Inv (N-Inv) (Sturm et al. 1999). The c-DNA-deduced amino-acid sequences of A/N-Inv show no similarity to sequences of acid Inv.

Much less is known about Suc metabolism in prokaryotes. SPS and SPP have recently been identified in *Anabaena* sp. (Porchia and Salerno 1996; Cumino et al. 2001, 2002) and in *Synechocystis* sp. PCC 6803 (Curatti et al. 1998; Lunn 2002). In contrast, SuS has been reported only in filamentous-nitrogen fixing cyanobacteria (Porchia et al. 1999; Curatti et al. 2000), in which it was shown to be involved in the cleavage of Suc in vivo and in the control of carbon flux into the N_2-fixing filament (Curatti et al. 2002). On the other hand, the hydrolysis of Suc by A/N-Inv activity was reported in heterocysts of *Anabaena variabilis* (Schilling and Ehrnsperger 1985) associated with nitrogen fixation and in *Scytonema* where it appeared not to respond to salt stress (Page-Sharp et al. 1999).

The present study describes the first isolation and functional characterization of prokaryotic alkaline Inv (A-Inv) and N-Inv genes from *Anabaena* sp. PCC 7120 (*inv*A and *inv*B). Moreover, *Anabaena Inv*A is the only A-Inv gene reported to date. Searches for the occurrence of these genes in complete genomes showed that A/N-Inv homologues are present in all cyanobacterial strains examined and in plants. Phylogenetic analysis suggests that modern plant A/N-Inv might have originated from an orthologous ancestral gene after the endosymbiotic origin of chloroplasts.

Materials and methods

Culture conditions

Cells of the cyanobacteria *Anabaena* sp. PCC 7120, *A. variabilis* PCC 7937, *Fischerella* sp. PCC 7520, *Gloeothece* sp. PCC 6909, *Nostoc* sp. PCC 7107, *Nostoc* sp. PCC 7413, *N. ellisosporum*, *Oscillatoria* sp. PCC 6304, *Synechococcus* sp. PCC 6301, *Synechococcus* sp. PCC 7942, *Synechococcus* sp. PCC 6714 and *Synechocystis* sp. PCC 6803, were cultivated in BG-11 liquid medium as described by Porchia et al. (1999). *Anabaena* sp. strain PCC 7120 was also grown in BG-11 (Curatti et al. 2002). *E. coli* DH5α and BL21(λDE3):pLysS (Novagen) strains were used in this study. *E. coli* was routinely grown in Luria-Bertani medium supplemented with 50 μg ml^{-1} carbenicillin.

Isolation and purification of A/N-Inv from *Anabaena* sp. PCC 7120

Packed cells (6–8 g FW), harvested from *Anabaena* cultures at exponential-phase, were resuspended, disrupted and centrifuged as described by Porchia and Salerno (1996). The supernatant fraction, referred to as crude extract, was loaded onto a column of DEAE-Sephacel (Amersham–Pharmacia) pre-equilibrated with 20 mM Hepes–NaOH (pH 8.0), containing 1 mM EDTA, 5 mM 2-mercaptoethanol (ME) and 20% (v/v) glycerol. This step was repeated using the same elution buffer at different pH values between 6.0 and 8.0. Inv activities were always detected in the non-bound proteins, which were pooled and immediately applied to a column of CM-cellulose (Bio-Rad) equilibrated with 10 mM potassium phosphate buffer (pH 6.0), containing 1 mM EDTA, 5 mM ME and 10% (v/v) glycerol. Proteins were eluted with a linear potassium phosphate (pH 6.0) gradient from 0.01 to 0.5 M in equilibration buffer. Fractions containing N-Inv activity were pooled, concentrated in an Amicon ultrafiltration cell and further purified by gel filtration through a Sephadex G-100 (Amersham–Pharmacia) column equilibrated with 50 mM Hepes–NaOH (pH 7) containing 100 mM KCl, 5 mM ME and 10% (v/v) glycerol. For relative molecular mass (M_r) determinations, a Sephadex G-100 column was calibrated with standard molecular markers: blue dextran (2×10^3 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa) and carbonic anhydrase (29 kDa). The M_r values of *Anabaena* Inv were estimated by chromatographing an aliquot of either the concentrated enzyme from the DEAE-Sephacel step (for the A-Inv enzyme) or from the CM-cellulose step (for the N-Inv enzyme), or of the recombinant enzymes produced in *E. coli*.

Enzyme assays

The pH optima were determined using 200 mM potassium phosphate buffer over a pH range from 5.0 to 9.0. Inv activity was determined in reaction mixtures containing 100 mM potassium phosphate buffer, 100 mM Suc and an appropriate volume of enzyme in a final volume of 50 μl. The assay was carried out at pH 6.8 to detect N-Inv, or at pH 8.0 in the presence of 20 mM $MgCl_2$ to detect only A-Inv in preparations that contained both A/N-Inv activities. The mixture was incubated at 30 °C for different times. The amount of reducing sugar liberated was measured using Somogyi–Nelson reagent or by coupling hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, and following the appearance of NADPH spectrophotometrically (Porchia et al. 1999). For K_m determinations, different Suc concentrations (between 5 and 400 mM) were added to the incubation mixture (Lee and Sturm 1996). Inv substrate specificity was tested with Suc, raffinose, stachyose, melezitose, 1-kestose, trehalose and maltose over a concentration range of about 5–100 mM. Proteins were quantified according to Bradford (1976) or by absorbance at 280 nm.

Amplification, cloning and identification of *Anabaena* Inv genes

Standard procedures were followed for the isolation of *Anabaena* genomic DNA (Cai and Wolk 1990) and plasmids (Sambrook and Russell 2001). Two DNA sequences of 1,407 bp and 1,452 bp (named 1407 *orf* and 1452 *orf*), homologous to *Daucus carota* neutral Inv (Dc-N-Inv) and *Lolium temulentum* N-Inv (Lt-N-Inv) were obtained by BLAST searches against the genome of the *Anabaena* sp. strain PCC 7120 (http//www.kazusa.or.jp/cyano/). Both DNA fragments were PCR-amplified with the following primer pairs: 5′CCGGATCCATGAAAACCCCTCCAATTAATCAGAAGTCTCTT3′ and 5′CGGTCGACATTAAAGACTACAACCAGGGCCGATAAAA3′; and 5′CGGGATCCATGCAAAAGCTAAACGGACTGCTAACAAATGACAT3′ and 5′CGGTCGACATTAACGAGATATAGAAGCATATACACTGCCAATTTCAAA3′, respectively. The amplification products were ligated between the *Bam*HI and *Xho*I sites of the expression vector pRSET-A (Invitrogen), and recombinant plasmids were named pR1407 and pR1452, respectively. The resulting constructs were introduced into *E. coli* strain BL21(λDE3):pLysS (Novargen) to produce recombinant proteins (named His_6::InvA and His_6::InvB, respectively), which were purified by Co^{2+} affinity chromatography (TALON resin; Clontech) and concentrated for further characterization studies.

Western immunoblots and immunotitration of Inv activities

Proteins were separated by SDS–PAGE on 12% polyacrylamide gels (Laemmli 1970) and visualized with Coomassie blue or electroblotted onto a nitrocellulose membrane (HyBond C; Amersham) as described by Renart and Sandoval (1984). The membranes were then probed with antibodies raised in rabbits against His_6::InvB (anti-An-InvB) according to Salerno et al. (1998). The concentration dependence of Inv activity inhibition by anti-An-InvB was determined by adding different volumes of antibodies to aliquots of recombinant purified proteins as previously described (Salerno et al. 1991). Parallel incubations with pre-immune serum were carried out as a control. After incubation, Inv activity was determined in the supernatant fluids.

Isolation of RNA and RT–PCR assay

RNA from *Anabaena* was isolated using the TRIZOL reagent (Gibco–BRL). RNA quality and PCR products were analyzed by electrophoresis in 1% agarose gels. For RT–PCR analysis, total RNA (3 μg) treated with DNase (RQ1 Rnase-free Dnase; Promega) was reverse-transcribed using MMLV (Moloney murine leukemia virus) reverse transcriptase (Promega) and specific primers described above. PCR reactions were run on a PTC-100 thermal cycler (model-96 V; MJ Research, Mass., USA) for 20 cycles of 94 °C (1 min), 55 °C (1 min), and 72 °C (1 min), and a single step at 72 °C for 5 min. Under these conditions, PCR amplification occurs in the linear range. A constitutively expressed gene from *Anabaena* PCC 7120 (*rnp*B) was used as a loading control as described by Zhu et al. (2001).

Sequence analysis

The sequences reported in this paper have been deposited in the EMBL database (accession numbers AJ311089 and AJ491788 to AJ491793). Other sequences were obtained from the non-redundant protein databases National Center for Biotechnology (http://www.ncbi.nlm.nih.gov), the Kazusa DNA Research Institute, and the Department of Energy Joint Genome Institute (http://www.jgi.doe.gov) by BLAST searches (Altschul et al. 1990) using as query characterized A/N-Inv genes of plants. All *orf*s scored as A/N-Inv homologues had *E*-values ranged from 10^{-150} to 10^{-78} when compared with genes of established biochemical function (An-InvA, An-InvB). No sequences could be retrieved with *E*-values higher than 10^{-78} up to 10^{-1}. Sequences used are from: *Arabidopsis thaliana* [At-A/N-InvA (AY120777), At-A/N-InvB (AC009323), At-A/N-InvC (AC020580), At-A/N-InvD (AY088388), At-A/N-InvE (AB012244), At-A/N-InvF (AC069273), At-A/N-InvG (AY065247), At-A/N-InvH (AC012393), At-A/N-InvI (NM_117019), At-A/N-InvJ (AL161515) and At-A/N-InvK (AL161586)], *Beta vulgaris* [Bv-N-Inv (AJ422050)], *Daucus carota* [Dc-N-Inv (Y16262)], *Lolium temulentum* [Lt-N-Inv (AP003143)], *Oryza sativa* (Os-A/N-Inv (AJ003114)], *Anabaena* sp. PCC 7120 [An-InvA (AJ491788) and An-InvB (AJ311089)], *Nostoc punctiforme* [Np-InvA (AJ491790) and Np-InvB (AJ491789)], *Prochlorococcus marinus* MIT9313 [PmMIT-Inv (AJ491792)], *Prochlorococcus marinus* MED4 [SmMED-Inv (AJ491791)], *Synechococcus marinus* WH8102 [Sm-Inv (AJ491793)], and *Synechocystis* sp. PCC 6803 [Sy-Inv (*sll*0626)]. Sequence alignments were generated with the CLUSTAL X (Thompson et al. 1997) software program (version 1.8), ProDom program, using the BLOSUM 62 matrix and Pfam database (Bateman et al. 1999). Dendrograms were compiled by using the neighbor-joining method (computed from 1,000 independent trials) of CLUSTAL X and the maximum parsimony algorithm of the PHYLIP package (Felsenstein 1993). Searches for sequences homologous to functionally characterized acid Inv from *D. carota* (CAA77267) and from *Saccharomyces cerevisiae* (NP_012104), *Bacillus subtilis* levanase (NP_390581) and *E. coli* sucrose hydrolase (NP_288933) were performed in all the cyanobacterium genomes sequenced to date.

Results

Presence of A/N-Inv in *Anabaena* sp. cells

Two An-A/N-Inv activity peaks were shown in *Anabaena* crude extracts in a pH-dependent activity assay, with maximum pHs in the ranges 6.5–6.9 and 7.6–8.0, respectively (Fig. 1a). This suggests the simultaneous presence of two Inv activities that may be ascribed to N-Inv and A-Inv. The two activities could not be separated when crude extracts were chromatographed on DEAE-Sephacel columns using elution buffers at different pHs. In all cases, A/N-Inv activities eluted with the non-bound protein fractions and showed a pH-dependent activity pattern similar to the crude extract (data not shown). These fractions were pooled and chromatographed through a CM-cellulose column, recovering the Inv activity in a single peak at 0.25 M potassium phosphate buffer. The pH optimum of this Inv activity was found to be in the range 6.4–6.8 (Fig. 1b), and the enzyme was therefore identified as an N-Inv. As no other fractions (either of the non-bound or bound protein) showed Inv activity (data not shown), we assumed that the A-Inv was very labile and susceptible to inactivation. N-Inv was further purified by gel filtration in a Sephadex G-100 column, reaching about 300-fold purification with a 0.1% recovery (data not shown). It should be noted that both *Anabaena* Inv showed specific activities about one order of magnitude lower than their plant counterparts.

Functional identification of *Anabaena* A/N-Inv genes

BLAST searches using as query Dc-N-Inv and Lt-N-Inv revealed two homologous *orf*s (named 1407 *orf* and 1452 *orf*) in the *Anabaena* sp. PCC 7120 genome, 54% identical to the plant N-Inv, that encode proteins of 53,158 Da and 54,720 Da, respectively. Both 1407 *orf* and 1452 *orf* were identified as functional Inv genes (named *inv*A and *inv*B, respectively) because extracts of *E. coli* harboring pR1407 and pR1452 produced recombinant proteins (named His_6::InvA and His_6::InvB; Fig. 2a) that exhibited A-Inv and N-Inv activity, respectively (Fig. 1c, d). Recombinant proteins and the enzymes partially purified from *Anabaena* also showed similar apparent K_ms for Suc, and inhibition by fructose and Tris–HCl (Table 1). Also, 1 mM *p*-hydroxymercuribenzoate strongly inhibited both *Anabaena* Inv (data not shown). The substrate specificities of the authentic *Anabaena* Inv and of the *inv*A and *inv*B products were determined by incubation with Suc, raffinose, stachyose, melezitose, 1-kestose, trehalose and maltose (all at 100 mM). Enzyme activity was observed only when Suc was used as a substrate. *Anabaena* A-Inv and N-Inv eluted from gel-filtration columns as polypeptides with M_rs of approximately 51 and 54 kDa, similar values to those of the recombinant proteins (Fig. 2b, c; Table 1). The purified Inv migrated as a single band in a denaturing

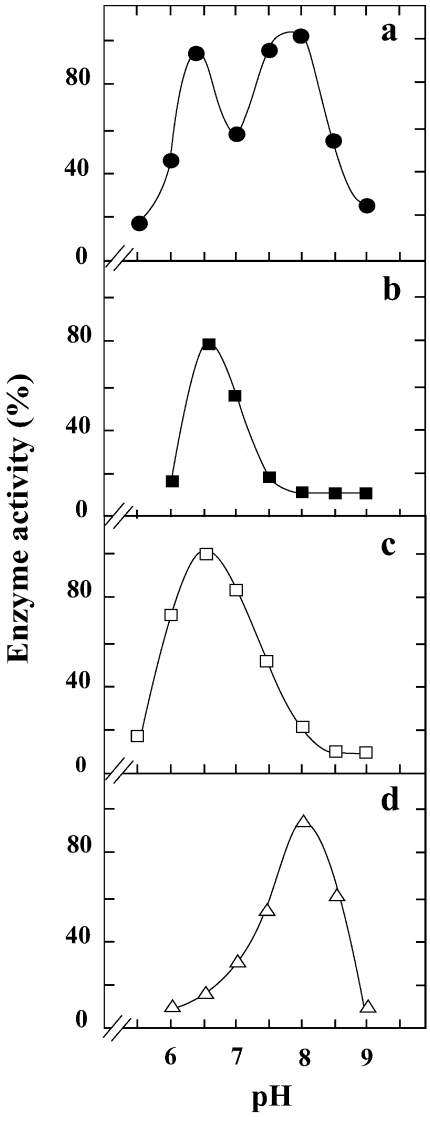

Fig. 1a–d Effect of pH on Inv activity in *Anabaena* sp. cells. Enzyme activity was measured in: **a** *Anabaena* crude extract, **b** partially purified N-Inv at the CM-cellulose purification step, **c** recombinant protein (His_6::InvB) and **d** recombinant protein (His_6::InvA)

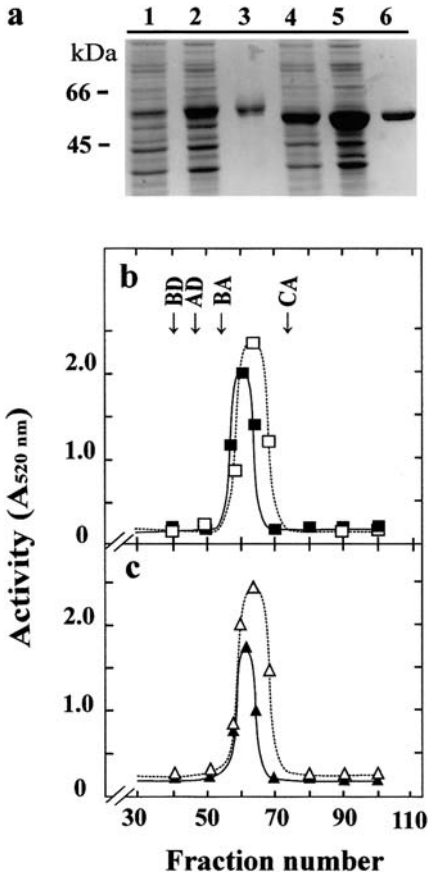

Fig. 2a–c Molecular mass determination of *Anabaena* Inv. **a** SDS–PAGE (12%) of recombinant proteins stained with Coomassie blue. *Lanes*: *1* crude extract from non-induced *E. coli* (pR1452); *2* crude extract from *E. coli* (pR1452), isopropyl β-D-thiogalactpyranoside (IPTG)-induced; *3* purified His_6::InvB; *4* crude extract from non-induced *E. coli* (pR1407); *5* crude extract from *E. coli* (pR1407) IPTG-induced; *6* purified His_6::InvA. **b** Sephadex G-100 chromatography of authentic *Anabaena* N-Inv (*filled squares*) or recombinant His_6::InvB (*open squares*). **c** Sephadex G-100 chromatography of authentic *Anabaena* A-Inv (*filled triangles*) or recombinant His_6::InvA (*open triangles*). The arrows in **b** and **c** indicate the position of standard markers: *BD* blue dextran (2×10^3 kDa), *AD* alcohol dehydrogenase (150 kDa), *AB* bovine albumin (66 kDa), *CA* carbonic anhydrase (29 kDa)

SDS–PAGE gel with an estimated M_r coincidental with the native values (Figs. 2a, 3a; Table 1). Additional immunological studies showed that anti-An-InvB revealed and immunotitrated both the recombinant and authentic A/N-An-Inv (Fig. 3 and data not shown). Remarkably, $MgCl_2$ (between 5 and 40 mM) only inhibited the neutral An-InvB (about 10–75% at pH 6.8 and 100% at pH 8.0). Taken together, these results lead us to conclude that *inv*A and *inv*B correspond to the *Anabaena* A-Inv and N-Inv genes, respectively.

Sequence analysis of *Anabaena* Inv protein

The *Anabaena inv*A and *inv*B genes encode 468- and 483-amino-acid polypeptides respectively. A comparison of the deduced amino-acid sequences of both genes with Lt-N-Inv and Dc-N-Inv (Gallagher and Pollock 1998; Sturm et al. 1999), the only plant A/N-Inv sequences functionally characterized, showed a high amino-acid identity (54–56%) throughout all the sequences (Fig. 4). A differential feature of plant N-Inv is an amino-terminal extension of about 78 and 178 amino acids for the *L. temulentum* and *D. carota* proteins, respectively.

Occurrence of Inv in cyanobacteria, and phylogenetic analysis

We have retrieved only A/N-Inv homologues from all the available sequence databases. BLAST searches (including all the microbial genomes sequenced at the time of writing), using as query An-InvA, An-InvB, Dc-N-Inv and Lt-N-Inv, and multiple sequence alignments,

Table 1 Biochemical characterization of *Anabaena* invertases

Characteristics	Enzymes purified from *Anabaena* cells		Recombinant enzymes		Plant invertases		
	A-Inv[a]	N-Inv	His_6::InvA	His_6::InvB	Dc-A-Inv[b]	Dc-N-Inv[b]	Other A/N-Inv[c]
Native M_r (kDa)	51 ± 2	54 ± 2	55 ± 2	58 ± 2	504	456	200–280, 60–120
Polypeptide M_r (kDa)	53 ± 1 (53.16)[d]	54 ± 1 (54.72)[d]	56 ± 2	58 ± 2	126	57	53–65
Optimal pH	7.8 ± 0.2	6.6 ± 0.3	7.8 ± 0.2	6.7 ± 0.2	8.0	6.8	7.0–7.5
K_m Suc (mM)	27 ± 3	10 ± 3	36 ± 6	15 ± 2	15.2	14.3	10–20
K_i Fructose (mM)	nd[e]	nd	30 ± 3	17 ± 2	28	13.4	1.2
K_i Tris (mM)	nd	nd	5.7 ± 0.4	1.4 ± 0.3	6.8	0.8	nd

[a]Enzyme activity measured at pH 8.0 in the presence of40 mM $MgCl_2$
[b]Carrot (*Daucus carota*) invertases; data from Lee and Sturm(1996), and Sturm et al. (1999)
[c]Data from Masuda et al. (1987),Chen and Black (1992), Van den Ende and Van Laere (1995), Ross et al. (1996), Vorster and Botha (1998), Gallagher and Pollock (1998)
[d]Deduced amino-acid sequence M_r
[e]Not determined

showed that A/N-Inv are restricted to cyanobacterial species and plants. A dendrogram generated by multiple alignment, using the Clustal method, showed a coincidental and unique A/N-Inv in the picoplanktonic open-ocean cyanobacteria (PmMIT-Inv, PmMED-Inv, SmWH-Inv) regardless of the query sequence used (about 33–36% identical to both *Anabaena* Inv; Fig. 5). In *N. punctiforme*, another filamentous nitrogen-fixing cyanobacterium, there are also two separate A/N-Inv, named InvA and InvB from their homology to the characterized *Anabaena* gene products. Cyanobacterial A/N-Inv clearly diverge from the plant homologues (Fig. 5). Qualitatively similar tree topologies were observed when using a maximum parsimony algorithm (not shown). Interestingly, no sequence with similarity to acid Inv, levanase, or sucrose hydrolase could be found in the cyanobacterial genomes sequenced to date.

The occurrence of A/N-Inv activity was also shown in crude extracts of cyanobacteria from different taxonomic groups (PCC sections I, III–V), either unicellular or filamentous, including nitrogen-fixing and non-fixing strains (Fig. 6).

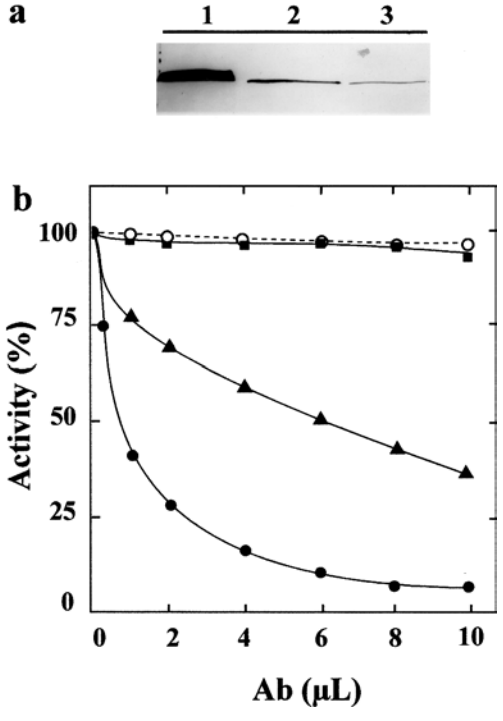

Fig. 3a, b Immunoanalysis of *Anabaena* Inv. **a** Immunoblot analysis using anti-An-InvB. *Lanes*: *1* purified His_6::InvB, *2* *Anabaena* Inv DEAE-Sephacel step, *3* *Anabaena* N-Inv Sephadex G-100 step. **b** Neutralization of Inv activity by anti-An-InvB. Purified His_6::InvB (*filled circles*), purified His_6::InvA (*filled triangles*), control incubation of His_6::InvB with pre-immune serum (*open circles*), and control incubation of His_6::InvA with pre-immune serum (*filled squares*)

Effect of the nitrogen source on the expression of *Anabaena invA* and *invB*

It has been reported that Inv activity increases in filaments of cyanobacteria grown diazotrophically and also that it is present in nitrate- and ammonium-grown cells (Schilling and Ehrnsperger 1985; Curatti et al. 2002). To determine if there is a differential expression of *inv*A and *inv*B in cells grown in different nitrogen sources, we measured A/N-Inv activities and analyzed the steady-state levels of their mRNAs (Fig. 7). An-InvA and An-Inv-B showed 3- and 6-fold higher activities, respectively, in cells under nitrogen-fixation conditions than in cells cultured with nitrate. Similar expression patterns were found when *inv*A and *inv*B mRNA contents were determined by RT–PCR.

Discussion

In this study, we report the first biochemical characterization of prokaryotic A/N Inv. The functional identification of two genes (*inv*A and *inv*B) in the *Anabaena* sp. PCC 7120 genome encoding An-InvA (An-A-Inv) and An-InvB (An-N-Inv) not only completes our knowledge of the enzymes involved in Suc metabolism in modern

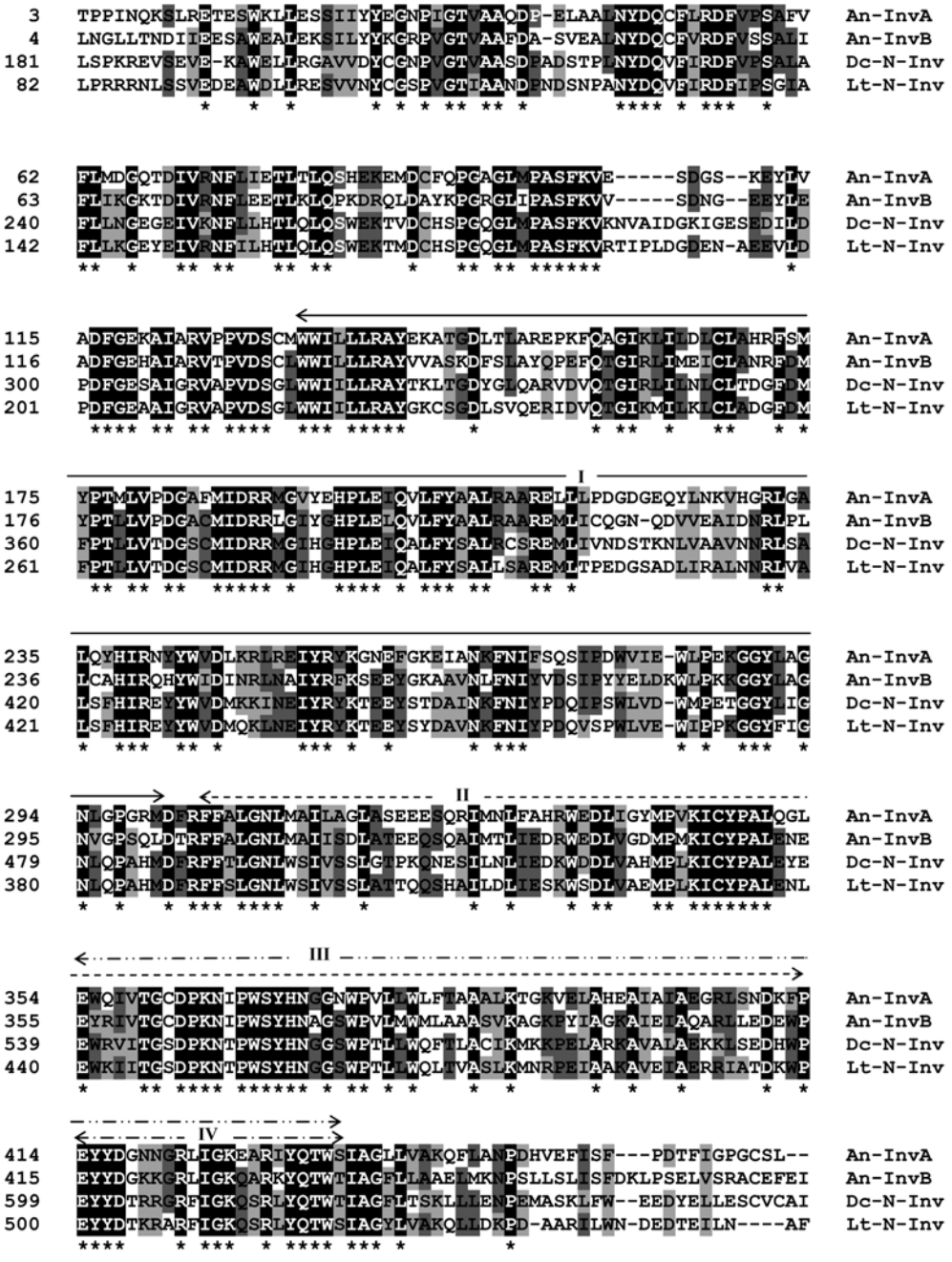

Fig. 4 Multiple sequence alignment of amino-acid sequences of alkaline/neutral invertase (A/N-Inv). *Anabaena* sp. PCC 7120 Inv (*An-InvA*, *An-InvB*), *D. carota* N-Inv (*Dc-N-Inv*) and *L. temulentum* N-Inv (*Lt-N-Inv*). Conserved motifs among members of the different families are indicated from *I* to *IV* [*I* β-glucosidase hydrolase glycosidase precursor family (——); *II* trehalase sequences (- - - -); *III* glycogen debranching enzyme family (–··–··), and *IV* sucrose-6-glycosyltransferase family (–·–·–)], which were calculated using the Pfam database and ProDom program. Identical residues are *black* and indicated with *asterisks*; conservative amino-acid substitutions are *gray-dashed*

cyanobacteria but also gives a better insight into the origin and evolution of this central pathway in plant life.

A comparison of a number of biochemical properties of A/N-Inv showed that An-A-Inv and An-N-Inv display many of the characteristics common to the orthologous plant enzymes, regarding the range of pH optimum, substrate specificity, K_m for Suc, K_i for Tris, product inhibition and polypeptide M_r (with the exception of Dc-A-Inv; Table 1). Even though cyanobacteria can be considered alkalophile prokaryotes, it has been shown that they have a limited ability to regulate their internal pH, and there is still no satisfactory understanding of the mechanisms involved in pH homeostasis (Giraldez-Ruiz et al. 1997). Thus, the discrimination between A-Inv and N-Inv is unlikely to be due to different physiological functions of the two isoforms. Also, a single amino-acid substitution can significantly change the pH optimum of Inv, suggesting that the presence of A-Inv and N-Inv in *Anabaena* cells might be ascribed to such event (Goetz and Roitsch 1999).

Unlike most plant A/N-Inv that were reported to be oligomers (Schaffer 1986; Masuda et al. 1987; Chen and Black 1992; Van den Ende and Van Laere 1995; Lee and Sturm 1996; Ross et al. 1996; Gallagher and Pollok 1998; Vorster and Botha 1998; Sturm et al. 1999), *Anabaena* native A-Inv and N-Inv are each composed of a single polypeptide chain with an M_r of about 53 and 55 kDa (Figs. 2, 3a; Table 1), being the smallest poly-

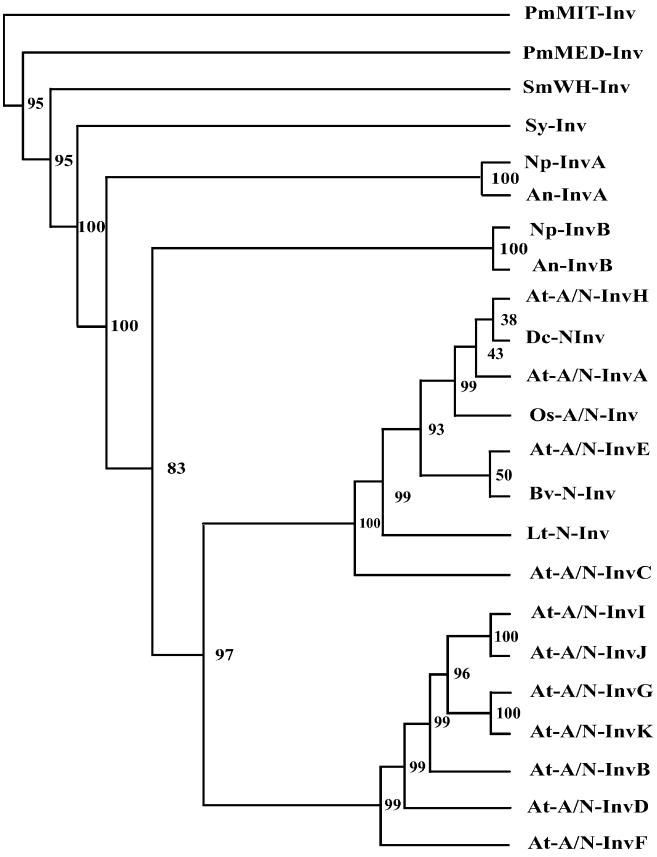

Fig. 5 Phylogenetic analysis of alkaline/neutral invertase (A/N-Inv). Neighbor-joining phylograms were constructed after sequence alignments of plant and cyanobacterial A/N-Inv sequences with the CLUSTAL X program using a BLOSSUM matrix and a bootstrap trial of 1,000. The graphical representation of the tree was generated using the Treeview16 program. Pm-MIT-Inv was employed as an outgroup

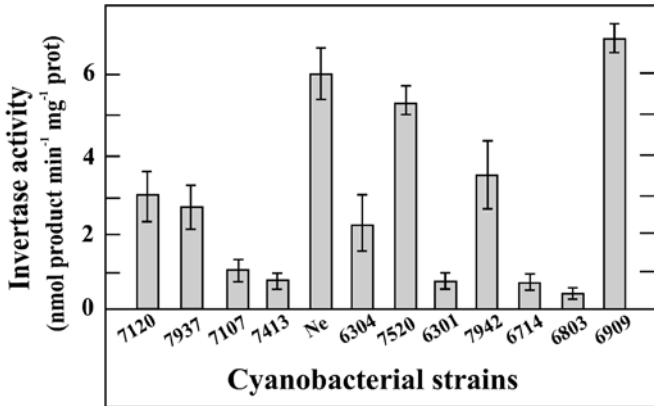

Fig. 6 Invertase (Inv) activity in cyanobacteria from different taxonomic groups. Crude extracts were prepared from cells of *Anabaena* sp. PCC 7120, *A. variabilis* PCC 7937, *Fischerella* sp. PCC 7520, *Gloeothece* sp. PCC 6909, *Nostoc* sp. PCC 7107, *Nostoc* sp. PCC 7413, *N. ellisosporum* (*Ne*), *Oscillatoria* sp. PCC 6304, *Synechococcus* sp. PCC 6301, *Synechococcus* sp. PCC 7942, *Synechococcus* sp. PCC 6714 and *Synechocystis* sp. PCC 6803, at exponential phase. Enzyme activity was measured at pH 7.0

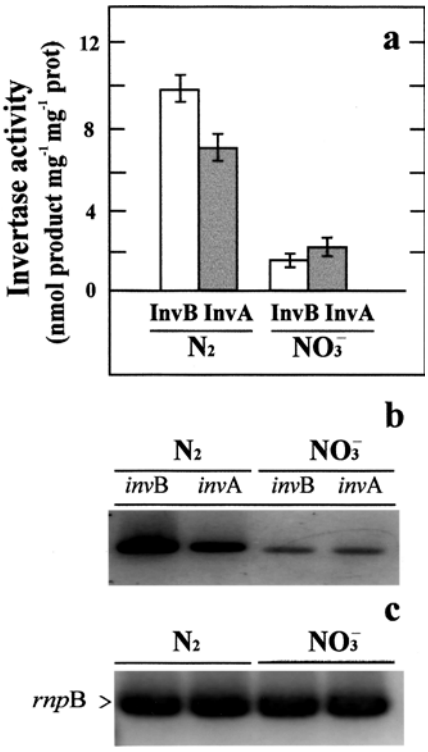

Fig. 7a–c Expression of *inv*A and *inv*B of *Anabaena* sp. PCC 7120 grown with different nitrogen sources. **a** Enzyme activities in crude extracts of *Anabaena* cells grown under N_2-fixing and nitrate-assimilation conditions. Data are the mean ± SE of three independent experiments. **b** RT–PCR from total RNA extracted from *Anabaena* cells grown under N_2-fixing or nitrate-assimilation conditions. **c** Amplification of *Anabaena rnp*B, used as a loading control

peptide with that enzyme activity. In plants, the sugar cane N-Inv is an exception that was shown to be also active as a 60-kDa monomer (Vorster and Botha 1998). This structural difference between plant and cyanobacterial enzymes has also been reported for other Suc-metabolism-related proteins such as SPS and SPP involved in *Anabaena* and *Synechocystis* Suc biosynthesis (Porchia and Salerno 1996; Curatti et al. 1998; Cumino et al. 2001, 2002). Whether this feature, in addition to the presence of extra N-terminal (in plant SPS and Inv) or C-terminal (in plant SPP) domains in comparison with the cyanobacterial protein orthologues, might contribute to the higher specific activity of the plant enzymes is still not understood. The fact that An-A/N-Inv activities are highly specific for Suc suggests that they are not β-fructofuranosidases, consistent with reported substrate specificities for plant A/N-Inv, and unlike all known acid Inv (Sturm 1999; Sturm et al. 1999). Both the K_m for Suc and the K_i for fructose are in the range of the concentrations of these sugars occurring in *Anabaena* cells (data not shown). Inhibition by fructose and, to a minor extent, by glucose (not shown) suggests that Inv activity could be regulated in vivo by the reaction products, as reported in plants (Masuda et al. 1987; Van den Ende and Van Laere 1995; Lee and Sturm 1996; Ross et al. 1996; Vorster and Botha 1998).

The differential inhibition of An-N-Inv by Mg^{2+} was not reported for plant A/N-Inv, for which the Mg^{2+} effect does not follow a general pattern (Morell and Copeland 1984; Van den Ende and Van Laere 1995; Lee and Sturm 1996). This result was useful because An-A-Inv activity, like that of some plant A/N-Inv (Sturm 1999), was rapidly lost during the purification from *Anabaena* cells and, consequently, its activity had to be assayed in enzyme preparations containing both An-A-Inv and An-N-Inv.

Two *orfs* of 1,407 bp and 1,452 bp present in the *Anabaena* sp. PCC 7120 genome were identified as the coding regions of Inv genes (*inv*A and *inv*B), as their expression in *E. coli* produced fully active A-Inv and N-Inv with biochemical and molecular characteristics similar to those of Inv purified from *Anabaena* cells (Table 1; Figs. 1, 2, 3). The deduced amino-acid sequence of *inv*A is about 60% identical to that of *inv*B, and they share a considerable homology (50–56%) with plant A/N-Inv (Fig. 4). Also, the amino-terminal extension (between 50 and 180 amino acids), which differs markedly in plant N-Inv (Sturm 1999; this paper), is absent in cyanobacterial Inv, indicating that it is not involved in the catalysis. On the other hand, with the exception of Lt-N-Inv, cyanobacterial and plant A/N-Inv sequences (including all the available putative sequences) do not conserve the four amino-acid residues (NDPN) of the pentapeptide NDPNG, a hallmark of the acid β-fructofuranosidases (Sturm and Chrispeels 1990; Sturm 1999), supporting the conclusion that this is not an essential sequence for A/N-Inv activity. In contrast, as in most plant A/N-Inv, the presence of sulfhydryl groups might be essential for the activity of *Anabaena* Inv because they are strongly inhibited by Hg^{2+} (Morell and Copeland 1984; Van den Ende and Van Laere 1995; Vorster and Botha 1998; Fig. 4). An-A-Inv and An-N-Inv share five conserved Cys and three conserved Met residues; two of the Cys and two of the Met residues are in similar positions within the polypeptide chains of Dc-N-Inv and Lt-N-Inv. Interestingly, conserved sequence motifs of members of the β-glucosidase hydrolase glycosidase precursor family, of three trehalase sequences, of members of the glycogen-debranching enzyme family, and of members of the sucrose-6-glycosyltransferase family, were found either in An-A-Inv and An-N-Inv or in plant A/N-Inv amino-acid deduced sequences (Fig. 4, motifs I–IV, respectively).

The analysis of the occurrence of A/N-Inv in complete genomes has been useful in providing a more comprehensive view of the origin and evolution of the Suc metabolic pathway. A/N-Inv homologues are present in all cyanobacterial genomes and in plant genomes, but not in non-cyanobacterial prokaryote genomes, examined to date. The presence of An-A-Inv homologues in open-ocean species (Fig. 5), phylogenetically located at the base of the cyanobacterial radiation (Palenik and Haselkorn 1992; Urbach et al. 1992) led us to conclude that A/N-Inv originated from an ancestral A-Inv like gene about 2–3.5 billion years ago, supporting the proposed origin of Suc metabolism based on the appearance of an ancestral SPS-like gene (Cumino et al. 2002). Also, the absence of SuS homologues in open-ocean strains (Cumino et al. 2002) led us to suggest that Suc hydrolysis only by A/N-Inv like proteins might be associated with the origin of Suc metabolism. Further gene duplications during cyanobacterial diversification might have originated two different A/N-Inv in filamentous nitrogen-fixing strains and plant A/N-Inv. In the *Arabidopsis* genome there are 11 putative A/N-Inv. All plant A/N-Inv cluster together in two sister clades, indicating that they might have originated by recent gene duplications of a common ancestor within the plant lineage (Fig. 5). However, molecular and functional characterization of plant A/N-Inv is only now beginning and conclusions are still limited by the relatively small number of sequences available (no plant A-Inv sequence is known to date).

In contrast to the knowledge of the physiological role of Suc in plants (Winter and Huber 2000), in cyanobacteria its role has not been elucidated yet. It has been suggested that Suc may act as a carbon carrier molecule during nitrogen fixation in filamentous heterocyst-forming cyanobacteria (Wolk et al. 1994), where A/N-Inv activity was shown to be predominantly located in the heterocysts (Schilling and Ehrnsperger 1985). Here, we have shown in *Anabaena* sp. PCC 7120 cells that both An-A-Inv and An-N-Inv are increased during culture under nitrogen-fixing conditions. The importance of alkaline Inv activity in nitrogen fixation has already been shown in developing soybean nodules where it appears to be the main enzyme of Suc breakdown (Morell and Copeland 1984). But the specific functions of the two *Anabaena* Inv isoforms await to be elucidated. Additionally, although with a lower expression level, An-A/N-Inv may account for the differences in Suc contents found in *Anabaena* (Fig. 7; Curatti et al. 2002) and in other filamentous heterocyst-forming or unicellular non-nitrogen-fixing cyanobacteria, grown with nitrate as nitrogen source (Fig. 7). A comparison of K_m values for Suc of *Anabaena* SuS (about 300 mM; Porchia et. al. 1999) and An-A-Inv and An-N-Inv (10–30 mM; Table 1) seems to indicate that in *Anabaena* cells Suc utilization is preferentially mediated by Inv when there is a high demand for hexoses. Thus, in addition to its role in nitrogen fixation, cyanobacterial A/N-Inv are proposed, as was reported for plant cytosolic Inv (ap Rees 1974), to be "maintenance" enzymes involved in providing Suc degradation substrates when the other Suc hydrolytic activities (acid Inv and SuS in the case of plants, or only SuS in the case of cyanobacteria) are low (Sturm 1999; Curatti et al. 2002). The fact that A/N-Inv activity was measured either in unicellular or filamentous, nitrogen- and non-nitrogen-fixing strains (Fig. 6) also strengthens this conclusion.

In view of the substantial evidence that plant chloroplasts have a cyanobacterial origin and that genes could be transferred from the chloroplast to the nuclear

genome (Douglas 1994; Martin and Herrmann 1998), it seems likely plants acquired A/N-Inv genes as part of Suc metabolism during the endosymbiotic origin of the chloroplast. The plastids did not retain the Suc biochemistry of the free-living ancestor and, instead of re-importing the gene products, they gave rise to a novel cytoplasmic pathway in the plant lineage (Cumino et al. 2002). Further comprehensive studies on A/N-Inv in other model organisms will help to support the phylogenetic conclusions as well as to promote understanding of the involvement of A/N-Inv in the regulation of Suc levels in relation to other physiological process.

Acknowledgements The authors thank M. Fernández and C. Fernández for technical assistance and Prof. Horacio Pontis for critical reading of the manuscript. This work is part of the PhD thesis of W.V. (Univ. Nac. de Mar del Plata). G.L.S. is a career investigator of the CONICET. This work was supported by grants from CONICET and UNMdP. W. Vargas and A. Cumino contributed equally to this work.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

ap Rees T (1974) Pathways of carbon breakdown in plants. In: Northcote DH (ed) MTP international reviews of science, Biochemistry, series I, vol 11, Plant biochemistry. Butterworth, London, pp 89–127

Avigad G, Dey PM (1997) Plant biochemistry. In: Dey PM, Harborne JB (eds) Carbohydrate metabolism: storage carbohydrates. Academic Press, London, pp 143–204

Bateman A, Birney E, Durbin R, Eddy SR, Finn RD, Sonnhammer EL (1999) Pfam 3.1:1313 multiple alignments and profile HMMs match the majority of proteins. Nucleic Acids Res 27:260–262

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of dye binding. Anal Biochem 72:143–147

Cai Y, Wolk CP (1990) Use of a conditional lethal gene in Anabaena sp. PCC 7120 to select for double recombinants and to entrap insertion sequences. J Bacteriol 172:3138–3145

Chen JQ, Black CC (1992) Biochemical and immunological properties of alkaline invertase isolated from sprouting soybean hypocotyls. Arch Biochem Biophys 295:61–69

Cumino A, Ekeroth C, Salerno GL (2001) Sucrose-phosphate phosphatase from Anabaena sp. strain PCC 7120: isolation of the protein and gene revealed significant structural differences from the higher-plant enzyme. Planta 214:250–256

Cumino A, Curatti L, Giarrocco L, Salerno GL (2002) Sucrose metabolism: Anabaena sucrose-phosphate synthase and sucrose-phosphate phosphatase define minimal functional domains shuffled during evolution. FEBS Lett 517:19–23

Curatti L, Folco E, Desplats P, Abratti G, Limones V, Herrera-Estrella L, Salerno G (1998) Sucrose-phosphate synthase from Synechocystis sp. PCC 6803: identification of the spsA gene and characterization of the enzyme expressed in E. coli. J Bacteriol 180:6776–6779

Curatti L, Porchia AC, Herrera-Estrella L, Salerno GL (2000) A prokaryotic sucrose synthase gene (susA) isolated from a filamentous nitrogen-fixing cyanobacterium encodes a protein similar to those of plants. Planta 211:729–735

Curatti L, Flores E, Salerno G (2002) Sucrose is involved in the diazotrophic metabolism of the heterocyst-forming cyanobacterium Anabaena sp. FEBS Lett 513:175–178

Douglas SE (1994) Chloroplast origins and evolution. In: Bryant DA (ed) Molecular biology of cyanobacteria. Kluwer, Dordrecht, pp 91–118

Felsenstein J (1993) PHYLIP (phylogeny inference package), version 3.5c. University of Washington, Seattle

Gallagher JA, Pollock CJ (1998) Isolation and characterization of a cDNA clone from Lolium temulentum encoding for a sucrose hydrolytic enzyme which shows alkaline/neutral invertase activity. J Exp Bot 49:789–795

Giraldez-Ruiz N, Mateo P, Bonilla I, Fernandez-Piñas F (1997) The relationship between intracellular pH growth characteristics and calcium in the cyanobacterium Anabaena sp. strain PCC7120 exposed to low pH. New Phytol 137:599–605

Goetz M, Roitsch T (1999) The different pH optima and substrate specificities of extracellular and vacuolar invertases from plants are determined by a single amino-acid substitution. Plant J 20:707–711

Hagemann M, Erdmann N (1997) Environmental stresses. In Rai AK (ed) Cyanobacterial nitrogen metabolism and environmental biotechnology. Narosa Publishing House, New Delhi, pp 156–220

Khmelenina VN, Kalyuzhnaya MG, Sakharousky VG, Suzina NE, Trotsenko YA, Gottschalk G (1999) Osmoadaptation in halophilic and alkaliphilic methanotrophs. Arch Microbiol 172:321–329

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277:680–685

Lee HS, Sturm A (1996) Purification and characterization of neutral and alkaline invertase from carrot. Plant Physiol 112:1513–1522

Levitt J (1980) Responses of plants to environmental stresses. Physiological ecology series, vol I. Academic Press, New York, pp 163–179

Lunn JE (2002) Evolution of sucrose synthesis. Plant Physiol 128:1490–1500

Martin W, Herrmann R (1998) Gene transfer from organelles to the nucleus: how much, what happens, and why? Plant Physiol 118:9–17

Masuda H, Takahashi T, Sugawara S (1987) The occurrence and properties of alkaline invertase in mature roots of sugar beets. Agric Biol Chem 51:2309–2314

Morell M, Copeland L (1984) Enzymes of sucrose breakdown in soybean nodules. Plant Physiol 74: 1030–1034

Page-Sharp M, Behm CA, Smith GD (1999) Involvement of the compatible solutes trehalose and sucrose in the response to salt of a cyanobacterial Scytonema species isolated from desert soils. Biochim Biophys Acta 1472:519–528

Palenik B, Haselkorn R (1992) Multiple evolutionary origins of prochlorophytes, the chlorophyll b-containing prokaryotes. Nature 355:265–267

Porchia AC, Salerno GL (1996) Sucrose biosynthesis in a prokaryotic organism: presence of two sucrose-phosphate synthases in Anabaena with remarkable differences compared with the plant enzymes. Proc Natl Acad Sci USA 93:13600–13604

Porchia AC, Curatti L, Salerno GL (1999) Sucrose metabolism in cyanobacteria: sucrose synthase form Anabaena sp. strain PCC 7119 is remarkably different from the plant enzymes with respect to substrate affinity and amino-terminal sequence. Planta 210:34–40

Potts M (2000) Nostoc. In: Whitton BA, Potts M (eds) The ecology of cyanobacteria. Kluwer, Dordrecht, pp 465–504

Reed RH, Richardson DL, Warr SRC, Stewart WDP (1984) Carbohydrate accumulation and osmotic stress in cyanobacteria. J Gen Microbiol 130:1–4

Renart J, Sandoval IV (1984) Western blots. Methods Enzymol 104:455–460

Ross HA, McRae D, Davies HV (1996) Sucrolytic enzyme activities in cotyledons of the faba bean. Plant Physiol 111:329–338

Salerno GL, Crespi MD, Zabaleta EJ, Pontis HG (1991) Sucrose-phosphate synthase from wheat. Characterization of peptides by immunoblotting analysis. Physiol Plant 81:541–547

Salerno GL, Pagnussat GC, Pontis HG (1998) Studies on sucrose-phosphate synthase from rice leaves. Cell Mol Biol 44:407–416

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Schaffer AA (1986) Invertases in young and mature leaves of Citrus sinensis. Phytochemistry 25:2275–2277

Schilling N, Ehrnsperger K (1985) Cellular differentiation of sucrose metabolism in Anabaena variabilis. Z Naturforsch 40:776–779

Smeekens S (2000) Sugar-induced signal transduction in plants. Annu Rev Plant Physiol Plant Mol Biol 51:49–81

Sturm A (1999) Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. Plant Physiol 121:1–7

Sturm A, Chrispeels MJ (1990) cDNA cloning of carrot extracellular β-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell 2:1107–1119

Sturm A, Hess D, Lee HS, Lienhard S (1999) Neutral invertase is a novel type of sucrose-cleaving enzyme. Physiol Plant 107:159–165

Thomson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Urbach E, Robertson DL, Chisholm SW (1992) Multiple evolutionary origins of prochlorophytes within the cyanobacterial radiation. Nature 355:267–270

Van den Ende W, Van Laere A (1995) Purification and properties of a neutral invertase from the roots of Cichorium intybus. Physiol Plant 93:241–248

Vorster DJ, Botha FC (1998) Partial purification and characterization of sugarcane neutral invertase. Phytochemistry 49:651–655

Winter H, Huber SC (2000) Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. Crit Rev Plant Sci 19:31–67

Wolk CP, Ernst A, Elhai J (1994) Heterocyst metabolism and development. In: Bryant DA (ed) Molecular biology of cyanobacteria. Kluwer, Dordrecht, pp 769–823

Zhu J, Jäeger K, Black T, Zarka K, Koksharova O, Wolk CP (2001) HcwA, an autolysin, is required for heterocyst maturation in Anabaena sp. strain PCC 7120. J Bacteriol 183:6841–6851