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ORIGINAL ARTICLE

Differential expression of alkaline and neutral invertases in response to environmental stresses: characterization of an alkaline isoform as a stress-response enzyme in wheat leaves

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Abstract It is well accepted that sucrose (Suc) metabolism is involved in responses to environmental stresses in many plant species. In the present study we showed that alkaline invertase (A-Inv) expression is up-regulated in wheat leaves after an osmotic stress or a low-temperature treatment. We demonstrated that the increase of total alkaline/neutral Inv activity in wheat leaves after a stress could be due to the induction of an A-Inv isoform. Also, we identified and functionally characterized the first wheat cDNA sequence that codes for an A-Inv. The wheat leaf full-length sequence encoded a protein 70% similar to a neutral Inv of *Lolium temulentum*; however, after functional characterization, it resulted to encode a protein that hydrolyzed Suc to hexoses with an optimum pH of 8, and, consequently, the encoding sequence was named *Ta-A-Inv*. By RT-PCR assays we demonstrated that *Ta-A-Inv* expression is induced in response to osmotic and cold stress in mature primary wheat leaves. We propose that Ta-A-Inv activity could play an important role associated with a more efficient cytosolic Suc hydrolysis during environmental stresses.

Keywords Alkaline invertase · Cold stress · Osmotic stress · Sucrose hydrolysis · Transcriptional regulation · Wheat

Abbreviations

A-Inv	Alkaline invertase
Ac-Inv	Acid invertase
A/N-Inv	Alkaline/neutral invertase
An-	Anabaena
FSE	Fructan synthesis enzymes
N-Inv	Neutral invertase
RS	Reducing sugars
Suc	Sucrose

Introduction

Water deficit and low temperatures are common adverse situations affecting land plant germination, growth and productivity. Environmental stresses elicit a complex of responses at the cellular, physiological and developmental levels (Levitt 1980). Particularly, some species exposed to drought or chilling temperatures alter their Suc and/or Suc-derived-oligosaccharide content to cope with these unfavorable conditions (Calderón and Pontis 1985; Hendry 1993; Spollen and Nelson 1994; Balibrea et al. 2000; Fernandes et al. 2004). Even though carbohydrate accumulation was proposed to play an important function in osmoprotection, it is likely that Suc also plays a role as a source of carbon for the biosynthesis of other osmoprotective substances and/or as a source of energy.

Suc metabolism has been widely studied in plants under adverse environmental conditions. It has been demonstrated that sucrose-phosphate synthase (SPS), responsible for the disaccharide synthesis, sucrose-synthase (SuS), which

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catalyzes a readily reversible reaction that could be involved in both the synthesis and cleavage of Suc, and acid invertases (Ac-Inv) that hydrolyze Suc in cell wall and vacuoles, modify their expression after stress treatments (Tognetti et al. 1989, 1990; Dejardin et al. 1999; Wang et al. 2000; Baud et al. 2004; Fernandes et al. 2004). By contrast, there are only few reports on the effect of stress conditions on alkaline/neutral invertase (A/N-Inv) expression. An increase in A/N-Inv activity was shown in cultured sweet potato cells under an osmotic stress (Wang et al. 2000) and in tomato and *Lupinus albus* leaves in response to salinity (Balibrea et al. 2000; Fernandes et al. 2004).

A/N-Invs are a group of intriguing enzymes present in oxygenic photosynthetic organisms (Sturm 1999; Vargas et al. 2003), which has been scarcely taken into account in biochemical, physiological and molecular studies in comparison to Ac-Invs. A/N-Invs exist as two different isoforms with optimum pH either close to 6.5 or to 8, named N-Inv or A-Inv, respectively. Both isoforms have been purified and biochemically characterized from several plant species and from cyanobacteria (Chen et al. 1992; Ross et al. 1996; Lee and Sturm 1996; Walker et al. 1997; Vorster and Botha 1998; Vargas et al. 2003). The first two sequences functionally characterized as coding for A/N-Inv were those corresponding to *L. temulentum* and carrot N-Inv (Gallagher and Pollock 1998; Sturm et al. 1999). Remarkably, in cyanobacteria A-Inv and N-Inv isoforms are encoded by different genes (Vargas et al. 2003). Multigene families of putative A/N-Invs have been detected in *Arabidopsis thaliana* and *Oryza sativa* genomes (Vargas et al. 2003; Ji et al. 2005) but only one encoding sequence (*CINV1*) of a cytosolic A-Inv isoform from *A. thaliana* and another from *O. sativa* (*OsNIN1*) were functionally characterized (Lou et al. 2007; Murayama and Handa 2007). Also recent results shed some light on the biological role of A/N-Inv. It was proposed that they could supply hexoses for several biosynthetic processes in the developing nodules in *Lotus japonicus* (Flemetakis et al. 2006) and play a regulatory role in fruit growth and development in *Prunus persica* plants (Nonis et al. 2007). In *A. thaliana*, an A-Inv was demonstrated to be involved in the control of root cell elongation mediated by sugars (Lou et al. 2007). But the most outstanding finding was the recent demonstration that A/N-Inv not only achieved the hydrolysis of Suc in the cytosol, as it was largely believed (Chen and Black 1992; Van den Ende and Van Laere 1995), but also some of them could be located into plant organelles (Murayama and Handa 2007; Vargas et al., unpublished).

In the present work we demonstrate the differential induction of an A-Inv activity in response to osmotic and cold stress in fully expanded wheat (*Triticum aestivum*) leaves. We also identified and functionally characterized a wheat cDNA sequence (*Ta-A-Inv*) encoding an A-Inv isoform with biochemical properties similar to those of the native stress-induced isoform and whose transcription resulted as up-regulated in response to low temperature and osmotic stress.

Materials and methods

Plant materials and bacterial growth

Wheat seeds (*Triticum aestivum*, cv. Pincén) were germinated and hydroponically grown with half-strength Hoagland's solution (Hoagland and Arnon 1950) in a controlled-environment room (27°C, 250 μmol/m^2/s photosynthetic photon flux density and a day-night regime of 16–8 h). Eight-day-old seedlings were exposed to osmotic (400 mM mannitol) or cold (4°C) treatment, while control plants were kept in Hoagland's solution at 27°C as described above. Fully expanded primary leaves were harvested, frozen in liquid nitrogen and stored at −80°C until further analyses.

Escherichia coli DH5α and BL21(λDE3):pLysS (Novagen) strains were used as a general host strain for cloning and protein expression, respectively. Both strains were routinely cultured in Luria–Bertani medium supplemented with the antibiotics required according to the plasmid they harbored (Sambrook and Russell 2001).

Carbohydrate extraction and determination

Harvested leaves were cut into 0.5 cm segments, lyophilized and dry weight (DW) was determined. Sugars were extracted three times by boiling the samples in alkaline water for 5 min and extracts were pooled for analysis. Reducing sugars (RS) were quantified with the Somogyi–Nelson reagent (Ashwell 1957), or by coupling hexokinase, phosphoglucose isomerase plus glucose-6-phosphate dehydrogenase, and following spectrophotometrically the appearance of NADPH (Jones et al. 1977). Suc content was estimated by measuring fructose and glucose after hydrolysis with Sucrase® (Megazyme International) (Puebla et al. 1999). Fructans were determined by the thiobarbituric acid reaction after Suc hydrolysis using Sucrase® and free monosaccharides destruction with NaOH (Puebla et al. 1999). Carbohydrate contents were expressed in dry weight (DW) basis because tissue water content is modified by the different treatments.

Protein crude extracts

Leaves from wheat seedlings grown under control conditions or exposed to a stress treatment were powdered with

mortar and pestle under liquid nitrogen. Proteins were extracted with 50 mM Hepes-NaOH (pH 7.5), 1 mM EDTA, 20 mM $MgCl_2$, 20% (*v*/*v*) glycerol, 0.01% (*v*/*v*) Triton, 20 mM β-mercaptoethanol and 1 mM PMSF, during 10 min at 0°C. Extracts were filtered through cheesecloth and centrifuged for 20 min at 20,000×*g*. The clarified supernatants (crude extracts) were immediately used for enzyme activity assays or for further purification steps.

Purification of A/N-Inv from wheat leaves

Crude extracts prepared from the leaves of control or stressed seedlings were loaded onto DEAE-Sephacel columns (1.0 × 20 cm) [Amersham-Pharmacia] pre-equilibrated with 50 mM Hepes-NaOH (pH 7.5), 1 mM EDTA, 5 mM β-mercaptoethanol and 20% (*v*/*v*) glycerol. Proteins were eluted with a 0–0.5 M NaCl lineal gradient in the equilibration buffer. Fractions containing A/N-Inv activity were pooled, concentrated in an Amicon ultrafiltration cell, rechromatographed through DEAE-Sephacel columns (0.5 × 20 cm) and eluted as described above. Fractions displaying A/N-Inv activity were pooled, concentrated and further purified by gel filtration through Sepharose-6B columns (1.0 × 100 cm) [Amersham-Pharmacia] pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 10% (*v*/*v*) glycerol, 5 mM β-mercaptoethanol and 150 mM KCl. Since the activity of both A/N-Inv isoforms was labile or susceptible to inactivation, fractions of the Sepharose-6B chromatography were collected in tubes containing bovine serum albumin (final concentration 5 mg/ml) to stabilize the enzymes, and those fractions displaying A/N-Inv activity were immediately pooled and concentrated. As a consequence of the enzyme instability, the purification of the native wheat A/N-Inv isoforms could only attain approximately 300–400 folds, and the obtained partially purified fractions were used for further studies.

Enzyme assays

A/N-Inv activity was routinely assayed in incubation mixtures (total volume 50 µl) containing 200 mM Suc, 200 mM Hepes-NaOH (pH 7.5) and an aliquot of the protein fraction to be tested. The mixture was incubated at 30°C for different times and the progress of the reaction was followed detecting the amount of reducing sugars as described above. For optimum pH determinations, 200 mM potassium phosphate buffer over a range from 5.0 to 9.0 was added to the reaction medium. Apparent K_m determinations were performed as reported by Vargas et al. (2003). The inhibitory effect of 10 mM Tris and 1 mM $CuCl_2$ on A/N-Inv was assayed in the conditions described above. Ac-Inv activity was determined in a reaction mixture containing 50 mM Suc and 100 mM acetic acid/sodium acetate buffer (pH 4.5). SPS, SuS, and fructan synthesis enzymes (FSE) activity were measured as previously described (Fernandes et al. 2004; Tognetti et al. 1990; Puebla et al. 1999). Protein concentration was quantified according to Bradford (1976).

Wheat A/N-Inv analysis on SDS-PAGE and immunodetection

Polypeptides were separated by SDS-PAGE on 12% polyacrylamide gels (Laemmli 1970) and stained with Coomassie blue or blotted onto a nitrocellulose membrane (HyBond C®, Amersham) as described by Renart and Sandoval (1984). Membranes were probed according to Salerno et al. (1998) with antibodies specific for A/N-Inv, raised in rabbits against recombinant An-InvB (anti-An-InvB) (Vargas et al. 2003), which did not recognize any *E. coli* polypeptide.

Identification and cloning of an A/N-Inv cDNA from wheat

L. temulentum N-Inv sequence (accession number AJ003114) was used as query to search homologous cDNA clones in *T. aestivum* ESTs (expressed sequence tags) on the UniGene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). Several EST sequences were recovered from different cDNA libraries, which allowed us to assemble one contig containing a full-length sequence encoding a putative A/N-Inv (initially referred as *Ta-A/N-Inv-like*, and then named *Ta-A-Inv* after characterization). Representative clones of *Ta-A/N-Inv-like* were only described for cDNA libraries derived from stress-induced wheat tissues and from roots (Guilleroux and Osbourn 2004; Mochida et al. 2006). The *Ta-A/N-Inv-like* cDNA sequence was amplified using PCR technology and the oligonucleotides: pInv-f (5′-GGATCCATGAAGAG AGTCTCGTCGCATGTCT-3′) and pInv-r (5′-AACTGCA GAAATTATTTGGTGTCGGATACTTGG-3′).

RT-PCR and *Ta-A-Inv* cDNA expression assays

Total RNA from fully expanded leaves from control or stressed seedlings (exposed to mannitol or to 4°C) was prepared using the TRIZOL® reagent (Gibco-BRL). RNA quality and PCR products were analyzed by electrophoresis on 1% agarose gels. cDNA synthesis was performed from RNA (5 µg) treated with DNase (RQ1 RNase-Free DNase®; Promega) and using MMLV-RT® (Moloney murine leukaemia virus) reverse transcriptase (Promega) and oligo-dT primers. DNA fragments were PCR-amplified with the pInv-f and pInv-r primers described above. For semi-quantitative RT-PCR experiments, PCR reactions were run for 25 cycles of 94°C (1 min), 55°C (1 min), and

72°C (1 min), and a single final step at 72°C for 5 min. Under these conditions, PCR amplification occurs in the linear range. The constitutively expressed coding gene for wheat ubiquitin (*UBI*, GenBank® accession number X56601) was used as an internal loading control.

Expression of the His6::Ta-A-Inv fusion protein in *E. coli* cells

Adapter sequences for *Bam*HI and *Pst*I restriction enzymes were added to the pInv-f and pInv-r oligonucleotides, respectively. The 1986-bp PCR product was cloned into *BamH*I and *Pst*I sites of the *E. coli* expression vector pRSET-A® (Invitrogen). Both DNA strands of the construct, named pRA::*Ta-A-Inv*, were sequenced to confirm the identity and the correct translation frame of the fusion. The construct was transferred to *E. coli* BL21(λDE3):pLysS (Novagen). The expression of the fusion protein was induced by adding 1 mM IPTG (isopropyl β-D-thiogalactoside) when the culture reached an A_{600} of about 0.5. After 16 h induction at 18°C, the cells were harvested and the fusion protein (His6::Ta-A-Inv) 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 His6::Ta-A-Inv were analyzed by SDS-PAGE and immunoblot experiments.

Characterization of His6::Ta-A-Inv activity products

The reaction mixtures (final volume 50 µl) contained 5 µl of [U-^{14}C]Suc (specific activity 5×10^6 cpm/µmol), 200 mM potassium phosphate buffer (pH 6.5–8.5) and aliquots of His6::Ta-A-Inv. The assays were carried out at 30°C for different times, and the reactions were stopped by heating at 100°C for 2 min. After desalting through mixed-bed ion-exchange columns, sugars were separated by chromatography and quantified determining the labeled sugars as previously described (Porchia et al. 1999).

Sequences analysis

Sequence comparisons were performed using nucleotide and deduced amino-acid sequences available in the databases at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The sequences used in this report are: *D. carota* N-Inv (Dc-N-Inv Y16262), *L. temulentum* N-Inv (Lt-N-Inv AP003143), *A. thaliana* A-Inv (CNIV1, At1g35580), *O. sativa* A/N-Inv (OsNIN1, AK103334) and *Anabaena* sp. PCC 7120 A-Inv and N-Inv (An-InvA, AJ491788, and An-InvB, AJ311089). Sequence alignments were generated with the CLUSTAL X (version 1.8) software (Thompson et al. 1997) and graphic representations of phylogenetic trees were performed using the TREEVIEW16 software. The presence of signals for subcellular localization in the deduced amino-acid sequences of A/N-Inv were predicted using TargetP (version 1.01), Predotar and PSORT softwares (http://www.cbs.dtu.dk/services/TargetP/, http://urgi.versailles.inra.fr/predotar/predotar.html, and http://psort.ims.u-tokyo.ac.jp/, respectively). The sequence reported in this paper (Ta-A-Inv) was deposited in the EMBL database under the accession number AM295169.

Results

Effect of osmotic and cold stress on Suc metabolism in wheat leaves

When wheat seedlings were exposed to 400 mM mannitol or to a low temperature (4°C), a differential effect on leaf soluble sugar content was obtained. While the level of Suc and RS (mainly glucose and fructose, not shown) diminished in the mannitol treated leaf blades, fructan content remained unchanged (Fig. 1). This result is in agreement with the activity level of FSE and Suc hydrolysis by A/N-Inv (Fig. 2a). In contrast, Suc, fructan and RS content increased after 72 h of cold stress (Fig. 1). As previously reported, the modification on carbohydrate content was paralleled by an increase in the activity of all Suc metabolism enzymes and FSE (Fig. 2b) (Tognetti et al. 1989, 1990). Moreover, the increase in A/N-Inv activity due to the effect of mannitol and low temperature was shown from 20 h onwards (Fig. 3a). A/N-Inv polypeptide level could account for these results, as it was higher in stressed than in control leaves, as shown by immunological analysis using anti-An-InvB (Fig. 3b).

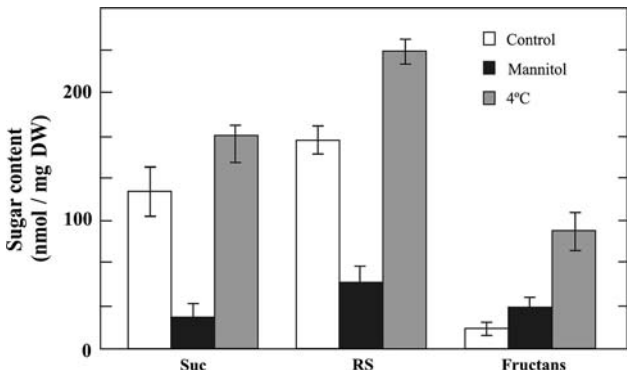

Fig. 1 Effect of osmotic and cold stress on soluble carbohydrate content in primary fully expanded wheat leaves. Sugar contents were determined 72 h after the addition of 400 mM mannitol or after the onset of the cold treatment (4°C). Values are the mean ± standard error of two independent experiments

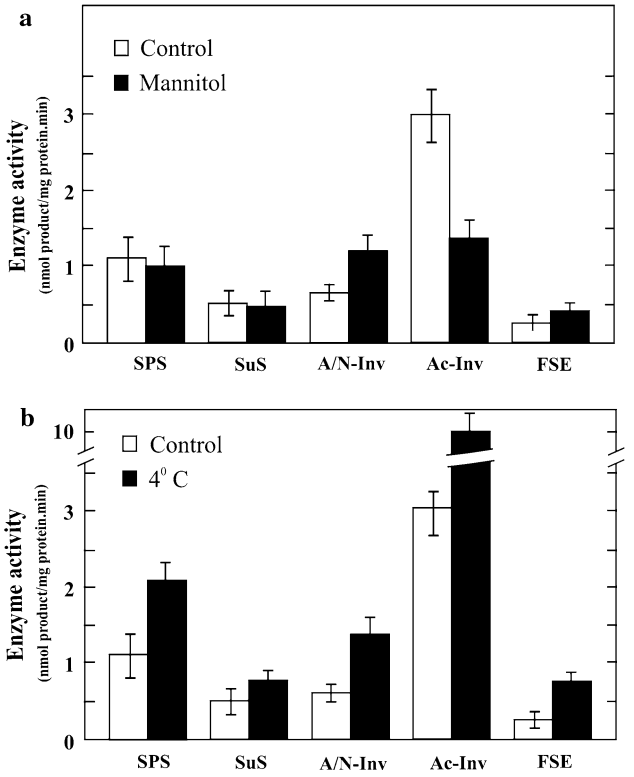

Fig. 2 Effect of osmotic and cold stress on Suc metabolism enzymes and FSE activity in primary fully expanded wheat leaves. Enzyme activities were determined 72 h after the addition of 400 mM mannitol (**a**) or after the onset of the cold treatment (4°C) (**b**). Ac-Inv was determined at pH 4.5 and total A/N-inv (A- plus N-Inv) activity was assayed at pH 7.5. Values are the mean ± standard error of three independent experiments

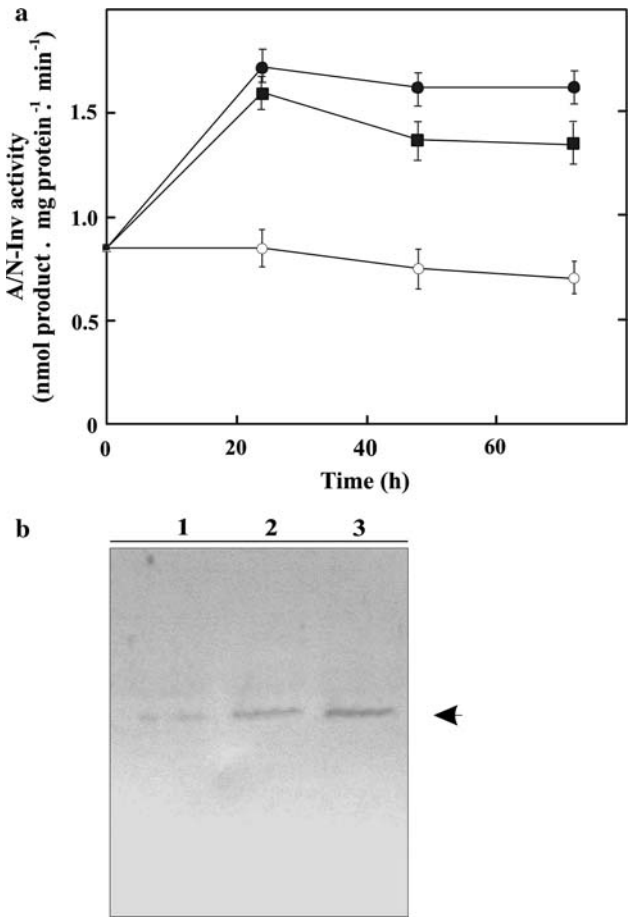

Fig. 3 Effect of osmotic and cold stress on A/N-Inv of wheat seedling leaves. **a** Time course of A/N-Inv activity in crude extracts from mannitol- or cold-treated wheat seedlings. Crude extracts were prepared from leaf blades harvested at different times after the onset of the treatment. Values are the mean ± standard error of three independent experiments. Control (*line with open circle*); 400 mM mannitol (*line with filled square*); 4°C (*line with filled circle*). **b** Immunoanalysis of A/N-Inv after 72-h treatment. Polypeptides from control (lane *1*), mannitol-treated (lane *2*) or cold-treated (lane *3*) seedlings were separated on a 12% SDS-polyacrylamide gel, blotted onto nitrocellulose membranes and probed with anti-An-InvB. Equal amount of protein (30 μg) was loaded in each lane. *Arrowhead* indicates the position of the expected M_r of a polypeptide corresponding to A/N-Inv from plants (Sturm 1999)

A/N-Inv isoforms from wheat leaves

To investigate the presence of A/N-Inv isoforms in wheat, leaf crude extracts from seedlings submitted to osmotic and cold stresses were chromatographed on DEAE-Sephacel columns. While for control leaf extracts A/N-Inv activity eluted in a single peak at 0.30 M NaCl, in the cases of mannitol-treated and 4°C-exposed seedlings, two A/N-Inv activity peaks (I and II) were obtained (maximal activities at 0.30 and 0.36 M NaCl, respectively) (Fig. 4a). We proved that the contribution of Ac-Inv activity to A/N-Inv activity measurements was negligible as it eluted under a single peak with a maximum at 0.23 M NaCl in all cases (as an example it is shown the chromatographic pattern for the cold-treated seedling extract in Fig. 4a, lower panel). Fractions with A/N-Inv activity eluted under peak I and II were separately rechromatographed in a similar column. Enzyme activity eluted from each column at the same NaCl concentrations as previously, confirming the presence of two different isoforms (not shown). Polypeptides present in fractions corresponding to peak I and II were separated by SDS-PAGE and immunorevealed with anti-An-InvB after blotting onto nitrocellulose membranes (Fig. 4b). A/N-Inv proteins eluted under peak I and peak II were named as isoform I and II, respectively. To further purify both isozymes, fractions under peak I and peak II (Fig. 4a) were concentrated and submitted to a gel filtration chromatography on Sepharose-6B columns. Fractions with A/N-Inv activity were pooled and concentrated for further biochemical characterization. Although both isoforms were specific for Suc hydrolysis (not shown) with apparent K_m between 19 and 21 mM, maximum activity for isoform II was obtained at pH 8, while for isoform I was at pH 6.5 (Fig. 8a).

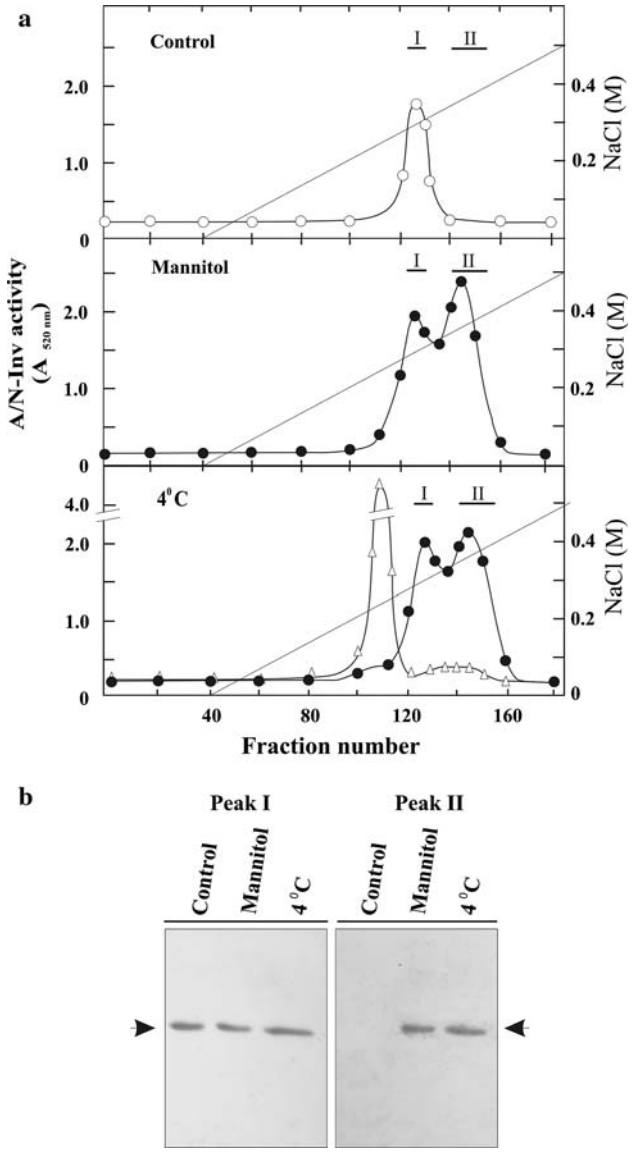

Fig. 4 Identification of A/N-Inv isoforms expressed in wheat seedling leaves. **a** Chromatography on DEAE-Sephacel columns of crude extracts from leaf blades. Protein extracts were prepared from control plants or from 24-h mannitol or cold-treated seedlings. Equal amount of protein was loaded onto each column. Proteins were eluted with a lineal NaCl gradient (0–0.5 M). Total A/N-Inv activity was assayed under standard assay conditions (pH 7.5). For comparison, the elution pattern of Ac-Inv activity (measured at pH 4.5) was included when cold-treated extracts were chromatographed. **b** Immunoblot analysis of A/N-Inv polypeptides after separation by SDS-PAGE of the fractions under peak I and II. Blots on nitrocellulose membranes were probed with anti-An-InvB. *Arrows* indicate the position of A/N-Inv from plants (Sturm 1999)

Identification of a putative A/N-Inv encoding sequence of wheat leaves

Since no full-length coding sequence for a wheat A/N-Inv had been reported, BLAST searches on ESTs databases using the Lt-N-Inv deduced amino-acid sequence as query were performed. Only two sequences, CJ726688 and CJ687871 (Mochida et al. 2006), recovered from EST database led us to assemble a 2,335-bp contig containing a full-length open reading frame (1,662 bp) similar to A/N-Inv. The open reading frame encodes a 553 amino-acids protein with a predicted molecular mass of 62,886 Da. The putative protein was 70% identical to Lt-N-Inv and was initially referred to as Ta-A/N-Inv-like. A comparison of the deduced amino-acid sequences of functionally characterized A/N-Inv present in the public domain (GenBank®) with Ta-A/N-Inv-like showed a high amino-acid identity throughout all the sequences except in the amino terminal region (Fig. S1 and not shown). The three conserved regions (1–3) proposed as useful for the identification of A/N-Inv (Sturm et al. 1999) were also present in Ta-A/N-Inv-like sequence. Moreover sequence analysis of *Ta-A/N-Inv-like* deduced amino acid sequence using subcellular targeting signal softwares (Predotar, TargetP and Psort) pointed to Ta-A/N-Inv-like as a cytosolic protein (Table 1). In addition phylogenetic reconstruction using all the functionally characterized A/N-Inv sequences, showed that Ta-A/N-Inv-like diverged from the subcellular isozymes but clustered with the CINV1 (Fig. 5), a recent characterized cytosolic A-Inv from *A. thaliana* (Lou et al. 2007).

Expression studies and functional characterization of Ta-A/N-Inv-like

RT-PCR assays showed that *Ta-A/N-Inv*-like transcripts were undetectable in leaves of control wheat seedlings, but they accumulated in leaf blades of mannitol- and cold-treated seedlings (Fig. 6). To functionally characterize the protein product of *Ta-A/N-Inv*-like expression, the PCR amplified 1,662-bp DNA fragment was expressed in *E. coli* under the control of a promoter inducible by IPTG (Fig. 7a). Remarkably, the purified recombinant protein (His6::Ta-A-Inv) was immunorevealed by anti-An-InvB and exhibited A/N-Inv activity (Fig. 7b, c). Further characterization showed that pH dependence and inhibition by Tris or $CuCl_2$ of the recombinant enzyme were similar to those for the A-Inv isoforms purified from wheat leaves (Fig. 8).

Discussion

In an attempt to complete the picture of the Suc metabolism response to osmotic and cold stress in wheat leaves, we investigated the activity levels of related enzymes and metabolites. In this article, we present data that clearly show that Inv isoforms with an optimum pH of about 8.0 (A-Inv) are increased in leaves of wheat seedlings after cold and osmotic treatments. We also identified and biochemically

Table 1 Prediction of the subcellular targeting for functionally characterized plant A/N-Inv

Protein	Prediction software		
	Predotar	Target P	PSORT
Ta-A-Inv	None	None	Cytosol
CINV1 (At1g35580)	None	None	Cytosol
Lt-N-Inv	Probable mitochondria	Mitochondria	Probable mitochondria
Dc-N-Inv	Probable mitochondria	Probable mitochondria	Probable mitochondria
OsNIN1	Mitochondria	Mitochondria	Mitochondria

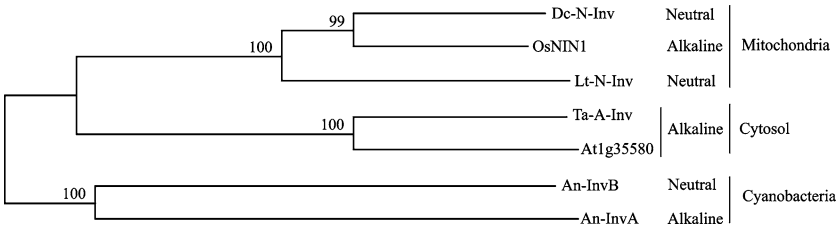

Fig. 5 Phylogenetic analysis of functionally characterized A/N-Inv. Neighbor-Joining phylograms were constructed after sequence alignments of plant and cyanobacterial A/N-Inv sequences using CLUSTAL X software and a bootstrap trial of 1000. The graphical representation was generated using TREEVIEW

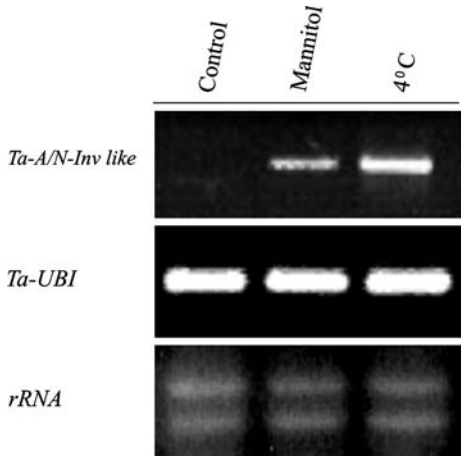

Fig. 6 Expression of *Ta-A-Inv* in wheat leaves of mannitol- and cold-treated seedlings. Semi-quantitative RT-PCR of *Ta-A-Inv* from RNA extracted from leaves of control and mannitol- or cold- treated wheat seedlings. Total RNA was isolated from fully expanded leaves after 24-h treatment. RNA (5 μg) was used for RT-PCR experiments using 25 cycles of amplification. The PCR products were analyzed on 1% agarose gels and visualized after ethidium bromide-staining. Wheat ubiquitin (*Ta-UBI*) was used as internal loading control

characterized a wheat A-Inv encoding sequence (*Ta-A-Inv1*) whose protein product has similar biochemical properties to native wheat stress-response A-Inv and its transcription is up-regulated in response to stresses.

The analysis of the effect of low temperature and mannitol on primary fully expanded wheat leaves revealed that the whole scenario for Suc accumulation and Suc enzyme levels is different in each situation (Figs. 1, 2). Similar to previous reports (Tognetti et al. 1989, 1990; Santoiani et al. 1993), chilling temperatures caused an increase in total Suc, RS and fructan content, in accordance with a higher Suc and polymer biosynthesis. It was proposed that fructans and Suc play a role as protective compounds in response to cold and drought (Pollock and Cairns 1991). Also, it was recently demonstrated in wheat that Suc could act as a signal molecule regulating the gene expression of FSE (Martinez-Noël et al. 2006, 2007). On the contrary, a mannitol treatment caused a decrease in total Suc and RS content, which was in agreement with a higher A/N-Inv activity. Remarkably, although differential responses in Suc metabolism were obtained, either in the cold or in the osmotic treatment A/N-Inv activity increased. Several lines of evidence support an important role of A/N-Invs associated with monosaccharide supply or high hexoses demanding processes, such as those related to energy production and biosynthetic pathways. Zagdanska (1995) reported that in wheat plants submitted to drought, respiration increases to support the energy-requiring processes onset during the stressful situation. Wang et al. (2000) suggested that A/N-Invs might be involved in the control of carbon partitioning and energy production in sweet potato cells under water deficit. It has been stated that in response to drought, plants could regulate the tension in the water column by removing water to or from "cellular reservoirs" in an energy-dependent mechanism (Netting 2002). An increase in A/N-Inv activity and in glucose consumption was also reported in *L. albus* plants exposed to salt stress, which was proposed to assure a continuous ATP and NAD(P)H supply required to avoid or repair salt damages (Fernandes et al. 2004). In the case of developing root nodules of *L. japonicus*, it was suggested that A/N-Invs are

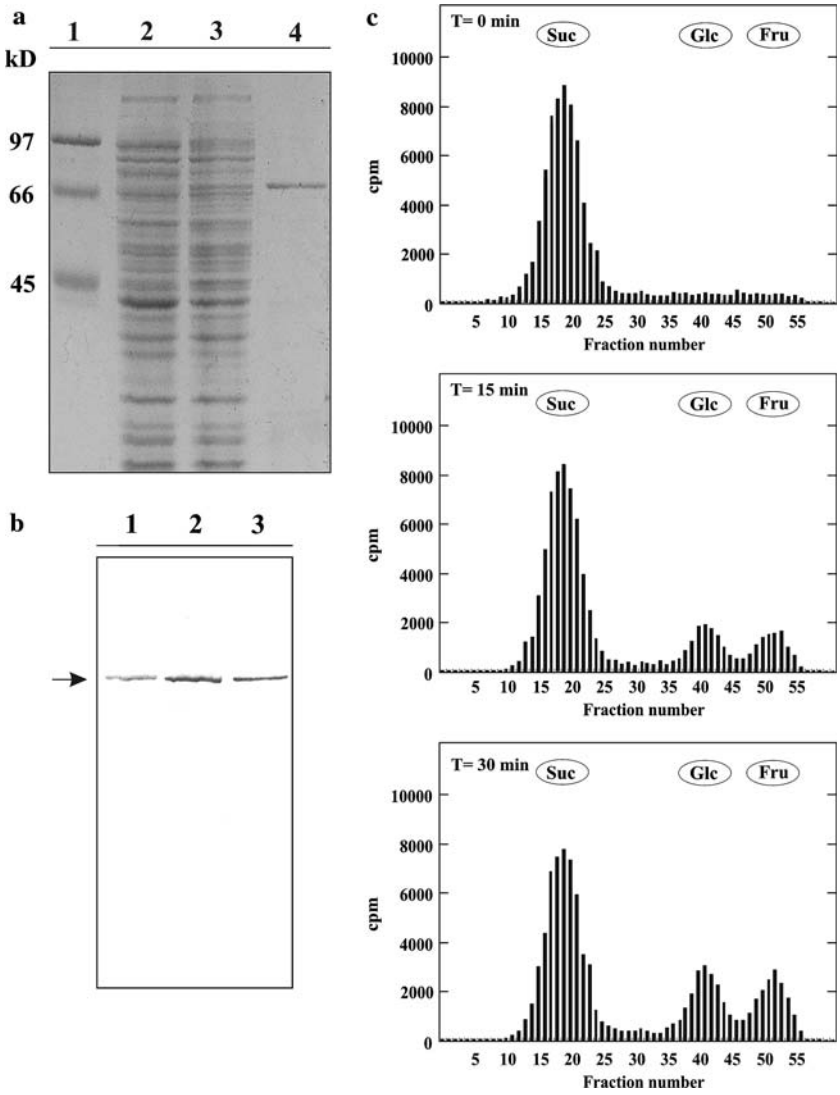

Fig. 7 Expression and purification of His6::Ta-A/N-Inv like. **a** SDS-PAGE on 10% polyacrylamide of *E. coli* protein extracts from uninduced (lane *2*) or IPTG-induced (lane *3*) cells, and of purified His6::Ta-A/N-Inv like (lane *4*). Polypeptides were stained with Coomassie blue. Molecular mass markers were loaded on lane *1*. **b** Immunoanalysis after SDS-PAGE. Polypeptides present in *E. coli* protein extracts of uninduced (lane *1*) and IPTG-induced (lane *2*) cells, and purified His6::Ta-A-Inv protein (lane *3*) were electroblotted onto nitrocellulose membrane and probed with anti-An-InvB. *Arrowhead* indicates the position expected for His6::Ta-A-Inv. **c** A/N-Inv activity of His6::Ta-A/N-Inv like. Activity was assayed in the presence of 50 mM [^{14}C]Suc plus 50 mM phosphate buffer pH 7.5 after 0, 15 and 30 min incubation at 30°C. The reaction products were identified and quantified by determining total radioactivity corresponding to [^{14}C]glucose and [^{14}C]fructose. Standard sugars were simultaneously chromatographed on parallel strips

associated to hexoses supply to different biosynthetic pathways (Flemetakis et al. 2006). In peach (*P. persica*), A/N-Inv activity was proposed to control sugar levels and the growth and development of the fruits (Nonis et al. 2007). Lou et al. (2007) clearly showed the importance of a cytosolic A-Inv in sugar-mediated root cell elongation. It was recently proposed in *A. thaliana* cells under salt stress that Suc hydrolysis products may be channeled to a complex metabolic network to produce amino acids and glycine betaine, well-known osmoprotectants in different plants species (Kim et al. 2007). In the case of wheat, it was described that an accumulation of amino acids and glycinebetaine is also part of the responses to environmental stresses such as water deficit and cold stress (Naidu et al. 1991; Sakamoto and Murata 2002). Our data on wheat seedling leaves led us to conclude that Suc hydrolysis by A/N-Invs is likely to be an early step of a more general response mechanism of plants to support a high-energy demand and the biosynthesis of compounds to overcome unfavorable situations.

To identify A/N-Invs involved in the response to osmotic and cold stress, we examined the occurrence of isoforms in leaves of wheat seedlings. We demonstrated that only A/N-Inv with optimum pH close to 6.5 (isoform I) is expressed in wheat leaves kept under control conditions (Figs. 4, 8). Moreover, the increase of A/N-Inv in stressed leaves (Figs. 2, 3) could be attributed to the appearance of a novel Inv isoform (stress-related isoform II) with optimum pH of ~8 (Figs. 4, 8), and similar properties [affinity for Suc and inhibition by Tris and $CuCl_2$ (Fig. 8)] to those previously reported for A/N-Invs (Chen et al. 1992; Ross et al. 1996; Lee and Sturm 1996; Walker et al. 1997; Vorster and Botha 1998; Vargas et al. 2003; Lou et al. 2007). Whether the presence of isoform II is due to post-translational modifications of already existing proteins cannot be discarded; however, the functional characterization of distinct genes coding for an A-Inv or an N-Inv in the cyanobacterium *Anabaena* (Vargas et al. 2003), with differential regulation and functions (Vargas and Salerno, unpublished) supports

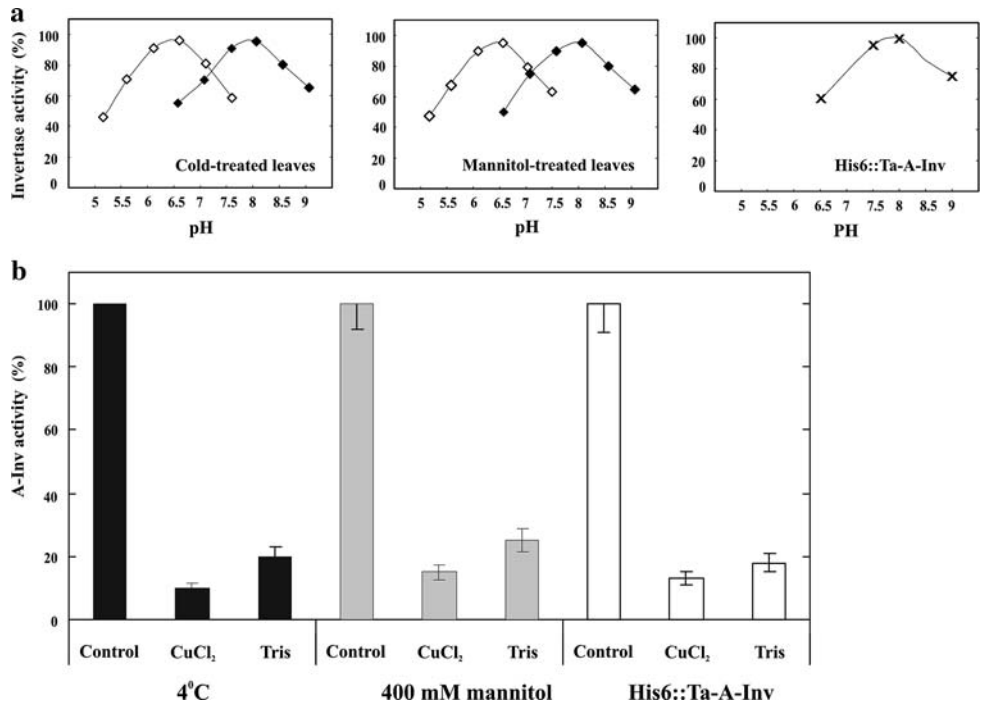

Fig. 8 Functional characterization of the recombinant His6::Ta-A/N-Inv. **a** Comparison of the pH dependence of A/N-Inv activity of the recombinant protein (*line with cross*) with the A/N-Inv isoforms (*line with open diamond* isoform I, *line with filled diamond* isoform II) partially purified from mannitol- or cold-treated wheat leaf blades (see Fig. 4a and "Materials and methods"). The activity of the native wheat enzymes was assayed in the presence of 50 mM phosphate buffer of different pHs and 50 mM Suc, and that of the purified His6::Ta-A/N-Inv was determined in the presence of 50 mM [^{14}C]Suc with buffers in the same pH range. **b** Effect of Tris and $CuCl_2$ on the activity of His6::Ta-A-Inv (*open bars*) and A-Inv isolated from mannitol- (*grey bars*) or cold-treated (*black bars*) wheat seedlings. Enzyme activity was assayed as described above at pH 8.0 in the presence of Tris (50 mM, pH 8), or $CuCl_2$ (1 mM). The reactions were carried out at 30°C for 30 min. Mean values ± standard error of two independent experiments are shown

the possibility of a differential regulation at the gene expression level and different physiological functions. This assumption is also supported by the identification and functional characterization of the encoding gene of a wheat A/N-Inv (*Ta-A-Inv*) that displays maximum activity around pH 8, and whose expression is up-regulated in response to cold or osmotic stress (Figs. 6, 7, 8), similar to isoform II.

After sequence analysis, we propose Ta-A-Inv is a cytosol-located enzyme as no subcellular targeting signal could be detected (Table 1). A preliminary phylogenetic analysis performed with the few A/N-Invs available sequences showed that Ta-A-Inv grouped with *A. thaliana* CINV1 (Fig. 5), the only cytosolic isoform functionally characterized with maximum activity at pH ~8.0 (Lou et al. 2007). OsNIN1, the A/N-Inv recently characterized in rice (Murayama and Handa 2007), is in a separate cluster. However, although this protein was described as an A-Inv, the pH dependence of OsNIN1 activity did not display the characteristic profile described for A/N-Invs (Chen et al. 1992; Ross et al. 1996; Lee and Sturm 1996; Walker et al. 1997; Vorster and Botha 1998; Vargas et al 2003; Lou et al. 2007). Conversely, OsNIN1 sequence is likely to be more related to plant functionally characterized N-Inv. In addition, sequence comparison showed the presence of a conserved motif (DGE/DG) only in An-A-Inv, CINV1 and Ta-A-Inv (Fig. S1, black shaded residues), the only well-characterized A/N-Inv with optimum pH ~8. Interestingly this motif is located in the region 2 of the protein, suggested by Sturm et al. (1999) as the possible substrate binding site of the enzyme. This structural difference between A- and N-Inv may introduce different acid/base properties in the putative catalytic site, which tempt us to speculate that it might be responsible for the difference in optimum pH between both isoforms. Further studies, such as active site identification and crystal structure, will help in clarifying this issue.

While a slight difference in optimum pH for two different isoforms of a given protein could be considered as a random variation, we are confident that it is not the case for A/N-Invs. It was demonstrated for Ac-Invs that one single amino acid substitution on the catalytic site of the enzyme could have strong consequences on the optimum pH of the enzyme (Goetz and Roisch 1999). This observation suggests that optimum pH may be rather a property associated to the amino acids involved in the catalytic site than to a random variation. Also, the fact that A-Inv and N-Inv were demonstrated to be encoded by individual genes (Sturm et al. 1999;

Gallagher and Pollock 1998; Vargas et al. 2003; Murayama and Handa 2007; Lou et al. 2007, this report) and that we showed a differential expression of A- and N-Inv isoforms in response to cold or osmotic stress points toward different physiological significance of the difference in optimum pH. It was demonstrated that in stressed guard cells the pH in the cytoplasm increases up to 0.3 units, probably reaching 7.6 units, and it was proposed that it may be a general response of plant cells to stressful situations (Irving et al. 1992; Blatt et al. 1998; Netting 2002). Therefore, the induction of a cytosol-located A-Inv in response to water deficit and cold stress might be associated with an increase in cytosolic pH and a higher demand for hexoses to produce energy or osmoprotective substances. Comparing the pH dependence of A/N-Inv activity of isoforms I and II (Fig. 8) it is clear that isoform II is almost twice more active at pH close to 8 than isoform I is, and consequently, the induction of an A-Inv confers a higher efficiency to hydrolyze Suc to the cell.

Although Suc occupies a key position in plant life, our knowledge on the function of A/N-Invs still remains limited. Our present contribution reinforces the differential role of the two isoform types and will favor further studies on their structural features and distinct physiological functions.

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