Planta (2008) 227:795–807
DOI 10.1007/s00425-007-0657-1

ORIGINAL ARTICLE

# New insights on sucrose metabolism: evidence for an active A/N-Inv in chloroplasts uncovers a novel component of the intracellular carbon trafficking

**Walter A. Vargas · Horacio G. Pontis · Graciela L. Salerno**

Received: 20 August 2007 / Accepted: 17 October 2007 / Published online: 22 November 2007
© Springer-Verlag 2007

**Abstract** The presence of sucrose (Suc) in plastids was questioned for several decades. Although it was reported some decades ago, neither Suc transporters nor Suc metabolizing enzymes were demonstrated to be active in those organelles. By biochemical, immunological, molecular and genetic approaches we show that alkaline/neutral invertases (A/N-Invs) are also localized in chloroplasts of spinach and *Arabidopsis*. A/N-Inv activity and polypeptide content were shown in protein extracts from intact chloroplasts. Moreover, we functionally characterized the *Arabidopsis At-A/N-InvE* gene coding for a chloroplast-targeted A/N-Inv. The *At-A/N-InvE* knockout plants displayed a lower total A/N-Inv activity in comparison with wild-type plants. Furthermore, neither A/N-Inv activity nor A/N-Inv polypeptides were detected in protein extracts prepared from chloroplasts of mutant plants. Also, the measurement of carbohydrate content, in leaves harvested either at the end of the day or at the end of the night period, revealed that the knockout plants showed a decrease in starch accumulation but no alteration in Suc levels. These are the first results demonstrating the presence of a functional A/N-Inv inside chloroplasts and its relation with carbon storage in *Arabidopsis* leaves. Taken together our data and recent reports, we conclude that the participation of A/N-Invs in the carbon flux between the cytosol and the plastids may be a general phenomenon in plants.

**Keywords** Alkaline/neutral invertase · *Arabidopsis* · Chloroplasts · Spinach subcellular localization · Sucrose hydrolysis

**Abbreviations**

| | |
|---|---|
| A/N-Inv | Alkaline/neutral invertase |
| Chl | Chlorophyll |
| Col 0 | *Arabidopsis thaliana* ecotype Columbia 0 |
| Inv | Invertase |
| gfp | Green fluorescent protein |
| Suc | Sucrose |
| UDP-Glc | UDP-glucose |
| UGPase | UDP-glucose pyrophosphorylase |

## Introduction

Plastids play central roles in the different stages of the plant life cycle. They are involved in important aspects of the plant biology, such as photosynthesis, fatty acid production, and starch metabolism (Tetlow et al. 2004; López-Juez and Pyke 2005; López-Juez 2007). Communication between plastids and the cytosol is a crucial process strictly coordinated inside the plant cells. In the case of photosynthesis, it is well known that an accurate cross-talk is established in order to regulate carbon partitioning between the cytosol and chloroplasts. Triose-phosphate is the main form of photosynthetic carbon transported to the cytosol, being the initial substrate for sucrose (Suc) biosynthesis. When Suc

**Electronic supplementary material** The online version of this article (doi:10.1007/s00425-007-0657-1) contains supplementary material, which is available to authorized users.

W. A. Vargas · H. G. Pontis · G. L. Salerno (✉)
Centro de Investigaciones Biológicas,
Fundación para Investigaciones Biológicas Aplicadas (FIBA),
C.C. 1348, 7600 Mar del Plata, Argentina
e-mail: gsalerno@fiba.org.ar

*Present Address:*
W. A. Vargas
Department of Plant Pathology and Microbiology,
Texas A&M University, College Station, TX 77843, USA

demand is satisfied in photosynthetic tissues, a feedback signaling mediated by a high cytosolic triose-phosphate/phosphate ratio prevents carbon transport towards the cytosol and starch synthesis is induced in chloroplasts. In this way, a close relationship between the regulation of carbon fixation, starch storage and Suc metabolism is established (Dennis and Blakeley 2000).

The presence of Suc in plastids has been described in several studies (Heldt and Sauer 1971; Wang and Nobel 1971; Santarius and Milde 1977; Stitt and Heldt 1981; Heineke et al. 1994; Winter et al. 1994; Fukushima et al. 2001). Because of its low level, plastidic Suc was severely debated and even it was argued to be an artifact of the isolation procedures since neither the presence of Suc transporters nor Suc metabolizing enzymes had been described in these organelles. By indirect evidence it has been recently shown that Suc could enter chloroplasts efficiently (Gerrits et al. 2001). Transgenic tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) plants expressing a plastid-targeted levansucrase gene were able to accumulate fructans in the plastids. Also, a reduction of up to 80% of total Suc content was obtained when a yeast invertase was expressed into potato tuber amyloplasts. Because of the low Suc levels detected inside the plastids, Gerrits et al. (2001) proposed that Suc might be quickly metabolized or exported out by some unknown manner, making even more intriguing the origin and fate of plastidic Suc.

Since several decades ago, it is generally accepted that photosynthetically-derived Suc originates primarily in the cytoplasm (Walker 1974). Suc-phosphate synthase (SPS, EC 2.4.1.14) and Suc-phosphate phosphatase (SPP, EC 3.1.3.24), enzymes responsible for Suc biosynthesis, were extensively studied. SPS and SPP are known to be cytosol-located proteins (Huber and Huber 1996; Winter and Huber 2000; Lunn et al. 2000; Salerno and Curatti 2003). However, different cellular locations were shown for Suc synthase (SuS, EC 2.4.1.13), which is usually assigned a role in Suc cleavage under most physiological conditions. SuS has the capacity to direct carbon towards other polysaccharide biosynthesis (i.e. starch, cellulose and callose) and respiration, and also it is involved in carbohydrate import by heterotrophic tissues (Amor et al. 1995; Winter and Huber 2000). SuS can be found in the cytosol, bound to the plasmatic membrane or associated to mitochondria, depending on the phosphorylation status of its polypeptides (Koch 2004; Subbaiah et al. 2006). In addition, invertases (Invs, EC 3.2.1.26) which catalyze the irreversible hydrolysis of Suc into hexoses, exist in several isoforms differentially located in the cell. Invs, which play an important role when there is a demand for carbon and energy, were classified according to the pH of maximum activity in: (1) acid invertases (acid-Invs, $\beta$-fructofuranosidases, optimum pH about 5.0) that are extracellularly or vacuolarly located, and (2) alkaline/neutral invertases (A/N-Invs, optimum pH between 6.5 and 8.0), which were poorly studied and thought to be exclusively localized into the cytosol (Chen and Black 1992; Van den Ende and Van Laere 1995; Ross et al. 1996; Lee and Sturm 1996; Walker et al. 1997; Vorster and Botha 1998). In contrast, recent reports point to a subcellular location of these proteins. By genome analysis, Ji et al. (2005) studied the different Inv isoforms in rice and proposed the presence of putative subcellular targeting signals in an A/N-Inv family of rice. Afterward and during the writing of the present report, Murayama and Handa (2007) reported that two cDNA sequences of rice (*OsNIN1* and *OsNIN3*) encode proteins that are transported into the mitochondria and into plastids, respectively. However, only the mitochondrial-targeted A/N-Inv (OsNIN1) was functionally characterized in vitro.

In this work, we present functional studies on chloroplast isoforms of A/N-Inv using a combination of biochemical, molecular and genetic approaches and we explore their possible biological roles. We showed A/N-Inv activity and polypeptides in spinach (*Spinacia oleracea*) and *Arabidopsis* (*Arabidopsis thaliana*) chloroplasts. Moreover, we functionally characterized the coding sequence for At-A/N-InvE, an *Arabidopsis* chloroplast-located A/N-Inv, confirmed its subcellular fate by transient expression experiments, and performed biochemical studies in *At-A/N-InvE* knockout plants. Our results reinforce recent findings in rice that describe the subcellular localization of A/N-Invs and suggest that it is a general feature in plants, bringing to light a novel point of control in the regulation of carbon partitioning between the cytosol and chloroplasts in leaves.

## Materials and methods

### Plant material

Spinach (*Spinacia oleracea*) plants were freshly obtained from the central market and leaves were properly washed previous to their processing. Two *Arabidopsis* lines were used in this study: *Arabidopsis* wild-type ecotype Columbia 0 (Col 0) and the SALK_138953 (SALK) mutant line, which contains a T-DNA insertion in the locus At5g22510, protein accession number NP_197643 (Alonso et al. 2003). Seeds from Col 0 and the knockout lines were obtained from the Arabidopsis Biological Resource Centre, Ohio State University. *Arabidopsis* seeds were germinated on 0.8% agar containing 0.5 × MS growth medium supplemented with 5% (w/v) Suc (Murashige and Skoog 1962). Plates were placed in a cold room (4°C) for 3 days to get a synchronized germination and then moved to a growth

chamber (CONVIRON, model CMP4030) at 22°C with a 12-h photoperiod. After 7 days the seedlings were transferred to pots containing a 1:1:1 mixture of perlite:vermiculite:sphagnum moss. For metabolic studies, plant growth was continued for 3 weeks at 22°C, in the same chamber with a short-day light/dark cycle (10 h/14 h) and watered with Hoagland's solution (Hoagland and Arnon 1950). The average light intensity on the top of the pots was ~150 µmol $m^{-2}$ $s^{-1}$. Three self-pollination, selection and growth rounds were necessary to carry out with SALK_138953 plants to obtain homozygosis in the knocked out locus. For phenotypic comparison between Col 0 and mutant plants, growth conditions and procedures were similar to those described by Boyes et al. (2001). For chloroplast preparation, plants were kept in darkness 20 h prior to harvest and processing to obtain a high yield of intact chloroplasts.

*Escherichia coli* and *Agrobacterium tumefaciens* growth

*Escherichia coli* strains [DH5α and BL21 (λDE3):pLysS (Novagen)] and *A. tumefaciens* strain CV2260 were routinely cultured in Luria-Bertani medium and supplemented with the required antibiotics according to the plasmid with which they were transformed (Sambrook and Russell 2001).

Carbohydrate assays

Harvested leaves were cut into 0.5 cm segments and soluble sugars were extracted by boiling the samples in 500 µl alkaline water. The extraction was repeated three times for 5 min at 100°C and the extracts were combined for the analysis. Suc content was quantified after hydrolysis with yeast invertase (Sigma), determining the amount of glucose and fructose by an enzyme coupled assay (Jones et al. 1977). After soluble sugar extraction, the remaining leaf samples were used for starch extraction by adding 500 µl of water and heating in an autoclave at 120°C for 1 h. Starch content was determined in the supernatant as the amount of glucose released after hydrolysis with α-amyloglucosidase (Sigma) in the presence of 100 mM sodium acetate buffer, pH 4.5. Glucose was analyzed using Somogyi-Nelson reagents (Ashwell 1957), or enzymatically with hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer et al. 1974). For qualitative starch staining, *Arabidopsis* plants were boiled in 80% (v/v) ethanol for 5 min. The leaf starch was stained using an iodine–iodide solution. Iodine excess was removed by washing the leaves with water and the plants were immediately photographed. Statistical analyses (ANOVA) were performed using the VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html) tools.

Preparation of total protein extracts from *Arabidopsis* and spinach leaves

Fresh leaves (1 g) were ground with a mortar and pestle in the presence of liquid nitrogen. The powder was homogenized with 2 ml of 50 mM Hepes–NaOH (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 20% (v/v) glycerol, 0.01% (v/v) Triton X-100, 20 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride 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 desalted through Sephadex G-50 columns and immediately used for enzyme activity assays.

Chloroplast isolation and protein extract preparations

Spinach and *Arabidopsis* leaves were ground with a mortar and pestle in the presence of 400 mM mannitol, 50 mM Hepes–NaOH (pH 7.5), 2 mM EDTA, 1 mM $MgCl_2$, 0.2% β-mercaptoethanol and 0.2% (w/v) BSA (grinding buffer) and following the protocol described by Mourioux and Douce (1981). Intact chloroplasts were separated after centrifugation during 15 min at 9,000×*g* on a pre-formed Percoll® gradient (50% [v/v] Percoll, 45 min 22,000×*g*, 4°C, brake off), collected and washed three times with grinding buffer. The integrity of chloroplasts was judged by microscopy. Isolated chloroplasts were ground with a pestle and mortar in the presence of glass powder and liquid nitrogen. Proteins were extracted as indicated above. The homogenate was centrifuged for 20 min at 12,000×*g* and the supernatant was desalted through a Sephadex G-50 column prior to enzyme assays.

Enzyme assays, chlorophyll and protein determination

A/N-Inv activity was routinely determined at 30°C in reaction mixtures containing 200 mM Hepes–NaOH (pH 7.5) and 200 mM Suc. The released reducing sugars were quantified using Somogyi-Nelson reagents (Ashwell 1957) or by enzyme coupled assays following spectrophotometrically the appearance of NADPH (Bergmeyer et al. 1974; Bernt and Bergmeyer 1974). UDP-Glc pyrophosphorylase (UGPase) activity was determined at 30°C in a reaction mixture containing 100 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 10 mM UDP-Glc, 10 mM sodium pyrophosphate. The rate of UDP-Glc pyrophosphorolysis was measured spectrophotometrically in the presence of phosphoglucomutase, glucose-6-phosphate dehydrogenase and NADP as previously described (Sowokinos 1981). NADP-dependent malate dehydrogenase activity was assayed at 30°C in the presence of 100 mM Hepes–NaOH (pH 7.5), 10 mM sodium malate and 10 mM NADP, and the reaction progress was followed as described previously (Scheibe and Stitt 1988). Fumarase

activity was assayed as the conversion of malate into fumarate in the same conditions described by Cooper and Beevers (1969), and the reaction progress was followed determining the absorbance at 240 nm. Chlorophyll (Chl) was extracted with methanol and its concentration was calculated according to the equation $\mu g_{Chl} = A_{665\ nm} \times 13.43$ (Mackinney 1941). Protein concentration was determined using the Bradford reagent (Bradford 1976).

Protein analysis on SDS-PAGE and immunodetection

Proteins 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 raised in rabbits against recombinant InvB (An-InvB) from *Nostoc* sp. PCC 7120 (Vargas et al. 2003), or against *Solanum lycopersicum* UGPase (kindly provided by Prof J. Sowokinos, University of Minnesota) as previously reported (Asatsuma et al. 2005). To visualize chloroplastic A/N-Inv by immunoblot analysis, the protein was loaded in each lane on an equal cytosolic marker basis according to Beers et al. (1992).

Total RNA preparation and expression assays

RNA from different *Arabidopsis* tissues was prepared using the TRIZOL® reagent (Gibco-BRL) according to the manufacturer. RNA quality and PCR products were analyzed by electrophoresis in 1% agarose gels. cDNA synthesis was performed from total RNA (5 μg) treated with DNase (RQ1 RNase-Free DNase®, Promega), using moloney murine leukaemia virus-reverse transcriptase (MMLV-RT®, Promega) and oligo-dT primers. PCR reactions to amplify *At-A/N-InvE* were performed using the primers AtInvEF (GGATC CATGGCAGCTTCAGAAACAGTTC) and AtInvER (TCTAGAAATAAATGGTTGTTGAGCTTTTTT). In the case of *At-A/N-InvH*, AtInvHF (CTCGAGATGAATGCC ATCACTTTTCTTG) and AtInvHR (ACTAGTTATAAG AATCTGAGACTTTGCTGC) were used as primers. The amplification of the constitutively expressed ubiquitin gene (*AtUbi*, accession number AK118678) was used as an internal loading control, using the primer pair UbiF (CTTCGTC AAAACCCTCACCGGCAAAA) and UbiR (GAGAAGA CCACCCCTAAGAGCAAGA). For expression analysis, semi-quantitative RT-PCR experiments were carried out. After reverse transcription of total RNA, PCR reactions were performed 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. Parallel reaction were performed to clone the amplification product corresponding to *At-A/NInvE* cDNA into pGEM®-T Easy vector system (Promega), resulting in the plasmid pG*At-A/N-InvE*. The identity of the cloned DNA fragment was ascertained by sequencing both DNA strands.

Construction, expression and enzyme assays of a $His_6$::At-A/N-InvE fusion protein

The AtInvEF primer for the amplification of *At-A/N-InvE* cDNA was designed with an adapter for *Bam*HI restriction enzyme. The plasmid pG*At-A/N-InvE* was *Bam*HI/*Sal*I digested and the DNA fragment corresponding to the At-A/N-InvE was sub-cloned between the sites *Bam*HI/*Xho*I of the expression vector pRSET-A (Invitrogen). The construct was transferred to *E. coli* BL21($\lambda$DE3):pLysS cells (Novagen) and the expression of the recombinant protein was induced with 1 mM isopropyl $\beta$-D-thiogalactoside (IPTG). After 16 h induction at 18°C, the cells were harvested and the fusion protein ($His_6$::At-A/N-InvE) was purified throughout $Co^{2+}$ affinity chromatography (TALON® resin, Clontech) and concentrated for further characterization. Cell extracts from IPTG-treated or non induced cells and purified $His_6$::At-A/N-InvE were analyzed by SDS-PAGE and immunoblot experiments. The enzyme activity of the recombinant protein was assayed in reaction mixtures (final volume 50 μl) containing 5 μl of [U-$^{14}$C]Suc (specific activity $5 \times 10^6$ cpm/μmol), 200 mM potassium phosphate buffer (pH 7.5) and aliquots of the purified $His_6$::At-A/N-InvE. The assays were carried out at 30°C for different times. 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, using Suc, glucose and fructose as standards (Porchia et al. 1999).

Transient expression experiments of At-A/N-InvE fused to green fluorescent protein (gfp)

The *At-A/N-InvE::gfp* fusion was constructed in the pCHF3 vector (kindly provided by E. Zabaleta). The *At-A/N-InvE* sequence was fused upstream and in frame with the *gfp-mut2* gene (Cormack et al. 1996) under the control of the cauliflower mosaic virus 35 S promoter (pCHF3::CaMV35S::*At-A/N-InvE*::*gfp*). In this construction the stop codon corresponding to *At-A/N-InvE* was substituted by the initial methionine codon of *gfp*. The DNA fragments used to generate the fusion were PCR amplified using the primers AtInvEF and AtInvER for *At-A/N-InvE* and the pair of primers GFPF (TCTAGAATGAGTAAAGGAGAAGAA CTT) and GFPR (GTCGACTTATTTGTATAGTTCATC CATGCC) to amplify *gfp*. The plasmid containing the gene fusion was transformed into *A. tumefaciens* strain CV2260

by the freeze–thaw methodology (Sambrook and Russell 2001). *Arabidopsis* leaves were infiltrated with *A. tumefaciens* cell suspensions according to Wroblewski et al. (2005). After 48 h, infiltrated leaves were used to isolate protoplasts by tissue digestion with 1% (w/v) cellulase (Onozuca R-10) and 0.25% (w/v) macerozyme R-10 as described by Mathur and Koncz (1998). The protoplasts were visualized with a confocal laser scanning microscope LSM Pascal Axioplan II (Carl Zeiss, Germany). Chlorophyll and gfp were excited at a wavelength of 488 and 543 nm, respectively. Emission of gfp was collected from 510 to 535 nm and Chl emission was recorded at wavelengths >560 nm.

### Sequence analyses

Sequences were obtained from the non-redundant protein databases of the 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. 1997) and using as query the functionally characterized A/N-Inv coding sequences from *Anabaena* (*Nostoc*) sp. PCC 7120 (Vargas et al. 2003). To perform the sequence alignments and phylogenetic analyses we retrieved sequences from genome databases whose cDNA were identified in EST libraries or their encoded proteins were functionally characterized. The sequences used in this work (accession number between parenthesis) belong to the following organisms: *A. thaliana* [At-A/N-InvA (NP_176049), At-A/N-InvB (NP_195212), At-A/N-InvC (NP_187302), At-A/N-InvD (NP_564177), At-A/N-InvE (NP_197643), At-A/N-InvF (NP_177345), At-A/N-InvG (NP_849750), At-A/N-InvH (NP_187233.2), At-A/N-InvI (NP_567347)], *Beta vulgaris* [Bv-A/N-Inv (AJ422050)], *Daucus carota* [Dc-A/N-Inv (Y16262)], *Lolium temulentum* [Lt-A/N-Inv (AJ003114)], *Lotus corniculatus* [Lcor-A/N-Inv (AJ717412)], *Oryza sativa* [OsNIN1 (AK103334), OsNIN2 (AK120720), OsNIN3 (AK121301), OsNIN4 (AAX95795), OsNIN5 (NP_001052830), OsNIN6 (AK070884), OsNIN7 (AK065562)], *Ipomoea trifida* [Itr-A/N-Inv (AAS79609)], *Solanum lycopesicum* [Sl-A/N-Inv (ABQ28669)], *Triticum aestivum* [Ta-A-Inv (AM295169)], *Nostoc* sp. PCC 7120 [AnInvA (AJ491788), AnInvB (AJ311089)], *Nostoc punctiforme* [NpInvA (AJ491790), NpInvB (AJ491789)], *Prochlorococcus marinus* MIT9313 [PmMIT9313 (AJ491792)], *P. marinus* subsp. pastoris [Pmpastoris (AJ491791)], *P. marinus* subsp. marinus [Pmmarinus (NP_874763)], *Synechococcus marinus* WH8102 [Sm8102 (AJ491793)], *Synechococcus* sp PCC 7942 [Sy7942 (ZP_00163518)], *Synechococcus* sp. PCC 6301 [Sy6301 (YP_171828)] and *Synechocystis* sp. PCC 6803 [Sy6803 (CAD33848)]. Sequence alignments were generated with CLUSTALX program version 1.8 (Thompson et al. 1997). Dendrograms were compiled by using the neighbor-joining method (computed from 1,000 independent trials) of CLUSTALX and the maximum parsimony algorithm of the PHYLIP package (Felsenstein 1993). The presence of signals for subcellular location in the deduced amino-acid sequences of A/N-Invs were predicted using TargetP (version 1.01), ChloroP and Predotar software (http://www.cbs.dtu.dk/services/TargetP/, http://www.cbs.dtu.dk/services/ChloroP/ and http://urgi.versailles.inra.fr/predotar/predotar.html, respectively).

## Results

### Subcellular plastid-targeting signals in plant A/N-Inv homologues

BLAST searches retrieved 34 sequences including A/N-Invs functionally characterized or putative proteins whose mRNA sequences were identified in EST libraries. The sequences displayed similarities between 30.6 and 78.4% (not shown). Sequence alignments and phylogenetic analyses grouped A/N-Invs in three main families (A, B and C) (Fig. S1 of Electronic Supplementary Material). All cyanobacterial homologues clustered as a separated group (family A), containing two subgroups $A_1$ and $A_2$ (corresponding to unicellular and filamentous cyanobacteria, respectively). As reported by Ji et al. (2005) and Murayama and Handa (2007), plant A/N-Invs that diverged in two different families (B and C), show an N-terminal extension as a differential feature. We found that the N-terminal extension of deduced sequences belonging to members of family B and C are about 80 and 150 amino acids, respectively. In agreement with previous reports (Ji et al. 2005; Murayama and Handa 2007) we determined that the difference in the N-terminal extension was due to the presence of subcelluar targeting signals in members of the family C (not shown). The Predotar, ChloroP and TagetP programs consistently predicted similar results. In addition to the subcellular targeting signals reported in *Arabidopsis* and rice putative A/N-Invs (Ji et al. 2005; Murayama and Handa 2007), we identified a putative mitochondrial targeting signal in Dc-A/N-Inv and Lt-A/N-Inv (not shown) and a possible plastid transit peptide in Bv-A/N-Inv and Itr-A/N-Inv sequences (Table 1). We also found that the identified putative transit peptides displayed the consensus motif and the phosphorylation site (indicated by an asterisk) required for translocation to chloroplast, P/G$X_n$R/K$X_n$S/T$X_n$S*/T* (Table 1) (Waegemann and Soll 1996). As it was reported for a chloroplastic $\alpha$-amylase from rice (Chen et al. 2004), there is no R/K residue in the consensus motif for the targeting plastid signal of Itr-A/N-Inv. However, it was recently

**Table 1** Structure analysis to predict transit peptides in A/N-Inv sequences

| A/N-Inv | N-terminal sequence |
|---|---|
| At-A/N-InvE | MAASETVLRVPLGSVSQSCYLASFFVNST**P**NLSF**K**PV**S**RNRK**T**VR |
| At-A/N-InvH | MNAITFLGNSTMIPSQCILRAFTRIS**P**SKYI**R**DT**S**FR**S**YPSRFSSCINQYRNADSDRIIRPTNAVPFCTDR |
| OsNIN3 | MNGQTPMGLAAAAAAAVR**P**CRR**R**LLS**S**A**T**AAAAAATVATATPLFPRCPHPHHHLHGRR |
| Bv-A/N-Inv | MTTAEALLSGSALWN**P**TFQSDIFLKP**K**L**T**H**S**GVFS |
| Itr-A/N-Inv | MHLIANSTIQHTPSN**P**HMQA**T**LL**S**FEVSSRAPIGIFYTSQLDHLKVKGKN |
| RU | MASSVLSSAAVATRSNVAQANMVAPFT**G**L**KS**AA**S**FPVSRKQNLDITSIASNGGRVQC |
| OE 33 | MAASLQAAATLMQ**P**TKL**RS**N**T**LQLKSNQSVSKA |
| Consensus motif **P/G**X$_n$**R/K**X$_n$**S/T**X$_n$**S***/**T*** | |

RU and OE33 depict the transit peptide for precursors of the small subunit of ribulose-bisphosphate carboxylase/oxygenase (RubisCO) and the oxygen evolving protein of 33 kDa, respectively

Amino acids involved in the formation of a putative phosphorylation motif (Waegemann and Soll 1995) are indicated in higher size and bold letters. * Underlined residues: probable phosphorylation sites

demonstrated that this residue is not essential for the targeting of *Arabidopsis* small subunit of ribulose-biphosphate carboxylase/oxygenase (RubisCO) into chloroplasts (Lee et al. 2006).

### Assessment of A/N-Inv isoforms inside spinach and *Arabidopsis* chloroplasts

The occurrence of A/N-Inv activity was ascertained in protein extracts of chloroplasts isolated from *Arabidopsis* and spinach leaves. Contamination from cytosol or mitochondria was very low in chloroplast preparations (Table 2). Plastid A/N-Inv activity corresponded to about 13 and 20% of total enzyme activity determined from spinach and *Arabidopsis* leaf extracts, respectively. The presence of the A/N-Inv polypeptide in chloroplasts was confirmed by immunodetection using anti-An-InvB antibodies (Vargas et al. 2003), which cross-reacts with plant A/N-Invs (Vargas et al. 2007) and revealed a single band (Fig. 1). Either for spinach or *Arabidopsis*, chloroplast extract was compared to a total protein leaf extract on an equal cytosolic marker UGPase basis. A comparison of lanes T (total leaf protein) and C (chloroplast proteins), where equal UGPase activity were loaded, indicates that only a small amount of the immunosignal detected for the plastid extract was due to cytosol contamination. To confirm that equal amounts of UGPase were loaded in each lane, membranes were probed with anti-UGPase antibodies.

### *At-A/N-InvE* encodes a plastid-directed A/N-Inv

To investigate the fate of the proteins encoded by the A/N-Inv *Arabidopsis* genes that contain sequences corresponding to putative chloroplast targeting signals (*At-A/N-InvE* and *At-A/N-InvH*), we first studied their expression pattern by semiquantitative RT-PCR experiments. The *At-A/N-InvE* transcripts were detected in every tissue assayed, while *At-A/N-InvH* was transcribed only in flowers (Fig. 2). Since *At-A/N-InvE* was the only gene coding for a putative

**Table 2** A/N-Inv activity recovery from isolated *Arabidopsis* and spinach chloroplasts

| Enzyme | Enzyme activity recovery (%) | |
|---|---|---|
| | Col 0 | Spinach |
| A/N-Inv | 13 ± 1 | 20 ± 2 |
| UGPase | 0.9 ± 0.2 | 4.8 ± 0.7 |
| NADP-MDH | 80 ± 5 | 97 ± 6 |
| Fumarase | Nd | Nd |

UGPase and fumarase activities were used as cytosol and mitochondrial contamination markers, respectively. NADP-MDH activity was used as a control for the efficiency of chloroplasts breakage. Results are mean values ± standard error of three independent experiments. Nd, not detectable

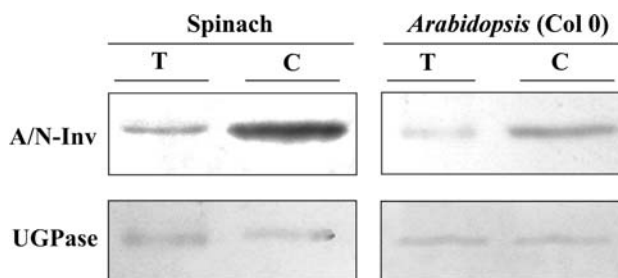


**Fig. 1** Occurrence of A/N-Inv polypeptides in *Arabidopsis* and spinach chloroplast extracts. Proteins from total crude extract (T) and chloroplast protein preparations (C) were separated by SDS-PAGE using 12% acrylamide gels and electroblotted onto nitrocellulose membranes for immunoassays. The amount of protein loaded in each lane was standardized according to equal UGPase activity (cytosol marker) present in the extracts (Beers et al. 1992). The blots were probed with polyclonal antibodies raised against An-InvB or UGPase

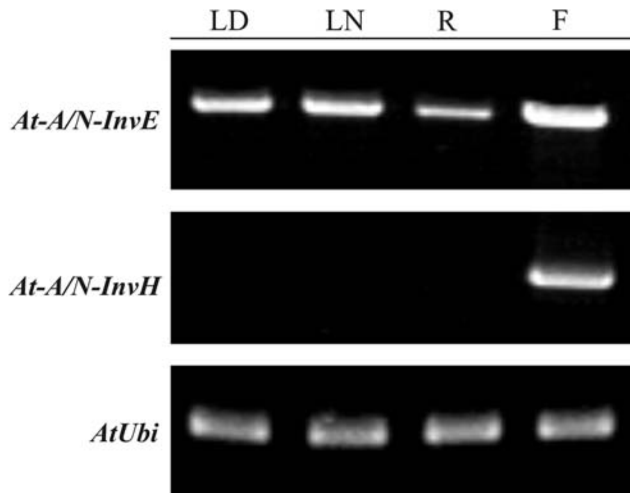


**Fig. 2** Expression of *At-A/N-InvE* and *At-A/N-InvH* in different *Arabidopsis* tissues. Semiquantitative RT-PCR experiments were carried out using isoform-specific primers. Gene expression was assayed in leaves (LD, leaves harvested in the midday; LN, leaves harvested in the midnight), roots (R) and flowers (F). RT-PCR products were analyzed on 1% agarose gels and visualized after ethidium bromide staining. Amplification of ubiquitin (*AtUbi*, AK118678) was used as an internal loading control

A/N-Inv with a putative transit peptide to chloroplast which was expressed in leaves, we assumed that its protein product (At-A/N-InvE) could correspond to the A/N-Inv detected in the protein extract from *Arabidopsis* chloroplasts.

Because of the importance of Suc metabolism and its metabolic cross-talk with chloroplasts, we focused our study on the characterization of the putative chloroplast At-A/N-InvE. Its amino acid deduced sequence was about 73% identical to OsNIN3, Lt-A/N-Inv and Dc-A/N-Invs (all containing subcellular targeting signals), and about 59% identical to Ta-A-Inv (not shown) (Gallagher and Pollock 1998; Sturm et al. 1999; Murayama and Handa 2007; Vargas et al. 2007). *At-A/N-InvE* encodes a predicted protein of 617 amino acids (69,240 Da) and contains a putative 45-amino-acid transit peptide. For biochemical characterization, *At-A/N-InvE* sequence was PCR-amplified, cloned, and expressed in *E. coli*. The purified His-tagged recombinant protein was assayed for A/N-Inv activity. The progress of the reaction was followed by chromatographic analysis of the products (glucose:fructose in a 1:1 ratio) (Fig. 3a). Immunoblots analysis using anti-An-InvB antibodies revealed the purified protein as a single band (Fig. 3b).

To study the in vivo subcellular targeting of At-A/N-InvE, the entire encoding sequence (excluding the stop codon) was fused upstream of the *gfp* gene (*At-A/N-InvE::gfp*). Transient expression experiments showed a green fluorescence pattern that co-localized with the red autofluorescence of chlorophyll (Fig. 3c). In the control (expression of *gfp* driven by the CaMV 35 S promoter) the green fluorescence was only detected in the cytosol (not shown). Taken together, these data demonstrate that *At-A/N-InvE* encodes a chloroplast-targeted protein which displays A/N-Inv activity.

### *At-A/N-InvE*-knockout plants lack chloroplast A/N-Inv

To confirm that At-A/N-InvE corresponded to the enzyme responsible for chloroplast A/N-Inv activity and was a plastid isoform, we analyzed *At-A/N-InvE* knockout mutant plants. The SALK_138953 *Arabidopsis* plants contain a T-DNA insertion in the second exon of the gene *At-A/N-InvE* (locus At5g22510) that impairs the expression of *At-A/N-InvE* (Fig. 4a). The loss of *At-A/N-InvE* expression in mutant plants was verified by RT-PCR assays (Fig. 4b). The comparison of phenotypes of the mutant (SALK) and wild-type (Col 0) plants, showed no difference in germination, the number of leaf or siliques per plant, seed production, and flowering time (Table 3). A/N-Inv activity determined in protein extracts from fully expanded leaves resulted about 30% lower in the mutant than in the Col 0 plants (Fig. 4c). Also, highly purified chloroplasts were prepared from SALK and Col 0 plants. Conversely to the wild-type plants, the knockout *Arabidopsis* plants did not display chloroplast A/N-Inv activity (Fig. 4d). This was in agreement with immunoblot analysis of the polypeptides present in chloroplast protein extracts from SALK plants, where no band was revealed with anti An-InvB antibodies (Fig. 4e). Further biochemical characterization of the SALK plants showed no difference in the total Suc content; however, a significative 33% lower starch content was obtained at the end of the day in comparison with the wild-type plants (Fig. 5a–c).

## Discussion

The cyanobacterial endosymbiotic origin of plant chloroplasts and the transfer of most cyanobacterial genes to the plant nucleus are generally accepted (Rujan and Martin 2001). Thus, Suc metabolism genes, which were proposed to have originated before the cyanobacterial phylogenetic radiation, seem to be lost efficiently from the ancestral chloroplasts because no homologue has been identified in sequenced plastid genomes (Salerno and Curatti 2003; Vargas et al. 2003). However, in contrast to the majority of protein product of cyanobacterial origin genes that were preferentially re-imported to the organelle, Suc metabolism proteins were believed to localize in the cytosol (Salerno and Curatti 2003). Particularly, A/N-Invs were commonly considered cytosolic enzymes in extant plants (Chen and Black 1992; Van den Ende and Van Laere 1995; Ross et al.

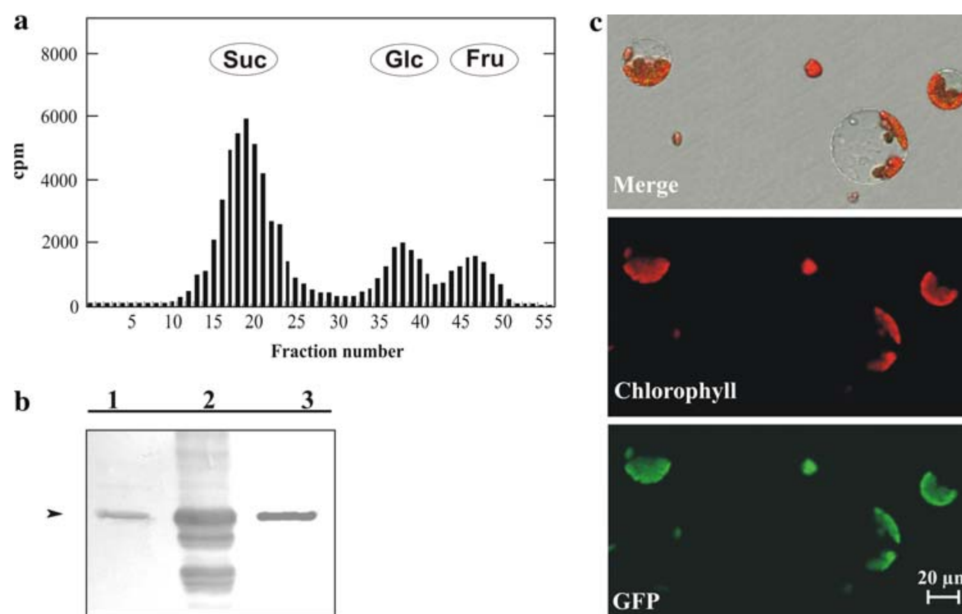


**Fig. 3** Functional characterization and subcellular localization of the protein product of *At-A/N-InvE*. **a** A/N-Inv activity determination of the purified $His_6$::At-A/N-InvE. Activity was determined using [$^{14}$C]Suc in a mixture buffered at pH 7.5. The reaction products were chromatographically analyzed. The position of standard sugars are indicated (Suc, sucrose; Glc, glucose; Fru, fructose). No activity was retrieved when it was assayed at pH 4.5. **b** Immunoanalysis after SDS-PAGE. The polypeptides present in non-induced (*lane 1*) and IPTG-induced (*lane 2*) *E. coli* cells, and the purified enzyme (*lane 3*), were separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with polyclonal antibodies against An-InvB. *Arrowhead* indicates the expected position for $His_6$::At-A/N-InvE. The immnodetection of a polypeptide in lane 1 may be ascribed to a leaky expression of the protein in *E. coli* cells. **c** Subcellular localization of At-A/N-InvE in *Arabidopsis* cells. The translational fusion of the gfp coding sequence and *At-A/N-InvE* was constructed downstream of the CaMV 35 S promoter in the plasmid pCHF3 (pCHF3::*At-A/N-InvE::gfp*). *Arabidopsis* leaves were infiltrated with cell suspensions of *A. tumefaciens* CV2266 carrying the plasmid pCHF3*::At-A/N-InvE::gfp*. After 48 h, protoplasts were prepared from infiltrated leaves and visualized under laser scanning confocal microscope

1996; Lee and Sturm 1996; Walker et al. 1997; Vorster and Botha 1998; Sturm 1999). But this statement should be definitively forsaken. Our study demonstrates not only that an *Arabidopsis* A/N-Inv is transported into chloroplasts but also that *Arabidopsis* and spinach chloroplasts exhibit A/N-Inv activity, complementing the recent findings of Murayama and Handa (2007) in rice with respect to the chloroplast fate of some A/N-Inv isoforms.

Structure and phylogeny analyses using amino acid-deduced sequences of A/N-Invs from cyanobacteria and from *A. thaliana*, *O. sativa*, *B. vulgaris*, *I. trifida*, *L. corniculatus*, *S. lycopersicum*, as well as from the functionally characterized sequences of *T. aestivum*, *L. temulentum* and *D. carota*, confirmed that plant A/N-Invs grouped in two separated families (Fig. S1 of Electronic Supplementary Material), in agreement with recent reports (Ji et al. 2005; Murayama and Handa 2007). In addition, we found the presence of subcellular targeting signals in A/N-Inv belonging to family C, including Dc-A/N-Inv, Bv-A/N-Inv, Lt-A/N-Inv and Itr-A/N-Inv. The detection of putative transit peptides seems to be a feature of some A/N-Inv sequences either of monocot or dicot plant species, which led us to suggest that organelle-targeted A/N-Invs might have emerged at early stages of plant differentiation (Table 1, Vargas et al. 2007). However, taking into account that A/N-Invs appear to be originated and confined to oxygenic photosynthetic organisms (Salerno and Curatti 2003; Vargas et al. 2003), the differential origin for A/N-Invs transported to mitochondria and plastids proposed by Murayama and Handa (2007) seems to be unlikely. An increased complexity in the role and regulation of Suc enzymes might have been adjusted after the endosymbiotic origin of chloroplasts providing the basis of the contemporary photosynthetic plant cell scenario.

The occurrence of Suc in the chloroplasts was controversial for decades. Several reports had previously described the presence of the disaccharide in the plastids (Heldt and Sauer 1971; Wang and Nobel 1971; Santarius and Milde 1977; Stitt and Heldt 1981; Heineke et al. 1994; Winter et al. 1994; Fukushima et al. 2001), but no active Suc metabolism enzymes had been characterized in those organelles. Stitt and Heldt (1981) described that isolated chloroplasts from spinach kept at 20°C hydrolyzed Suc into glucose and fructose. Recently, Gerrits et al. (2001) indirectly demonstrated a substantial influx of Suc into plastids and proposed a high rate of degradation for the disaccharide. This hydrolysis may be due to the action of an A/N-Inv, with maximum activity at a pH range similar to that of

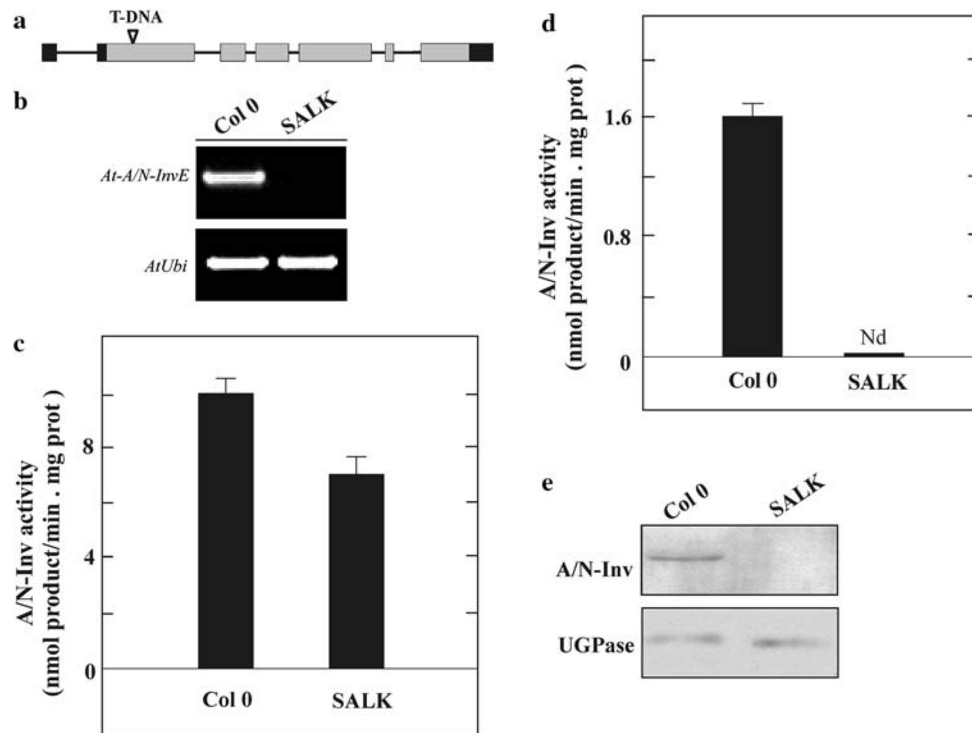


**Fig. 4** A/N-Inv in *At-A/N-InvE* knockout *Arabidopsis* plants. **a** Structure of the *At-A/N-InvE* gene: seven exons and six introns. The coding and untranslated regions are depicted by *grey* and *black* boxes, respectively. The *triangle* shows the T-DNA insertion site in the SALK_138953 mutant line. **b** *At-A/N-InvE* expression in wild-type (Col 0) and mutant *Arabidopsis* plants. Expression was assayed by semiquantitative RT-PCR with total RNA extracted from leaves. RT-PCR products were analyzed on 1% agarose gels and visualized after ethidium bromide staining. Amplification of ubiquitin encoding sequence (*AtUbi*, AK118678) was used as an internal loading control. **c** A/N-Inv activity in crude extracts prepared from mature leaves of wild-type and *At-A/N-InvE*-knockout *Arabidopsis* plants. Values are the mean ± standard error of three independent experiments. **d** Determination of A/N-Inv activity in protein extracts of chloroplasts from wild-type and *At-A/N-InvE* knockout plants. Enzymatic activity was corrected considering the cytosolic contamination, which was evaluated with the UGPase activity. Values are the mean ± standard error of three independent experiments. Nd: not detected. **e** Immunodetection of A/N-Inv polypeptides in protein extracts of chloroplast isolated from wild-type and *At-A/N-InvE* knockout *Arabidopsis* plants. Proteins were electrophoresed on 12% SDS-acrylamide gels, electroblotted onto nitrocellulose membranes and probed with polyclonal antibodies anti An-InvB or UGPase. The amount of protein loaded in each lane was equivalent to equal UGPase activity. Similar results were obtained in independent experiments. Col 0: *Arabidopsis* wild-type plants. SALK: *Arabidopsis At-A/N-InvE* knockout plants (SALK_138953)

the stroma (pH between 7 and 8 depending on the physiological status of the cell; Hauser et al. 1995; Sturm 1999).

Whereas recent reports predicted plastid and mitochondria A/N-Inv isoforms (Murayama and Handa 2007; Vargas et al. 2007), only a mitochondrial-located A/N-Inv (OsNIN1) of *O. sativa* was functionally characterized (Murayama and Handa 2007). The present biochemical and functional genomic studies go beyond those findings and definitively prove that Suc hydrolysis can take place inside the chloroplasts by the action of an A/N-Inv encoded in the cell nucleus (Table 2; Fig. 1). The *A. thaliana At-A/N-InvE* was confirmed to be targeted into chloroplasts (Fig. 3c) and the gene product was functionally characterized after heterologous expression in *E. coli* cells (Fig. 3a,b). Our data contribute to set up a new picture for Suc metabolism where the occurrence of organelle-targeted A/N-Inv isoforms is a more general phenomenon in plants. The differential A/N-Inv subcellular localization suggests distinct physiological functions for the isoenzymes, giving more intricate levels to Suc metabolism regulation and to carbon trafficking control.

To gain knowledge on the physiological relevance of chloroplast A/N-Inv, we analyzed *Arabidopsis* mutant plants (SALK_138953) impaired in the expression of *At-A/N-InvE*. These plants were shown to exhibit lower total A/N-Inv and, particularly, neither A/N-Inv polypeptide nor activity were detected in their chloroplasts (Fig. 4), leading to the conclusion that *At-A/N-InvE* encodes the protein localized in these plastids (Table 2). The analysis of the impact on carbohydrate content of *At-A/N-InvE* knockout plants showed that At-A/N-InvE is not crucial for the accumulation of Suc in leaves; however, its activity seems to be linked to photosynthetic starch accumulation (Fig. 5).

A strong body of evidence indicates a close interplay between Suc and starch metabolism. Chloroplast starch content depends on a high regulation of the enzymes related

**Table 3** Phenotype characteristics of wild-type (Col 0) and SALK_138953 *Arabidopsis thaliana* plants

| | Col 0 | SALK |
|---|---|---|
| Germination (%) | 89 ± 5 | 92 ± 6 |
| Hypocotyl and cotyledon emergence (days)[a] | 5 ± 1 | 5 ± 1 |
| Leaf (number/plant)[b] | 12 ± 2 | 14 ± 3 |
| Inflorescence emergence (days)[c] | 25 ± 2 | 24 ± 3 |
| Silique production (number/plant)[d] | 150 ± 15 | 135 ± 20 |
| Seed production (mg/plant)[e] | 120 ± 10 | 125 ± 15 |

[a] Average time from date of sowing

[b] Number of leaves at the moment of inflorescence emergence. Average of 10 plants ± SD

[c] Time when the first flower bud was visible. Average of 10 plants ± SD

[d] Determined after completion of flowering. Average of 10 plants ± SD

[e] Reported as a dry mass after seed desiccation. Average of 10 plants ± SD

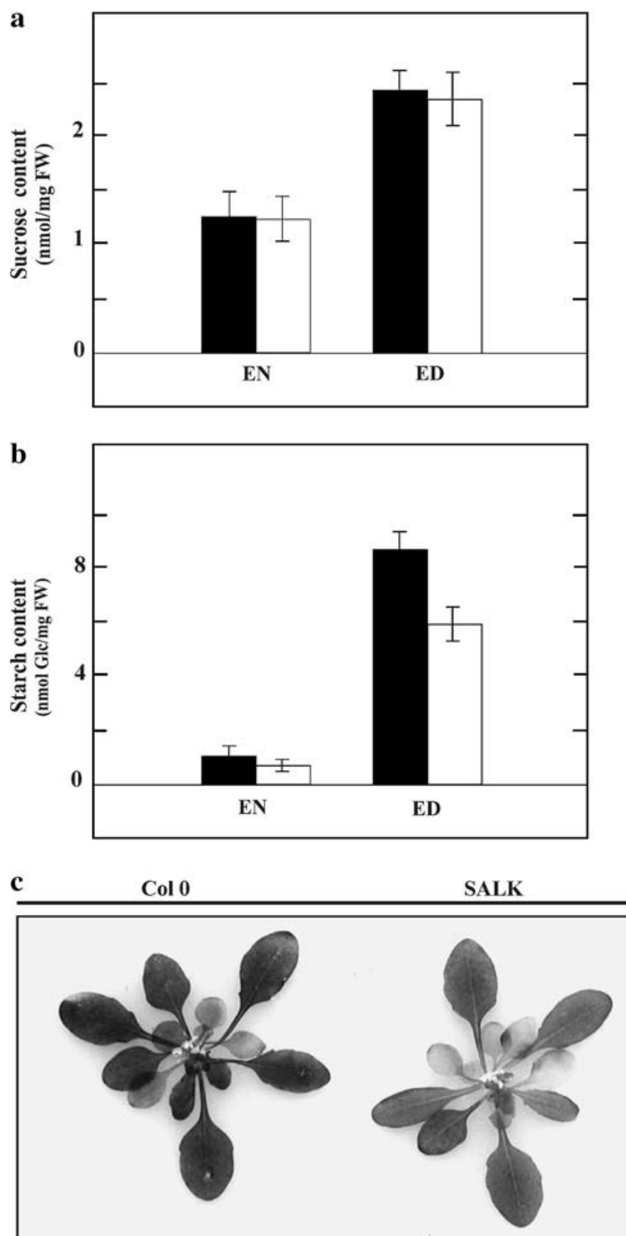


**Fig. 5** Effect of *At-A/N-InvE* knockout on Suc and starch accumulation in *Arabidopsis* plants. **a** and **b**, Suc and starch content, respectively. Mature leaves from wild-type *Arabidopsis* (Col 0) or SALK_138953 (*At-A/N-InvE* knockout mutant) plants were harvested at the end of the day (ED) and at the end of the night (EN). Values are the mean ± standard error of three independent experiments. The analysis of variance ($P < 0.03$) indicated a significant difference in starch content between Col 0 (*black boxes*) and SALK (*empty boxes*) plants harvested at the end of the day. **c** In situ determination of starch accumulation. Col 0 and SALK plants were harvested at the end of the day and starch was stained with the iodine reagent

to its biosynthesis and degradation (Martin and Smith 1995; Smith et al. 2005). Suc levels control not only starch accumulation but also can affect its degradation. Moreover, a direct correlation was found between the cell Suc content and the activation state of ADP-glucose pyrophosphorylase, a key enzyme for starch biosynthesis in amylosplasts and chloroplasts (Tiessen et al. 2002; Hendriks et al. 2003). On the other hand, starch degradation can be regulated by light and the carbohydrate status of the cell (Sharkey et al. 2004; Smith et al. 2005). At night, maltose export from chloroplasts is an essential step in starch-to-Suc conversion, and it was hypothesized that plants sense this conversion and alter starch/Suc ratios by tracking flux through the cytosolic hexokinase (Sharkey et al. 2004). Since in many cases a lag in starch degradation was observed until there was a drop in leaf Suc content, it was suggested that high sugar contents have a (still unknown) feedback effect on starch degradation other than Pi/triose-phosphate exchange (Paul and Foyer 2001). However, to date the molecular mechanisms and signalling pathways by which Suc mediates starch content regulation still remain unknown.

Suc hydrolysis by an A/N-Inv inside the chloroplasts is likely to be part of a novel mechanism controlling chloroplast-cytosolic carbon partitioning. It is well known that Suc is an important signal molecule in plant cells that controls different processes in the plant life (Paul and Foyer 2001; Sheen et al. 1999; Koch 2004). One of the most well-accepted sensing mechanisms involves Suc hydrolysis followed by glucose and fructose phosphorylation by hexokinase (Koch 2004). The occurrence of hexokinases associated with organelles (chloroplasts, mitochondria, and nucleus) was already related with sugar sensing and regulatory functions (Rolland et al. 2002; Cho et al. 2006). The presence of a functional hexokinase (Hxk2) in chloroplast stroma was already demonstrated in tobacco (Giese et al. 2005). We identified a sequence in the *Arabidopsis* genome

(At1g47840.1) as coding for a putative hexokinase (61% identical to Hxk2) containing a transit peptide to chloroplasts (not shown). This, together with our data presented above, points to Suc playing a sensing role also inside the chloroplasts. This assumption supports the hypothesis that chloroplastic Suc hydrolysis might be a source of glucose and fructose sensed through hexokinase, which may contribute to control carbon partitioning and Suc/starch ratio. However, it must be also considered that a dynamic Suc transport across the chloroplast membrane with a high efflux activity, repressed in response to an increased external Suc concentration (Edelman et al. 1971), would also favor a possible channeling of any cytosolic Suc excess toward the chloroplast. Thus, when Suc demands in the cytosol would be satisfied, any transient increase of the disaccharide content may be directed back into the chloroplast to be hydrolyzed by A/N-Inv and regulate starch accumulation.

In addition, a possible regulatory mechanism involving protein–protein interaction between chloroplastic A/N-Inv with other proteins should be taken into account. A physical interaction was recently reported between a cytosolic A/N-Inv and a phosphatidylinositol monophosphate kinase, and that interaction was described to negatively regulate the sugar-mediated root growth in *Arabidopsis* plants (Lou et al. 2007). Since a high sequence similarity was reported for A/N-Inv from different plant species (Vargas et al. 2003; Ji et al. 2005; Vargas et al. 2007), it is likely that the motifs involved in such interactions might be conserved in the different isoforms. Then, regulatory functions of chloroplastic A/N-Inv isoforms in starch/Suc ratio might not be associated only with catalysis but also with protein–protein interactions that control enzymatic activities involved in carbon trafficking.

Even though the results presented in this report are focused on the study of the presence of A/N-Inv in chloroplasts, the occurrence of these enzymes in other plastids also deserves to be discussed. It is well known that during ontogeny all plastids derive from embryonic proplastids (López-Juez and Pyke 2005; López-Juez 2007). Also, similar molecular and biochemical processes, such as protein recognition and transport across membranes, are common to different plastids (Kessler and Schnell 2006). Gerrits et al. (2001) showed that Suc is also taken up by amyloplasts and suggested a substantial flux and metabolism of Suc in these organelles. The expression of *At-A/N-InvE* in roots and flowers, and of *At-A/N-InvH* in flowers (Fig. 2) points to the involvement of their gene products in the hydrolysis of Suc in plastids other than chloroplasts, such as the amyloplasts.

Organelle A/N-Invs introduce a new challenge for plant biologists. The recent findings propose a more intricate intracellular carbon trafficking in plants, encouraging scientists to revise carbon partitioning regulation.

## References


Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, E Hom, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301:653–657

Altschul SF, Meadden T, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Poc Natl Acad Sci USA 92:9353–9357

Asatsuma S, Sawada C, Itoh K, Okito M, Kitajima A, Mitsui T (2005) Involvement of α-amylase I-1 in starch degradation in rice chloroplasts. Plant Cell Physiol 46:858–869

Ashwell G (1957) Colorimetric analysis of sugars. In: Colowik SP, Kaplan NO (eds) Methods enzymol, vol 3. Academic, New York, pp 73–105

Beers EP, Moreno TN, Callis J (1992) Subcellular localization of ubiquitin and ubiquitinated proteins in *Arabidopsis thaliana*. J Biol Chem 267:15432–15439

Bergmeyer HU, Bernt E, Schmidt F, Stork H (1974) D-Glucose: determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis, vol 3. Academic, New York, pp 1196–1201

Bernt E, Bergmeyer HU (1974) D-Fructose. In: Bergmeyer HU (ed) Methods of enzymatic analysis, vol 3. Academic, New York, pp 1304–1307

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Görlach J (2001) Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. Plant Cell 13:1499–1510

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 7:248–254

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

Chen M-H, Huang L-F, Li H-M, Chen Y-R, Yu S-M (2004) Signal peptide-dependent targeting of a rice α-amylase and cargo proteins to plastids and extracellular compartments of plant cells. Plant Physiol 135:1367–1377

Cho Y-H, Yoo S-D, Sheen J (2006) Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell 127:579–589

Cooper TG, Beevers H (1969) Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. J Biol Chem 244:3507–3513

Cormack BP, Valdivia RH, Falkow S (1996) FACS-optimized mutants of the green fluorscent protein (gfp). Gene 173:33–38

Dennis DT, Blakeley SD (2000) Carbohydrate metabolism. In: Buchanan BB, Gruissem W, Jones RL (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, pp 630–675

Edelman J, Schoolar AI, Bonnor HW (1971) Permeability of sugarcane chloroplasts to sucrose. J Exp Bot 22:534–545
Felsenstein J (1993) PHYLIP (Phylogeny inference package), version 3.5c. University of Washington, Seattle
Fukushima E, Arata Y, Endo T, Sonnewald U, Sato F (2001) Improved salt tolerance of transgenic tobacco expressing apoplastic yeast-derived invertase. Plant Cell Physiol 42:245–249
Gallagher JA, Pollock CJ (1998) Isolation and characterization of a cDNA clone from *Lolium temulentum* encoding for a Suc hydrolytic enzyme which shows alkaline/neutral invertase activity. J Exp Bot 49:789–795
Gerrits N, Turk SCHJ, van Dun KPM, Hulleman SHD, Visser RGF, Weisbeek PJ, Smeekens SCM (2001) Sucrose metabolism in plastids. Plant Physiol 125:926–934
Giese JO, Herbers K, Hoffmann M, Klösgen RB, Sonnewald U (2005) Isolation and functional characterization of a novel plastidic hexokinase from *Nicotiana tabacum*. FEBS Lett 579:827–831
Hauser M, Eichelman H, Oja V, Heber U, Laik A (1995) Stimulation by light of rapid pH regulation in the chloroplast stroma in vivo as indicated by $CO_2$ solubilization in leaves. Plant Physiol 108:1059–1066
Heineke D, Wildenberger K, Sonnewald U, Willmitzer L, Heldt HW (1994) Accumulation of hexoses in leaf vacuoles: studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. Planta 194:29–33
Heldt HW, Sauer F (1971) The inner membrane of the chloroplast envelope as site of specific metabolite transport. Biochim Biophys Acta 234:83–91
Hendriks JH, Kolbe A, Gibon Y, Stitt M, Geigenberger P (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of *Arabidopsis* and other plant species. Plant Physiol 133:838–849
Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil, Univ Calif Agric Exp Stn, Berkeley, CA, Circular No 347, pp 1–39
Huber SC, Huber JL (1996) Role and regulation of sucrose-phosphate synthase in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47:431–444
Ji X, Van den Ende W, Van Laere A, Cheng S, Bennett J (2005) Structure, evolution, and expression of the two invertase gene families of rice. J Mol Evol 60:615–634
Jones MGK, Outlaw WH, Lowry OH (1977) Enzymatic assay of $10^{-7}$ to $10^{-4}$ moles of Suc in plant tissue. Plant Physiol 60:379–383
Kessler F, Schnell DJ (2006) The function and diversity of plastid protein import pathways: a multilane GTPase highway into plastids. Traffic 7:248–257
Koch K (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opin Plant Biol 7:235–246
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
Lee DW, Lee S, Lee G-j, Lee KH, Kim S, Cheong GW, Hwang I (2006) Functional characterization of sequence motifs in the transit peptide of *Arabidopsis* small subunit of rubisco. Plant Physiol 140:466–483
Lee HS, Sturm A (1996) Purification and characterization of neutral and alkaline invertase from carrot. Plant Physiol 112:1513–1522
López-Juez E (2007) Plastid biogenesis, between light and shadows. J Exp Bot 58:11–26
López-Juez E, Pyke KA (2005) Plastids unleashed: their development and their integration in plant development. Int J Dev Biol 49:557–577
Lou Y, Gou J-Y, Xue H-W (2007) PIP5K9, an *Arabidopsis* phosphatidylinositol monophosphate kinase, interacts with a cytosolic invertase to negatively regulate sugar-mediated root growth. Plant Cell 19:163–181
Lunn J, Ashton A, Hatch M, Heldt H (2000) Purification, molecular cloning, and sequence analysis of Suc-6-phosphate phosphohydrolase from plants. Proc Natl Acad Sci USA 97:12914–12919
Mackinney G (1941) Absortion of light by chlorophyll solutions. J Biol Chem 140:315–322
Martin C, Smith AM (1995) Starch biosynthesis. Plant Cell 7:971–985
Mathur J, Koncz C (1998) Protoplasts isolation, culture and regeneration. In: Martínez-Zapater JM, Salinas J (eds) *Arabidopsis* protocols. Methods in molecular biology, vol 8. Humana Press, Totowa, NJ, pp 35–42
Mourioux G, Douce R (1981) Slow passive diffusion of orthophosphate between intact isolated chloroplasts and suspending medium. Plant Physiol 67:470–473
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15:473–497
Murayama H, Handa S (2007) Genes for alkaline/neutral invertase in rice: alkaline/neutral invertases are located in plant mitochondria and also in plastids. Planta 225:1193–1203
Paul MJ, Foyer CH (2001) Sink regulation of photosynthesis. J Exp Bot 52:1383–1400
Porchia AC, Fiol DF, Salerno GL (1999) Differential synthesis of sucrose and trehalose in *Euglena gracilis* cells during growth and salt stress. Plant Sci 149:43–49
Renart J, Sandoval IV (1984) Western blots. In: Jacoby WB (eds) Methods enzymol, vol 104, Academic, Orlando, pp 455–460
Rolland F, Moore B, Sheen J (2002) Sugar sensing and signaling in plants. Plant Cell 14(Suppl):S185–S205
Ross HA, McRae D, Davies HV (1996) Sucrolytic enzyme activities in cotyledons of the faba bean (developmental changes and purification of alkaline invertase). Plant Physiol 111:329–338
Rujan T, Martin W (2001) How many genes in *Arabidopsis* come from cyanobacteria? An estimate from 386 protein phylogenies. Trends Genet 17:113–120
Salerno GL, Pagnussat GC, Pontis HG (1998) Studies on Suc-phosphate synthase from rice leaves. Cell Mol Biol 44:404–416
Salerno G, Curatti L (2003) Origin of sucrose metabolism in higher plants: when, how and why? Trends Plant Sci 8:63–69
Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
Santarius KA, Milde H (1977) Sugar compartmentation in frost-hardy and partially deharded cabbage leaf cells. Planta 136:163–166
Scheibe R, Stitt M (1988) Comparison of NADP-malate dehydrogenase activation, QA reduction and O2 evolution in spinach leaves. Plant Physiol Biochem 26:473–481
Sharkey TD, Laporte M, Lu Y, Weise S, Weber APM (2004) Engineering plants for elevated $CO_2$: a relationship between starch degradation and sugar sensing. Plant Biol 6:280–288
Sheen J, Zhou L, Jang JC (1999) Sugars as signaling molecules. Curr Opin Plant Biol 2:410–718
Smith AM, Zeeman SC, Smith SM (2005) Starch degradation. Annu Rev Plant Biol 56:73–98
Sowokinos J (1981) Pyrophosphorylases in *Solanum tuberosum*. Plant Physiol 68:924–929
Stitt M, Heldt HW (1981) Physiological rates of starch breakdown in isolated intact spinach chloroplasts. Plant Physiol 68:755–761
Sturm A (1999) Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. Plant Physiol 121:1–7
Sturm A, Hess D, Lee HS, Lienhard S (1999) Neutral invertase is a novel type of Suc-cleaving enzyme. Physiol Plant 107:159–165
Subbaiah CC, Palaniappan A, Duncan K, Rhoads DM, Huber SC, Sachs MM (2006) Mitochondrial localization and putative signaling function of sucrose synthase in maize. J Biol Chem 281:15625–15635
Tetlow IJ, Morell MK, Emes MJ (2004) Recent developments in understanding the regulation of starch metabolism in higher plants. J Exp Bot 55:2131–2145

Thompson JD, Gibson TJ, Plewniak FM, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 15:4876–4882

Tiessen A, Hendriks JHM, Stitt M, Branscheid A, Gibon Y, Farré EM, Geigenberger P (2002) Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: a novel regulatory mechanism linking starch synthesis to the sucrose supply. Plant Cell 14:2191:2213

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

Vargas W, Cumino AC, Salerno GL (2003) Cyanobacterial alkaline/neutral invertases. Origin of Suc hydrolysis in the plant cytosol? Planta 216:951–960

Vargas WA, Pontis HG, Salerno GL (2007) 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. Planta 226:1535–1545

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

Waegemann K, Soll J (1996) Phosphorylation of the transit sequence of chloroplast precursor proteins. J Biol Chem 271:6545–6554

Walker DA (1974) Chloroplast and cell. The movement of certain key substances, etc. across the chloroplast envelope. In: Northcote DH (ed) MTP International review of science, biochemistry series one, vol 11. Butterworth, London, pp 1–49

Walker RP, Winters AL, Pollock CJ (1997) Purification and characterization of invertases from leaves of *Lolium temulentum*. New Phytol 135:259–266

Wang CT, Nobel PS (1971) Permeability of pea chloroplastst alcohols and aldoses as measured by reflection coefficients. Biochim Biophys Acta 241:200–212

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

Winter H, Robinson DG, Heldt HW (1994) Subcellular volumes and metabolite concentrations in spinach leaves. Planta 193:530–535

Wroblewski T, Tomczak A, Michelmore R (2005) Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. Plant Biotech J 3:259–273