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Summary

- Many plants accumulate proline, a compatible osmolyte, in response to various environmental stresses such as water deficit and salinity. In some stress responses, plants generate hydrogen peroxide (H_2O_2) that mediates numerous physiological and biochemical processes. The aim was to study the relationship between stress-induced proline accumulation and H_2O_2 production.

- Using pharmacological and reverse genetic approaches, the role of NADPH oxidases (Rboh) in the induction of proline accumulation was investigated in *Arabidopsis thaliana* in response to stress induced by either 200 mM NaCl or 400 mM mannitol.

- Stress from NaCl or mannitol resulted in a transient increase in H_2O_2 content accompanied by accumulation of proline. Dimethylthiourea, a scavenger of H_2O_2, and diphenylene iodonium (DPI), an inhibitor of H_2O_2 production by NADPH oxidase, were found to significantly inhibit proline accumulation in these stress conditions. DPI also reduced the expression level of Δ^1-pyrroline-5-carboxylate synthetase, the key enzyme involved in the biosynthesis of proline. Similarly less proline accumulated in KO-mutants lacking either *AtRbohD* or *AtRbohF* than in wild type in response to the same stresses.

- Our data demonstrate that AtRbohs contribute to H_2O_2 production in response to NaCl or mannitol stress to increase proline accumulation in *A. thaliana*.

Key-words: Abiotic stresses; Arabidopsis thaliana; cell signalling; hydrogen peroxide; NADPH-oxidases (Rboh); proline metabolism.

Introduction

In their natural environments, plants commonly encounter a variety of abiotic constraints like drought and salinity (Nakashima *et al*., 2009). To overcome these constraints, plants have developed a variety of adaptive mechanisms that allow them to perceive external signals and to optimize adaptive responses. One of these mechanisms is osmotic adjustment through the accumulation of large quantities of osmolytes as it allows plants to avoid water deficit stress by maintaining water uptake. Glycine betaine, polyols, sugars and free amino acids are examples of such osmolytes (Chen & Jiang, 2010; Slama *et al.*, 2015).

Proline is the most common free amino acid to accumulate in plants subjected to water deficit stress. Proline has multifunctional roles though which do not necessarily relate to the osmotic balance (for review see Szabados & Savouré, 2010). Proline may stabilize protein complexes, scavenge free radicals and be a source of carbon and nitrogen for growth after stress relief. Proline biosynthesis and degradation are involved in regulating intracellular redox potential and storage as well as the transfer of energy and reducing power (Sharma *et al*., 2011; Szabados & Savouré, 2010). The beneficial effect of proline on plant growth after stress is likely to be the result of changes in proline metabolism rather than the accumulation of the amino acid itself (Sharma *et al*., 2011; Szabados & Savouré, 2010).

The proline content of plant cells depends on tight regulation of its proline biosynthesis and catabolism. Housekeeping levels of proline biosynthesis occur in the cytosol, but stress-induced biosynthesis is thought to be localized in chloroplasts (Székely *et al*., 2008). When under water-deficit stress, proline is mainly synthesized from glutamate. The bifunctional pyrroline-5-carboxylate synthetase (P5CS) reduces glutamate to glutamyl-5-semialdehyde, which is spontaneously converted to pyrroline-5-carboxylate (P5C). P5C is then reduced to proline by P5C reductase (P5CR). Degradation of proline takes place in mitochondria via the sequential action of proline dehydrogenase (ProDH) and P5C dehydrogenase. The rate-limiting steps in proline biosynthesis and degradation are catalyzed by P5CS and ProDH, respectively. Two closely related P5CS-encoding genes were identified in *Arabidopsis thaliana*. *P5CS1* is induced by drought and salt stress (Savouré *et al*., 1995; Yoshiba *et al*., 1995), while *P5CS2* is expressed in dividing cells in cell suspension cultures (Strizhov *et al*., 1997), in meristematic and reproductive tissues (Székely *et al*., 2008), and in response to biotic stress such as incompatible plant-pathogen interactions (Fabro *et al*., 2004). Similarly, *A. thaliana* has two genes for ProDH, *ProDH1* and *ProDH2* (Kiyosue *et al*., 1996; Verbruggen *et al*., 1996; Funck *et al*., 2010). *ProDH1* is thought to encode the main isoform

involved in proline degradation (for review see Servet *et al*., 2012). The expression of *ProDH1* is down-regulated by osmotic stress and upregulated by proline (Kiyosue *et al*., 1996; Verbruggen *et al*., 1996).

The regulation of *P5CS* and *ProDH* expression has been studied to identify components of signalling pathways that control proline accumulation. Under non-stress conditions, phospholipase D functions as a negative regulator of proline biosynthesis in *Arabidopsis* (Thiery *et al*., 2004), whereas calcium signalling and phospholipase C (PLC) trigger *P5CS1* transcription and proline accumulation during salt stress (Parre *et al*., 2007). *P5CS1* expression has also been found to be stimulated by light (Hayashi *et al*., 2000) and nitric oxide (Zhao *et al.*, 2009) and it is abscisic acid (ABA) independent under water-deficit stress (Savouré *et al*., 1997; Sharma & Verslues, 2010). Recently Leprince *et al.* (2015) demonstrated that phosphatidylinositol 3-kinase is involved in the regulation of proline catabolism through transcriptional regulation of *ProDH1*.

Another common plant response to all types of environmental constraints is the accumulation of ROS, which are toxic at high concentrations but at lower concentrations may act as signal molecules in the control of various cellular processes. H_2O_2 is a ROS produced by plant plasma membrane-localized NADPH oxidases, known as respiratory burst oxidase homologues (Rbohs). Rbohs reduce molecular oxygen to superoxide by oxidising NADPH via FAD and two hemes. The superoxide primary product is then converted into H_2O_2 by superoxide dismutase (Sagi & Fluhr, 2001; Sagi & Fluhr, 2006). The *Arabidopsis* genome contains 10 NADPH oxidase-encoding genes, designated *AtRbohA* to *J*, that exhibit different patterns of expression throughout plant development and in response to environmental factors (Fluhr, 2009; Marino *et al.*, 2012). For instance, *AtRbohA*, *B* and *C* are only expressed in roots, especially in the elongation zone. *AtRbohC* was specifically identified as playing a role in root hair development (Foreman *et al*., 2003). *AtRbohH* and *J* are reported to be expressed only in pollen. Both *AtRbohD* and *AtRbohF* are expressed in all plant organs and are the main isoforms involved in pathogen defence responses (Torres *et al*., 2002), ABA-induced stomatal closure (Kwak *et al*., 2003), jasmonic acid signalling regulated by transcription factor MYC2 (Maruta *et al*., 2011) and ROS-dependent regulation of Na^+/K^+ homeostasis under salt stress (Ma *et al*., 2012). *AtRbohD* has also been demonstrated to mediate rapid systemic signalling triggered by multiple abiotic stresses (Miller *et al*., 2009) and to be required for salt acclimation signalling mediated by heme oxygenase in *Arabidopsis* (Xie *et al*., 2011). It was reported that mild salt stress causes a rapid and transient accumulation of ROS in *A. thaliana*

(peak I after 1 h) followed by a second oxidative burst (peak II after 6 h) (Xie *et al*., 2011). The conclusion is that HY1 heme oxygenase plays an important role in salt acclimation signalling and requires the participation of AtRbohD-derived ROS from peak II. More recently, it has been reported that AtRbohF fulfils a crucial role in protecting shoot cells from transpiration-dependent accumulation of excess Na^+ (Jiang *et al*., 2012). Rbohs are thus key regulators of ROS production with pleiotropic functions in plants.

It is possible that there is a link between quantitative changes in ROS and proline. Exogenous H_2O_2 treatment led to a significant accumulation of proline in coleoptiles and radicles of maize seedlings due to the induction of biosynthetic P5CS enzyme activity and a decrease in catabolic ProDH enzyme activity (Yang *et al*., 2009). Fabro *et al*. (2004) have also demonstrated that ROS can mediate the activation of *AtP5CS2* and proline accumulation during biotic stress. However, reports on the relationship between endogenous ROS increase and proline accumulation under osmotic stress are still limited and the link between NADPH oxidases and proline accumulation has never been addressed. In the present study, the role of ROS-generating NADPH-oxidase enzymes in proline accumulation in response to salt or mannitol stress was investigated in *Arabidopsis thaliana* seedlings.

Materials and Methods

Growth conditions and stress treatments

Arabidopsis thaliana (L.) Heynh ecotype Columbia-0 (Col-0) was used as the wild-type in this study. Homozygous *Arabidopsis thaliana* transposon insertion mutant lines *atrbohd-3* (European Arabidopsis Stock Centre code N9555) and *atrbohf-3* (European Arabidopsis Stock Centre code N9557) and double mutant *atrbohd/f* (European Arabidopsis Stock Centre code N9558) (Torres *et al*., 2002) were ordered from the European *Arabidopsis* Stock Centre. Homozygous T-DNA insertion lines SALK_070610 (*atrbohd*; seventh exon insertion) and SALK_059888 (*atrbohf*; third intron insertion) were ordered from the Salk collection (Pogany *et al*., 2009) and used as controls for the transposon insertion lines.

Surface-sterilized seeds of wild-type (Col-0) and *Arabidopsis* mutant plants were sown onto grids placed on 0.5 × Murashige and Skoog (MS) solid agar medium in Petri dishes according to Parre *et al.* (2007). After 24 h at 4°C to break dormancy, seedlings were allowed to grow at 22°C under continuous light (90 µmol photons m^{-2} s^{-1}). Twelve-day-old *Arabidopsis* seedlings were exposed to H_2O_2 (5 to 120 mM), paraquat (PQ, also called methyl viologen, 5 to 120 µM), 200 mM NaCl or 400 mM mannitol for 24 h. To alter ROS levels, the seedlings were

pre-incubated for 4 h without or with H_2O_2 scavenger dimethylthiourea (DMTU, 5 to 40 mM) or flavoenzyme inhibitor diphenylene iodonium (DPI, 5 to 40 µM) in 0.5 × liquid MS medium and then exposed to either NaCl or mannitol for 24 h.

Determination of ion content

Whole plants were harvested, rinsed twice in pure water and quickly blotted. Samples were dried at 60 °C until they reached constant weight then ground. Ions were extracted from samples in 0.5% HNO_3. Na^+ and K^+ were assayed by flame emission photometry (Corning, UK).

Proline content measurements

Free proline content was measured according to the Bates method (Bates *et al*., 1973). Powdered plant samples (50 to 100 mg) were homogenized in 1.5 ml of 3% sulfosalicylic acid and centrifuged at 14,000 × *g* for 10 min at 4 °C. To 1 ml of this extract, 1 ml of acid-ninhydrin and 1ml of glacial acetic acid were added. The reaction mixture was incubated at 100°C for 1 h then placed on ice to stop the reaction. In the presence of proline, the reaction produces a red chromogen. The chromogen was extracted with 2 ml of toluene and the absorbance of the resulting upper phase was read at 520 nm. Proline content of samples was calculated by referring to a standard curve drawn from absorbance readings from samples containing known concentrations of proline.

H_2O_2 content measurements

H_2O_2 content was determined in a horseradish peroxidase-based spectrophotometric assay following the protocol described by Oracz *et al*. (2009). Plant samples (300 mg FW) were ground in a mortar on ice in 1 ml of 0.2 M perchloric acid. After 15 min of centrifugation at 13,000 × *g* at 4°C, the resulting supernatant was neutralized to pH 7.5 with 4 M KOH and then centrifuged at 13,000 × *g* at 4°C. The concentration of H_2O_2 in the supernatant was immediately determined by adding peroxidase with substrates 3-dimethylaminobenzoic acid and 3-methyl-2-benzothiazolidone hydrazone. The increase in absorbance at 590 nm was monitored for 15 min after the addition of peroxidase at 25 °C and compared to a calibration curve obtained with known amounts of fresh H_2O_2.

Histochemical detection of H_2O_2

The production of H_2O_2 was visualized *in vivo* by 2',7'-dichlorofluorescein diacetate (H_2DCFDA) or DAB staining methods. *Arabidopsis* roots were collected after 6-h treatments and immersed in 25 µM H_2DCFDA for 15 min in the dark and then washed with 20 mM potassium phosphate buffer pH 6. Fluorescent signals were visualized using a Zeiss ApoTome microscope (excitation, 488 nm; emission, 525 nm). DAB staining was performed as described by Torres *et al*. (2002). Leaves from PQ-treated seedlings were detached and vacuum infiltrated with DAB solution (1 mg ml^{-1} DAB-HCl, pH 3.8). DAB forms a reddish-brown polymer in the presence of H_2O_2 formation. After staining, leaves were cleared in 96% boiling ethanol and observed using a binocular microscope. For both staining methods, digital images were obtained with an AxioCam camera and AxioVision software (Zeiss).

Cytochemical detection of H_2O_2

Cytochemical detection of H_2O_2 was carried out according to the method described by Bestwick *et al*. (1997). Leaf samples were collected from treated and control seedlings and incubated in freshly prepared 5 mM cerium chloride for 30 min. The leaves were then fixed in a solution containing 4% glutaraldehyde and 1.5% paraformaldehyde for 1 h, post-fixed for 45 min in 1% osmium tetroxide and then embedded in Eponaraldite resin after dehydration in an ethanol series. Sections (60 to 80 nm thick) were cut with a LKB 2128 ultramicrotome, mounted on uncoated copper grids, and stained with 5% uranyl acetate. Sections were observed using a Zeiss912 Omega transmission electron microscope. Digital images were obtained using a Veleta Camera (2kx2k, Olympus) and iTem software (Zeiss).

RT-PCR and qRT-PCR Analysis

Total RNA was extracted from 100 mg of plant tissue ground in liquid nitrogen using a mixer mill (MM301, Retsch, Germany). The powder was suspended in 0.5 mL extraction buffer (0.2 M Tris-HCl, 0.5% (v/v) SDS, 0.25 M NaCl, 25 mM EDTA) and mixed with 0.5 mL of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v). The aqueous phase was extracted 3 times with phenol/chloroform/isoamylalcohol. Total RNA was then precipitated overnight on ice with 2 M LiCl. After centrifugation (15 min, 10,000 ×x *g*), the pellet was resuspended in 2 M LiCl and left to precipitate for 6 h on ice. Traces of DNA were removed by DNase treatment. RNA quantified by measuring the absorbance at 260 nm using a Nanovue® spectrophotometer (GE Healthcare Life Science). First-strand cDNA was obtained from 1.5

µg of total RNA using RevertAidTM reverse transcriptase synthesis kit (Fermentas) and oligo(dT)23 as primer.

For RT-PCR, cDNAs were amplified using Taq polymerase and gene-specific primers (Supporting Information Table S1). *APT1* (adenine phosphoribosyltransferase 1; At1g27450) gene transcripts were amplified as a control. Amplified PCR fragments were visualized using ethidium bromide stained 2% (w/v) agarose gels. RT-PCR signals were quantified using the ImageJ 1.48 software (National Institutes of Health, Bethesda, MD).

For quantitative PCR 5 µL of diluted cDNA was used with 10 µL of Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, France) and gene-specific primers (Supporting Information Table S1) in a Mastercycler® ep *realplex* (Eppendorf, France). Critical thresholds (Ct) were calculated using the Realplex 2.0 software (Eppendorf, France). For each gene, a standard curve made with dilutions of cDNA pools was used to calculate the reaction efficiencies, and relative expressions were calculated according to Hellemans *et al.* (2007) with *APT1* (At1g27450) and *AT5G* (At5g13440) as housekeeping genes. A mixture of cDNAs corresponding to each sample was used as reference. All qRT-PCR experiments were carried out with three biological replicates.

Western blots

Total proteins were subjected to SDS-PAGE using 8% acrylamide resolving gels and electroblotted onto a PVDF membrane. Rabbit polyclonal antibodies raised against P5CS or ProDH were used as primary antibodies (Thierry *et al*., 2004; Parre *et al*., 2007). Blots were incubated with secondary horseradish peroxidase-conjugated antibodies (GE Healthcare Life Sciences). The highly sensitive ECL Prime detection system (GE Healthcare) was used to quantify proteins using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD) after densitometric scanning of autoradiography films. Alternatively the Storm 840 FluorImager (Molecular Dynamics) was used to visualise and quantify proteins on blots.

Statistical analysis

A one-way analysis of variance (ANOVA) at $P < 0.05$ significance level was performed using the SPSS program for Windows. The Duncan post-hoc test was used to test significant differences between treatments.

Results

Kinetics of ROS and proline accumulation in wild-type *A. thaliana* seedlings

To investigate ROS and proline accumulation in response to stress induced by either salt or mannitol, 12-day-old *A. thaliana* seedlings were exposed to 200 mM NaCl or 400 mM mannitol for up to 24 h. In both cases proline started to accumulate after 6 h. A maximum 16-fold increase in proline content was reached after 18 h of NaCl or mannitol stress (Fig. 1). A transient increase in H_2O_2 content was also observed in stressed seedlings after 6 h. H_2O_2 content had returned to a basal level after 12 h.

Effect of H_2O_2 and paraquat on proline accumulation in *A. thaliana*

The kinetics of H_2O_2 and proline accumulation in response to either NaCl or mannitol stress showed that proline had already started to increase 6 h after stress was applied when the transient increase in H_2O_2 content was also observed (Fig. 1). To determine whether H_2O_2 could be involved in proline accumulation, we first investigated whether adding different concentrations of exogenous H_2O_2 would affect proline accumulation in *A. thaliana*. Results showed that proline accumulation is induced by exogenous H_2O_2 application with 10 mM H_2O_2 having the maximum effect (Fig. 2a).

Paraquat (PQ) is a herbicide which induces superoxide anions and subsequently H_2O_2 generation *in situ*.. Like H_2O_2, PQ also stimulated proline accumulation in Arabidopsis seedlings in a dose dependent manner with 40 µM PQ having the maximum effect (Fig. 2b). Leaves treated with high concentrations of PQ such as 80 or 100 µM PQ started to bleach after 48 h of treatment due to the high amount of H_2O_2 generated (data not shown) even though proline contents were not higher than at 40 µM PQ. DAB staining revealing the presence of H_2O_2 in leaves demonstrates that exogenously applied PQ gives rise to H_2O_2 *in situ* in a dose dependent manner (Fig. 2c). Taken together, these results indicate a possible causal relationship between H_2O_2 and proline production. However, much less proline accumulated in the presence of 10 mM H_2O_2 (about 5 times less) or 40 µM PQ (about 8 times less) than with NaCl or mannitol stress (Fig. 2a, 2b and Fig.1).

Effect of DMTU, a chemical scavenger of H_2O_2, on proline accumulation in response to stress.

To investigate whether the induction of proline accumulation by salt or mannitol requires H_2O_2, we treated *A. thaliana* seedlings with DMTU, a chemical trap for H_2O_2. As shown in

Fig. 3a, addition of up to 40 mM DMTU inhibited proline accumulation in a dose-dependent manner in seedlings treated with NaCl and to a lesser extent in those treated with mannitol.

Subcellular localisation of H_2O_2 accumulation

Multiple sources of H_2O_2 production inside plant cells have been described (Petrov & Van Breusegem, 2012). To investigate the subcellular localization of H_2O_2 accumulation in leaves of *A. thaliana* seedlings, a cytochemical approach using cerium perhydroxide was taken (Bestwick *et al.*, 1997). No visible cerium perhydroxide deposits were observed at the subcellular in *A. thaliana* leaves growing under normal growth conditions, indicating the absence of H_2O_2 using this detection method (Fig. 4). However, both salt and mannitol triggered H_2O_2 accumulation in cell walls of mesophyll cells. Importantly no cerium perhydroxide deposits were observed in the cytosol, chloroplasts or mitochondria indicating that no major H_2O_2 accumulation occurred in these organelles.

NADPH oxidases are essential for proline accumulation in response to NaCl and mannitol stresses

Stress-induced H_2O_2 accumulated in the cell wall and apoplasm. The main source of cell wall H_2O_2 is the plasma membrane-bound NADPH oxidase (Petrov & Van Breusegem, 2012). We therefore hypothesized that NADPH oxidase activity is required for proline accumulation in response to salt and mannitol stress. We investigated this using a pharmacological approach. Treatments of *A. thaliana* seedlings with DPI, an inhibitor of flavin enzymes such as NADPH oxidases, resulted in a dose-dependent decrease of proline accumulation induced by salt and mannitol stress (Fig. 3b). Addition of various concentrations of DPI did not affect proline accumulation in seedlings growing under normal growth conditions. For further experiments we used 20 µM DPI which diminished proline accumulation by approximately 60%. To aid interpretation of results, the pharmaceutical toxicity of DPI was also tested in seedlings. As shown in Fig. 5, the reduction of proline accumulation caused by the addition of 20 µM DPI to NaCl- and mannitol-treated seedlings for 24 h was totally reversed after washing the seedlings and putting them back in growth medium. In addition no leaf injury or lethality was observed whatever the treatment.

We monitored H_2O_2 production in root tips by adding H_2DCFDA, which reacts with H_2O_2 in living cells to produce fluorescent DCF. As observed in whole seedlings, H_2O_2 is detected in root tips 6 h after exposure to either NaCl or mannitol (Fig. 6a). In contrast H_2O_2 was not

detected in root tips incubated with DPI prior to treatment with either NaCl or mannitol prevented, suggesting H_2O_2 production was inhibited.

The rate limiting steps in proline biosynthesis and degradation in *Arabidopsis* are catalyzed by P5CS and ProDH respectively. To further investigate the role of NADPH oxidase in proline metabolism, steady-state transcript levels of *AtP5CS1* and *AtProDH1* were investigated. Semi-quantitative RT-PCR analysis showed that DPI treatment significantly decreased *P5CS1* transcript levels in response to salt and to a lesser extent to mannitol (Fig. 6c). Steady-state transcript levels of *ProDH1* gene were not altered by DPI. Protein levels of P5CS and ProDH were also investigated by Western blot analyses. In wild-type *A. thaliana* plants, 200 mM NaCl and 400 mM mannitol both caused an increase in P5CS protein content compared with untreated plants (Fig. 6d). However, the increase in P5CS protein levels by salt or mannitol was markedly suppressed by pre-treating seedlings with DPI. In contrast to ProDH, whose protein levels were not affected by the different conditions, a good correlation was observed between *P5CS* transcript and protein levels and proline content.

Proline accumulation in *atrboh* mutants

If ROS produced by NADPH oxidase is involved in regulating proline accumulation due to NaCl and mannitol stress, then KO mutants defective in NADPH oxidase would be expected to accumulate less proline than wild type. Of the ten *Arabidopsis* NADPH oxidase genes identified, *AtRbohD* and *AtRbohF* are the only ones expressed in all plant organs and they are implicated in abiotic stress physiology (Fluhr, 2009; Marino *et al.*, 2012). *Atrbohd-3* and *atrbohf-3* mutants were therefore selected for our study. Semi-quantitative RT-PCR using wild-type and mutant plants revealed that *rbohd-3* and *rbohf-3* homozygous plants contained no detectable levels of *AtRbohD* and *AtRbohF* transcripts in response to salt treatment (data not shown). In both *atrbohd-3* and *atrbohf-3* mutants, the transient increase in H_2O_2 levels at 6 h in response to NaCl and mannitol stresses was not as large as the wild-type increase (Fig. 7). After cerium perhydroxide staining no visible cerium perhydroxide deposits were observed in the leaves of *atrbohd* mutant seedlings grown under mannitol or salt stress, indicating the absence of detectable levels of H_2O_2 (Supporting Information Fig. S1).

The two NADPH oxidase-deficient *atrbohd* and *atrbohf* mutants were examined for the accumulation of proline and P5CS protein in response to either mannitol or salt stress. As expected, application of 200 mM NaCl or 400 mM mannitol induced proline accumulation in wild-type *Arabidopsis* plants (Fig. 8a). Consistent with the results of DPI treatment in wild-

type (see Fig. 6), *atrbohd-3* and *atrbohf-3* mutants accumulated less proline and less P5CS protein than wild-type in the presence of 200 mM NaCl (Fig. 8a, b). The results of 400 mM mannitol treatments were similar although less pronounced (Fig. 8a, b). Similar results for proline accumulation were observed with independent alleles of *atrbohd* and *atrbohf* (T-DNA insertion lines SALK_070610 and SALK_059888 respectively) (Supporting Information Fig. S2). In the double *atrbohd/f* mutant proline levels reached similar levels to those in the single mutants in response to mannitol stress (Fig. 8). The amount of proline that accumulated in response to NaCl in the double mutant was slightly higher but was not as high as the level induced in wild type. It was noted that the decrease in proline and P5CS accumulation was less pronounced in the *atrboh* mutants than in seedlings treated with DPI (Fig. 5 and 6). Perhaps NADPH oxidases other than AtRbohD and AtRbohF or other sources of H_2O_2 are involved in the regulation of proline biosynthesis. To further demonstrate a role for H_2O_2, we reversed the effect of *atrboh* mutation by applying exogenous H_2O_2. Addition of 10 mM H_2O_2 restored the levels of proline and P5CS protein accumulation in mannitol-treated *atrbohd-3*, *atrbohf-3* and double *atrbohd/f* seedlings (Fig. 8).

Discussion

Plant stress tolerance involves diverse mechanisms such as signal perception and transduction, osmolyte accumulation, ion homeostasis, growth regulation and cellular protection from damage triggered by reactive oxygen species (ROS). It is widely accepted that H_2O_2 and other ROS are also important signalling molecules in the activation of defence genes in response to biotic stress (Foyer & Noctor, 2009, Bartoli *et al*., 2012). The connection between ROS and proline in response to biotic stress has already been highlighted by Fabro *et al.* (2004). Nevertheless little is known about the involvement of ROS in the regulation of proline metabolism in response to abiotic stresses. The aim of our work was to evaluate the roles of H_2O_2 in the regulation of proline metabolism in response to two different abiotic stresses in *A. thaliana*. Our results showed that both NaCl and mannitol stress induced proline accumulation and a transient increase in H_2O_2 content (Fig. 1). The timing of these changes might suggest that H_2O_2 could act as a secondary messenger involved in triggering proline biosynthesis. This hypothesis is supported by the observation that treatment with H_2O_2 or PQ also promoted proline accumulation (Fig. 2). However these effects are not as strong as those caused by physiological NaCl or mannitol stresses. The fact that much less proline accumulated in response to H_2O_2 and PQ than to the stress conditions is in itself intriguing.

One possibility is that additional signalling pathways are involved in the full stress response. Phospholipase D enzymes are putative signalling components as they have been previously shown to negatively regulate proline accumulation in non-stress or mild stress conditions (Thiery *et al.*, 2004). Following this reasoning, phospholipase D activity may need to be inhibited in order to elicit a full proline stress response by H_2O_2 treatment. As H_2O_2 is able to increase proline accumulation and DMTU is an effective inhibitor of proline accumulation induced by NaCl or mannitol, our data suggest that the stress-induced proline accumulation observed in *A. thaliana* seedlings is mediated at least partly by H_2O_2.

ROS, such as singlet oxygen ($^1O_2$), H_2O_2, and hydroxyl radical ($OH^{\cdot}$), are produced during normal aerobic metabolism in different cell compartments such as cell walls, plasma membranes, chloroplasts, mitochondria and peroxisomes (Dat *et al*., 2000). The production of H_2O_2 has been repeatedly demonstrated at the subcellular level by using $CeCl_3$ techniques, for example, during abscisic acid signalling (Hu *et al*., 2006), pathogen attack (Bestwick *et al*., 1997), responses to ozone (Pellinen *et al*., 1999), drought (Hu *et al*., 2006), anoxia (Blokhina *et al*., 2001), and heavy metal excess (Romero-Puertas *et al*., 2004). Our observation based on $CeCl_3$ detection revealed that either salt or mannitol stress induced H_2O_2 accumulation in walls of mesophyll cells (Fig. 4). H_2DCFDA fluorescence analysis also revealed an accumulation of H_2O_2 in Arabidopsis root tips in response to either NaCl or mannitol stress (Fig. 6). This is consistent with a report that H_2O_2 accumulation was detected along the plasma membrane of maize leaves challenged with abscisic acid and water-deficit stress (Hu *et al*., 2006). Using $CeCl_3$, we did not detect any of H_2O_2 in chloroplasts or in any other organelle of *A. thaliana* leaf cells after 6 h of either salt or mannitol treatment. The apoplastic oxidative burst and resultant H_2O_2 accumulation in the extracellular space is characteristic of plant cells exposed to biotic and abiotic stresses (Bartoli *et al*., 2012). Studies of different plant species have demonstrated the action of plasma membrane-bound NADPH oxidases in the apoplastic ROS-producing system during early oxidative bursts which is critical in plant signalling and development, including in defence, root hair development, stomatal closure, and early responses to salt stress (Torres *et al*., 2002; Foreman *et al*., 2003; Kwak *et al*., 2003; Leshem *et al*., 2007). DPI is a commonly used potent inhibitor of flavin enzymes such as NADPH oxidase. We found that DPI efficiently inhibited H_2O_2 production in *A. thaliana* seedling roots exposed to NaCl or mannitol (Fig. 6a), strongly suggesting that at least some of the H_2O_2 production induced by salt or mannitol originates from NADPH oxidase.

In response to either salt or mannitol stress, *P5CS1* proline biosynthesis transcript and protein levels increased at the same time as proline accumulated. Pretreatment of *A. thaliana* seedling with DPI reduced the salt- or mannitol-induced proline accumulation in a dose-dependent manner (Fig. 6). In addition, DPI significantly diminished *P5CS1* transcript and protein accumulation. The reaction catalyzed by NADPH oxidase uses O_2 to generate superoxide ($O_2^{\cdot-}$), which is then converted into H_2O_2 by apoplastic superoxide dismutase. H_2O_2, as a nonpolar molecule, can easily pass through the plasma membrane probably through aquaporins. H_2O_2 has important roles as a signalling molecule in the regulation of a variety of biological processes. Possibly by redox changes H_2O_2 might directly or indirectly activate unknown signalling components, such as transcription factors, to regulate the transcription of proline biosynthesis genes.

The role of Rbohs in the regulation of proline metabolism was further investigated by reverse genetic approach using *atrbohd* and *atrbohf* KO mutants. Compared to wild type, the strong reduction of H_2O_2 production after 6 h in the *atrbohd* mutant in response to NaCl and mannitol, observed to a lesser extent in the *atrbohf*, could be considered to be consistent with *AtRbohD* being the most highly expressed member of the *AtRboh* gene family in response to salt (Leshem *et al*., 2007). Furthermore the cytochemical detection of H_2O_2 indicated that H_2O_2 production during salt and mannitol stress is associated with *AtRbohD* expression.

Our study shows that proline accumulation was 20-fold higher in salt-treated wild-type compared to control wild-type plants; however, it was respectively only 5-fold and 9-fold higher in salt-treated *atrbohd* and *atrbohf* seedlings compared to their controls. The double *atrbohd/f* mutant surprisingly produced slightly more proline in response to NaCl than the corresponding single mutants possibly because the double mutant is more sensitive to NaCl. Similarly, a much lower P5CS level was observed in the *atrbohd* mutant, compared to only a marginal reduction in *atrbohf* mutant compared to wild-type plants. Taken together, these results indicate that these NADPH oxidase isoforms are involved in proline accumulation during salt stress with AtRbohD having a prominent role. It was noted that the decrease in proline accumulation was more pronounced in wild-type seedlings treated with DPI than in *atrboh* mutants. This result would implicate other NADPH oxidases in the proline accumulation response. Indeed according to qPCR measurements, *AtRbohA*, *AtRbohB* and *AtRobohC* transcripts are more abundant in *atrbohd* and *atrbohf* mutants than in wild-type under stress, which is possibly preliminary evidence of a feed-back mechanism in these mutants (Supporting Information Fig. S3).

Recently, it has been demonstrated that ROS generated by AtrbohF has a specific and predominant role in regulating Na^+ accumulation and soil-salinity tolerance (Jiang *et al.*, 2012). When grown in saline soil, *atrbohf* mutant accumulated higher levels of Na^+ than wild-type plants. In addition, Ma *et al.* (2012) have suggested that AtrbohF acts redundantly with AtrbohD in regulating Na^+/K^+ homeostasis. These independent observations lead us to postulate that the difference in proline accumulation in the wild-type and *atrboh* mutants plants could be due to impaired Na^+/K^+ regulation. No differences in Na^+ and K^+ content were found between *atrbohd, atrbohf* and wild-type plants subjected to NaCl treatment for 24 h (Supporting Information Fig. S4). However the double *atrbohdf* mutant contained less Na^+ but had a higher Na^+/K^+ ratio. Differences in Na^+ accumulation in *atrboh* single mutants between our study and the study of Jiang *et al.* (2012) were probably due to large differences in growth and stress conditions in the two experimental systems, such as continuous light versus 16 h light/8 h dark cycles or short versus long durations of stress. However the regulation of proline accumulation by Rboh in Arabidopsis was probably mainly due to the osmotic stress component of salt stress rather than to the ionic component, because Rboh was involved in the response to both NaCl and mannitol stress. Lastly, since the generation of ROS by Rboh causes changes in the cell redox potential, we postulate that redox-sensitive signalling components or transcription factors may be activated and to influence the expression of proline biosynthesis genes.

In conclusion, our results shed new light on the regulation of proline metabolism in response to abiotic stresses showing the involvement of NADPH oxidase and H_2O_2. We show that H_2O_2 is involved in proline accumulation induced by salt and mannitol stresses. First, proline accumulation was preceded by elevated H_2O_2 levels, and scavenging of H_2O_2 by DMTU abolished proline accumulation. Second, we have presented evidence that NADPH oxidases are the potential source of the observed stress-induced H_2O_2 generation. Third, the absence of H_2O_2 production in cell walls and the accumulation of less proline in *atrbohd* and *atrbohf* KO mutants in response to NaCl and mannitol provides convincing genetic evidence that the corresponding NADPH oxidase isoforms contribute to proline accumulation.

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Figure legends

Fig. 1. NaCl and mannitol stresses trigger transient increases in H_2O_2 and proline accumulation.

Twelve-day-old wild-type *A. thaliana* seedlings grown on 0.5 x MS solid medium were transferred to 0.5 × MS liquid medium for treatment. Plants were then exposed to 200 mM NaCl (triangles), 400 mM mannitol (squares) or neither (circles) for 24 h. Open and closed symbols indicate proline and H_2O_2 measurements respectively. The results shown are the means of at least three independent experiments (± SE).

Fig. 2. H_2O_2 induces proline accumulation.

Proline accumulation in wild-type *A. thaliana* seedlings treated with H_2O_2 (a) and paraquat (b) for 24 h. Means (± SE) of at least three independent experiments with different letters are significantly different at $P < 0.05$. (c) H_2O_2 was visualized by using DAB staining in *A. thaliana* leaves exposed to different concentrations of paraquat for 24 h.

Fig. 3. The NADPH oxidase inhibitor DPI and the H_2O_2 scavenger DMTU affect proline accumulation induced by salt or mannitol stress.

Twelve-day-old *A. thaliana* seedlings grown on 0.5 × MS solid medium were transferred to 0.5 × MS liquid medium for treatment. Plants were preincubated with various concentrations of DMTU (a) or DPI (b) for 4 h and then exposed to 200 mM NaCl (grey bars), 400 mM mannitol (black bars) or neither (white bars) for 24 h. Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$.

Fig. 4. NaCl and mannitol stresses trigger apoplastic H_2O_2 accumulation in *A. thaliana* leaves.

Twelve-day-old seedlings grown on 0.5 × MS solid medium were transferred to 0.5 × MS liquid medium. Subcellular localization of H_2O_2 was detected by $CeCl_3$ staining of leaves of wild-type *A. thaliana* grown under normal conditions (control, a, d) or in the presence of either 200 mM NaCl (b, e) or 400 mM mannitol (c, f) for 6 h. Arrows indicate electron-dense deposits of cerium perhydroxides formed in the presence of H_2O_2 and $CeCl_3$. Ch, chloroplast; CW, cell wall; M, mitochondria; S, starch; V, vacuole.

Fig. 5. Inhibition of proline accumulation by DPI can be reversed.

A. thaliana seedlings were pre-treated with 20 µM DPI as described in the legend of Fig. 3 and then treated with either 200 mM NaCl or 400 mM mannitol for 24 h (a). Seedlings were then washed twice and transferred onto NaCl or mannitol medium for another 24 h (b). Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$.

Fig. 6. DPI inhibition of NADPH oxidase activity affects proline metabolism induced by either salt or osmotic stress.

A. thaliana seedlings were prepared and treated as previously described in the legend of Fig. 3. (a) Sites of H_2O_2 production visualized by using 2',7'-dichlorofluorescein diacetate (H_2DCFDA) in wild-type *A. thaliana* root tips pre-treated with 20 µM DPI and grown under normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 6 h. (b) Proline accumulation in wild-type seedlings pre-treated with 20 µM DPI and grown under normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 24 h. Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$. (c) RT-PCR analysis of *P5CS1* and *ProDH1* expression. RT-PCR was performed using total RNAs extracted from seedlings treated for 24 h as described in (b). *APT1* transcripts were amplified as an internal control. Lower panels, quantification of the expression of *P5CS1* and *ProDH1* normalized to *APT1*. (d) Western blot of P5CS and ProDH proteins from seedlings treated for 24 h as described in (b). Rubisco revealed by Ponceau-S staining of all protein was used as a loading control.

Fig. 7. AtRbohD and AtRbohF are required for transient H_2O_2 accumulation induced by salt or mannitol stress.

A. thaliana seedlings were prepared and treated as described in the legend of Fig. 3. H_2O_2 accumulation was measured in seedlings of wild-type and the transposon insertion mutants *atrbohd-3* and *atrbohf-3* at 6 h after stress treatment with either 200 mM NaCl or 400 mM mannitol. Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$.

Fig. 8. AtRbohD and AtRbohF are essential for proline accumulation in response to either NaCl or mannitol.

A. thaliana seedlings were prepared and treated as previously described in the legend of Fig. 3. (a) Proline accumulation was determined in transposon insertion mutant lines *atrbohd-3*, *atrbohf*-3 single mutants and in *atrbohd/f* double mutant in comparison to wild-type grown under normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 24 h. Proline content was also measured in wild type and mutant seedlings grown in the same condition after application of 10 mM H_2O_2. Means (± SE) of three biological replicates with different letters were significantly different at $P < 0.05$. (b) Western blot of P5CS proteins from seedlings treated as described in (a). Rubisco revealed by Ponceau-S staining of all proteins was used as loading control. Quantification of the western blot normalized to Rubisco is shown as bar graph. C, control; N, NaCl; M, mannitol.

Supporting Information

Fig. S1. Early apoplastic H_2O_2 accumulation is mediated through plasma membrane–bound NADPH oxidases in leaves of *A. thaliana* in response to either NaCl or mannitol.

Fig. S2. Proline accumulation in *atrboh* insertion lines.

Fig. S3. qRT-PCR analysis of differential expression of three *AtRboh* genes in wild-type and *atrbohd* and *atrbohf* mutants.

Fig. S4. Changes in Na^+ and K^+ content and Na^+/K^+ ratios in Arabidopsis wild-type (WT) and *atrbohd* and *atrbohf* mutants upon NaCl stress.

Table S1: List of primers used for RT-PCR and qRT-PCR analysis.

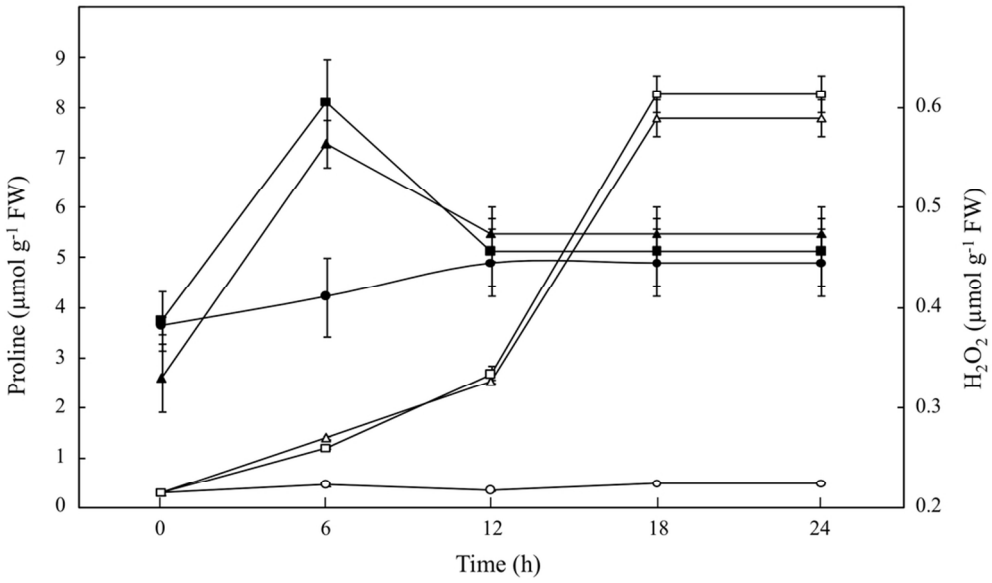

Fig. 1. NaCl and mannitol stresses trigger transient increases in H2O2 and proline accumulation. Twelve-day-old wild-type A. thaliana seedlings grown on 0.5 x MS solid medium were transferred to 0.5 × MS liquid medium for treatment. Plants were then exposed to 200 mM NaCl (triangles), 400 mM mannitol (squares) or neither (circles) for 24 h. The results shown are the means of at least three independent experiments (± SE).

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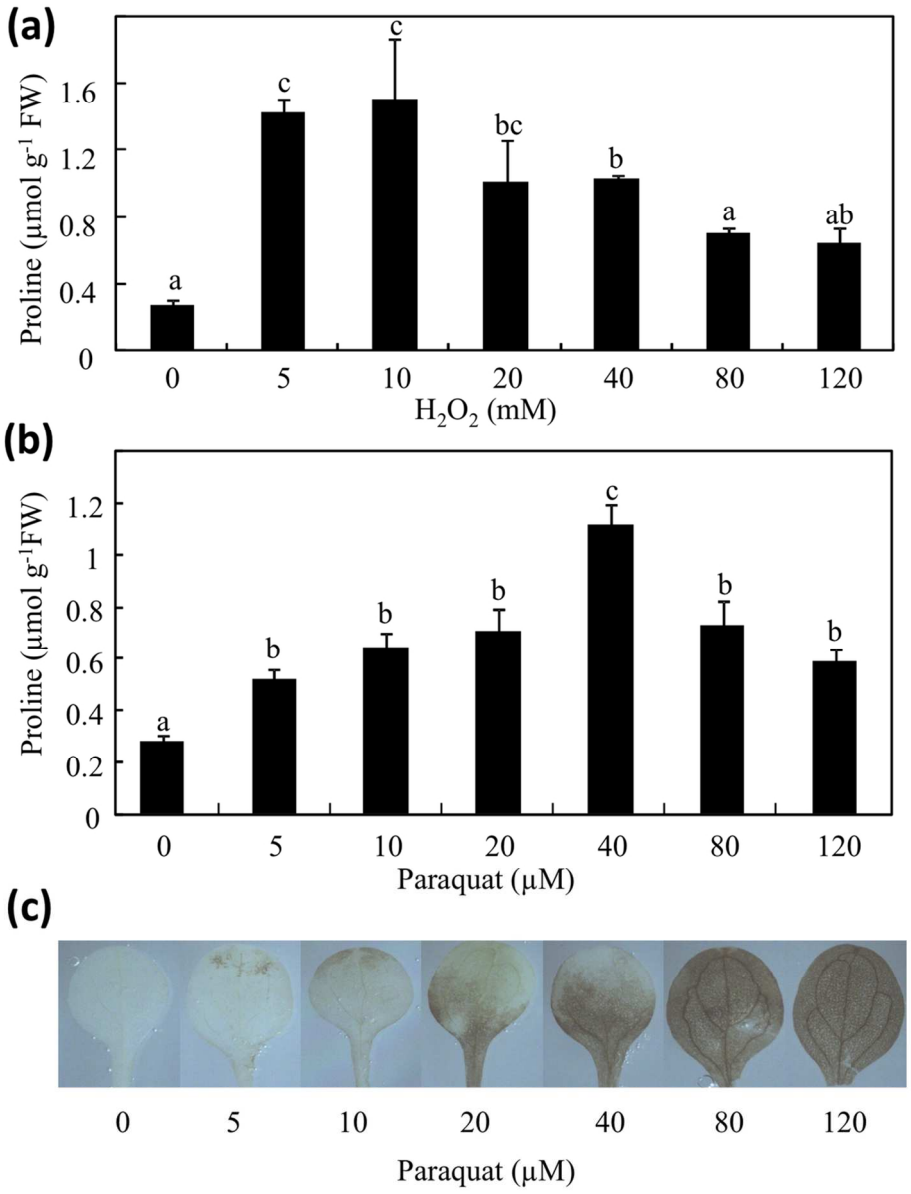

Fig.2. H2O2 induces proline accumulation.
Proline accumulation in wild-type A. thaliana seedlings treated with H2O2 (a) and paraquat (b) for 24 h. Means (± SE) of at least three independent experiments with different letters are significantly different at P < 0.05. (c) H2O2 was visualized by using DAB staining in A. thaliana leaves exposed to different concentrations of paraquat for 24 h.

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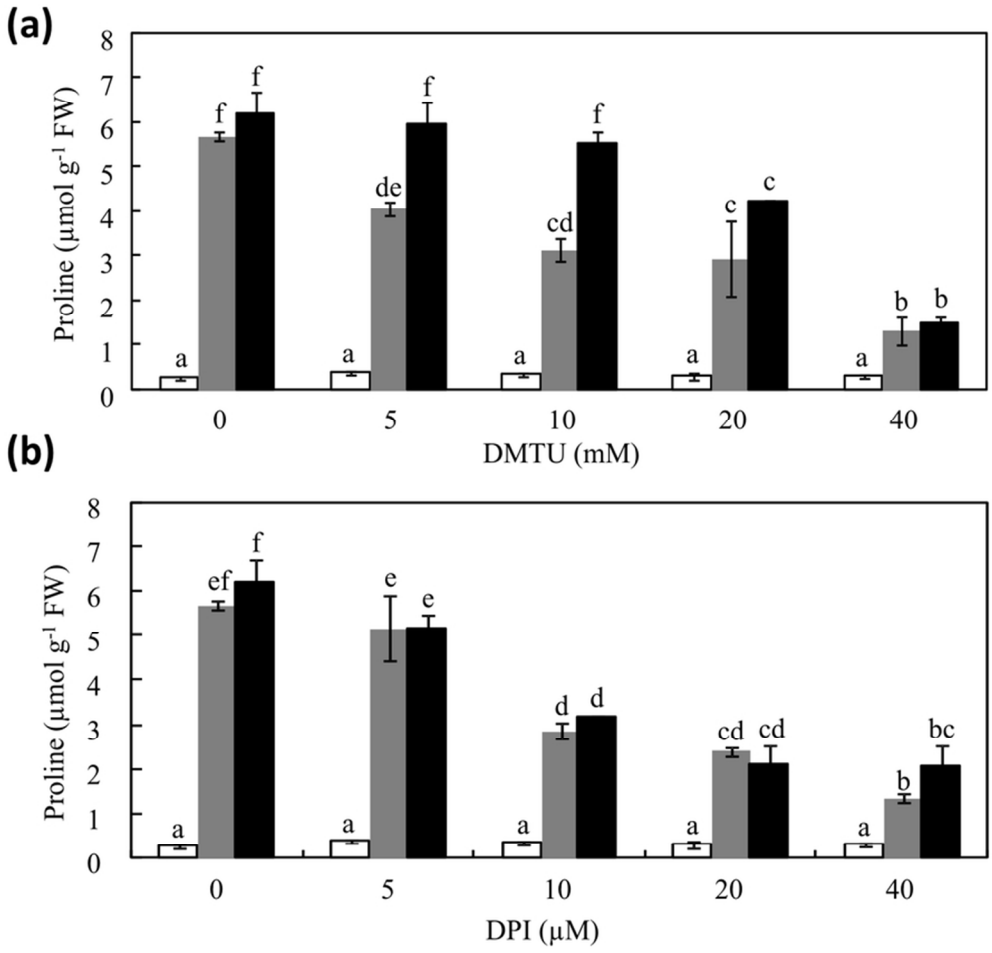

Fig. 3. The NADPH oxidase inhibitor DPI and the H2O2 scavenger DMTU affect proline accumulation induced by salt or mannitol stress.
Twelve-day-old A. thaliana seedlings grown on 0.5 × MS solid medium were transferred to 0.5 × MS liquid medium for treatment. Plants were preincubated with various concentrations of DMTU or DPI for 4 h and then exposed to 200 mM NaCl (grey bars), 400 mM mannitol (black bars) or neither (white bars) for 24 h. Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$.

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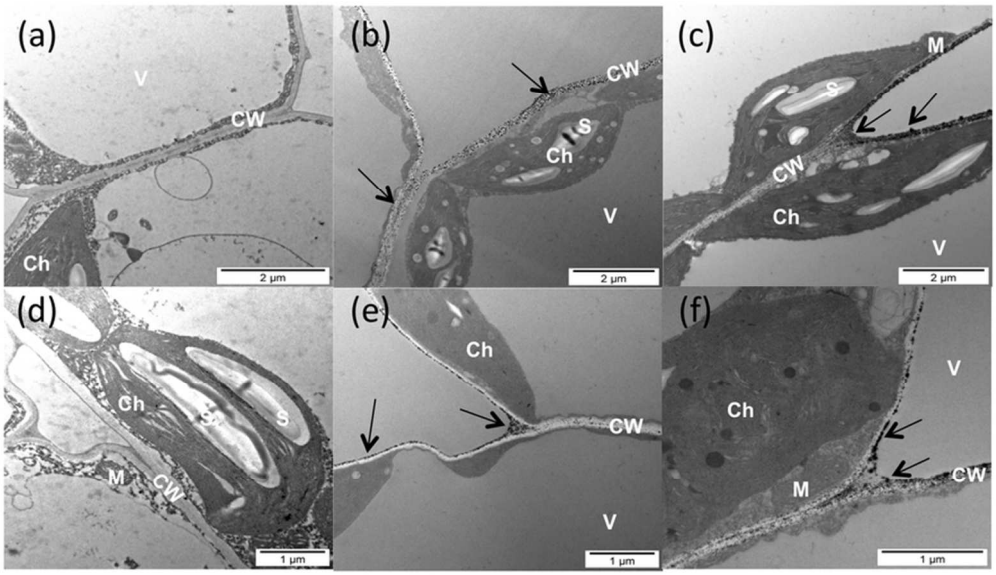

Fig. 4. NaCl and mannitol stresses trigger apoplastic H2O2 accumulation in A. thaliana leaves. Twelve-day-old seedlings grown on 0.5 × MS solid medium were transferred to 0.5 × MS liquid medium. Subcellular localization of H2O2 was detected by CeCl3 staining of leaves of wild-type A. thaliana grown under normal conditions (control, a, d) or in the presence of either 200 mM NaCl (b, e) or 400 mM mannitol (c, f) for 6 h. Arrows indicate electron-dense deposits of cerium perhydroxides formed in the presence of H2O2 and CeCl3. Ch, chloroplast; CW, cell wall; M, mitochondria; S, starch; V, vacuole.

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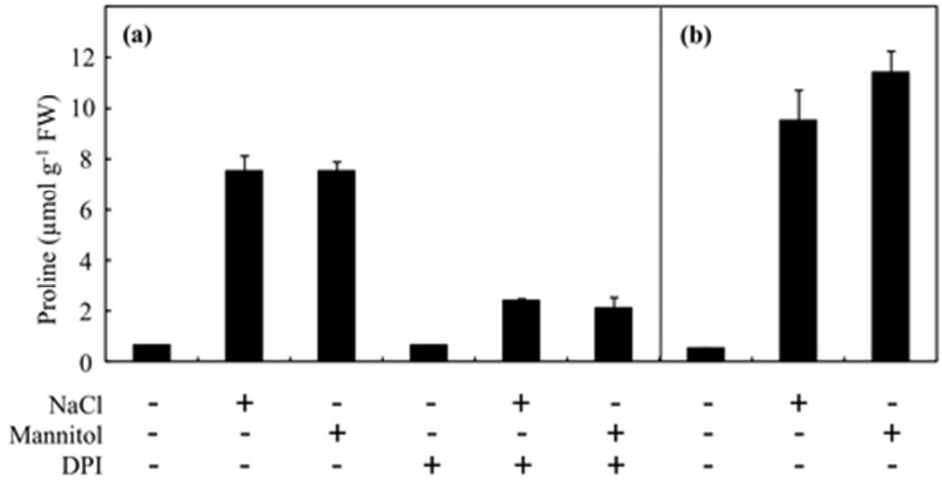

Fig. 5. Inhibition of proline accumulation by DPI can be reversed.
A. thaliana seedlings were pre-treated with 20 µM DPI as described in the legend of Fig. 3 and then treated with either 200 mM NaCl or 400 mM mannitol for 24 h (a). Seedlings were then washed twice and transferred onto NaCl or mannitol medium for another 24 h (b). Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$.

40x20mm (300 x 300 DPI)

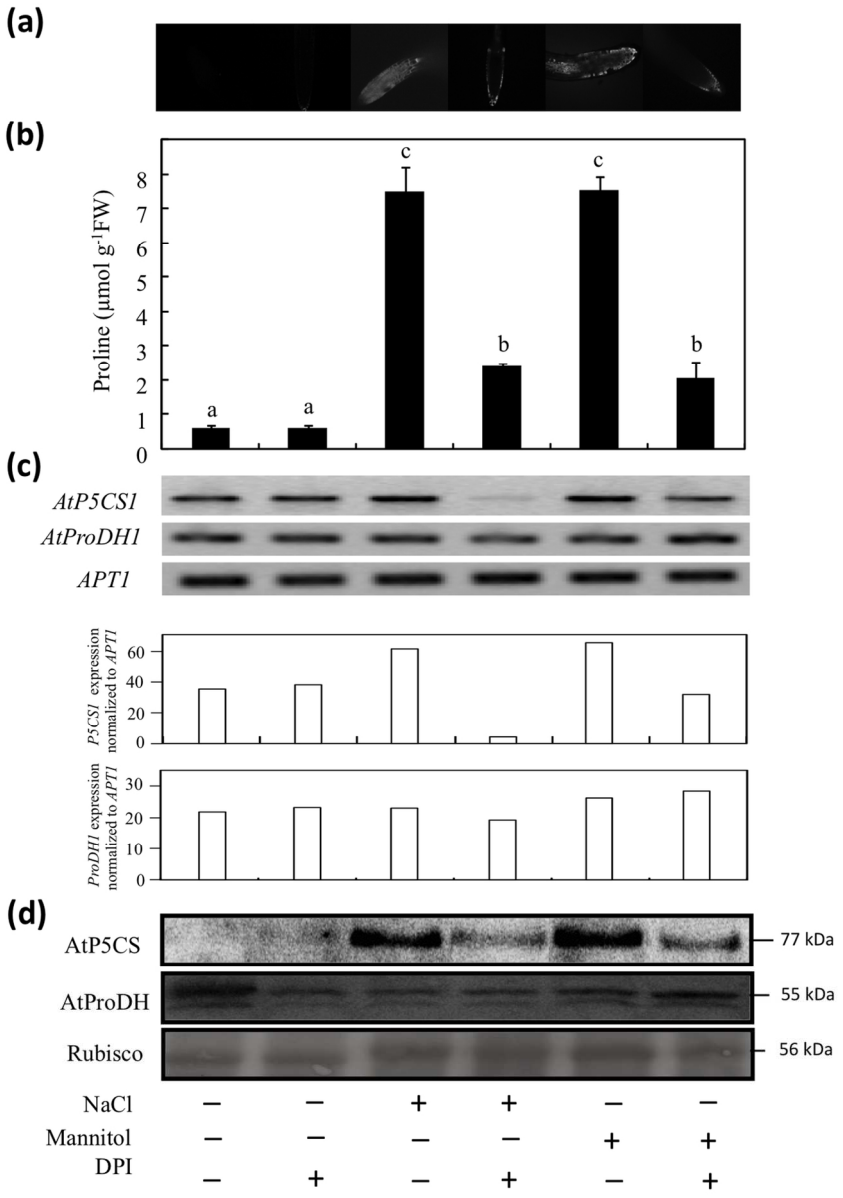

Fig. 6. DPI inhibition of NADPH oxidase activity affects proline metabolism induced by either salt or osmotic stress.

A. thaliana seedlings were prepared and treated as previously described in the legend of Fig. 3. (a) Sites of H2O2 production visualized by using 2',7'-dichlorofluorescein diacetate (H2DCFDA) in wild-type A. thaliana root tips pre-treated with 20 µM DPI and grown under normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 6 h. (b) Proline accumulation in wild-type seedlings pre-treated with 20 µM DPI and grown under normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 24 h. Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$. (c) RT-PCR analysis of P5CS1 and ProDH1 expression. RT-PCR was performed using total RNAs extracted from seedlings treated for 24 h as described in (b). APT1 transcripts were amplified as an internal control. Lower panels, quantification of the expression of P5CS1 and ProDH1 normalized to APT1. (d) Western blot of P5CS and ProDH proteins from seedlings treated for 24 h as described in (b). Rubisco revealed by Ponceau-S staining of all protein was used as a loading control.

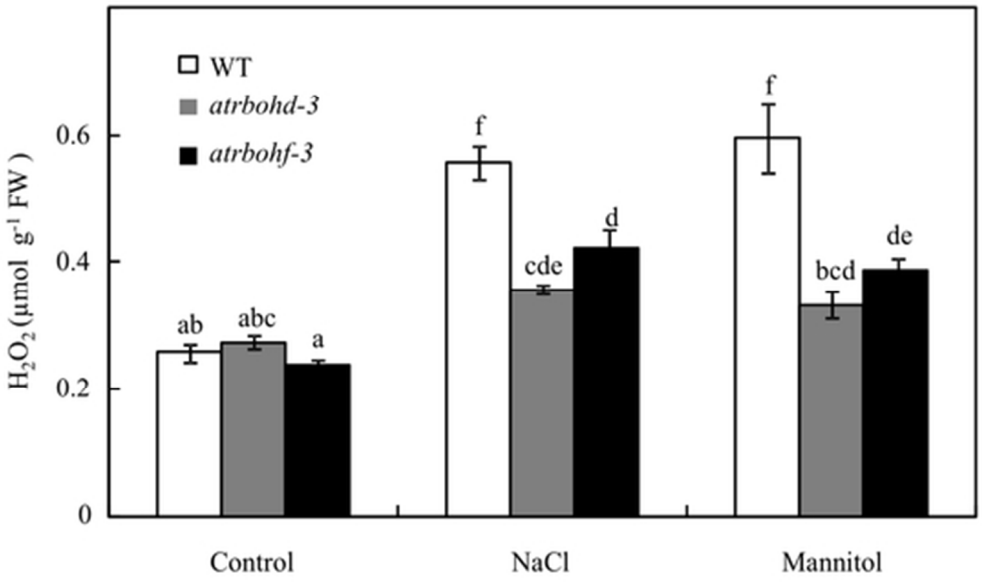

Fig. 7. AtRbohD and AtRbohF are required for transient H2O2 accumulation induced by salt or mannitol stress.

A. thaliana seedlings were prepared and treated as described in the legend of Fig. 3. H2O2 accumulation was measured in seedlings of wild-type and the transposon insertion mutants atrbohd-3 and atrbohf-3 at 6 h after stress treatment with either 200 mM NaCl or 400 mM mannitol. Means (± SE) of three independent experiments with different letters are significantly different at $P < 0.05$.

46x26mm (300 x 300 DPI)

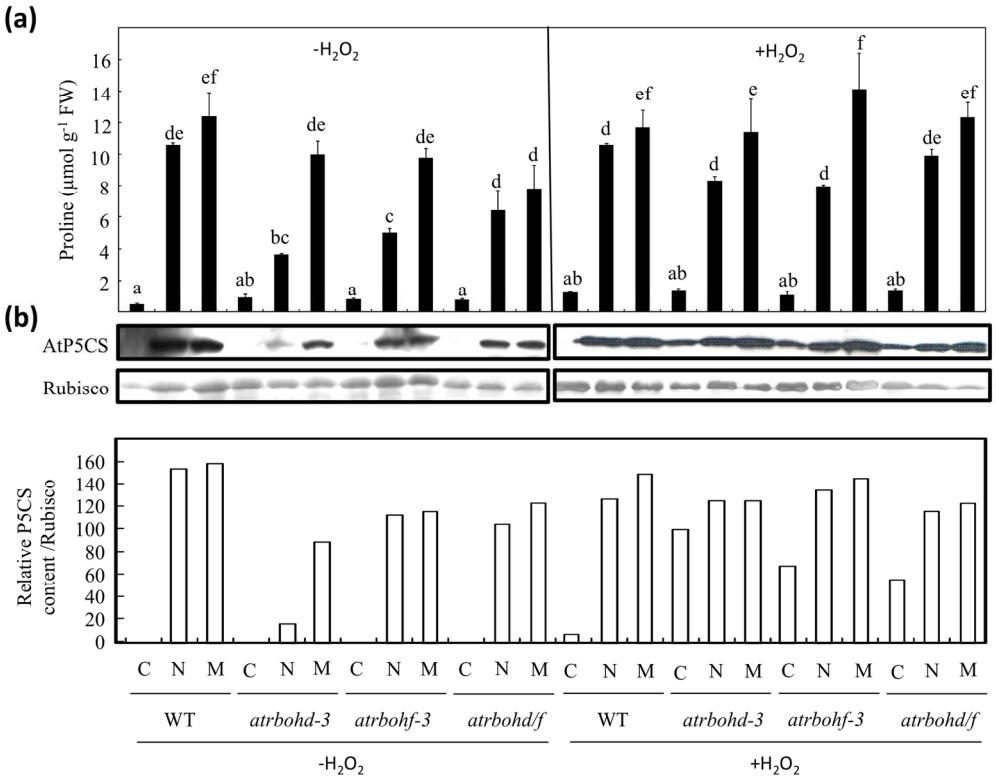

Fig. 8. AtRbohD and AtRbohF are essential for proline accumulation in response to either NaCl or mannitol. A. thaliana seedlings were prepared and treated as previously described in the legend of Fig. 3. (a) Proline accumulation was determined in transposon insertion mutant lines atrbohd-3, atrbohf-3 single mutants and in atrbohd/f double mutant in comparison to wild-type grown under normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 24 h. Proline content was also measured in wild type and mutant seedlings grown in the same condition after application of 10 mM H2O2. Means (± SE) of three biological replicates with different letters were significantly different at $P < 0.05$. (b) Western blot of P5CS proteins from seedlings treated as described in (a). Rubisco revealed by Ponceau-S staining of all proteins was used as loading control. Quantification of the western blot normalized to Rubisco is shown as bar graph. C, control; N, NaCl; M, mannitol.

78x60mm (600 x 600 DPI)