Planta
DOI 10.1007/s00425-012-1794-8

ORIGINAL ARTICLE

A mitochondrial alkaline/neutral invertase isoform (A/N-InvC) functions in developmental energy-demanding processes in Arabidopsis

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Received: 13 August 2012 / Accepted: 21 October 2012

Abstract Recent findings demonstrate that alkaline/neutral invertases (A/N-Invs), enzymes that catalyze the breakdown of sucrose into glucose and fructose, are essential proteins in plant life. The fact that different isoforms are present in multiple locations makes them candidates for the coordination of metabolic processes. In the present study, we functionally characterized the encoding gene of a novel A/N-Inv (named A/N-InvC) from Arabidopsis, which localizes in mitochondria. *A/N-InvC* is expressed in roots, in aerial parts (shoots and leaves) and flowers. A detailed phenotypic analysis of knockout mutant plants (*invc*) reveals an impaired growth phenotype. Shoot growth was severely reduced, but root development was not affected as reported for *A/N-InvA* mutant (*inva*) plants. Remarkably, germination and flowering, two energy demanding processes, were the most affected stages. The effect of exogenous growth regulators led us to suggest that A/N-InvC may be modulating hormone balance in relation to the radicle emergence. We also show that oxygen consumption is reduced in *inva* and *invc* in comparison with wild-type plants, indicating that both organelle isoenzymes may play a fundamental role in mitochondrion functionality. Taken together, our results emphasize the involvement of mitochondrial A/N-Invs in developmental processes and uncover the possibility of playing different roles for the two isoforms located in the organelle.

Keywords Alkaline/neutral invertases · Mitochondrial isoforms · Respiration · Germination · Flowering

Abbreviations

ABA	Abscisic acid
A/N-Inv	Alkaline/neutral invertase
A/N-InvA or A/N-InvC	At-A/N-InvA or At-A/N-InvC: Arabidopsis alkaline/neutral invertase A, or C
GA	Gibberellic acid 3
Inv	Invertase
inva or *invc*	Arabidopsis mutant lacking alkaline/neutral invertase A or C
Suc	Sucrose
wt	Arabidopsis wild-type plants, ecotype Columbia 0

Introduction

Sucrose (Suc) occupies a crucial role in plant life not only as a source of carbon and energy but also as a signal transduction molecule (Smeekens et al. 2010). Photosynthetically derived Suc originates primarily in the cytosol through the concomitant action of Suc-phosphate synthase and Suc-phosphate phosphatase. In contrast, different cellular locations were shown for sucrose synthase (SuS), one of the enzymes involved in Suc degradation in vivo, such as in the cytosol, bound to the plasmatic membrane or

Electronic supplementary material The online version of this article (doi:10.1007/s00425-012-1794-8) contains supplementary material, which is available to authorized users.

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Published online: 08 November 2012

associated to mitochondria (Koch 2004; Subbaiah et al. 2006). Suc cleavage by SuS yields fructose and nucleotide sugars involved in other polysaccharide biosynthesis (i.e., starch, cellulose and callose) and respiration (Amor et al. 1995; Winter and Huber 2000). In addition, invertases (Invs), which catalyze the irreversible hydrolysis of Suc into hexoses, exist in several isoforms differentially located in the cell. Invs play an important role when there is a demand for carbon and energy. There are two classes of invertases, initially differentiated by their in vitro optimum pHs and, more recently, grouped into two different protein families. Acid invertases (fructofuranosidases of the glycoside hydrolase family 32) are glycoproteins located in the vacuole or in the cell wall, with a characteristic optimum pH between 4.5 and 5.0. Additionally, alkaline/neutral invertases (A/N-Invs) are non-glycosylated proteins grouped in the glycoside family 100, with optimum pHs in the range of 6.5–8.0 (Sturm 1999; Roitsch and González 2004; Lammens et al. 2009). A/N-Invs, barely studied in the past because of their low and unstable activity, were historically described as exclusively cytosolic proteins. Recent reports have contributed to a growing body of evidence that suggests that cytosolic A/N-Invs are involved in carbon distribution, cellular differentiation, tissue development, and responses to environmental stresses (Qi et al. 2007; Jia et al. 2008; Barratt et al. 2009; Welham et al. 2009; Yao et al. 2009). Also, surprising findings about the presence of functional A/N-Inv isoforms inside plant organelles revived interest in their investigation (Vargas and Salerno 2010). The first breakthrough was to show that not all A/N-Inv isoforms are located in the cytosol but some of them are targeted to subcellular compartments, as chloroplasts, mitochondria, and nuclei (Murayama and Handa 2007; Lou et al. 2007; Vargas et al. 2008; Szarka et al. 2008). The targeting of A/N-Invs to mitochondria is likely to be a general feature, not only because it was shown in rice (Murayama and Handa 2007) and in Jerusalem artichoke tubers (Szarka et al. 2008) but also because putative mitochondrial targeting signals have been found in A/N-Inv sequences from carrot, *Lolium temulentum*, and *Arabidopsis thaliana* (Vargas et al. 2008; Vargas and Salerno 2010). Szarka et al. (2008) proposed that in the mitochondria there is a functional connection between the organelle sugar transport and the matrix localized A/N-Inv activity. In this model, Suc is taken up by mitochondria from the cytosol, cleaved to glucose and fructose by an A/N-Inv in the matrix, and the monosaccharides are subsequently transported back to the cytosol.

A nine-member A/N-Inv gene family (*At-A/N-InvA* to -*InvI* genes) was described in the *A. thaliana* genome (Vargas et al. 2003; Ji et al. 2005). Reverse genetic experiments using Arabidopsis knockout mutants suggest that At-A/N-InvE, a chloroplast-located isoform, is involved in the control of carbon balance between the cytosol and plastids linked to starch accumulation. These results imply a novel scenario for carbon movement within the photosynthetic cell (Vargas et al. 2008). Also, some isoforms could be involved in signaling functions, as was demonstrated for At-A/N-InvG, a cytosol protein that also co-localizes in membranes and the nucleus with a phosphatidyl monophosphate 5 kinase (PIP5K9) (Lou et al. 2007). Recently, Xiang et al. (2011) showed that *At-A/N-InvA* knockout plants (*inva*) have a more severe growth phenotype than those of *At-A/N-InvG* null mutants. The absence of either of these A/N-Invs was associated with a higher expression of genes encoding antioxidative proteins, while transient overexpression of *At-A/N-InvA* and *At-A/N-InvG* in leaf mesophyll protoplasts down-regulates the oxidative stress-responsive ascorbate peroxidase 2 (APX2) promoter (Xiang et al. 2011). Thus, it was suggested that A/N-Invs might contribute to mitochondrial reactive oxygen species homeostasis.

In the present work, we investigate the role of Arabidopsis *At-A/N-InvC* (hereafter *A/N-InvC*) by showing its mitochondrial localization and analyzing the phenotype of knockout plants (*invc*). The occurrence of A/N-InvC with a different physiological role to that of A/N-InvA (the other mitochondrial isoform) points to an increasing complexity in the regulation of the intracellular communication that coordinates plant growth and developmental processes.

Materials and methods

Plant material and phenotype analyses

Three Arabidopsis lines were used in this study: Arabidopsis wild-type ecotype Columbia 0 (Col-0, wt), and mutants SALK_109830 (*inva*) and SALK_080181 (*invc*), which contain a T-DNA insertion in the locus At1g56560 and At3g06500, respectively (Alonso et al. 2003). Seeds, obtained from the Arabidopsis Biological Resource Centre, Ohio State University, were germinated on Murashige-Skoog (MS) medium with Gamborg's vitamins (Duchefa, Netherlands) agar medium plates in a cold room (4 °C) for three days to get a synchronized germination and then moved to a growth chamber (CONVIRON, model CMP4030) at 23 °C with 16-h daylight. After 7 days, the seedlings were transferred to pots containing a 1:1:1 mixture of perlite:vermiculite:sphagnum moss and watered with ATS (*A. thaliana* salts) solution (Estelle and Somerville 1987). The average light intensity on the top of the pots was ~ 150 µmol m^{-2} s^{-1}. Plants were watered by subirrigation as required, usually every 2–3 days, depending on growth stage. For phenotypic comparison between wt and mutant plants, growth conditions and procedures

were similar to those described by Boyes et al. (2001). Statistical analyses (ANOVA) were performed using the Instat tool.

Preparation of total protein extracts, enzyme assay, and immunoblotting analysis

The aerial parts of Arabidopsis 12-day-old seedlings (0.3 g) were ground with a 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 X-100, 20 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride for 10 min at 0 °C. Extracts were 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.

A/N-Inv activity was routinely determined at 30 °C in reaction mixtures containing 200 mM Hepes–NaOH (pH 6.5) and 200 mM Suc. The released reducing sugars were quantified using Somogyi-Nelson reagents (Vargas et al. 2007). Protein concentration was determined using the Bradford reagent (Bradford 1976).

For immunodetection analysis, polypeptides were separated by SDS-PAGE on 8 % polyacrylamide gels and stained with Coomassie blue or blotted onto a nitrocellulose membrane (Hybond-C®, Amersham) as described by Vargas et al. (2007). Membranes were immunorevealed with antibodies raised in rabbits against recombinant InvB (An-InvB) from *Nostoc* (*Anabaena*) sp. PCC 7120 (Vargas et al. 2003).

Nucleic acid isolation and RT-PCR assays

Total DNA from *A. thaliana* was isolated from young leaves (20–40 mg) using the hexadecyltrimethylammonium bromide (CTAB) method (Stewart and Via 1993). 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 (2 µg) treated with DNase (RQ1 RNase-Free DNase, Promega), using moloney murine leukemia virus-reverse transcriptase (MMLV-RT, Promega) and random primers. PCR reactions to amplify *At-A/N-InvC* and *At-A/N-InvA* (hereafter *A/N-InvC* and *A/N-InvA*) were performed using the primers InvC-fw (GAGAATTGATGTTCAAACAGGG), InvC-rv (ATGATAAAGCACTTAGGCGA), InvA-fw (TTTGGTGAATCAGCTATTGG), and InvA-rv (CTCTTGTAACGAGAAATCTCC). The amplification of the constitutively expressed SAND family gene (SAND, At2g28390) was used as an internal loading control, using the primer pair SAND-fw (TCTTCTATGTTGGGTCACAC) and SAND-rv (GCAAGAACACTTCATTCTCC). In semi-quantitative RT-PCR analysis, after reverse transcription from total RNA, PCR reactions were performed for 30–39 cycles of 95 °C (30 s), 60 °C (30 s) and 72 °C (30 s), and a single final step at 72 °C for 5 min. With these conditions, PCR amplification occurred in the linear range. Parallel reactions were performed to clone the amplification product corresponding to *A/N-InvC* cDNA into pGEM®-T Easy vector system (Promega), resulting in the plasmid pG*A/N-InvC*. The identity of the cloned DNA fragment was ascertained by sequencing both DNA strands.

Construction, expression, and assays of the recombinant His_6::A/N-InvC fusion protein

For the amplification of *A/N-InvC* cDNA, the primers InvCGFP-fw (CGAGCTCATGAACAGTAGAAGCTGTATCTG) and InvCGFP-rv (GGGGTACCAGATCTTGGAGGTGTGAAAC) were designed with an adapter for *Sac*I and *Kpn*I restriction enzyme, respectively. The plasmid pG*A/N-InvC* was *Sac*I/*Kpn*I digested, and the DNA fragment corresponding to the *A/N-InvC* was sub-cloned between the sites *Sac*I/*Kpn*I of the expression vector pRSET-A (Invitrogen). The construct was transferred to *E. coli* BL21(λDE3):pLysS cells (Novagen), and the expression of the recombinant protein was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG). After 4-h induction at 30 °C, cells were harvested and the fusion protein (His_6::A/N-InvC) was purified throughout Co^{2+} affinity chromatography (TALON® resin, Clontech), dialyzed, and concentrated for further characterization. Aliquots of cell extracts from IPTG-treated or non-induced cells, and purified His_6::A/N-InvC were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Enzyme activity of the recombinant protein was assayed with aliquots of the purified His_6::A/N-InvC as described above and at different pHs.

Expression experiments of A/N-InvC fused to the green fluorescent protein

The *A/N-InvC*::*gfp* fusion was constructed in the pCHF3 vector. The *A/N-InvC* sequence was fused upstream and in frame with the *gfpmut2* gene (Cormack et al. 1996) under the control of the cauliflower mosaic virus 35S promoter. In this construction the stop codon corresponding to *A/N-InvC* was substituted by the initial methionine codon of *gfp*. The DNA fragments used to generate the fusion were PCR amplified using the primer pairs InvCGFP-fw and InvCGFP-rv for *A/N-InvC*, and GFPF (TCTAGAATGAGTAAAGGAGAAGAACTT) and GFPR (GTCGACTTATTTGTATAGTTCATCCATGCC), to amplify *gfp*.

The plasmid containing the gene fusion (pCHF3::CaMV35S::*A/N-InvC*::*gfp*) was transformed into *A. tumefaciens* (GV3101 pMP90 strain) by electroporation. For plant transformation, the floral dip method was used (Clough and Bent 1998). Seeds obtained from the transformed plants were plated on MS with Gamborg's vitamins medium supplemented with 50 µg/ml kanamycin to select transgenic plants. Arabidopsis 12-day-old plants were incubated in medium containing 10 µM MitoTracker Red (Molecular Probes Division, Invitrogen) for 20 min, and then washed twice with PBS buffer (sodium-phosphate buffer pH 7.5). Images of transformed root cells were observed using a confocal laser scanning microscope (Nikon C1 Plus with Eclipse Ti Inverted Microscope).

Germination assays

All germination assays were performed on MS with Gamborg's vitamins medium (pH 5.8), solidified with 0.8 % agar. Before plating, seeds were surface-sterilized in 5 % (v/v) commercial bleach and 1 % SDS for 15–20 min and rinsed four to five times with sterile water. After a 4 days' stratification period at 4 °C in the dark, plates containing 50–100 seeds, with or without abscisic acid (ABA) or gibberellic acid 3 (GA), were incubated in a growth chamber at 23 °C with a 16 h/8 h light/dark cycle. Germination, defined as radicle emergence from the seed coat, was scored for 3 days. Data correspond to four independent experiments.

Oxygen consumption assay

Oxygen consumption assay was performed as previously described (Bartoli et al. 2006). Respiration of detached leaves was monitored using the LD2/3 leaf disc electrode (Hansatech, UK). Leaves were placed in the oxygen electrode chamber to finally measuring respiration.

Results

Expression of *A/N-InvC* in different Arabidopsis tissues

It was recently reported that *At-A/N-InvA* (hereafter *A/N-InvA*) and *At-A/N-InvG* are the most abundantly expressed genes of the mitochondrial and cytosolic subgroups, respectively (Barratt et al. 2009). Our search in the GENEVESTIGATOR database (https://www.genevestigator.com/gv/user/gvLogin.jsp) revealed a discrepancy: the encoding gene of At-A/N-InvC (hereafter A/N-InvC), an uncharacterized putative mitochondrial isoform, exhibits a higher expression level than that of *A/N-InvA,* which was recently reported to encode a mitochondrial isoform (Xiang et al. 2011) (Fig. 1, supplemental material). To investigate this controversy, we first studied the expression pattern of both *A/N-InvA* and *A/N-InvC* by semiquantitative RT-PCR experiments. *A/N-InvC* transcripts were detected in every Arabidopsis tissue assayed (Fig. 1). Indeed, *A/N-InvC* expression was higher than that of *A/N-InvA* in the aerial part of seedlings, roots and flowers, consistent with data presented in Supplemental Figure 1.

A/N-InvC encodes a functional A/N-Inv

Because many questions arise regarding the role of A/N-Invs and its integration into intermediary metabolism, we focused our study on the characterization of the *A/N-InvC* sequence that encodes a predicted 664-amino-acid protein (~74.7 kDa) containing, at the N-terminus, a putative mitochondrial transit peptide of 71 amino acids (MitoProt II-v1.101). For functional characterization, the *A/N-InvC* sequence was PCR-amplified, cloned, and expressed in *E. coli*. The purified His_6::A/N-InvC recombinant protein exhibits A/N-Inv activity, which is higher at pH between 6 and 6.5. In addition, a protein of expected size was immunodetected by anti-A/N-InvB polyclonal antibodies (Fig. 2).

A/N-InvC is a mitochondrial protein

To examine the subcellular localization of the A/N-InvC protein, we prepared a plasmid construct containing the entire encoding sequence of A/N-InvC fused in frame to the GFP (Green Fluorescent Protein) encoding gene driven by a 35S promoter of the CaMV (Cauliflower Mosaic Virus). This construct was used to transform Arabidopsis plants. Observations of transgenic root cells under confocal microscopy clearly show that the green fluorescence pattern due to GFP fused protein nicely co-localizes with the red pattern due to a mitochondrion-selective probe (Mito Tracker), as can be seen in the merge (yellow) picture (Fig. 3).

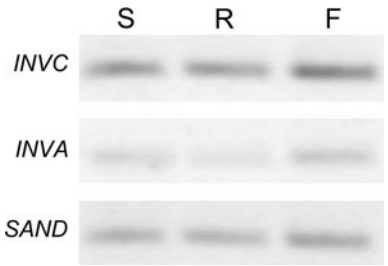

Fig. 1 Expression of *A/N-InvA* and *A/N-InvC* in different Arabidopsis tissues. Semiquantitative RT-PCR experiments were carried out using isoform-specific primers. Gene expression was assayed in the aerial part of seedlings (*S*), roots (*R*) and flowers (*F*). RT-PCR products were analyzed on 1 % agarose gels and visualized after ethidium bromide staining. Amplification of *SAND* family (*SAND*, At2g28390) was used as an internal loading control

invc null mutant plants show a delayed germination and flowering

To study the function of A/N-InvC, we analyzed Arabidopsis knockout mutant plants (*invc*) containing a T-DNA insertion in the first exon of the encoding gene (Fig. 2a, supplemental material). Genomic PCR genotyping was used to identify homozygous plants for the T-DNA insertion in *A/N-InvC*, using T-DNA-flanking primers (CLP, CRP and LBP), according to http://signal.salk.edu/tdnaprimers.2.html (Fig. 2b, supplemental material). The loss of *A/N-InvC* expression was verified by RT-PCR assays (Fig. 4a). For comparison, in every experiment, analysis of the *A/N-InvA* mutant (*inva*) was included. *A/N-InvC* expression was similar in *inva* and wt plants suggesting that both mitochondrial invertases are expressed independently. On the other hand, A/N-Inv activity level, determined in total protein extracts, was similar in *invc* and wt seedlings. Enzyme activity in *inva* was about 30 % lower than in wt plants (Fig. 4b), as previously reported (Xiang et al. 2011).

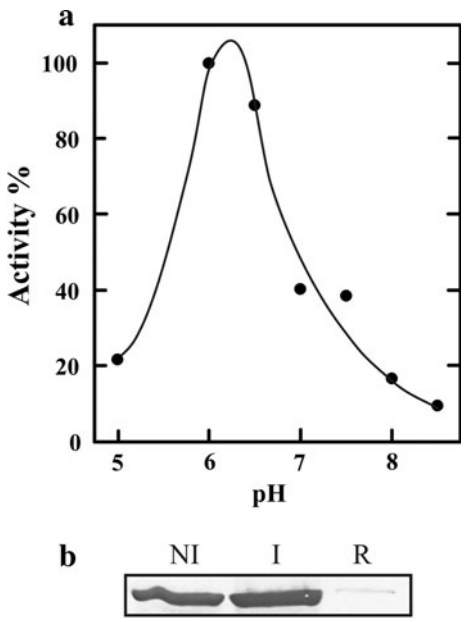

Fig. 2 Functional characterization of the protein product of *A/N-InvC*. **a** A/N-Inv activity determination of the purified His_6:: A/N-InvC as a function of the pH. **b** Immunoanalysis after SDS-PAGE. The polypeptides present in non-induced (*NI*) or IPTG-induced (*I*) *E. coli* cells, and the recombinant purified enzyme (*R*), were submitted to SDS-PAGE, blotted onto nitrocellulose membranes and probed with polyclonal antibodies against An-InvB (Vargas et al. 2003). The immunodetection of a polypeptide in lane NI may be ascribed to a leaky expression of the protein in *E. coli* cells

The growth of *invc* plants was analyzed by monitoring the time when the different organs emerged after transferring seeds to the growth chamber (Boyes et al. 2001). In Figs. 5 and 6, we summarized a description of phenotypes of *invc* in comparison with *inva* and wt plants, in both plate- and soil-based analysis. The *invc* plants were delayed in almost all developmental stages of growth. Germination and flowering were the most affected stages. To analyze in detail how the absence of A/N-InvC alters the germination, a kinetic experiment was conducted (Fig. 6a). The 50 % germination time (G50) for the *invc* mutant is over 44 h, an 8-h delay in comparison with the wt (Fig. 6a). In contrast, *inva* mutant and wt seeds germinate simultaneously.

In contrast to *inva*, *invc* seedlings have no alteration in root growth compared with wt. In both, *inva* and *invc* plants, a significantly reduced shoot growth could be observed compared with wt plants (Fig. 6b, j). However, leaf development is not altered in both mutants (Fig. 6c, h, i).

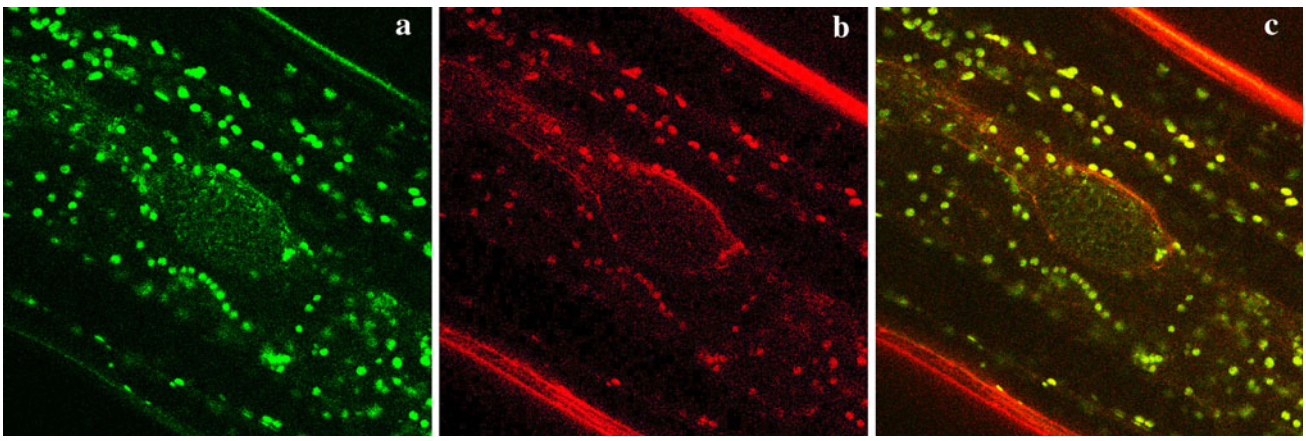

Fig. 3 Subcellular localization of A/N-InvC in Arabidopsis root cells. The translational fusion of the GFP encoding sequence and A/N-InvC was constructed downstream of the CaMV 35S promoter in the plasmid pCHF3 (pCHF3::CaMV35S::*A/N-InvC*::*gfp*). Arabidopsis plants were transformed by floral dip, with *A. tumefaciens* (GV3101 pMP90 strain) harboring the plasmid pCHF3::CaMV35S::*A/N-InvC*::*gfp*. From selected transformed seedlings, roots were visualized under laser scanning confocal microscope. **a** GFP fluorescence; **b** visualization of mitochondria with Mito-Tracker; **c** merged image, superimposing the images of GFP and the Mito-Tracker probe

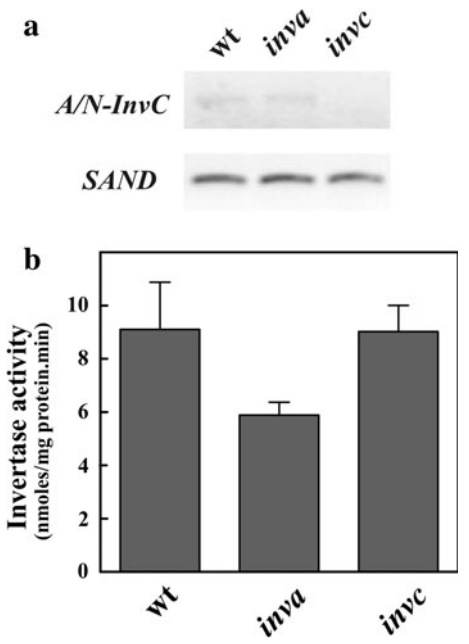

Fig. 4 *A/N-InvC* expression in *invc* and *inva* mutant plants. **a** Semi-quantitative RT-PCR was carried out with total RNA extracted from seedlings. Products were analyzed on 1 % agarose gels and visualized after ethidium bromide staining. Amplification of *SAND* family (*SAND*, At2g28390) was used as an internal loading control. **b** A/N-Inv activity in total extracts prepared from the aerial part of seedlings of *invc*, wild-type (wt), and *inva* plants. Values are the mean ± standard error of three independent experiments

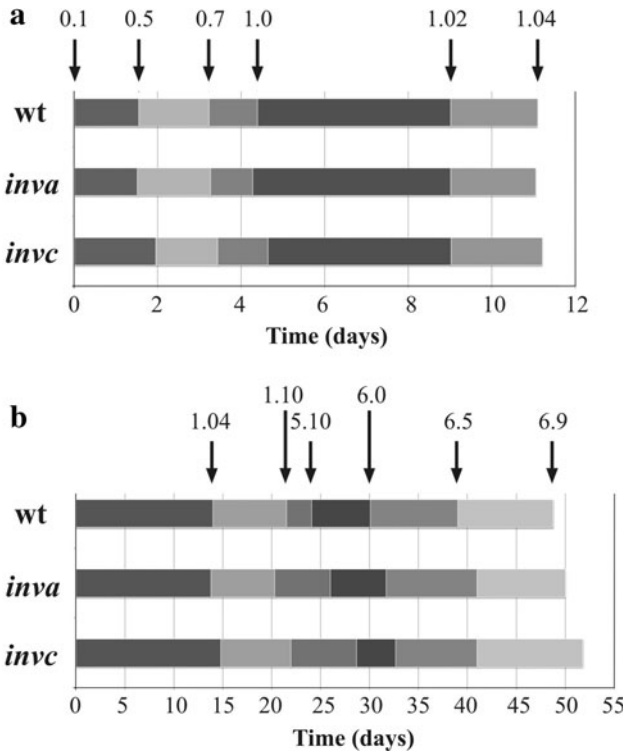

Fig. 5 Growth analysis of *invc* Arabidopsis mutant. Comparison of the length of different growth stages in *invc* with respect to *inva* and wild-type (wt) plants. Growth stage progression determined in the plate-based early analysis (**a**) or in the soil-based analysis (**b**). *Arrows* define the time (days after sowing) at which Col-0 plants (wt) reached the growth stages indicated with numbers, according to Boyes et al. (2001): 0.1, imbibition; 0.5, radicle emergence; 0.7, hypocotyl and cotyledons emerged from seed coat; 1.0, cotyledons opened fully; 1.02, two rosette leaves >1 mm in length; 1.04, four rosette leaves >1 mm in length; 1.10, ten rosette leaves >1 mm in length; 5.10, first flower buds visible; 6.0, first flower open; 6.5, midflowering; and 6.9, flowering complete. *Boxes* represent the time elapsed between the occurrences of successive growth stages. Junctions between *boxes* of different shading indicate the occurrence of a growth stage

In comparison with wt plants, *invc* mutant plants present a significant delay in stages 6.00, 6.50, and 6.90, which span the period of flower production (Fig. 5b). This is reflected in a delayed flowering time (Fig. 6e, f, g) that was assessed in three different ways: (1) when floral buds became visible in the center of the rosette (DTF1, Fig. 6e); (2) when the main shoot had elongated to 1 cm (DTF2, Fig. 6f); and (3) when the first flower opened (DTF3, Fig. 6g). Although *invc* plants flowered later than the wt ones (stages 6.00 and 5.10, respectively), the duration of this developmental stage was similar in both plants (19 d). *inva* plants showed a less pronounced flowering delay. In addition, the number of siliques produced by *invc* is similar to that of wt plants (Fig. 6k).

Since the delayed seed germination could be a consequence of a hormone alteration, we analyzed the effect of exogenous application of ABA and GA. Germination of *invc* seeds is less responsive to ABA treatment than that of the wt seeds (Fig. 7a). Interestingly, exogenous GA was able to rescue the delayed germination of *invc* seeds in a dose-dependent manner (Fig. 7b, and not shown).

To determine whether the lack of A/N-InvC is important for the organelle function, we measured mitochondrial respiration in *inva* and *invc* leaves in the dark. The lack of either A/N-InvC or A/N-InvA affects mitochondrial function, showing about 50 % reduction in oxygen consumption (Fig. 8).

Discussion

Recent findings have demonstrated the presence of A/N-Invs in organelles in addition to the cytosol, and their importance in plant development and stress tolerance (Barratt et al. 2009; Vargas and Salerno 2010; Vargas et al. 2012). This subcellular localization opened the possibility to uncover novel physiological functions for these proteins and highlighted the involvement of Suc in organelle metabolism.

The occurrence of mitochondrial A/N-Inv isoforms, first suggested by Ji et al. (2005), was experimentally demonstrated by different approaches in rice (OsNIN1),

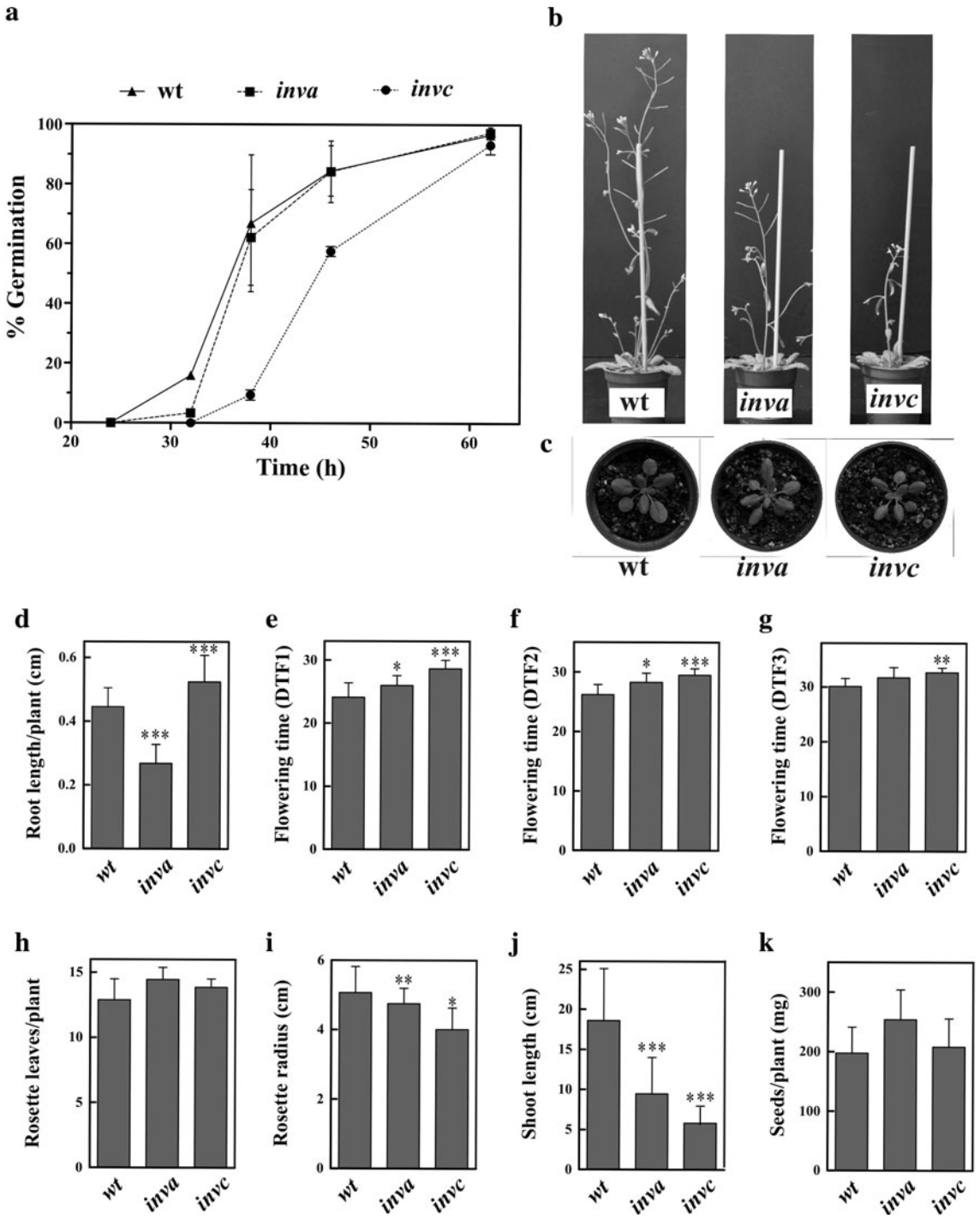

Fig. 6 Phenotype of *invc* mutant Arabidopsis plants in comparison with wt and *inva*. **a** Germination percentage. Seed germination was examined at indicated times and data were collected from four biological replicates. Average of 150 seeds ± SD. **b**, **c** Soil-grown phenotypes of wt, *inva* and *invc* knockout Arabidopsis plants. **d** Root length determined 4 d after sowing. Average of 40 seedling ± SD. **e** Flowering time as days to flower determined when floral buds became visible in the center of the rosette (DTF1). Average of 15 plants ± SD. **f** Flowering time as days to flower determined when the main shoot had elongated to 1 cm (DTF2). Average of 15 plants ± SD. **g** Flowering time as days to flower determined when the first flower opened (DTF3). Average of 15 plants ± SD. **h** Number of rosette leaves when the main shoot had elongated to 1 cm. Average of 15 plants ± SD. **i** Rosette radius determined 24 d after sowing. Average of 15 plants ± SD. **j** Shoot length determined 33 d after sowing. Average of 15 plants ± SD. **k** Amount of seed per plant reported as a dry mass after seed desiccation. Average of 15 plants ± SD. Significant statistically differences are indicated with *asterisk* ($P < 0.05$, $P < 0.01$, $P < 0.001$)

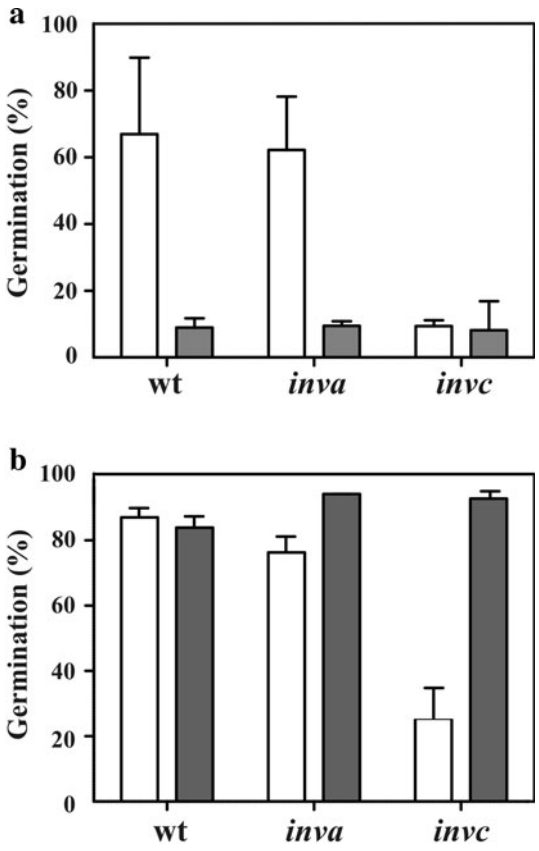

Fig. 7 Effect of ABA and GA on germination of Arabidopsis *inv* mutant seeds. Wild-type, *inva*, and *invc* seeds were germinated during 38 h on agar plates with the addition of 0.75 μM ABA (**a**) or 10 μM GA (**b**). Control, *white bars*; with hormone addition, *gray bars*

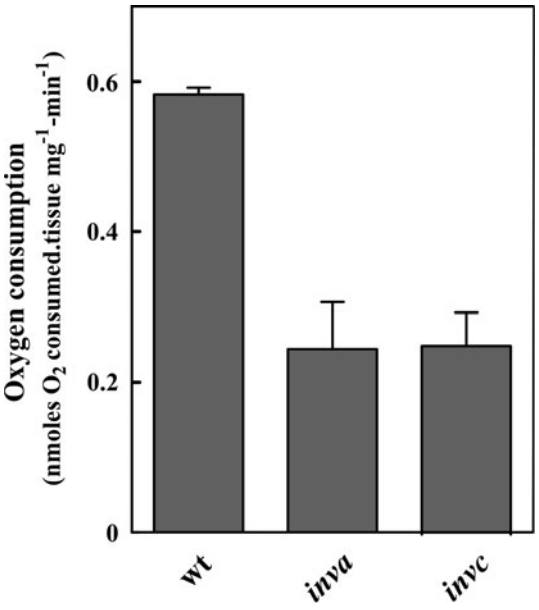

Fig. 8 Respiration measurement in *inva* and *invc* plants. Oxygen consumption was measured in the dark using detached leaves from wild-type (wt), *invc* and *inva* knockout Arabidopsis plants. Values represent mean and error bars represent SD ($n = 16$) from four independent experiments

Jerusalem artichokes, and recently, in Arabidopsis (A/N-InvA) (Murayama and Handa 2007; Szarka et al. 2008; Xiang et al. 2011). In the present study, the occurrence of an additional mitochondrial isoform (A/N-InvC) in Arabidopsis emphasizes the relevance of organellar A/N-Invs and uncovers a more intricate network of communication between mitochondria and other cell compartments to regulate plant growth and development.

The overall expression of *A/N-InvC* is higher than that of the *A/N-InvA* gene, in agreement with results obtained through the GENEVESTIGATOR browser, and contrasting with the report of Xiang et al. (2011). *A/N-InvC* is expressed in every tissue analyzed, similarly to *A/N-InvA* (Fig. 1). We do show that *A/N-InvC* also encodes an active A/N-Inv (Fig. 2) that localize in mitochondria (Fig. 3), which further reinforces the concept that Invs could participate in the organelle function. However, although knocking out *A/N-InvC* affects the growth phenotype of Arabidopsis plants (Figs. 5, 6), it seems not to affect total A/N-Inv activity (Fig. 4). This apparent contradiction may lead to several speculations. Some explanations, among others, may be that A/N-InvC activity makes a minor contribution to total activity, and/or it is not fully extracted when plant material from either the wt or mutant plants were homogenized, being total A/N-Inv activity level similar in both plant tissues, and/or other isoforms counteract the A/N-InvC absence.

The analysis of *invc* Arabidopsis mutant plants showed an impaired growth phenotype, with a severely reduced shoot growth. However, root development is not affected (Fig. 6), as occurs in *inva* plants (Xiang et al. 2011). Of even further importance, we have found that *invc* seeds showed a delayed germination and plants exhibited a late flowering phenotype that could be thought to be a consequence of a hormone alteration. It is well known that ABA is a positive regulator of dormancy, while gibberellins release dormancy and promote the completion of germination, counteracting the effects of ABA (Holdsworth et al. 2008). Thus, we assayed the effect of exogenous application of both hormones to evaluate their effect on the germination of mutant seeds, demonstrating that exogenous GA is able to rescue the delayed germination of *invc* seeds (Fig. 7). This leads us to suggest that the lack of A/N-InvC may cause the delay in radicle emergence by modulating hormone balance. We hypothesize that A/N-InvC itself or one of its activity products may repress ABA signaling and/or activate the GA signal transduction pathways.

The phenotype of *invc* emphasizes the involvement of mitochondrial A/N-Invs in development processes and points to different roles for A/N-InvC and A/N-InvA, both located in the organelle. On the other hand, only *invc* plants showed alteration in germination but both *A/N-Inv* knockout mutants displayed a late flowering. Remarkably, these

are highly energy-requiring processes. The determination of leaf respiration showed that there is a reduced oxygen consumption in both *inva* and *invc* plants (Fig. 8), indicating that they have dysfunctional mitochondria. Therefore, both mitochondrial isoforms (A/N-InvC and A/N–InvA) should play a fundamental role in the respiration process. These proteins could continuously and efficiently supply glucose to hexokinase, associated with the outer mitochondrial membrane (Graham et al. 2007), contributing to the steady-state recycling of ADP (Xiang et al. 2011), and supporting oxidative phosphorylation. This mechanism could be important to avoid a limitation in respiration due to low ATP synthesis, and thus, the subsequent H_2O_2 accumulation in the plant cells (Camacho-Pereira et al. 2009). Even more, Szarka et al. (2008) reported that the A/N-Inv activity, rather than the sugar uptake process, might control the respiration rate and ATP generation. They have demonstrated that sugars are transported independently of mitochondrial respiration and membrane potential, as neither KCN (an inhibitor of the mitochondrial respiratory chain) nor the uncoupler 2,4-dinitrophenol produces any effect on the uptake of sugars by mitochondria. The increased mitochondrial reactive oxygen species (ROS) levels observed in *inva* plants (Xiang et al. 2011) could play a role in signaling pathways by triggering nuclear gene expression responses, by a still poorly understood process called mitochondrial retrograde regulation. The involvement of ROS in this regulation and it association with the disruption of the mitochondrial function have already been reported (Rhoads and Subbaiah 2007; Rhoads 2011). However, ROS accumulation is likely to be due to a general response to mitochondrial dysfunction, which does not explain the differences between *inva* and *invc* phenotypes. Moreover, the presence of more than one A/N-Inv isoform in mitochondria suggests that a tight regulation should be operating in the intercommunication between the organelle, the cytosol, and the nucleus. Population heterogeneity of higher-plant mitochondria in structure and function was reported more than a decade ago (Dai et al. 1998). Mitochondria not only vary in morphology and behavior between different plant tissues but also between the same types of cells at different development stages (Lo et al. 2004). Also differential capacity of root and leaf mitochondria to achieve a given function, i.e., to reduce nitrite to NO, appears to be common among higher plants (Gupta et al. 2005). Thus, it could be thought that different A/N-Inv isoforms may be playing different roles in specific mitochondrial populations.

In summary, Suc, synthesized in the cytosol, and/or the hexose produced by its hydrolysis may enter into different metabolic pathways. Mitochondrial A/N-InvC and A/N-InvA (Xiang et al. 2011) might regulate Suc level in the organelle or at its membrane level, and sugars and ROS might be acting in mitochondrial retrograde regulation. Glucose, supplied by A/N-Inv isoforms to hexokinase, may either contribute to the recycling of ADP to support oxidative phosphorylation and thus, to control respiration and/or regulate the expression of nuclear genes that encode mitochondrial proteins through transcription factors, such as ABI4 (ABscisic acid Insensitive factor 4), shown to be involved in mitochondrial retrograde regulation (Giraud et al. 2009).

The occurrence of two A/N-Invs localized in mitochondria uncovers a new piece of an intricate puzzle, which offers exciting avenues for untangling the myriad of signals that couple key functions of the different plant cell compartments.

Acknowledgments We are very thankful to C. Fernández and M. Vidal for technical assistance. This research was funded by grants from CONICET (PIP 134 and PIP 102), Universidad Nacional de Mar del Plata (EXA 553/11), and FIBA.

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