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Kilani Ben Rejeb, Delphine Lefebvre-De Vos, Isabel Le Disquet, Anne-Sophie Leprince ...+5 more authors

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1 **Hydrogen peroxide produced by NADPH oxidases increases proline accumulation**
2 **during salt or mannitol stress in *Arabidopsis thaliana***
3
4 Kilani Ben Rejeb[1,2], Delphine Lefebvre-De Vos[1], Isabel Le Disquet[1], Anne-Sophie Leprince[1],
5 Marianne Bordenave[1], Régis Maldiney[1], Asma Jdey[2], Chedly Abdelly[2], Arnould Savouré[1]
6
7 [1]Sorbonne Universités, UPMC Univ Paris 06, Adaptation des Plantes aux Contraintes
8 Environnementales, Case 156, 4 place Jussieu, F-75005, Paris, France ; [2]Laboratoire des
9 Plantes Extrêmophiles, Centre de Biotechnologie de Borj-Cedria (CBBC), BP 901, Hammam-
10 Lif, 2050, Tunisia.
11
12 *Corresponding author:
13 *Arnould Savouré, email: arnould.savoure@upmc.fr*
14 *Phone: +33 (0)1 44 27 26 72, Fax: +33 (0)1 44 27 61 51*
15
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Results:	1427		.
Discussion:	1468		
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18

19 **Summary**

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

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

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

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

37

38

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

41 **Introduction**

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

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

58 The proline content of plant cells depends on tight regulation of its proline
59 biosynthesis and catabolism. Housekeeping levels of proline biosynthesis occur in the cytosol,
60 but stress-induced biosynthesis is thought to be localized in chloroplasts (Székely *et al.*,
61 2008). When under water-deficit stress, proline is mainly synthesized from glutamate. The
62 bifunctional pyrroline-5-carboxylate synthetase (P5CS) reduces glutamate to glutamyl-5-
63 semialdehyde, which is spontaneously converted to pyrroline-5-carboxylate (P5C). P5C is
64 then reduced to proline by P5C reductase (P5CR). Degradation of proline takes place in
65 mitochondria via the sequential action of proline dehydrogenase (ProDH) and P5C
66 dehydrogenase. The rate-limiting steps in proline biosynthesis and degradation are catalyzed
67 by P5CS and ProDH, respectively. Two closely related P5CS-encoding genes were identified
68 in *Arabidopsis thaliana*. *P5CS1* is induced by drought and salt stress (Savouré *et al.*, 1995;
69 Yoshiba *et al.*, 1995), while *P5CS2* is expressed in dividing cells in cell suspension cultures
70 (Strizhov *et al.*, 1997), in meristematic and reproductive tissues (Székely *et al.*, 2008), and in
71 response to biotic stress such as incompatible plant-pathogen interactions (Fabro *et al.*, 2004).
72 Similarly, *A. thaliana* has two genes for ProDH, *ProDH1* and *ProDH2* (Kiyosue *et al.*, 1996;
73 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*

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

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

123

124 **Materials and Methods**

125 **Growth conditions and stress treatments**

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

134 Surface-sterilized seeds of wild-type (Col-0) and *Arabidopsis* mutant plants were sown onto
135 grids placed on 0.5 × Murashige and Skoog (MS) solid agar medium in Petri dishes according
136 to Parre *et al.* (2007). After 24 h at 4°C to break dormancy, seedlings were allowed to grow at
137 22°C under continuous light (90 µmol photons m^{-2} s^{-1}). Twelve-day-old *Arabidopsis* seedlings
138 were exposed to H_2O_2 (5 to 120 mM), paraquat (PQ, also called methyl viologen, 5 to 120
139 µM), 200 mM NaCl or 400 mM mannitol for 24 h. To alter ROS levels, the seedlings were

140 pre-incubated for 4 h without or with H_2O_2 scavenger dimethylthiourea (DMTU, 5 to 40 mM)
141 or flavoenzyme inhibitor diphenylene iodonium (DPI, 5 to 40 µM) in 0.5 × liquid MS
142 medium and then exposed to either NaCl or mannitol for 24 h.
143
144 **Determination of ion content**
145 Whole plants were harvested, rinsed twice in pure water and quickly blotted. Samples were
146 dried at 60 °C until they reached constant weight then ground. Ions were extracted from
147 samples in 0.5% HNO_3. Na^+ and K^+ were assayed by flame emission photometry (Corning,
148 UK).
149
150 **Proline content measurements**
151 Free proline content was measured according to the Bates method (Bates *et al.*, 1973).
152 Powdered plant samples (50 to 100 mg) were homogenized in 1.5 ml of 3% sulfosalicylic acid
153 and centrifuged at 14,000 × *g* for 10 min at 4 °C. To 1 ml of this extract, 1 ml of acid-
154 ninhydrin and 1ml of glacial acetic acid were added. The reaction mixture was incubated at
155 100°C for 1 h then placed on ice to stop the reaction. In the presence of proline, the reaction
156 produces a red chromogen. The chromogen was extracted with 2 ml of toluene and the
157 absorbance of the resulting upper phase was read at 520 nm. Proline content of samples was
158 calculated by referring to a standard curve drawn from absorbance readings from samples
159 containing known concentrations of proline.
160
161 **H_2O_2 content measurements**
162 H_2O_2 content was determined in a horseradish peroxidase-based spectrophotometric assay
163 following the protocol described by Oracz *et al.* (2009). Plant samples (300 mg FW) were
164 ground in a mortar on ice in 1 ml of 0.2 M perchloric acid. After 15 min of centrifugation at
165 13,000 × *g* at 4°C, the resulting supernatant was neutralized to pH 7.5 with 4 M KOH and
166 then centrifuged at 13,000 × *g* at 4°C. The concentration of H_2O_2 in the supernatant was
167 immediately determined by adding peroxidase with substrates 3-dimethylaminobenzoic acid
168 and 3-methyl-2-benzothiazolidone hydrazone. The increase in absorbance at 590 nm was
169 monitored for 15 min after the addition of peroxidase at 25 °C and compared to a calibration
170 curve obtained with known amounts of fresh H_2O_2.
171
172 **Histochemical detection of H_2O_2**

173 The production of H_2O_2 was visualized *in vivo* by 2',7'-dichlorofluorescein diacetate
174 (H_2DCFDA) or DAB staining methods. *Arabidopsis* roots were collected after 6-h treatments
175 and immersed in 25 µM H_2DCFDA for 15 min in the dark and then washed with 20 mM
176 potassium phosphate buffer pH 6. Fluorescent signals were visualized using a Zeiss ApoTome
177 microscope (excitation, 488 nm; emission, 525 nm). DAB staining was performed as
178 described by Torres *et al.* (2002). Leaves from PQ-treated seedlings were detached and
179 vacuum infiltrated with DAB solution (1 mg ml^{-1} DAB-HCl, pH 3.8). DAB forms a reddish-
180 brown polymer in the presence of H_2O_2 formation. After staining, leaves were cleared in 96%
181 boiling ethanol and observed using a binocular microscope. For both staining methods, digital
182 images were obtained with an AxioCam camera and AxioVision software (Zeiss).
183

184 **Cytochemical detection of H_2O_2**

185 Cytochemical detection of H_2O_2 was carried out according to the method described by
186 Bestwick *et al.* (1997). Leaf samples were collected from treated and control seedlings and
187 incubated in freshly prepared 5 mM cerium chloride for 30 min. The leaves were then fixed in
188 a solution containing 4% glutaraldehyde and 1.5% paraformaldehyde for 1 h, post-fixed for
189 45 min in 1% osmium tetroxide and then embedded in Eponaraldite resin after dehydration in
190 an ethanol series. Sections (60 to 80 nm thick) were cut with a LKB 2128 ultramicrotome,
191 mounted on uncoated copper grids, and stained with 5% uranyl acetate. Sections were
192 observed using a Zeiss912 Omega transmission electron microscope. Digital images were
193 obtained using a Veleta Camera (2kx2k, Olympus) and iTem software (Zeiss).
194

195 **RT-PCR and qRT-PCR Analysis**

196 Total RNA was extracted from 100 mg of plant tissue ground in liquid nitrogen using a mixer
197 mill (MM301, Retsch, Germany). The powder was suspended in 0.5 mL extraction buffer (0.2
198 M Tris-HCl, 0.5% (v/v) SDS, 0.25 M NaCl, 25 mM EDTA) and mixed with 0.5 mL of
199 phenol/chloroform/isoamylalcohol (25:24:1, v/v/v). The aqueous phase was extracted 3 times
200 with phenol/chloroform/isoamylalcohol. Total RNA was then precipitated overnight on ice
201 with 2 M LiCl. After centrifugation (15 min, 10,000 ×x *g*), the pellet was resuspended in 2 M
202 LiCl and left to precipitate for 6 h on ice. Traces of DNA were removed by DNase treatment.
203 RNA quantified by measuring the absorbance at 260 nm using a Nanovue®
204 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.

239 **Results**

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

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

247

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

249 The kinetics of H_2O_2 and proline accumulation in response to either NaCl or mannitol stress
250 showed that proline had already started to increase 6 h after stress was applied when the
251 transient increase in H_2O_2 content was also observed (Fig. 1). To determine whether H_2O_2
252 could be involved in proline accumulation, we first investigated whether adding different
253 concentrations of exogenous H_2O_2 would affect proline accumulation in *A. thaliana*. Results
254 showed that proline accumulation is induced by exogenous H_2O_2 application with 10 mM
255 H_2O_2 having the maximum effect (Fig. 2a).
256 Paraquat (PQ) is a herbicide which induces superoxide anions and subsequently H_2O_2
257 generation *in situ*.. Like H_2O_2, PQ also stimulated proline accumulation in Arabidopsis
258 seedlings in a dose dependent manner with 40 µM PQ having the maximum effect (Fig. 2b).
259 Leaves treated with high concentrations of PQ such as 80 or 100 µM PQ started to bleach
260 after 48 h of treatment due to the high amount of H_2O_2 generated (data not shown) even
261 though proline contents were not higher than at 40 µM PQ. DAB staining revealing the
262 presence of H_2O_2 in leaves demonstrates that exogenously applied PQ gives rise to H_2O_2 *in*
263 *situ* in a dose dependent manner (Fig. 2c). Taken together, these results indicate a possible
264 causal relationship between H_2O_2 and proline production. However, much less proline
265 accumulated in the presence of 10 mM H_2O_2 (about 5 times less) or 40 µM PQ (about 8 times
266 less) than with NaCl or mannitol stress (Fig. 2a, 2b and Fig.1).

267

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

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

272 Fig. 3a, addition of up to 40 mM DMTU inhibited proline accumulation in a dose-dependent
273 manner in seedlings treated with NaCl and to a lesser extent in those treated with mannitol.
274

275 **Subcellular localisation of H_2O_2 accumulation**

276 Multiple sources of H_2O_2 production inside plant cells have been described (Petrov & Van
277 Breusegem, 2012). To investigate the subcellular localization of H_2O_2 accumulation in leaves
278 of *A. thaliana* seedlings, a cytochemical approach using cerium perhydroxide was taken
279 (Bestwick *et al.,* 1997). No visible cerium perhydroxide deposits were observed at the
280 subcellular in *A. thaliana* leaves growing under normal growth conditions, indicating the
281 absence of H_2O_2 using this detection method (Fig. 4). However, both salt and mannitol
282 triggered H_2O_2 accumulation in cell walls of mesophyll cells. Importantly no cerium
283 perhydroxide deposits were observed in the cytosol, chloroplasts or mitochondria indicating
284 that no major H_2O_2 accumulation occurred in these organelles.
285

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

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

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

305 detected in root tips incubated with DPI prior to treatment with either NaCl or mannitol
306 prevented, suggesting H_2O_2 production was inhibited.
307 The rate limiting steps in proline biosynthesis and degradation in *Arabidopsis* are catalyzed by
308 P5CS and ProDH respectively. To further investigate the role of NADPH oxidase in proline
309 metabolism, steady-state transcript levels of *AtP5CS1* and *AtProDH1* were investigated.
310 Semi-quantitative RT-PCR analysis showed that DPI treatment significantly decreased *P5CS1*
311 transcript levels in response to salt and to a lesser extent to mannitol (Fig. 6c). Steady-state
312 transcript levels of *ProDH1* gene were not altered by DPI. Protein levels of P5CS and ProDH
313 were also investigated by Western blot analyses. In wild-type *A. thaliana* plants, 200 mM
314 NaCl and 400 mM mannitol both caused an increase in P5CS protein content compared with
315 untreated plants (Fig. 6d). However, the increase in P5CS protein levels by salt or mannitol
316 was markedly suppressed by pre-treating seedlings with DPI. In contrast to ProDH, whose
317 protein levels were not affected by the different conditions, a good correlation was observed
318 between *P5CS* transcript and protein levels and proline content.
319

320 **Proline accumulation in *atrboh* mutants**

321 If ROS produced by NADPH oxidase is involved in regulating proline accumulation due to
322 NaCl and mannitol stress, then KO mutants defective in NADPH oxidase would be expected
323 to accumulate less proline than wild type. Of the ten *Arabidopsis* NADPH oxidase genes
324 identified, *AtRbohD* and *AtRbohF* are the only ones expressed in all plant organs and they are
325 implicated in abiotic stress physiology (Fluhr, 2009; Marino *et al.*, 2012). *Atrbohd-3* and
326 *atrbohf-3* mutants were therefore selected for our study. Semi-quantitative RT-PCR using
327 wild-type and mutant plants revealed that *rbohd-3* and *rbohf-3* homozygous plants contained
328 no detectable levels of *AtRbohD* and *AtRbohF* transcripts in response to salt treatment (data
329 not shown). In both *atrbohd-3* and *atrbohf-3* mutants, the transient increase in H_2O_2 levels at
330 6 h in response to NaCl and mannitol stresses was not as large as the wild-type increase (Fig.
331 7). After cerium perhydroxide staining no visible cerium perhydroxide deposits were
332 observed in the leaves of *atrbohd* mutant seedlings grown under mannitol or salt stress,
333 indicating the absence of detectable levels of H_2O_2 (Supporting Information Fig. S1).
334 The two NADPH oxidase-deficient *atrbohd* and *atrbohf* mutants were examined for the
335 accumulation of proline and P5CS protein in response to either mannitol or salt stress. As
336 expected, application of 200 mM NaCl or 400 mM mannitol induced proline accumulation in
337 wild-type *Arabidopsis* plants (Fig. 8a). Consistent with the results of DPI treatment in wild-

338 type (see Fig. 6), *atrbohd-3* and *atrbohf-3* mutants accumulated less proline and less P5CS
339 protein than wild-type in the presence of 200 mM NaCl (Fig. 8a, b). The results of 400 mM
340 mannitol treatments were similar although less pronounced (Fig. 8a, b). Similar results for
341 proline accumulation were observed with independent alleles of *atrbohd* and *atrbohf* (T-DNA
342 insertion lines SALK_070610 and SALK_059888 respectively) (Supporting Information
343 Fig. S2). In the double *atrbohd/f* mutant proline levels reached similar levels to those in the
344 single mutants in response to mannitol stress (Fig. 8). The amount of proline that accumulated
345 in response to NaCl in the double mutant was slightly higher but was not as high as the level
346 induced in wild type. It was noted that the decrease in proline and P5CS accumulation was
347 less pronounced in the *atrboh* mutants than in seedlings treated with DPI (Fig. 5 and 6).
348 Perhaps NADPH oxidases other than AtRbohD and AtRbohF or other sources of H_2O_2 are
349 involved in the regulation of proline biosynthesis. To further demonstrate a role for H_2O_2, we
350 reversed the effect of *atrboh* mutation by applying exogenous H_2O_2. Addition of 10 mM
351 H_2O_2 restored the levels of proline and P5CS protein accumulation in mannitol-treated
352 *atrbohd-3, atrbohf-3* and double *atrbohd/f* seedlings (Fig. 8).
353
354 **Discussion**
355 Plant stress tolerance involves diverse mechanisms such as signal perception and
356 transduction, osmolyte accumulation, ion homeostasis, growth regulation and cellular
357 protection from damage triggered by reactive oxygen species (ROS). It is widely accepted
358 that H_2O_2 and other ROS are also important signalling molecules in the activation of defence
359 genes in response to biotic stress (Foyer & Noctor, 2009, Bartoli *et al.*, 2012). The connection
360 between ROS and proline in response to biotic stress has already been highlighted by Fabro *et*
361 *al.* (2004). Nevertheless little is known about the involvement of ROS in the regulation of
362 proline metabolism in response to abiotic stresses. The aim of our work was to evaluate the
363 roles of H_2O_2 in the regulation of proline metabolism in response to two different abiotic
364 stresses in *A. thaliana*. Our results showed that both NaCl and mannitol stress induced proline
365 accumulation and a transient increase in H_2O_2 content (Fig. 1). The timing of these changes
366 might suggest that H_2O_2 could act as a secondary messenger involved in triggering proline
367 biosynthesis. This hypothesis is supported by the observation that treatment with H_2O_2 or PQ
368 also promoted proline accumulation (Fig. 2). However these effects are not as strong as those
369 caused by physiological NaCl or mannitol stresses. The fact that much less proline
370 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.

404 In response to either salt or mannitol stress, *P5CS1* proline biosynthesis transcript and protein
405 levels increased at the same time as proline accumulated. Pretreatment of *A. thaliana* seedling
406 with DPI reduced the salt- or mannitol-induced proline accumulation in a dose-dependent
407 manner (Fig. 6). In addition, DPI significantly diminished *P5CS1* transcript and protein
408 accumulation. The reaction catalyzed by NADPH oxidase uses O_2 to generate superoxide
409 ($O_2^{\cdot -}$), which is then converted into H_2O_2 by apoplastic superoxide dismutase. H_2O_2, as a
410 nonpolar molecule, can easily pass through the plasma membrane probably through
411 aquaporins. H_2O_2 has important roles as a signalling molecule in the regulation of a variety of
412 biological processes. Possibly by redox changes H_2O_2 might directly or indirectly activate
413 unknown signalling components, such as transcription factors, to regulate the transcription of
414 proline biosynthesis genes.
415 The role of Rbohs in the regulation of proline metabolism was further investigated by reverse
416 genetic approach using *atrbohd* and *atrbohf* KO mutants. Compared to wild type, the strong
417 reduction of H_2O_2 production after 6 h in the *atrbohd* mutant in response to NaCl and
418 mannitol, observed to a lesser extent in the *atrbohf*, could be considered to be consistent with
419 *AtRbohD* being the most highly expressed member of the *AtRboh* gene family in response to
420 salt (Leshem *et al*., 2007). Furthermore the cytochemical detection of H_2O_2 indicated that
421 H_2O_2 production during salt and mannitol stress is associated with *AtRbohD* expression.
422 Our study shows that proline accumulation was 20-fold higher in salt-treated wild-type
423 compared to control wild-type plants; however, it was respectively only 5-fold and 9-fold
424 higher in salt-treated *atrbohd* and *atrbohf* seedlings compared to their controls. The double
425 *atrbohd/f* mutant surprisingly produced slightly more proline in response to NaCl than the
426 corresponding single mutants possibly because the double mutant is more sensitive to NaCl.
427 Similarly, a much lower P5CS level was observed in the *atrbohd* mutant, compared to only a
428 marginal reduction in *atrbohf* mutant compared to wild-type plants. Taken together, these
429 results indicate that these NADPH oxidase isoforms are involved in proline accumulation
430 during salt stress with AtRbohD having a prominent role. It was noted that the decrease in
431 proline accumulation was more pronounced in wild-type seedlings treated with DPI than in
432 *atrboh* mutants. This result would implicate other NADPH oxidases in the proline
433 accumulation response. Indeed according to qPCR measurements, *AtRbohA*, *AtRbohB* and
434 *AtRobohC* transcripts are more abundant in *atrbohd* and *atrbohf* mutants than in wild-type
435 under stress, which is possibly preliminary evidence of a feed-back mechanism in these
436 mutants (Supporting Information Fig. S3).

437 Recently, it has been demonstrated that ROS generated by AtrbohF has a specific and
438 predominant role in regulating Na^+ accumulation and soil-salinity tolerance (Jiang *et al.*,
439 2012). When grown in saline soil, *atrbohf* mutant accumulated higher levels of Na^+ than wild-
440 type plants. In addition, Ma *et al.* (2012) have suggested that AtrbohF acts redundantly with
441 AtrbohD in regulating Na^+/K^+ homeostasis. These independent observations lead us to
442 postulate that the difference in proline accumulation in the wild-type and *atrboh* mutants
443 plants could be due to impaired Na^+/K^+ regulation. No differences in Na^+ and K^+ content were
444 found between *atrbohd*, *atrbohf* and wild-type plants subjected to NaCl treatment for 24 h
445 (Supporting Information Fig. S4). However the double *atrbohdf* mutant contained less Na^+
446 but had a higher Na^+/K^+ ratio. Differences in Na^+ accumulation in *atrboh* single mutants
447 between our study and the study of Jiang *et al.* (2012) were probably due to large differences
448 in growth and stress conditions in the two experimental systems, such as continuous light
449 versus 16 h light/8 h dark cycles or short versus long durations of stress. However the
450 regulation of proline accumulation by Rboh in Arabidopsis was probably mainly due to the
451 osmotic stress component of salt stress rather than to the ionic component, because Rboh was
452 involved in the response to both NaCl and mannitol stress. Lastly, since the generation of
453 ROS by Rboh causes changes in the cell redox potential, we postulate that redox-sensitive
454 signalling components or transcription factors may be activated and to influence the
455 expression of proline biosynthesis genes.
456 In conclusion, our results shed new light on the regulation of proline metabolism in response
457 to abiotic stresses showing the involvement of NADPH oxidase and H_2O_2. We show that
458 H_2O_2 is involved in proline accumulation induced by salt and mannitol stresses. First, proline
459 accumulation was preceded by elevated H_2O_2 levels, and scavenging of H_2O_2 by DMTU
460 abolished proline accumulation. Second, we have presented evidence that NADPH oxidases
461 are the potential source of the observed stress-induced H_2O_2 generation. Third, the absence of
462 H_2O_2 production in cell walls and the accumulation of less proline in *atrbohd* and *atrbohf* KO
463 mutants in response to NaCl and mannitol provides convincing genetic evidence that the
464 corresponding NADPH oxidase isoforms contribute to proline accumulation.
465
466
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476
477
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615

616 **Figure legends**

617 **Fig. 1.** NaCl and mannitol stresses trigger transient increases in H_2O_2 and proline
618 accumulation.
619 Twelve-day-old wild-type *A. thaliana* seedlings grown on 0.5 x MS solid medium were
620 transferred to 0.5 × MS liquid medium for treatment. Plants were then exposed to 200 mM
621 NaCl (triangles), 400 mM mannitol (squares) or neither (circles) for 24 h. Open and closed
622 symbols indicate proline and H_2O_2 measurements respectively. The results shown are the
623 means of at least three independent experiments (± SE).
624

625 **Fig. 2.** H_2O_2 induces proline accumulation.
626 Proline accumulation in wild-type *A. thaliana* seedlings treated with H_2O_2 (a) and paraquat
627 (b) for 24 h. Means (± SE) of at least three independent experiments with different letters are
628 significantly different at $P < 0.05$. (c) H_2O_2 was visualized by using DAB staining in *A.*
629 *thaliana* leaves exposed to different concentrations of paraquat for 24 h.
630

631 **Fig. 3.** The NADPH oxidase inhibitor DPI and the H_2O_2 scavenger DMTU affect proline
632 accumulation induced by salt or mannitol stress.
633 Twelve-day-old *A. thaliana* seedlings grown on 0.5 × MS solid medium were transferred to
634 0.5 × MS liquid medium for treatment. Plants were preincubated with various concentrations
635 of DMTU (a) or DPI (b) for 4 h and then exposed to 200 mM NaCl (grey bars), 400 mM
636 mannitol (black bars) or neither (white bars) for 24 h. Means (± SE) of three independent
637 experiments with different letters are significantly different at $P < 0.05$.
638

639 **Fig. 4.** NaCl and mannitol stresses trigger apoplastic H_2O_2 accumulation in *A. thaliana*
640 leaves.
641 Twelve-day-old seedlings grown on 0.5 × MS solid medium were transferred to 0.5 × MS
642 liquid medium. Subcellular localization of H_2O_2 was detected by $CeCl_3$ staining of leaves of
643 wild-type *A. thaliana* grown under normal conditions (control, a, d) or in the presence of
644 either 200 mM NaCl (b, e) or 400 mM mannitol (c, f) for 6 h. Arrows indicate electron-dense
645 deposits of cerium perhydroxides formed in the presence of H_2O_2 and $CeCl_3$. Ch, chloroplast;
646 CW, cell wall; M, mitochondria; S, starch; V, vacuole.
647

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

649 *A. thaliana* seedlings were pre-treated with 20 µM DPI as described in the legend of Fig. 3
650 and then treated with either 200 mM NaCl or 400 mM mannitol for 24 h (a). Seedlings were
651 then washed twice and transferred onto NaCl or mannitol medium for another 24 h (b). Means
652 (± SE) of three independent experiments with different letters are significantly different at $P <$
653 0.05.
654
655 **Fig. 6.** DPI inhibition of NADPH oxidase activity affects proline metabolism induced by
656 either salt or osmotic stress.
657 *A. thaliana* seedlings were prepared and treated as previously described in the legend of Fig.
658 3. (a) Sites of H_2O_2 production visualized by using 2',7'-dichlorofluorescein diacetate
659 (H_2DCFDA) in wild-type *A. thaliana* root tips pre-treated with 20 µM DPI and grown under
660 normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 6 h. (b)
661 Proline accumulation in wild-type seedlings pre-treated with 20 µM DPI and grown under
662 normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for 24 h.
663 Means (± SE) of three independent experiments with different letters are significantly
664 different at $P < 0.05$. (c) RT-PCR analysis of *P5CS1* and *ProDH1* expression. RT-PCR was
665 performed using total RNAs extracted from seedlings treated for 24 h as described in (b).
666 *APT1* transcripts were amplified as an internal control. Lower panels, quantification of the
667 expression of *P5CS1* and *ProDH1* normalized to *APT1*. (d) Western blot of P5CS and ProDH
668 proteins from seedlings treated for 24 h as described in (b). Rubisco revealed by Ponceau-S
669 staining of all protein was used as a loading control.
670
671 **Fig. 7.** AtRbohD and AtRbohF are required for transient H_2O_2 accumulation induced by salt
672 or mannitol stress.
673 *A. thaliana* seedlings were prepared and treated as described in the legend of Fig. 3. H_2O_2
674 accumulation was measured in seedlings of wild-type and the transposon insertion mutants
675 *atrbohd-3* and *atrbohf-3* at 6 h after stress treatment with either 200 mM NaCl or 400 mM
676 mannitol. Means (± SE) of three independent experiments with different letters are
677 significantly different at $P < 0.05$.
678
679 **Fig. 8**. AtRbohD and AtRbohF are essential for proline accumulation in response to either
680 NaCl or mannitol.

681 *A. thaliana* seedlings were prepared and treated as previously described in the legend of Fig.
682 3. (a) Proline accumulation was determined in transposon insertion mutant lines *atrbohd-3*,
683 *atrbohf*-3 single mutants and in *atrbohd/f* double mutant in comparison to wild-type grown
684 under normal conditions or in the presence of either 200 mM NaCl or 400 mM mannitol for
685 24 h. Proline content was also measured in wild type and mutant seedlings grown in the same
686 condition after application of 10 mM H_2O_2. Means (± SE) of three biological replicates with
687 different letters were significantly different at $P < 0.05$. (b) Western blot of P5CS proteins
688 from seedlings treated as described in (a). Rubisco revealed by Ponceau-S staining of all
689 proteins was used as loading control. Quantification of the western blot normalized to
690 Rubisco is shown as bar graph. C, control; N, NaCl; M, mannitol.
691
692
693 **Supporting Information**
694 **Fig. S1.** Early apoplastic H_2O_2 accumulation is mediated through plasma membrane–bound
695 NADPH oxidases in leaves of *A. thaliana* in response to either NaCl or mannitol.
696
697 **Fig. S2.** Proline accumulation in *atrboh* insertion lines.
698
699 **Fig. S3.** qRT-PCR analysis of differential expression of three *AtRboh* genes in wild-type and
700 *atrbohd* and *atrbohf* mutants.
701
702 **Fig. S4.** Changes in Na^+ and K^+ content and Na^+/K^+ ratios in Arabidopsis wild-type (WT) and
703 *atrbohd* and *atrbohf* mutants upon NaCl stress.
704
705 **Table S1:** List of primers used for RT-PCR and qRT-PCR analysis.
706

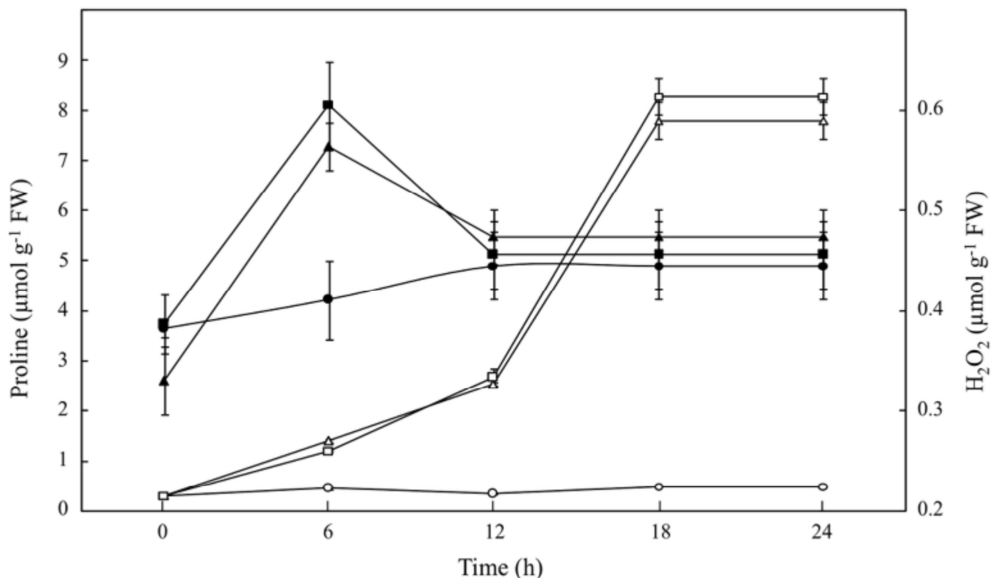

Fig. 1. NaCl and mannitol stresses trigger transient increases in H2O2 and proline accumulation. Twelve-day-old wild-type A. thaliana seedlings grown on 0.5 x MS solid medium were transferred to 0.5 × MS liquid medium for treatment. Plants were then exposed to 200 mM NaCl (triangles), 400 mM mannitol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

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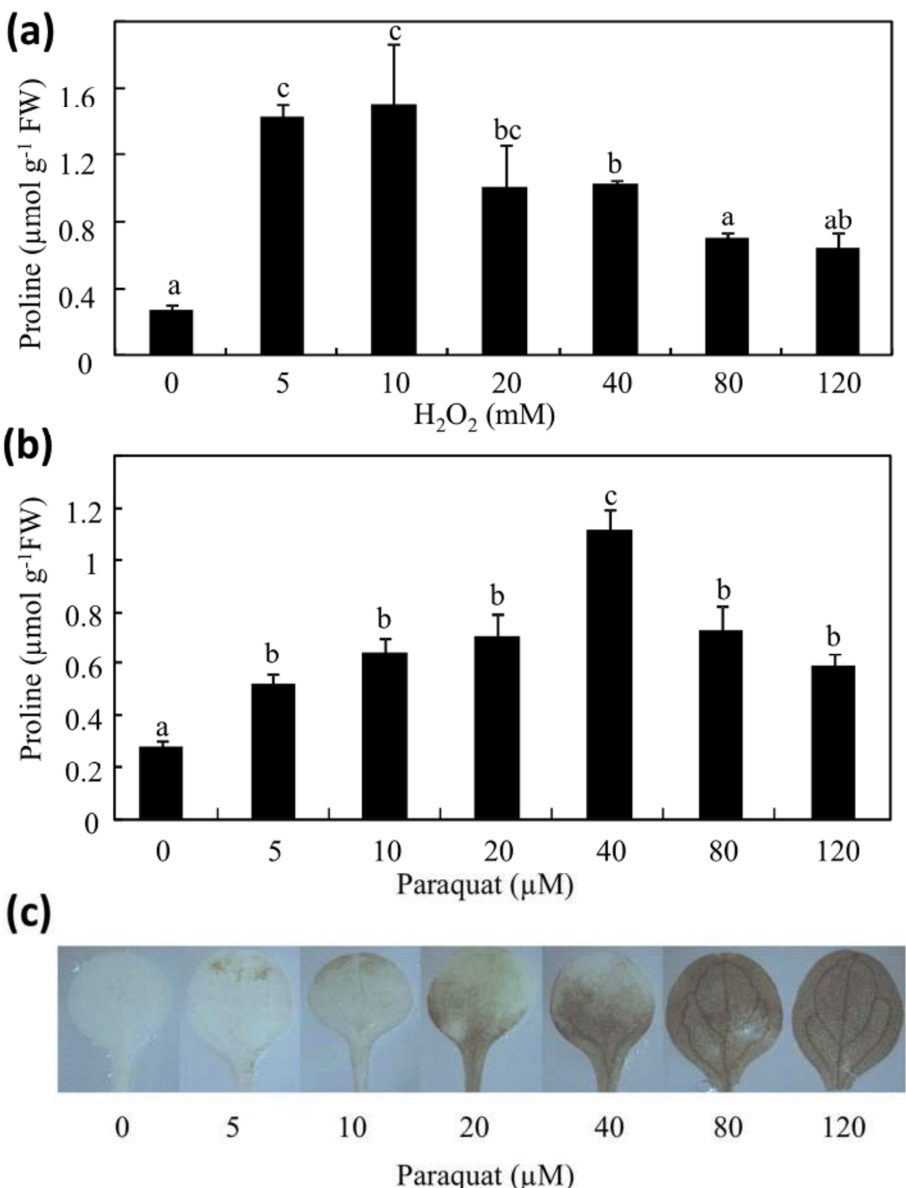

Fig.2. H2O2 induces proline accumulation.
Proline accumulation in wild-type A. thaliana seedlings treated with H2O2 (a) and paraquat (b) for 24 h. Means (± SE) of at least three independent experiments with different letters are significantly different at P < 0.05. (c) H2O2 was visualized by using DAB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

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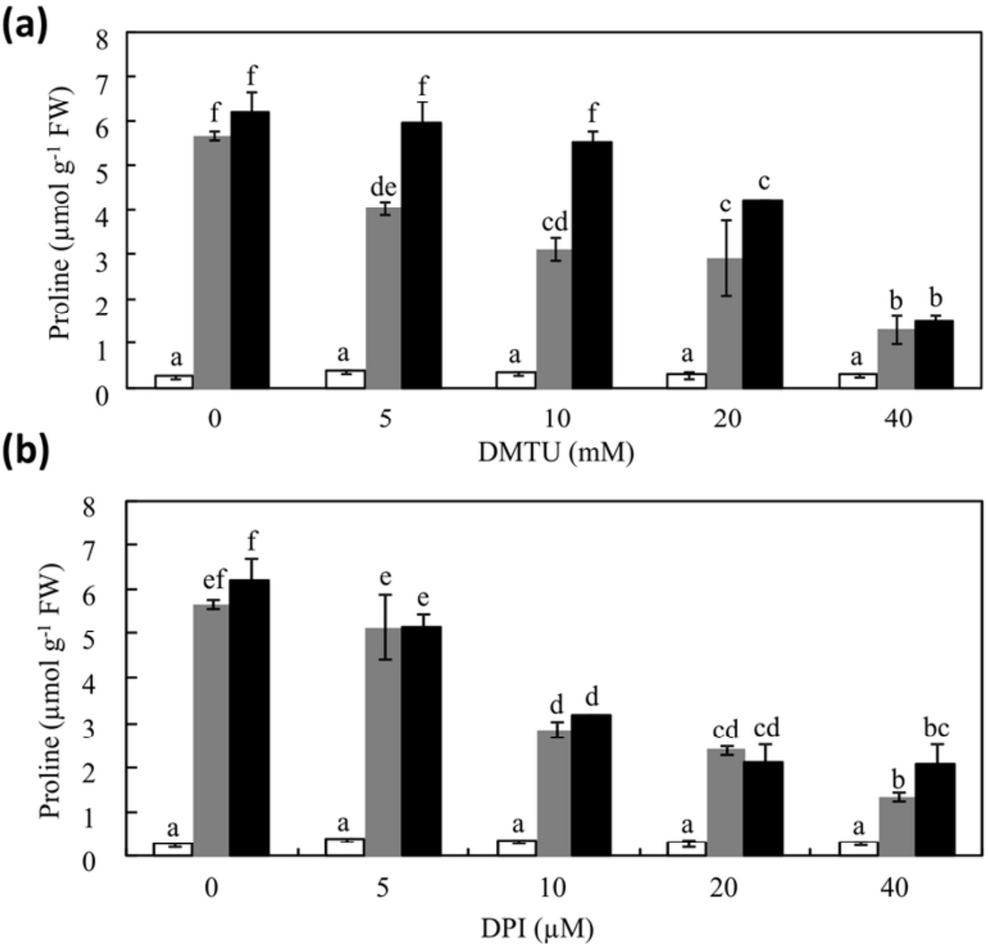

Fig. 3. The NADPH oxidase inhibitor DPI and the H2O2 scavenger DMTU affect proline accumulation induced by salt or mannitol stress.
Twelve-day-old A. thaliana seedlings grown on 0.5 × MS solid medium were transferred to 0.5 × MS liquid medium for treatment. Plants were preincubated with various concentrations 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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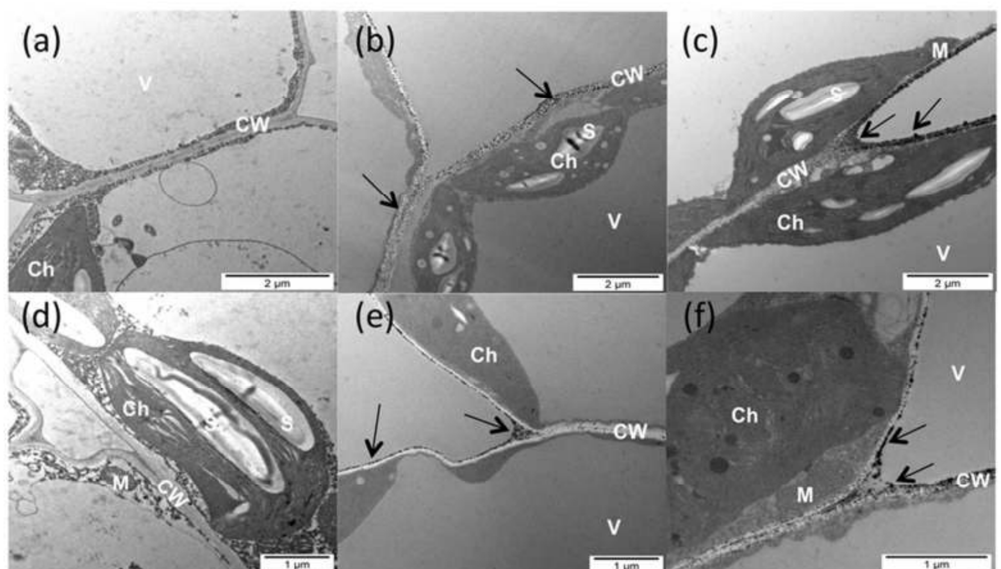

Fig. 4. NaCl and mannitol stresses trigger apoplastic H2O2 accumulation in A. thaliana leaves. Twelve-day-old seedlings grown on 0.5 × MS solid medium were transferred to 0.5 × MS liquid medium. Subcellular localization of H2O2 was detected by CeCl3 staining of leaves of wild-type A. thaliana grown under 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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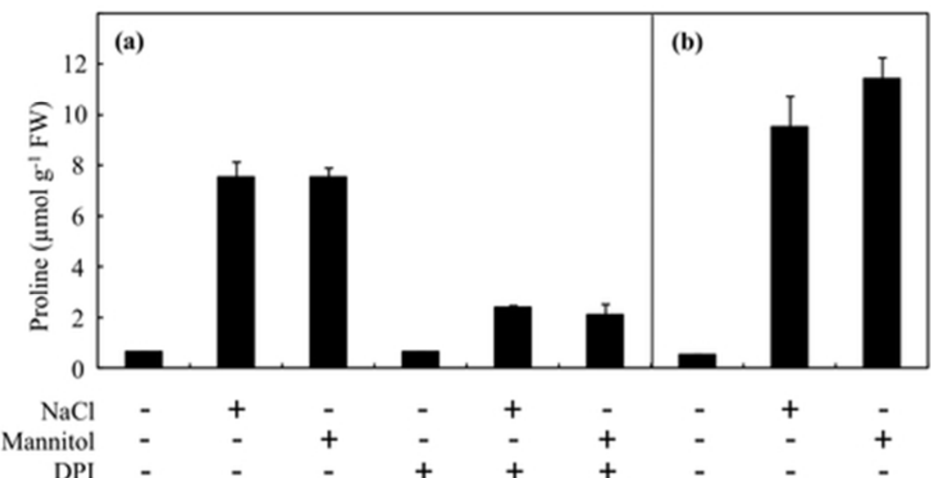

Fig. 5. Inhibition of proline accumulation by DPI can be reversed.
A. thaliana seedlings were pre-treated with 20 µM DPI as described in the legend of Fig. 3 and then treated with either 200 mM NaCl or 400 mM mannitol for 24 h (a). Seedlings were then washed twice and transferred onto NaCl or mannitol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

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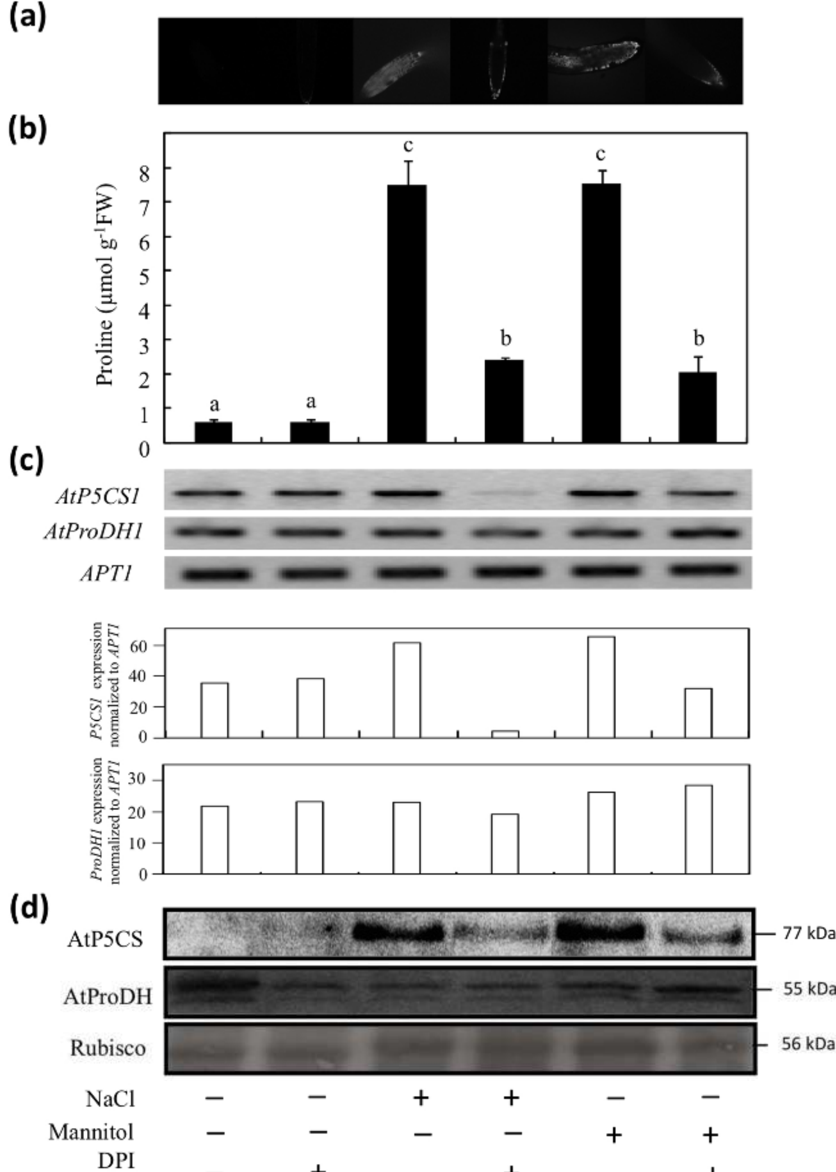

Fig. 6. DPI inhibition of NADPH oxidase activity affects proline metabolism induced by either salt or osmotic stress.

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.

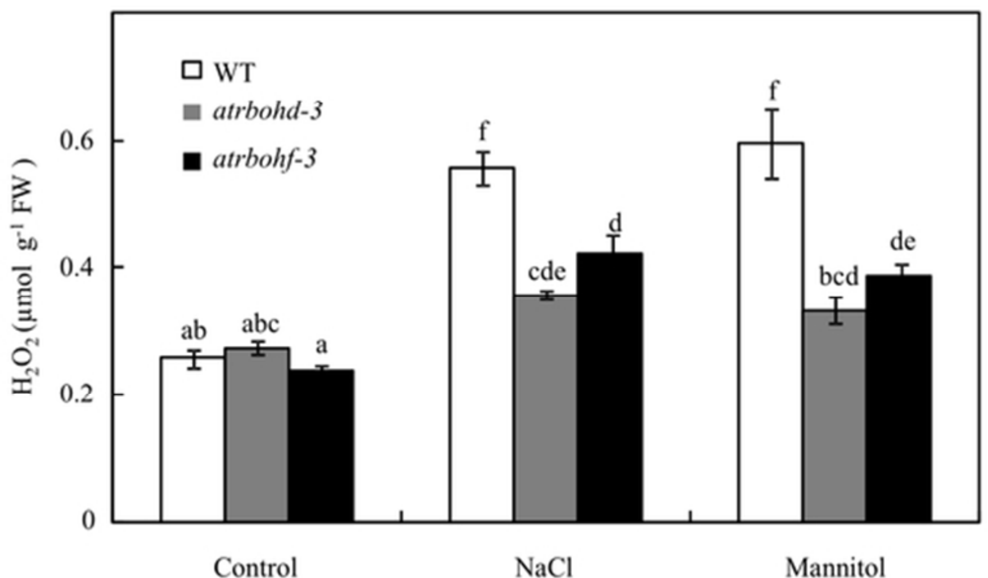

Fig. 7. AtRbohD and AtRbohF are required for transient H2O2 accumulation induced by salt or mannitol stress.
A. thaliana seedlings were prepared and treated as described in the legend of Fig. 3. H2O2 accumulation was measured in seedlings of wild-type and the transposon insertion mutants atrbohd-3 and 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.

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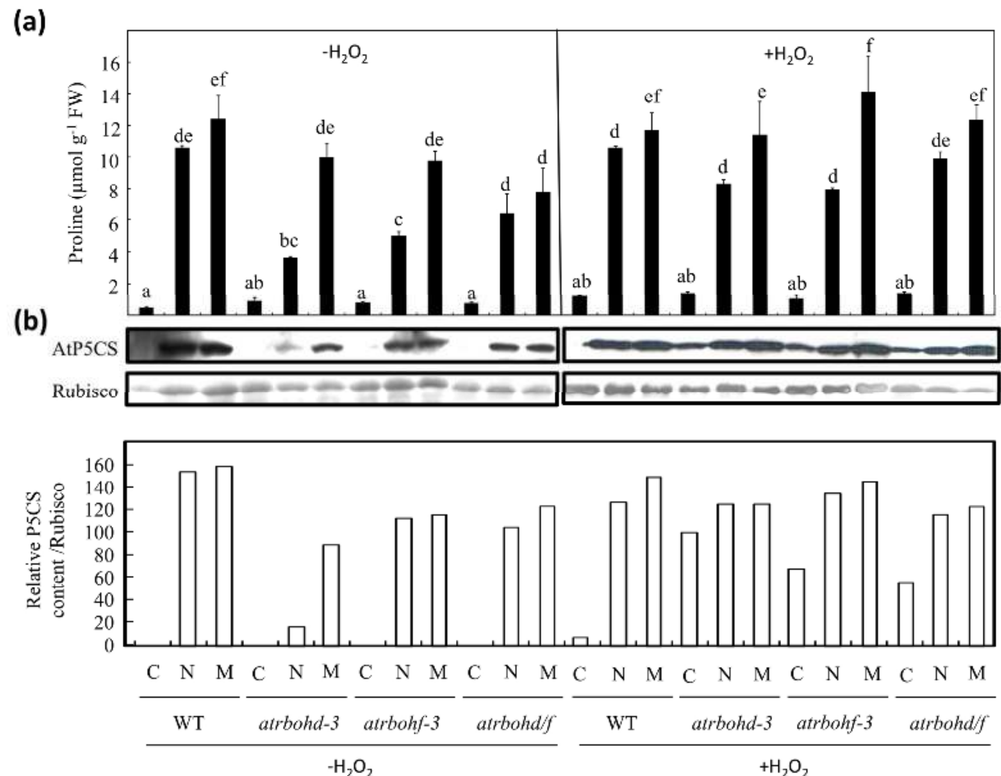

Fig. 8. AtRbohD and AtRbohF are essential for proline accumulation in response to either NaCl or mannitol. A. thaliana seedlings were prepared and treated as previously described in the legend of Fig. 3. (a) Proline accumulation was determined in transposon insertion mutant lines atrbohd-3, atrbohf-3 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.

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