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RESEARCH PAPER

Exploring the neutral invertase–oxidative stress defence connection in *Arabidopsis thaliana*

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Abstract

Over the past decades, considerable advances have been made in understanding the crucial role and the regulation of sucrose metabolism in plants. Among the various sucrose-catabolizing enzymes, alkaline/neutral invertases (A/N-Invs) have long remained poorly studied. However, recent findings have demonstrated the presence of A/N-Invs in various organelles in addition to the cytosol, and their importance for plant development and stress tolerance. A cytosolic (At-A/N-InvG, At1g35580) and a mitochondrial (At-A/N-InvA, At1g56560) member of the A/N-Invs have been analysed in more detail in *Arabidopsis* and it was found that *At-A/N-InvA* knockout plants show an even more severe growth phenotype than *At-A/N-InvG* knockout plants. The absence of either A/N-Inv was associated with higher oxidative stress defence gene expression, while transient overexpression of *At-A/N-InvA* and *At-A/N-InvG* in leaf mesophyll protoplasts down-regulated the oxidative stress-responsive ascorbate peroxidase 2 (*APX2*) promoter. Moreover, up-regulation of the *APX2* promoter by hydrogen peroxide or abscisic acid could be blocked by adding metabolizable sugars or ascorbate. A hypothetical model is proposed in which both mitochondrial and cytosolic A/N-Invs can generate glucose as a substrate for mitochondria-associated hexokinase, contributing to mitochondrial reactive oxygen species homeostasis.

Key words: *Arabidopsis thaliana*, ascorbate peroxidase, hexokinase, mitochondria, neutral invertase, oxidative stress.

Introduction

Sucrose (Suc) and its cleavage products glucose (Glc) and fructose (Fru) are central molecules for cellular biosynthesis and signal transduction throughout the plant life cycle (Smeekens *et al.*, 2010). In general, high Glc levels favour cell division and expansion, while high Suc levels are associated with differentiation and maturation (Weber *et al.*, 1995). Suc needs to be cleaved before it can be used as a carbon and energy source, and this is catalysed by two types of enzymes: sucrose synthases (Susys) reversibly hydrolyse Suc to UDPGlc and Fru, whereas invertases (Invs) catalyse an irreversible hydrolysis to Glc and Fru.

Invertases can be further classified into two major groups according to their pH optimum: the acid invertases (Ac-Invs) and neutral/alkaline invertases (A/N-Invs). Ac-Invs are glycosylated proteins occurring in the vacuole (Vac-Invs) or in the apoplast (Cw-Invs) and belong to glycoside hydrolase family 32 (GH32). A/N-Invs, on the other hand, are non-glycosylated and are classified in family GH100

Abbreviations: A/N-Inv, neutral/alkaline invertase; APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; cA/N-Inv, cytosolic neutral/alkaline invertase; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Fru, fructose; Glc, glucose; HXK, hexokinase; IM, inner membrane; IMS, intermembrane space; Inv, invertase; MeJa, methyl jasmonate; mtHXK, mitochondrial hexokinase; Mtl, mannitol; mtA/N-Inv, mitochondrial neutral/alkaline invertase; OM, outer membrane; ROS, reactive oxygen species; SE, standard error; SOD, superoxide dismutase; Suc, sucrose; Susy, sucrose synthase; wt, wild type.

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(Lammens *et al.*, 2009). A/N-Invs have been poorly studied at the native protein level, mainly because of apparent protein instability and their low expression levels in plant tissues. Only a few groups have succeeded in a complete purification and characterization of this type of enzyme (Chen and Black, 1992; Van den Ende and Van Laere, 1995; Lee and Sturm, 1996; Ross *et al.*, 1996; Walker *et al.*, 1997). A peculiar characteristic of A/N-Invs is the inhibition of their activity by TRIS, a commonly used buffer (Van den Ende and Van Laere, 1995). Possibly, TRIS mimicks a so far unidentified inhibitor acting *in planta*. A/N-Inv genes have been studied in *Arabidopsis thaliana* (Ji *et al.*, 2005), *Lolium temulentum* (Gallagher and Pollock, 1998), *Daucus carota* (Sturm *et al.*, 1999), *Beta vulgaris* (Gonzalez and Cejudo, 2007), *Oryza sativa* (Murayama and Handa, 2007), and *Anabaena cyanobacterium* (Vargas *et al.*, 2003).

For decades, A/N-Invs were believed to occur exclusively in the cytosol, although proper localization data to corroborate this assumption were not available. Phylogenetic and *in silico* analyses, however, suggested the existence of two A/N-Inv subfamilies with different subcellular localization: an α-group with a predicted mitochondrial or plastidic localization and a β-group with a predicted cytosolic localization (Ji *et al.*, 2005). Murayama and Handa (2007) were the first to demonstrate the presence of A/N-Invs experimentally in (or attached to) mitochondria and plastids of rice (*OsNIN1* and *OsNIN3*), while Vargas *et al.* (2007) provided additional evidence for a chloroplast-targeted A/N-Inv in *Arabidopsis* (*At-A/N-InvE*; At5g22510). Similarly, some Susy forms have been localized in mitochondria (Subbaiah *et al.*, 2006). AtSUS2 seems to be localized in (or attached to) plastids of the embryo (Núñez *et al.*, 2008). The unexpected presence of A/N-Invs and Susy forms in organelles and the observed interaction of an A/N-Inv (*At-A/N-InvG*) with a phosphatidyl monophosphate 5 kinase (*PIP5K9*; Lou *et al.*, 2007) suggest that some of these proteins are involved in signalling functions, stimulating further research in this area (Vargas and Salerno, 2010). Recently, it was convincingly demonstrated in *Arabidopsis* that cytosolic A/N-Invs (cA/N-Invs) are indispensable for normal plant growth and development (Barratt *et al.*, 2009; Welham *et al.*, 2009) as postulated in previous studies (Qi *et al.*, 2007; Jia *et al.*, 2008; Yao *et al.*, 2009). Clearly, most recent reports have focused on the cA/N-Invs of the β-group in *Arabidopsis* and rice, and on a chloroplastic A/N-Inv (of the α-group in *Arabidopsis*: Vargas *et al.*, 2007), while the mitochondrial A/N-Invs (mtA/N-Invs) have received little attention.

When plants are exposed to abiotic and biotic stresses, reactive oxygen species (ROS) homeostasis is disturbed, resulting in oxidative stress (Asada, 1999; Mittler, 2002; Neill *et al.*, 2002). Chloroplasts, peroxisomes, and mitochondria are the major sources of ROS production in plant cells (Møller, 2001; Mittler *et al.*, 2004; Blokhina and Fagerstedt, 2010). Superoxide dismutase (SOD) is the first line of defence against oxidative stress by catalysing the dismutation of superoxide ($O_2\cdot^-$) to molecular oxygen and hydrogen peroxide (H_2O_2) (Okamoto *et al.*, 2001). Three types of SOD isoenzymes have been reported in various plant species: Mn-SOD (e.g. MSD1), Cu/Zn-SOD (e.g. CSD1), and Fe-SOD (e.g. FSD1). Mn-SOD is located in mitochondria while Cu/Zn-SOD and Fe-SOD are chloroplastic (Alscher *et al.*, 2002). In addition, both catalases (CATs) and ascorbate peroxidases (APXs)—the latter using ascorbate (AsA) as a substrate—play crucial roles in H_2O_2 scavenging processes, in concert with the different enzymes of the so-called Halliwell–Asada pathway which represents one of the most important antioxidant systems of the cytosol (Noctor and Foyer, 1998), chloroplasts (Arora *et al.*, 2002), and mitochondria (Mittova *et al.*, 2004). Recently, a specific vacuolar antioxidant mechanism has been proposed to work in concert with the well-known cytosolic, chloroplastic, and mitochondrial antioxidant mechanisms in plants (Van den Ende and Valluru, 2009; Bolouri-Moghaddam *et al.*, 2010). Moreover, a scenario is emerging in which the catalytic activity of mitochondria-associated hexokinase (HXK) regulates ROS levels and perhaps also the signalling pathways leading to antioxidant defence responses (Camacho-Pereira *et al.*, 2009; Bolouri-Moghaddam *et al.*, 2010).

A mutant affected in the *At-A/N-InvG* gene (*CINV1*, At1g35580) is severely affected in root growth under osmotic stress (Qi *et al.*, 2007). Vargas *et al.* (2008) also reported extensive increases in A/N-Inv enzymatic activities and gene expression under osmotic and cold stress in wheat leaves. Since all biotic and abiotic stresses are believed to result in oxidative stress responses *in planta* (Mittler *et al.*, 2004), this led to the hypothesis that both organellar and cytoplasmic A/N-Invs could be directly or indirectly linked to oxidative stress defence responses in plants.

Gene expression analyses (derived from the *Arabidopsis* eFP browser http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi and Fig. S7 in Barratt *et al.*, 2009) indicated that *At-A/N-InvA* (At1g56560) and *At-A/N-InvG* (At1g35580) are the most abundantly expressed *A/N-Inv* genes of the α (mitochondrial) and β (cytosolic) subgroups, respectively. Here, the subcellular localization of the *At-A/N-InvA* and *At-A/N-InvG* gene products in *Arabidopsis* leaf protoplasts is reported. Analysis of T-DNA knockout plants and transient overexpression in protoplasts further suggested for the first time a direct connection between A/N-Inv activities and the expression levels of genes involved in oxidative stress defence. More particularly, the results suggest that both cytosolic and mitochondrial A/N-Invs might contribute to delivering Glc as a substrate for HXK, in this way contributing to ROS homeostasis.

Materials and methods

Amplification of target clones

Total RNA was extracted with Trizol Reagent from leaves of 30-day-old *Arabidopsis* plants. RT-PCR (reverse transcriptase PCR) was carried out according to the supplier's instructions (Access RT-PCR System, Promega, USA).

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia was used in all these experiments. *At-A/N-InvA* (Salk_109830, Salk_015233) and *At-A/N-InvG* (Salk_095807) knockout seeds were acquired from the

Nottingham Arabidopsis Stock Center. *Arabidopsis* seeds were surface-sterilized and sown in soil or on vertically placed plates containing half-strength Murashige and Skoog (MS) medium. Plants were grown under 12 h–12 h conditions (75 μmol s^{-1} m^{-2}), 21 °C, and 50–60% humidity. MS medium was supplemented with 0, 1, 5, and 10% (w/v) Suc or mannitol (Mtl), respectively.

Transient expression of GFP fusion proteins

Full-length (*At-A/N-InvA*::*GFP*) and N-terminal (amino acids 1–31; *At-A/N-InvAn*::*GFP*) versions of *At-A/N-InvA* were amplified from cDNA with the primer pairs At-A/N-InvAFw and At-A/N-InvARev and with At-A/N-InvAnFw and At-A/N-InvAnRev (Supplementary Table S1 available at *JXB* online), respectively. Both fragments were digested with the *Bgl*II and *Stu*I restriction endonucleases, and subsequently ligated in-frame with the green fluorescent protein (GFP) tag in the expression vector HBT35S::GFP::NOSter (Sheen, 1993). Similarly, full-length (*At-A/N-InvG*::*GFP*) and N-terminal (amino acids 1–31; *At-A/N-InvGn*::*GFP*) versions of At-A/N-InvG were cloned in the same GFP expression vector with *Bam*HI and *Stu*I restriction sites. GFP itself, containing no targeting signal, was used as control.

The constructs were subsequently transformed into *Arabidopsis* protoplasts as described elsewhere (Yoo *et al.*, 2007). After 12 h of incubation under low light, the *Arabidopsis* protoplasts were treated with 0.5 μM MitoTracker Orange (Molecular Probes Division, Invitrogen) for 10 min. GFP images of transformed protoplasts were captured by confocal microscopy (Olympus, Germany).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR; FastSYBR Green Master Mix Protocol; Applied Biosystems) was carried out to evaluate the expression level of genes involved in antioxidative defence in leaves of 30-day-old wild-type (wt) and knockout plants with/without oxidative (H_2O_2) stress. Total RNA was extracted as described above. The first cDNA strands were synthesized from 1 μg of total RNA using a Reverse Transcription System (Promega, USA), and *MSD1* (At3g10920), *FSD1* (At4g25100), *CSD1* (At1g08830), *CAT2* (At4g35090), *APX2* (At3g09640), *HXK1* (At4g29130), *At-A/N-InvA* (At1g56560), and *At-A/N-InvG* (At1g35580) expression was followed. The PCR program comprised an initial denaturation for 2 min at 95 °C and amplification by 45 cycles of 3 s at 95 °C and 30 s at 58 °C. The housekeeping genes *Actin2* (At3g18780) and *UBQ10* (At4g05320) were used as references for normalization. The primer sequences are provided in Supplementary Table S1 at *JXB* online. All qRT–PCR experiments were performed in biological triplicate reactions and the graph values are means with standard error (SE).

APX2 luciferase reporter assay

The primers APX2proFw and APX2proRev (Supplementary Table S1) were used to PCR amplify the promoter region of the *APX2* gene (–2000 bp relative to the translation start site) using genomic DNA of *A. thaliana* var. Columbia (isolated by a DNeasy Plant Mini Kit, Qiagen). The PCR product was digested with *Bam*HI and *Nco*I, and cloned into the luciferase reporter vector (pUC-Luc). *Arabidopsis* protoplasts were isolated and transfected by a modified polyethylene glycol method as described (Yoo *et al.*, 2007). Typically, 0.1 ml of protoplast suspension (10^6 protoplasts ml^{-1}) was co-transfected with 20 μg of DNA of three plasmids containing *At-A/N-InvA*/*At-A/N-InvG* or a control plasmid, *APX2*::*LUC* as reporter, and *UBQ10*::*GUS* as internal control. The transfected protoplasts were incubated for 4 h before collection under different conditions [30 mM Glc, 30 mM Suc, 30 mM Mtl, 30 mM AsA adjusted to pH 5.7 with KOH, 200 μM H_2O_2, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 200 μM H_2O_2 plus 30 mM Suc, and 10 μM abscisic acid (ABA)].

Heterologous expression of At-A/N-InvA and At-A/N-InvG in Escherichia coli

At-A/N-InvA and *At-A/N-InvG* were cloned into the pBAD TOPO TA vector. Transformation, heterologous expression, and protein extraction were carried out according to the manufacturer's instructions (pBAD TOPO TA Expression Kit, Invitrogen). The A/N-Inv activities for the pH optima (pH 5.0–11.0) and the TRIS inhibition experiments (pH 8.2) were assayed in 100 μl reaction mixtures containing 100 mM Suc and 50 mM potassium phosphate buffer, with protein concentrations adjusted to 10 μg ml^{-1} (Sedmak and Grossberg, 1977). The reaction mixtures were incubated at 30 °C for 15, 30, 45, and 60 min, and the reactions were stopped by keeping an aliquot for 10 min in a water bath at 90 °C. Similar reaction conditions were used to determine the K_ms of the recombinant At-A/N-InvA and At-A/N-InvG enzymes, at varying Suc concentrations (1–50 mM range). After centrifugation and appropriate dilution with 0.02% (w/v) Na-azide (to prevent microbial growth), 25 μl was injected onto a HPAEC-PAD column as described (Van den Ende and Van Laere, 1996). Only data from the linear range were used, ensuring that <10% of the original substrate was consumed. All experiments were repeated in duplicate or triplicate. The amount of Fru in the reaction mixtures was quantified by the external standard method and used for calculating the Inv activities and K_ms (Hanes plots and Michaelis–Menten kinetics) as described before (Ji *et al.*, 2007).

Total A/N-Inv activity measurements on Arabidopsis leaves

Plant material was blended in an equal volume of extraction buffer [50 mM TEA pH 8.5, 0.1% (w/v) Polyclar, 10 mM $NaHSO_3$, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM mercaptoethanol, and 0.02% (w/v) Na-azide]. After centrifugation (5 min, 13 000 rpm), $(NH_4)_2SO_4$ was added to 80% saturation. After 30 min incubation on ice, the sample was centrifuged (5 min, 13 000 rpm). The pellet was washed three times by adding 800 μl of 80% $(NH_4)_2SO_4$-saturated TEA buffer (50 mM, pH 8.5), followed by centrifugation. The pellet was dissolved in 50 μl of TEA buffer. Total A/N-Inv activity was determined in 100 μl reaction mixtures containing 100 mM Suc in 50 mM TEA buffer pH 8.5 also containing 0.02% (w/v) Na-azide. Reaction mixtures were incubated at 30 °C for 10, 20, and 30 min, and the reactions were stopped by keeping an aliquot for 10 min in a water bath at 90 °C. Further analysis was as described above.

Results

Phylogenetic relationships within the A/N-Inv gene family

Figure 1 shows an unrooted phylogenetic tree with nine putative A/N-Invs (At-A/N-InvA–I) from *Arabidopsis* together with other functionally characterized plant A/N-Invs. The α and β clusters are clearly separated. Within the α cluster, two further subgroups can be discerned (LtINV, OsNIN3, BvINV, and At-A/N-InvE on one hand; OsNIN1, DcINV, At-A/N-InvC, At-A/N-InvH, and At-A/N-InvA on the other hand). Members of the latter subgroup localize in mitochondria as demonstrated experimentally (At-A/N-InvA, this study; OsNIN1, Murayama and Handa, 2007) or as predicted by Target P (DcInv, At-A/N-InvH, and At-A/N-InvC). The other subgroup harbours OsNIN3 and At-A/N-InvE (At5g22510) which are localized in plastids (Murayama and Handa, 2007; Vargas *et al.*, 2008). BvINV is also predicted to be localized in plastids. However, LtINV is predicted to localize in mitochondria.

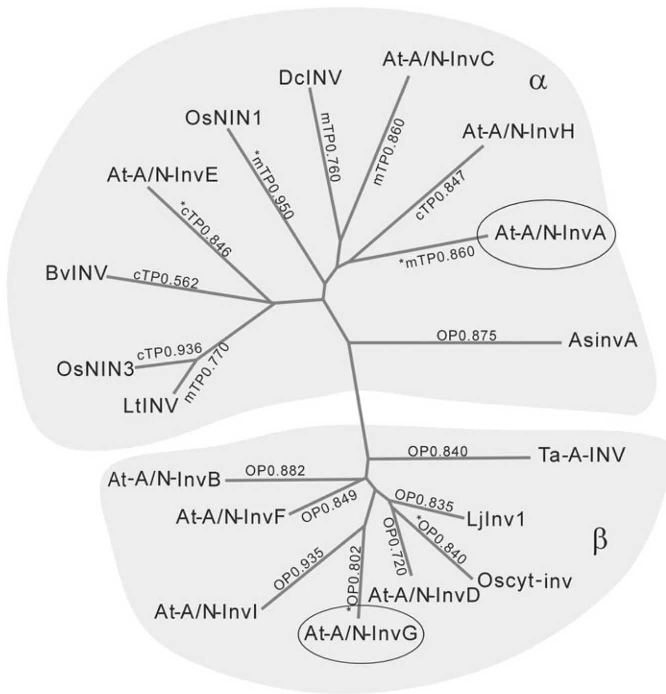

Fig. 1. Phylogenetic tree of A/N-Invs. An unrooted phylogenetic tree containing nine *Arabidopsis* A/N-Invs (At-A/N-Inv A–I) and other functionally studied A/N-Invs, drawn by Clustal W2. The α and β type of A/N-Invs can be discriminated. At-A/N-InvA and At-A/N-InvG, studied in this manuscript, are encircled. At-A/N-InvA, At1g56560; At-A/N-InvG, At1g35580 (Qi *et al.*, 2007; Lou *et al.*, 2007); At-A/N-InvF, At1g72000; At-A/N-InvH, At3g05820; At-A/N-InvC, At4g06500; At-A/N-InvI, At4g09510 (Barratt *et al.*, 2009); At-A/N-InvE, At5g22510 (Vargas *et al.*, 2008); At-A/N-InvD, At1g22650; At-A/N-InvB, At4g34860; AsinvA, *Anabaena* sp. PCC 7120 (Vargas *et al.*, 2003); BvINV, *Beta vulgaris* (González and Cejudo, 2007); DcINV, *Daucus carota* (Sturm *et al.*, 1999); LjInv1, *Lotus japonicus* (Flemetakis *et al.*, 2006); LtINV, *Lolium temulentum* (Gallagher and Pollock, 1998); OsNIN1 and OsNIN3, *Oryza sativa* (Murayama and Handa, 2007; Jia *et al.*, 2008); Ta-A/N-INV, *Triticum aestivum* (Vargas *et al.*, 2007). Referring to the (putative) subcellular localization of the enzymes, the confidence levels generated by target P are indicated. mTP, mitochondrial; cTP, chloroplastic; OP, other (presumably cytosolic); *, proven by localization studies. For a more extensive phylogenetic tree of (putative) At-A/N Invs, refer to the supplementary data of Vargas *et al.* (2008).

The cytosolic At-A/N-InvG is in the β cluster. All the members of this cluster are believed to be cytosolic proteins based on computational prediction. Besides At-A/N-InvF, At-A/N-InvD, At-A/N-InvB, Oscyt-Inv (Jia *et al.*, 2008), LjInv1 (Flemetakis *et al.*, 2006), Ta-A/N-Inv (Vargas *et al.*, 2007), and At-A/N-InvI (Barratt *et al.*, 2009) are well-characterized A/N-Invs.

At-A/N-InvA is located in mitochondria

To test the organellar localization of At-A/N-InvA and At-A/N-InvG in the α and β subgroups as predicted by Ji *et al.* (2005) and Murayama and Handa (2007), full-length and N-terminal versions of *At-A/N-InvA* and *At-A/N-InvG* in-frame with GFP were designed. Confocal microscopy was used to localize the resulting GFP fusion proteins (and a GFP only control) in *Arabidopsis* protoplasts, incubated for 12 h under dim light. In each case, 50 cells were investigated and >95% showed the subcellular localization as described. As expected based on the phylogenetic tree, the protoplasts expressing At-A/N-InvGn::GFP (Fig. 2c) and At-A/N-InvG::GFP (Fig. 2b), as well as the GFP control (Fig. 2a), showed fluorescence in the cytosol. In contrast, At-A/N-InvA::GFP (Fig. 2d) and At-A/N-InvAn::GFP (Fig. 2e) were mainly detected in small vesicle-like structures surrounding the chloroplasts. A clear overlap with the MitoTracker Orange marker demonstrated the mitochondrial location of At-A/N-InvA and suggests that the targeting signal for mitochondrial localization is in the N-terminal part of the protein. HXK1 (At4g29130) associated with the mitochondrial outer membrane (OM; Kim *et al.*, 2006) was used as an additional control for mitochondrial localization (Fig. 2f). The results show that At-A/N-InvA and HXK1 occur remarkably close to each other.

Enzymatic activities of recombinant At-A/N-InvA and At-A/N-InvG, and inhibition by TRIS

To confirm their catalytic activities, At-A/N-InvA and At-A/N-InvG were heterologously expressed in *E. coli.* Fully functional recombinant enzymes were obtained. The pH optima, the K_m, and the inhibitory effect of TRIS at pH 8.2 were determined. At-A/N-InvA shows a neutral pH optimum at about pH 7.5, and its activity is rather stable over a wide pH range (from pH 5.0 to 10.0: Fig. 3a). In contrast, the pH optimum of At-A/N-InvG is ~9.5 (Fig. 3a), indicating that it can be classified as an alkaline invertase. A typical inhibitory effect of TRIS on At-A/N-InvA activity was found (Fig. 3b), as observed for other A/N-Invs (Van den Ende and Van Laere, 1995, and references therein). In contrast, the At-A/N-InvG activity increased at the lower TRIS concentrations (0.5–4 mM range; Fig. 3b), 25–30 mM TRIS is needed for 50% inhibition, and the activity is strongly inhibited by 50 mM TRIS. The estimated K_m for At-A/N-InvG was 8.4 mM, compared with 17 mM for At-A/N-InvA (Fig. 3c). This is in the same range as the K_ms of other A/N-Invs (Vargas *et al.*, 2003).

Growth phenotype and total A/N-Inv activities of atinva *and* atinvg *knockout plants*

T-DNA insertion lines for *At-A/N-InvA* (Salk_109830 and Salk_015233, termed *atinva*) and *At-A/N-InvG* (Salk_095807, termed *atinvg*) (Fig. 4a) were grown on half-strength MS medium supplemented with 0, 1, and 5% (w/v) Suc or Mtl, and growth was compared with that of wt plants up to 15 d post-germination (Fig. 4b). The Mtl treatments resulted in more severe phenotypes compared with the Suc treatments. While Suc and Mtl both cause osmotic stress, Mtl is probably imported much more slowly and cannot be further

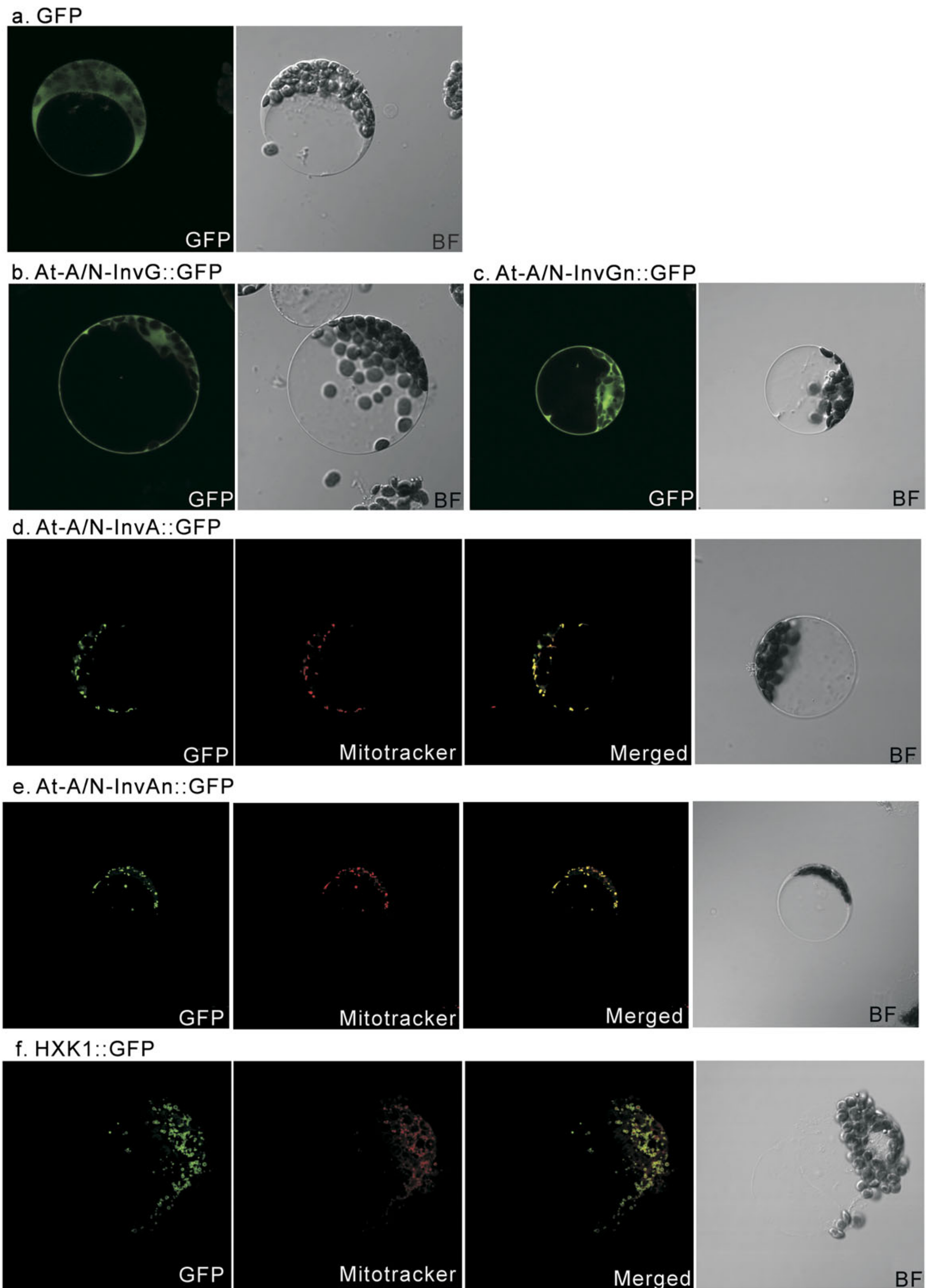

Fig. 2. Transient expression of fusion proteins in *Arabidopsis* protoplasts. (a) GFP fluorescence and bright field (BF) of GFP control protein; (b) GFP fluorescence and BF of At-A/N-InvG::GFP fusion protein; (c) GFP fluorescence and BF of At-A/N-InvGn::GFP fusion protein; (d) GFP, mitoTracker fluorescence, merge of GFP and Mitotracker, and BF of At-A/N-InvA::GFP fusion protein; (e) GFP, mitoTracker fluorescence, merge of GFP and mitoTracker, and BF of At-A/N-InvAn::GFP fusion protein; (f) GFP, mitoTracker fluorescence, merge of GFP and mitoTracker, and BF of HXK1::GFP fusion protein.

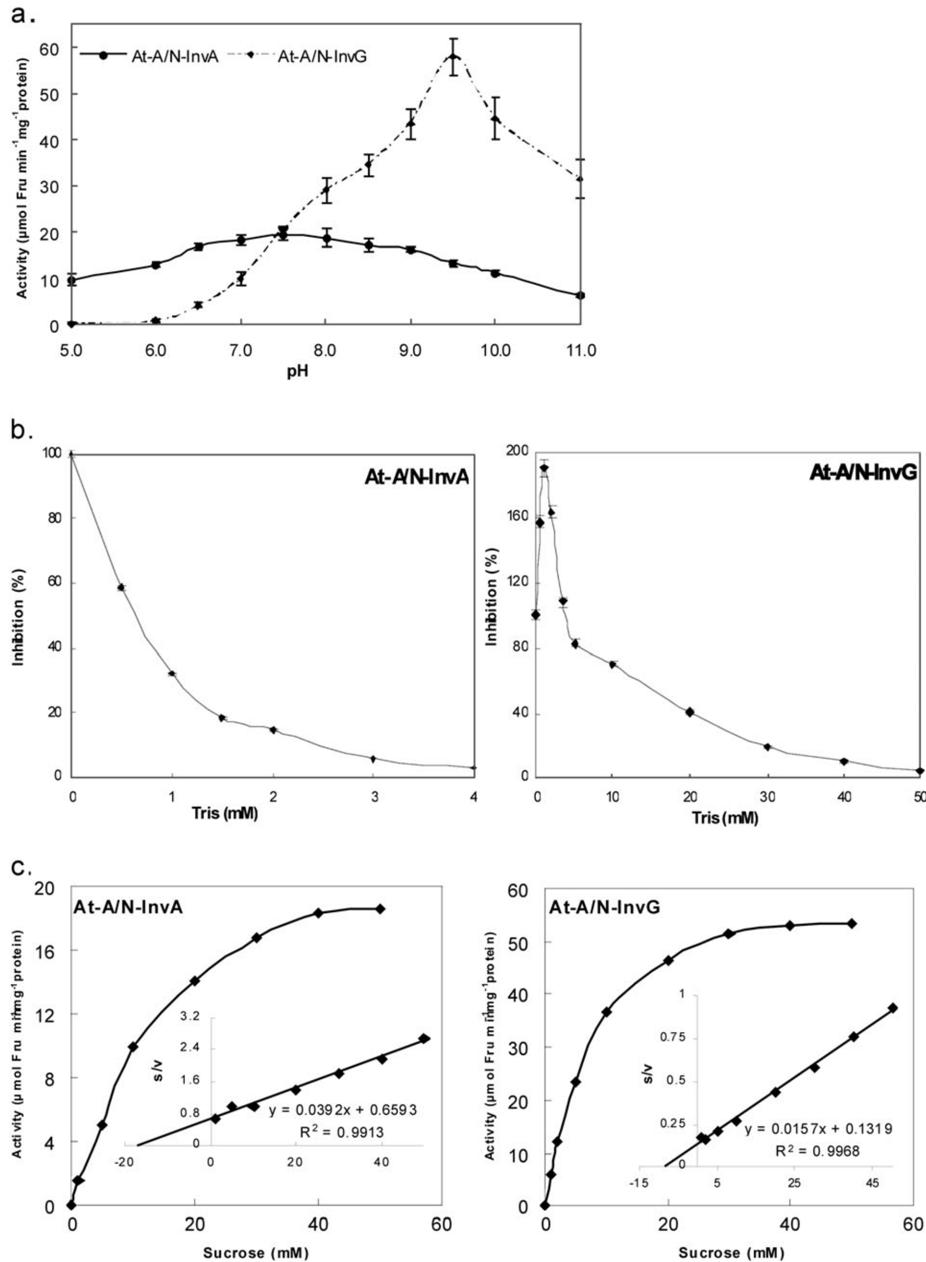

Fig. 3. Properties and kinetics of the recombinant At-A/N-InvA and At-A/N-InvG. (a) Invertase activity of At-A/N-InvA and At-A/N-InvG as a function of the pH (5.0–11.0). Reaction conditions: 100 mM Suc, 30 °C, 30 min. Vertical bars represent the SE for n=3. (b) Invertase activity of At-A/N-InvA and At-A/N-InvG (pH 8.2) as a function of TRIS concentration. Vertical bars represent the SE for n=3. (c) Substrate–velocity plots for Fru production by At-A/N-InvA (pH 7.5) and At-A/N-InvG (pH 9.5) at varying Suc concentrations (1–50 mM). Reaction time: 30 min. Reaction temperature: 30 °C. The corresponding linear Hanes plots are shown as inserts.

metabolized once taken up. Consistent with the results of Barratt *et al.* (2009), *atinvg* showed a severe root growth defect and a weaker leaf growth compared with the wt, at 0, 1, and 5% Suc and Mtl. Surprisingly, *atinva* knockout plants showed an even more severe growth phenotype compared with *atinvg*, affected in both leaf growth and root development (Fig. 4b). However, the main root length of *atinva* knockouts was relatively less affected at 1% Suc, suggesting that mitochondrial At-A/N-InvA activity is more important under conditions of both sugar starvation (0% Suc) and excess sugar or osmotic stress (5% Suc). Root lengths of wt, *atinva*, and *atinvg* plants (MS agar plate, 1.5% Suc, 15 d post-germination) were recorded. Fifty plants of each type were selected. The mean root length of *atinva* knockouts is remarkably shorter (44% of the wt), while *atinvg* knockout roots are less affected (Fig. 4c). Soil-grown *atinva* plants also showed a significantly reduced leaf and shoot development compared with *atinvg* and wt plants (Fig. 4d).

A/N-Invs were extracted from freshly harvested leaf material of *atinva*, *atinvg*, and wt plants. Total A/N-Inv activities

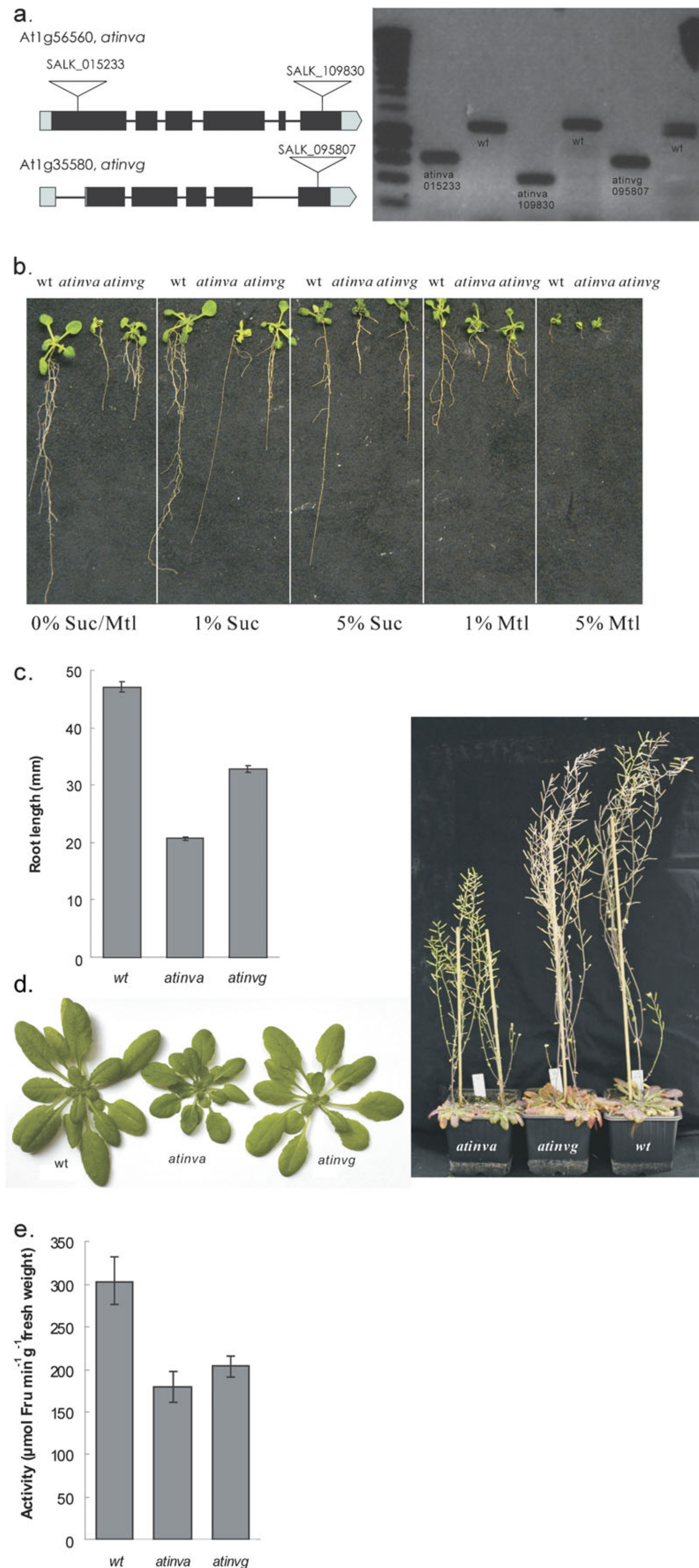

Fig. 4. Phenotypes of wt, *atinva*, and *atinvg* knockout plants. (a) Gene and PCR data (primer pairs: LBb1.3, LP, and RP, according to http://signal.salk.edu/tdnaprimers.2.html) of wt, *atinva* (Salk_109830, Salk_015233), and *atinvg* (Salk_095807). (b) Seedling phenotypes (15 d after germination) of wt, *atinva*, and *atinvg* at 0, 1, and 5% Suc and Mtl, respectively. (c) Root length of wt, *atinva*, and *atinvg* plants (15 d after germination). Vertical bars represent the SE for n=3. (d) Soil-grown phenotypes of wt, *atinva*, and *atinvg* plants (5 and 14 weeks, respectively). (e) Relative total A/N-Inv activities of *atinva* and *atinvg* compared with the wt leaves (5 weeks). Vertical bars represent the SE for n=3.

were measured at pH 8.5 (to minimize side activities of acid invertases). By comparison with wt plants, the total A/N-Inv activities of *atinva* and *atinvg* were ~40% and 35% lower (Fig. 4e). Knocking out only one out of nine A/N-Invs considerably affects both the growth phenotype and the total A/N-Inv activity, suggesting that both At-A/N-InvA and At-A/N-InvG make up a considerable part of the total activity. Alternatively, the At-A/N-InvA and At-A/N-InvG proteins themselves (independently of their catalytic activities) might play a role in controlling the total A/N-Inv activity and overall plant development, as suggested before by Lou *et al.* (2007) for At-A/N-InvG.

Expression of genes involved in oxidative stress defence

A clear connection between mitochondria-associated HXK (mtHXK) activity and ROS homeostasis was recently demonstrated (Camacho-Pereira *et al.*, 2009; Bolouri-Moghaddam *et al.*, 2010). It was hypothesized that A/N-Invs might assist in delivering Glc to mtHXK. Therefore, it was decided to investigate the expression levels of *At-A/N-InvA*, *At-A/N-InvG*, *HXK1*, and the oxidative stress-responsive *MSD1*, *FSD1*, *CSD1*, *CAT2*, and *APX2* genes in response to H_2O_2 by qRT-PCR. For this purpose, detached wt, *atinva*, and *atinvg* mature leaves were incubated in water or 20 mM H_2O_2 for 10 h.

Except for *FSD1*, all transcript levels significantly increased by the application of exogenous H_2O_2 in wt leaves (Table 1). Addition of H_2O_2 to the mutants generally resulted in even higher transcript levels, but untreated mutant leaves already showed enhanced basal levels of oxidative stress-associated gene expression. In line with previous observations (Fryer *et al.*, 2003; Costa *et al.*, 2010), *APX2* seems to be the most sensitive to H_2O_2 treatment. Therefore, this gene was investigated further, and an *APX2* promoter–luciferase (LUC) construct was created as an oxidative stress reporter for cellular assays (see Discussion).

The effect of transient *At-A/N-InvA* and *At-A/N-InvG* overexpression was analysed in *Arabidopsis* mesophyll protoplasts incubated for 4 h in the light or in darkness (Table 2) with addition of Glc (30 mM), Suc (30 mM), Mtl (30 mM), H_2O_2 (200 μM), ABA (10 μM), AsA (30 mM), DCMU (10 μM), and a combination of H_2O_2 and Suc (HS). Both H_2O_2 and ABA activated the *APX2* promoter in transfected wt protoplasts (Table 2). In contrast, the addition of the metabolizable sugars Glc and Suc as well as AsA down-regulated *APX2* promoter activity, while Mtl had no effect. Interestingly, the addition of Suc together with H_2O_2 resulted in a less pronounced activation of the *APX2* promoter compared with H_2O_2 alone. Furthermore, overexpressing *At-A/N-InvA* and *At-A/N-InvG* down-regulated the *APX2* promoter, in both the control and treated conditions. DCMU as an inhibitor of photosynthesis worked as an inhibitor of *APX2* promoter activity in the light, and as an activator in the dark (Table 2).

Discussion

Recently, cytosolic A/N-Invs have been recognized as important regulators of plant growth and development,

Table 1. qRT-PCR data of antioxidative gene expression for wt and knockout plants, with (+H_2O_2) or without H_2O_2 treatment

Actin2 (At3g18780) and *UBQ10* (At4g05320) were used as reference genes. The values in the table are fold changes in transcript levels normalized to the two reference genes with respect to the wt control. Means ±SE (*n*=3).

	wt	*atinva*	*atinvg*	wt+H_2O_2	*atinva*+ H_2O_2	*atinvg*+H_2O_2
APX2	1.00±0.03	3.78± 0.43	4.67±0.29	16.77±2.48	13.95±1.18	9.25±1.20
CAT2	1.00±0.02	1.55±0.12	1.50±0.09	4.73±1.05	2.78±0.25	2.25±0.18
CSD1	1.00± 0.03	1.80±0.12	1.18±0.14	2.86±0.42	2.26±0.30	2.45±0.42
FSD1	1.00±0.01	1.62±0.08	2.66±0.39	1.42±0.58	2.06±0.07	1.12±0.15
MSD1	1.00±0.04	1.31±0.15	1.63±0.10	2.30±0.24	1.71±0.21	1.65±0.21
HXK1	1.00±0.02	1.29±0.12	1.42±0.18	2.46±0.33	2.12±0.16	2.45±0.17
At-A/N-InvA	1.00±0.03	0.15±0.03	1.04±0.19	2.05 ±0.53	0.18±0.02	1.14±0.02
At-A/N-InvG	1.00±0.03	1.13±0.09	0.10±0.01	2.91±0.32	1.41±0.10	0.14±0.01

Table 2. *APX2* promoter luciferase assay of protoplasts derived from wt plants with or without overexpression of *At-A/N-InvA* or *At-A/N-InvG* under different treatments (30 mM Glc, 30 mM Suc, 30 mM AsA (adjusted to pH 5.7 with KOH), 30 mM Mtl, 200 μM H_2O_2, 10 μM DCMU, 200 μM H_2O_2+30 mM Suc (+HS), and 10 μM ABA)

The experiment was executed with (L) or without (D) light. The values are fold changes normalized to the wt control. Means ±SE (*n*=3).

	Control	+Glc	+H_2O_2	+Suc	+AsA	+ABA	+Mtl	+DCMU	+HS
wt L	1.00±0.04	0.71±0.11	3.28±1.27	0.64±0.09	0.74±0.05	1.70±0.13	1.13±0.27	0.42±0.04	1.52±0.04
At-A/N-InvA L	0.41±0.06	0.34±0.06	0.96±0.39	0.38±0.08	0.31±0.04	1.04±0.19	0.36±0.07	0.29±0.04	0.54±0.07
At-A/N-InvG L	0.35±0.04	0.21±0.03	0.78±0.21	0.26±0.03	0.27±0.01	0.97±0.11	0.28±0.04	0.23±0.03	0.30±0.06
wt D	1.00±0.04	0.60±0.17	2.45±0.47	0.82±0.08	0.77±0.02	3.48±0.91	1.17±0.14	1.88±0.54	2.12±0.46
At-A/N-InvA D	0.76±0.18	0.53±0.12	1.52±0.30	0.62±0.05	0.65±0.03	2.76±0.65	0.74±0.10	1.23±0.63	1.18±0.12
At-A/N-InvG D	0.54±0.10	0.33±0.14	1.01±0.08	0.36±0.07	0.46±0.12	1.50±0.45	0.50±0.11	0.85±0.37	0.56±0.18

possibly involved in metabolic signalling processes, especially under stress conditions (Barratt *et al.*, 2009; Vargas and Salerno, 2010). However, to date no research has been dedicated to studying the links between A/N-Invs and oxidative stress defence. In the model plant *A. thaliana*, At-A/N-InvA and At-A/N-InvG are expressed proteins belonging to α and β subgroups (Fig. 1) and with a different localization (Fig. 2). The optimal pH and TRIS inhibition differed strongly between the two recombinant enzymes, but the physiological implications of these differences, if any, remain unclear. Depending on conditions, both At-A/N-InvA and At-A/N-InvG knockouts showed smaller roots (Fig. 4b, c), which are typical phenotypes for plants suffering from oxidative stress, limited nitrate availability or signalling (Foreman *et al.*, 2003; Pnueli *et al.*, 2003; Rizhsky *et al.*, 2003; Qi *et al.*, 2007). In *atinva* seedlings grown on 1% Suc, typically resulting in optimal growth, the phenotype was less prominent compared with 0% and 5% Suc, respectively (Fig. 4b). These findings are in line with previous observations suggesting that soluble sugars can help protect plants prior to or under oxidative stress (Sulmon *et al.*, 2006; Nishizawa *et al.*, 2008; Ramel *et al.*, 2009; Bolouri-Moghaddam *et al.*, 2010). When plants are stressed, the steady-state level of ROS usually increases, but ROS (specifically H_2O_2) can also act as a signal for turning on stress-related genes (Mittler *et al.*, 2004). Exogenous application of H_2O_2 is widely used to induce oxidative stress-related gene expression. Table 1 shows the induction of several of these marker genes. Intriguingly, the H_2O_2 treatment also induced *At-A/N-InvA* and *At-A/N-InvG* gene expression (Table 1). In particular, the *APX2* promoter responds significantly and can be used as an oxidative stress reporter. Indeed, it is widely accepted that the *APX2* promoter acts as an integrator of various stress-related stimuli including high light, the redox status of the plastoquinone pool associated with photosynthesis, cellular H_2O_2 levels, as well as responses to ABA and osmotic stress (Karpinski *et al.*, 1999; Shigeoka *et al.*, 2002; Fryer *et al.*, 2003; Ball *et al.*, 2004; Chang *et al.*, 2004; Rossel *et al.*, 2004, 2006; Bechtold *et al.*, 2008; Barba-Espin *et al.*, 2010). Besides the fact that control of *APX2* expression and leaf water status is mediated by ABA, a certain concentration of APX2 seems necessary for ABA sensing and response (Fryer *et al.*, 2003).

Consistent with the results under light (Table 2), DCMU, an inhibitor of photosystem II, abolishes the production of chloroplastic H_2O_2 and subsequently the induction of APX2 in *Arabidopsis* leaves (Karpinski *et al.*, 1999; Chang *et al.*, 2004). These observations confirm the idea that *APX2* promoter activity, at least to some extent, can be used as a rough indicator of endogenous H_2O_2 levels (Fryer *et al.*, 2003). In the dark, chloroplastic ROS production is hampered and cytosolic H_2O_2 levels are thus more likely to be determined by the level of mitochondrial ROS production. Nonetheless, an up-regulation of APX2 expression upon addition of H_2O_2 and ABA, and down-regulation by metabolizable sugars is still observed in the dark albeit to a lesser extent (Table 2), suggesting that the decrease in *APX2* promoter activity reflects the decreasing mitochondrial ROS production in the At-A/N-Inv-overexpressing cells.

The importance of A/N-Inv activity may reside in regulating the Suc concentration in the cytosol and/or in organelles, or in the production of Glc which could be sensed by mitochondria-associated HXKs (Rolland *et al*,. 2006; Li *et al.*, 2007; Bolouri-Moghaddam *et al.*, 2010) or serve as a substrate for HXK to control mitochondrial ROS production (Camacho-Pereira *et al.*, 2009). The increased expression of antioxidant defence-related genes in *atinva* and *atinvg* knockout plants, the reported increased expression of A/N-Invs and HXK under stress conditions (Vargas *et al.*, 2008; Table 1), and the decrease in *APX2* promoter activity in protoplasts overexpressing At-A/N-InvA and At-A/N-InvG strongly suggest that A/N-Invs are part of the antioxidant system involved in cellular ROS homeostasis. Lou *et al.* (2007) demonstrated that At-A/N-InvG (AtCYTINV1 in their terminology) interacts with an *Arabidopsis* phosphatidylinositol monophosphate kinase (PIP5K9), and that it can localize in the nucleus, in contrast to the exclusive cytosolic localization that was observed here in *Arabidopsis* protoplasts. Similarly, it was suggested that AtHXK1 may be involved in a nuclear protein complex that directly modulates specific target genes in a Glc-dependent manner (Cho *et al.*, 2009). Therefore, one hypothesis could be that the presence of At-A/N-InvG in the nucleus may be associated with the production of Suc-derived Glc for the AtHXK1-mediated control of gene expression (Vargas and Salerno, 2010). However, the fact that Glc probably can diffuse freely through nuclear pores argues against the need for a Suc-hydrolysing enzyme activity in the nucleus.

Arabidopsis mesophyll protoplasts are widely used as a versatile cellular system for transient gene expression analysis (Yoo *et al.*, 2007). However, protoplasts immediately initiate new cellulose biosynthesis requiring massive amounts of UDPGlc derived from the activity of sucrose synthase (Fujii *et al.*, 2010). Since this requires an enormous investment of ATP and carbon skeletons, mesophyll protoplasts have a tendency to enter into sugar starvation conditions quite rapidly, and as such are sensitive to additional (oxidative) stresses such as exogenous H_2O_2 and ABA (Table 2). Exogenously supplied metabolizable sugars and AsA can counteract the oxidative stress as judged from the decreased *APX2* gene expression (Table 2). The increase in APX2 expression upon addition of 30 mM Mtl, however, suggests that this non-metabolizable sugar might generate additional stress, although Qi *et al.* (2007) reported that Mtl could also enhance endogenous At-A/N-InvG activity levels.

Sugar supplementation or enhanced Glc production by overexpressing A/N-Invs might help to increase the number of mitochondria, the respiration rate, and ATP generation (Giegé *et al.*, 2005), both in the light and in the dark (Table 2). However, it cannot be excluded that sugar-mediated feedback regulation of photosynthesis occurs in the light, thus partly decreasing the chloroplast-generated H_2O_2. Recently, a chloroplastic A/N-Inv has been suggested as a putative player in this process (Tamoi *et al.*, 2010). However, this reasoning seems inconsistent with the fact

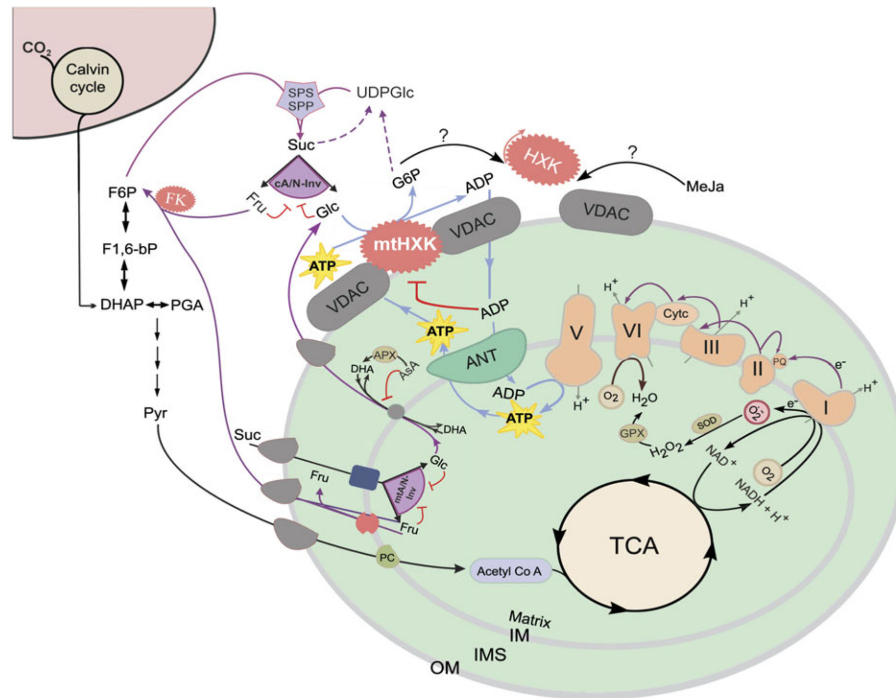

Fig. 5. Hypothetical model showing the putative role of A/N-Invs and mtHXKs in oxidative defence-related processes in plant mitochondria. Cytosolic Suc can serve as a substrate for cA/N-Invs. However, Suc can also enter the matrix via a Suc transporter in the inner membrane (IM). Glc and Fru are subsequently produced by mtA/N-Inv. Glc is transported back into the intermembrane space (IMS) through a Glc transporter that is regulated by AsA and through the outer membrane (OM) to serve as a substrate for HXK bound to the OM (termed mtHXK). mtHXK contributes to a steady-state ADP recycling via voltage-dependent anion channels (VDACs) and adenine nucleotide transporters (ANTs) to regulate H_2O_2 formation in the electron transport chain (ETC) on the IM. mtHXK activity is inhibited by ADP. It is not known whether methyl jasmonate (MeJa) and glucose 6-phosphate (G6P) can induce the detachement of mtHXK from the OM, as observed in animals. The G6P generated by mtHXK (as well as chloroplastic triose phosphates), can enter glycolysis, and the pyruvate (Pyr) produced crosses the IM via Pyr carboxylase (PC) to enter the Krebs (TCA) cycle. Alternatively, G6P can be used to produce UDPGlc for the resynthesis of carbohydrates (e.g. Suc or cellulose). Fru resulting from mtA/N-Inv or cA/N-Inv activity can be transformed into fructose 6-phosphate (F6P) by fructokinase (FK), which can enter glycolysis or can be used for Suc synthesis by sucrose phosphate synthetase (SPS) and sucrose phosphate phosphatase (SPP). Mitochondrial SOD and glutathione peroxidase (GPX) can assist in ROS-scavenging processes within the matrix. AsA is synthesized within the IMS, and can serve as a substrate for APX, to produce dehydroxyascorbate (DHA), which can be imported into the mitochondrial matrix by a transporter in the IM which also can function as a Glc transporter, regulated by AsA. Acetyl CoA, acetyl coenzyme A; Cytc, cytochrome *c*; DHAP, dihydroxyacetone phosphate; F1,6-bP, fructose 1,6-bisphosphate; PGA, phosphoglycolic acid; Q, plastoquinone pool; TCA, tricarboxylic acid.

that somewhat weaker but otherwise similar results were recorded in the dark (Table 2), suggesting that mitochondrial ROS-producing processes are important both in the light and in the dark, as recently discussed in other manuscripts (Dinakar *et al.*, 2010; Rossouw *et al.*, 2010; Nunes-Nesi *et al.*, 2011).

It has been demonstrated that cytosolic HXK isoforms are typically associated with the mitochondrial OM (Graham *et al.*, 2007; Balasubramanian *et al.*, 2007, Fig. 2f). It is suggested here that a continuous and efficient supply of Glc to mtHXK is necessary to maintain its activity at a rather constant level, which itself is needed to control the flux through the mitochondrial electron transport chain, influencing mitochondrial ROS production (Fig. 5; Camacho-Pereira *et al.*, 2009). In particular, it has been demonstrated that mtHXK can contribute to the steady-state recycling of ADP (ADP production by mtHXK, bound to the mitochondrial OM; ADP consumption through oxidative phosphorylation) which regulates H_2O_2 formation in the electron transport chain on the mitochondrial inner membrane (IM; Fig. 5). Importantly, this mitochondrial ADP recycling mechanism leads to a decrease in the mitochondrial membrane potential while an inhibition of the associated mtHXK causes an increase in H_2O_2 production. Thus, a tightly bound OM mtHXK produces ADP that can be transported through both mitochondrial membranes to reach the F_0F_1ATP synthase complex (Fig. 5). In conclusion, a tightly bound OM mtHXK could provide a basal level of ADP for ATP synthesis, preventing overpolarization of the mitochondrial IM and consequent accumulation of ROS, including H_2O_2. This mechanism would be particularly useful when other sources of ADP become limiting for oxidative phosphorylation. Interestingly, both methyl jasmonate (MeJa) and Glc-6-P (G6P) are known to induce the detachment of mammalian HXK from the mitochondrial OM (da-Silva *et al.*, 2004; Goldin *et al.*, 2008), but additional research is needed to

verify whether MeJa and G6P fulfil similar roles in the vicinity of plant mitochondria (Fig. 5).

What could be the source of the Glc substrate for HXKs in leaf mesophyll cells? First, Glc can originate from the activity of cA/N-Invs (Fig. 5). Secondly, cytosolic Glc might result from starch breakdown during the night, and, finally, it is proposed here that Glc could originate from the action of mtA/N-Invs (Fig. 5). Indeed, it was demonstrated in *Helianthus tuberosus* that mtA/N-Inv is present in the mitochondrial matrix (Szarka *et al.*, 2008). In this species, separate, bidirectional Suc, Glc, and Fru transporters are presumably present in the IM (Szarka *et al.*, 2008; Fig. 5). The mitochondrial OM contains numerous porins (e.g. voltage-dependent anion channels; Fig. 5) and is permeable to all molecules of ≤5000 Da (Colombini, 1979). Therefore, cytosolic Suc could enter through these porins and enter the matrix via a Suc transporter in the IM (Fig. 5), leading to the formation of Glc and Fru by the activity of mtA/N-Inv. It could be hypothesized that Glc is transported back into the intermembrane space (IMS; Fig. 5) and through pores of the OM to serve as a substrate for mtHXK (Fig. 5). Intriguingly, the backflow of Glc into the IMS might be tightly regulated by AsA (Fig. 5). Indeed, AsA very specifically inhibits the Glc transporter and not the Suc and Fru transporters in the IM (Fig. 5; Szarka *et al.*, 2008). In case mitochondrial AsA contents become too low (e.g. as a result of oxidative stress), Glc outflow and mtHXK activity are promoted, ADP recycling is stimulated, ATP synthesis-related limitation of respiration is avoided, and subsequent H_2O_2 release is reduced/avoided (Camacho-Pereira *et al.*, 2009). This mtHXK/mtA/N-Inv-controlled regulatory mechanism might be less critical when sufficient AsA and antioxidant-related enzymes are present to prevent ROS accumulation. Therefore, consistent with the data presented here, it is suggested that the inhibition of Glc outflow by AsA can be considered as an efficient feedback mechanism to control mtHXK activity and mitochondrial ROS production (Fig. 5).

Consistent with the proposed A/N-Inv/mtHXK model, it is well known that A/N-Inv enzymes are inhibited by their own hexose products (Van den Ende and Van Laere, 1995), which provides an elegant system to synchronize the A/N-Inv activities (cytosolic and/or mitochondrial) to mtHXK activities. Similar mechanisms, involving chloroplastic HXKs and A/N-Invs, might also occur in chloroplasts.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Names and sequence of the primers used in this manuscript.

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