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RESEARCH PAPER

The phosphatidylcholine-hydrolysing phospholipase C NPC4 plays a role in response of *Arabidopsis* roots to salt stress

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Abstract

Phosphatidylcholine-hydrolysing phospholipase C, also known as non-specific phospholipase C (NPC), is a new member of the plant phospholipase family that reacts to environmental stresses such as phosphate deficiency and aluminium toxicity, and has a role in root development and brassinolide signalling. Expression of *NPC4*, one of the six NPC genes in *Arabidopsis*, was highly induced by NaCl. Maximum expression was observed from 3 h to 6 h after the salt treatment and was dependent on salt concentration. Results of histochemical analysis of P_{NPC4}*:GUS* plants showed the localization of salt-induced expression in root tips. On the biochemical level, increased NPC enzyme activity, indicated by accumulation of diacylglycerol, was observed as early as after 30 min of salt treatment of *Arabidopsis* seedlings. Phenotype analysis of *NPC4* knockout plants showed increased sensitivity to salinity as compared with wild-type plants. Under salt stress *npc4* plants had shorter roots, lower fresh weight, and reduced seed germination. Expression levels of abscisic acid-related genes *ABI1*, *ABI2*, *RAB18*, *PP2CA*, and *SOT12* were substantially reduced in salt-treated *npc4* plants. These observations demonstrate a role for NPC4 in the response of *Arabidopsis* to salt stress.

Key words: *Arabidopsis thaliana*, diacylglycerol, phospholipase C, salt stress.

Introduction

Phospholipases are key components of the plant phospholipid signalling network. Besides their metabolic functions they play a key role in signal transduction mechanisms in plant cells. This phospholipid signalling network includes in particular phosphoinositide-specific phospholipase C (PI-PLC), phospholipase D (PLD), and phospholipases A_1 and A_2 (PLA_1 and PLA_2). Stimulation of the signalling network is involved in many responses of plants to both biotic and abiotic adverse environmental factors such as wounding, fungal and bacterial attack, drought, cold, and salt stress (Li *et al.*, 2009; Xue *et al.*, 2009; Munnik, 2010).

A link between water stress (salt, osmotic, and drought stress) and the phospholipid signalling network, particularly PLC and PLD, has been shown many times. Recently

Abbreviations: ABA, abscisic acid; ABI, ABA-insensitive; bodipy, boron-dipyrromethene (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene); BY-2, Bright Yellow 2; CBL, calcineurin B-like; CIPK24, CBL-interacting protein kinase 24; COR78, cold-regulated 78; DAG, diacylglycerol; DGK, diacylglycerol kinase; DR5, direct repeat5; GUS, β-glucuronidase; IP_3, inositol 1,4,5-trisphosphate; LTI78, low-temperature-induced 78; MS, Murashige–Skoog, NHX, Na^+/H^+ exchanger; NPC, non-specific phospholipase C; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA, phospholipase A; PC-PLC, phosphatidylcholine-hydrolysing phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PP2CA, protein phosphatase 2CA; RAB18, responsive to ABA 18; RD29, desiccation-responsive 29; SIGNAL, Salk Institute Genome Analysis Laboratory; SOS, salt overly sensitive; SOT, sulphotransferase; TOR, target of rapamycin; TSPO, tryptophan-rich sensory protein; UBQ10, ubiquitin 10; UPL, Universal Probe Library; VBI-0, Virginia Bright Italia 0; WT, wild-type; X-Gluc, 5-bromo-4-chloro-3-indolyl glucuronide.

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Munnik and Vermeer (2010) described osmotic stress-induced PI-PLC signalling in detail, whereas Hong *et al.* (2010) presented data on PLD and phosphatidic acid (PA) signalling in response to drought and salinity. Rapid accumulation of both the PI-PLC substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) and the product inositol 1,4,5-trisphosphate (IP_3) as a response to water stress was described in, for example, *Arabidopsis*, tobacco, and rice. The importance of PI-PLC in water stress-related processes was confirmed by PI-PLC gene manipulation (Wang *et al.*, 2008; Georges *et al.*, 2009). The involvement of PLDα1, α3, δ, and ϵ in salt, osmotic, and drought stress was also demonstrated (Mane *et al.*, 2007; Hong *et al.*, 2008*a*, *b*; Bargmann *et al.*, 2009).

Besides PI-PLC, phospholipase C hydrolysing phosphatidylcholine (PC-PLC), also termed non-specific phospholipase C (NPC), has been described. This enzyme that generates diacylglycerol (DAG) through glycerophospholipid hydrolysis [mainly phosphatidylcholine (PC)] has been characterized in animals (Exton, 1994) and is well known and characterized in bacteria (Titball, 1993). Based on the amino acid sequence similarity with the bacterial Gram-negative (non-haemolytic) PC-PLC family, Nakamura *et al.* (2005) identified six putative PC-PLC genes in *Arabidopsis* designated *NPC1–NPC6*. In that work, NPC4 was expressed in *Escherichia coli*, and NPC4 protein revealed Ca^{2+}-independent phospholipase activity that preferred PC over phosphatidylethanolamine (PE) as the substrate. NPC4 showed very low activity for PIP_2 as a substrate. To date, PC-PLC activity was reported to have a metabolic function in DAG exchange from phospholipid to galactolipids in plants (Andersson *et al.*, 2005; Gaude *et al.*, 2008; Tjellström *et al.*, 2008). Down-regulation of PC-PLC in response to elicitor signalling has been described previously. A rapid decrease of DAG levels in tobacco VBI-0 cells was found after treatment with the elicitor cryptogein from *Phytophthora cryptogea* (Scherer *et al.*, 2002). Similarly, inhibition of PC-PLC activity was observed after treatment of both tobacco Bright Yellow 2 (BY-2) cells and pollen tubes with aluminium (Pejchar *et al.*, 2010). The role of NPC3 and NPC4 in root development and brassinolide signalling was also shown in *Arabidopsis* (Wimalasekera *et al.*, 2010). Recently Reddy *et al.* (2010) expressed *NPC3* in *E. coli* and showed that purified NPC3 protein has lysophosphatidic acid phosphatase activity.

The aim of this work was to investigate the function of *Arabidopsis* NPCs further in relation to salt stress. Here it is shown that NPC4 is a component of the salt stress response in *Arabidopsis*.

Materials and methods

Plant material

Arabidopsis thaliana Columbia (Col-0) seeds were obtained from Lehle seeds and used as wild-type (WT) controls. Two T-DNA insertion lines were used in experiments: *npc4-1* (SALK_046713) from the Salk Institute Genome Analysis Laboratory (SIGNAL) collection (Alonso, 2003) and *npc4-2* (GK-571E10) from the GABI-KAT collection (Rosso *et al.*, 2003). For the first characterization of these mutants, see Wimalasekera *et al.* (2010).

Salt treatment

T-DNA mutants and WT *Arabidopsis* plants were grown on agar plates containing 4.4 g l^{-1} MS (Murashige–Skoog) basal salts, sucrose (10 g l^{-1}), MES (0.5 g l^{-1}), inositol (0.1 g l^{-1}), 1% (w/v) agar (pH 5.8) supplemented with 50 mM or 100 mM NaCl. Seeds were surface sterilized using 30% (v/v) bleach solution for 10 min and rinsed five times with sterile water. After planting seeds on agar (45 seeds per plate for weighing and 13 seeds per plate for root growth analysis), the plates were transferred for 4 d to the dark at 4 °C in order to synchronize germination. The plants were grown in a horizontal (weight) or vertical (root growth) position in a growth chamber at 22 °C under long day conditions (16 h/8 h light/dark cycle) and weighted or measured after 14 d of cultivation. Documentation was done by scanning (Canon CanoScan 8800F). Root measurements were done using JMicroVision 1.2.7 software.

Germination

The same basal medium as in the growth experiment with 45 seeds per plate and four replicates and with 150 mM NaCl was used in the germination test. The growth conditions were continuous light at 23 °C. Germinated seeds were counted at 24, 30, 36, and 42 h after transferring seeds from 4 °C.

Hydroponic cultivation

The seeds were surface sterilized and stratified as described above and sown onto rollers cut from *Grodan®* Master slab saturated with nutrient solution. Plants were cultivated in containers of 2.5 l in modified half-strength Hoagland's solution (Hoagland and Arnon, 1950). The nutrient concentrations were as follows: 0.5 mM $NH_4H_2PO_4$, 3 mM KNO_3, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 24.5 μM ferric citrate, 0.45 μM KI, 4.85 μM H_3BO_3, 5.92 μM $MnSO_4 \cdot 4H_2O$, 0.7 μM $ZnSO_4.7H_2O$, 0.1 μM $Na_2MoO_4 \cdot 2H_2O$, 0.01 μM $CuSO_4 \cdot 5H_2O$, 0.01 μM $CoCl_2 \cdot 6H_2O$, 10.02 μM Na_2EDTA, 10 μM $FeSO_4 \cdot 7H_2O$, 55.51 μM myo-inositol, 0.81 μM nicotinic acid, 0.49 μM pyridoxin, 2.97 μM thiamin. The solution was replaced for the first time when plants were 2 weeks old, and thereafter once a week. Aeration of the solution was carried out for 15 min every 3 h using an aquarium air pump. A 10 h/14 h light/dark cycle at 75% relative humidity and day/night temperatures of 22 °C and 20 °C, respectively, were used.

Quantitative RT-PCR

Hydroponically grown 5-week-old plants at the stage of leaf rosettes were used for expression analysis. For the determination of the basal expression of the studied genes, the root and leaf samples were collected immediately prior to the exchange of the nutrient solution. The original nutrient solution was replaced with a fresh solution with or without salt. NaCl concentrations were 25, 50, and 100 mM. Root and leaf samples were collected 1, 2, 3, 6, 12, and 36 h after changing the solution and instantly frozen in liquid nitrogen. RNA was isolated using a Spectrum Plant Total RNA Kit (Sigma-Aldrich), a Turbo DNA-free Kit (Applied Biosystems) was used for DNA removal, and a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used for cDNA synthesis. The reverse transcription reaction was primed with anchored-oligo$(DT)_{18}$ primer. Quantitative PCR was performed with a LightCycler® 480 system (Roche) using the LightCycler® Probes Master with the corresponding hydrolysis probe (UPL Roche) to detect the expression of *NPC* isoforms and reference genes, and LightCycler® 480 SYBR Green I Master (Roche) for the other genes. *Actin2* and *UBQ10* were used as reference genes

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for the normalization of target gene expression. Fold change in expression of the target gene was calculated using the equation (Pfaffl, 2001):

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(control-sample)}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(control-sample)}}$$

Primers and the probes are described in Supplementary Tables S1 and S2 available at *JXB* online.

Histochemical β-glucuronidase (GUS) staining

Construction of promoter:GUS plants was described previously (Wimalasekera *et al.*, 2010). The histochemical GUS assay (Jefferson *et al.*, 1987) was carried out on seedlings and 5-week-old plants. T_2 seeds of P_{NPC3}:*GUS* and P_{NPC4}:*GUS* were grown on agar plates under the same conditions as described in 'Salt treatment' (see above). Ten-day-old seedlings were transferred to a 12-well plate containing 1 ml of half-strength Hoagland's solution with 2% (w/v) sucrose with or without 100 mM NaCl. After 24 h incubation, the plants were immersed in X-Gluc buffer [2 mM X-Gluc, 50 mM $NaPO_4$ pH 7.0, 0.5% (v/v) Triton-X, 0.5 mM K-ferricyanide] for 16 h at 37 °C. Chlorophyll of the green parts was removed by repeated washing in 80% (v/v) ethanol. To determine NaCl-mediated expression of *NPC4* in adult plants, P_{NPC3}:*GUS* and P_{NPC4}:*GUS* were grown hydroponically for 5 weeks and exposed to 100 mM NaCl for 4 h. The staining procedure was the same as in the case of seedlings. Observations were done on a Nikon SMZ 1500 zoom stereoscopic microscope coupled to a Nikon DS-5M digital camera.

PC-PLC activity in salt-treated Arabidopsis *seedlings*

Seven-day-old *Arabidopsis* seedlings (five seedlings for each sample) were transferred from agar plates to 900 μl of water and labelled with 0.66 μg ml^{-1} of fluorescent phosphatidylcholine (bodipy-PC, D-3771, Invitrogen, USA) for 10 min. Then, 100 μl of NaCl solution was added to obtain final concentrations of 10–100 mM and seedlings were incubated on an orbital shaker at 23 °C in the dark for different times. Lipids were extracted by the modified method of Bligh and Dyer (1959) by addition of 4 ml of methanol/chloroform 2/1 (v/v) and 2 ml of 0.1 M KCl 30 min later. Samples were centrifuged for 15 min at 420 *g*. The lower phase was evaporated to dryness by a vacuum evaporator and redissolved in ethanol. Samples were applied on HP-TLC silica gel-60 plates (Merck KGaA, Darmstadt, Germany) by an ATS4 sampler (Camag, Muttenz, Switzerland). After 10 min of saturation, plates were developed in the horizontal developing chamber (Camag) in a mobile phase of acetone/chloroform 1/1 (v/v). Plates were dried, scanned using a Fuji FLA-7000 fluorescence scanner (Fujifilm, Tokyo, Japan), and analysed by Kodak ds 1D software. Identification of the spot corresponding to bodipy-DAG was based on comparison with the bodipy-DAG standard prepared as described previously (Pejchar *et al.*, 2010).

Results

Expression of the NPC gene family in response to salt

Arabidopsis plants were grown hydroponically for 5 weeks and afterwards were treated with Hoagland medium supplemented with 100 mM NaCl for 1, 2, 3, 6, 12, and 36 h. Expression of all members of the NPC gene family was measured by quantitative RT-PCR in both roots and leaves of control non-treated plants and salt-treated plants. A slight increase in NPC6 gene expression in roots was observed after 6 h of treatment (Fig. 1). Similarly, gene

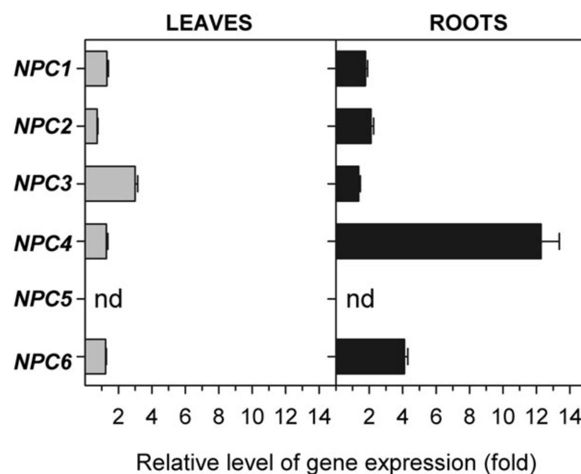

Fig. 1. Expression pattern of the NPC genes in NaCl-treated plants. The transcript levels of NPC genes were measured by quantitative real-time PCR in leaves and roots of non-treated plants and in plants treated with 100 mM NaCl for 6 h. *Actin2* and *UBQ10* were used as internal controls. The expression of each gene in non-treated controls was set to 1. Data represent the means ±SE, *n*=3 discrete samples from one biological experiment. This experiment was repeated three times with similar results. NPC, non-specific phospholipase C; nd, not determined.

expression of *NPC2* was slightly increased after 12 h of salt treatment (data not shown). However, the most meaningful rise in gene expression was shown by *NPC4*. Quantification of expression of *NPC5* was not possible because the transcript level in both non-treated and treated plants was too low in roots and leaves. This is consistent with published observations (Nakamura *et al.*, 2005; Wimalasekera *et al.*, 2010) where *NPC5* transcript was found only in flowers.

In order to investigate the expression pattern of *NPC4* further, *Arabidopsis* plants were also subjected to lower NaCl concentrations (25 mM and 50 mM) for identical amounts of time to those used in previous experiments. The results showed very distinct time and concentration dependence (Fig. 2). In both leaves and roots there was a transient increase in expression observed after 3–6 h of treatment. Expression in roots was about twice as high as that in leaves. In roots treated with 25 mM NaCl for 6 h the expression of *NPC4* was three times higher than *NPC4* expression in control plants. Treatment with 50 mM NaCl for 6 h led to a 5-fold increase in the expression level of *NPC4* in roots. Treatment with 100 mM NaCl induced an increase in expression in roots as early as after 1 h of the treatment, and the expression remained high for all sampled times. A rapid and transient increase of *NPC4* expression may indicate a signalling function for NPC4.

Activity of NPC in salt-treated Arabidopsis *seedlings*

A method based on using fluorescently labelled PC (bodipy-PC) to determine the activity of NPC in tobacco cells was recently reported (Pejchar *et al.*, 2010; Wimalasekera *et al.*, 2010). A similar approach was employed to determine the

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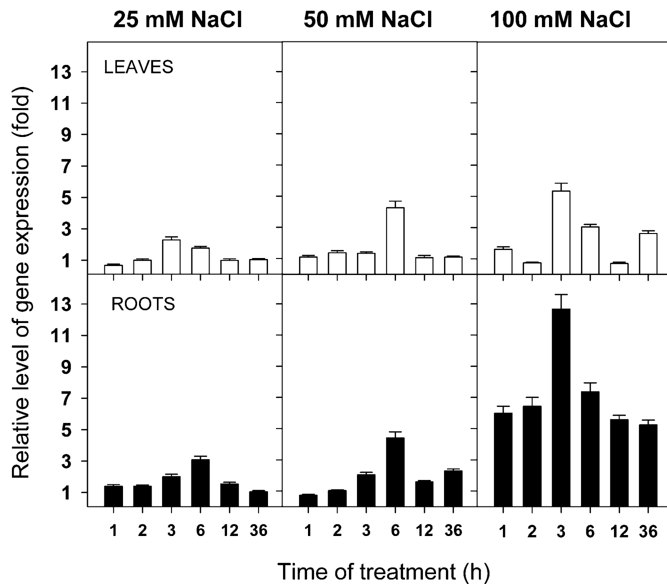

Fig. 2. Detailed analysis of NPC4 expression in NaCl-treated plants. The transcript levels of the NPC4 gene were measured by quantitative real-time PCR in leaves and roots of non-treated plants and in plants treated with different concentrations of NaCl (25–100 mM) for different times (1–36 h). *Actin2* and *UBQ10* were used as internal controls. The expression of *NPC4* in non-treated controls at the respective times was set to 1. Data represent the means ±SE, *n*=2 discrete samples from one biological experiment. This experiment was repeated twice with similar results.

activity of NPC in *Arabidopsis* seedlings. In order to find out if the observed increase in *NPC4* expression is recognizable at the level of enzyme activity, 1-week-old *Arabidopsis* seedlings were labelled with bodipy-PC and then treated with different NaCl concentrations (10–100 mM NaCl) for 90 min. The quantity of bodipy-DAG, the product of NPC activity, doubled after 90 min of 10 mM NaCl treatment and increased 4-fold after 100 mM NaCl treatment. (Fig. 3A). *Arabidopsis* seedlings were also treated with 100 mM NaCl for different times (15, 30, 60, and 90 min). Changes in NPC activity were already detectable after 30 min of NaCl treatment. Thus, it is obvious that salt-stressed *Arabidopsis* seedlings respond by the rapid alteration of the NPC activity and that this effect is caused even by very mild salt stress (10 mM NaCl). Rapid changes in expression of *NPC4* in 7-day-old seedlings were also determined after 4 h of 100 mM salt treatment and 2.5-fold (±0.3) increase found.

Expression of NPC4 *is localized in root tips*

To gain insights into the localization of expression of *NPC4* in roots after salt treatment, the expression pattern was investigated using promoter:GUS plants. Histochemical analysis of P_{NPC4}:*GUS* plants confirmed that the expression pattern under salt stress was changed only in the roots (Fig. 4; data from other plant organs are not shown). In 10-day-old seedlings the expression was located predominantly in the root tips of the main and lateral roots of both control and

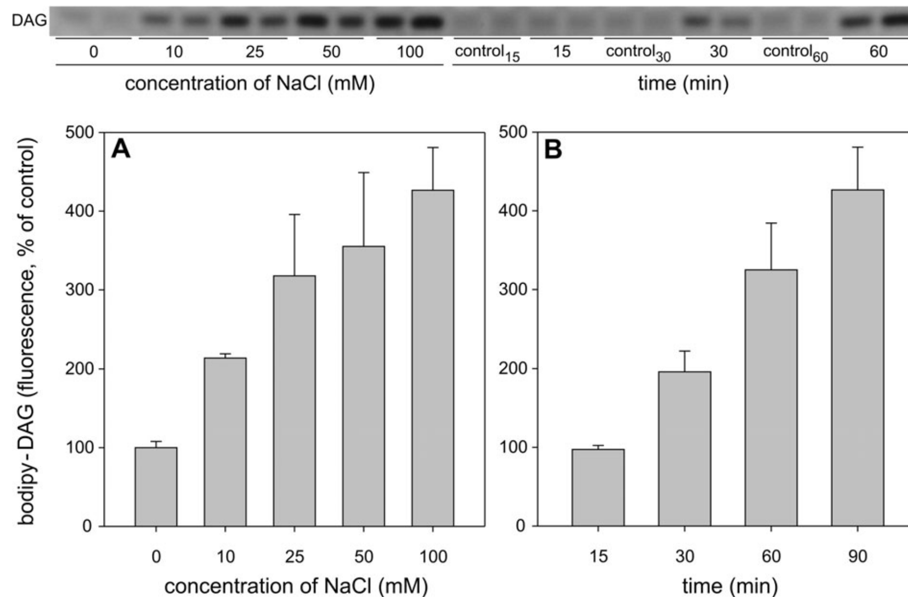

Fig. 3. Effect of NaCl on DAG production in *Arabidopsis* seedlings. *Arabidopsis* seedlings were grown on agar plates for 7 d. Prior to treatment seedlings were removed from agar and incubated with bodipy-PC for 10 min in water. (A) Seedlings were treated for 90 min with 0–100 mM NaCl. Lipids were extracted, separated by high-performance thin-layer chromatography (HP-TLC) and quantified. The quantity of bodipy-DAG in control non-treated seedlings was set to 100%. Data represent means ±SE from independently analysed parallel samples. This experiment was repeated three times with similar results. (B) Seedlings were treated with 100 mM NaCl. Lipids were extracted at the time intervals indicated, separated by HP-TLC, and quantified. The quantity of bodipy-DAG in control non-treated seedlings was set to 100%. Data represent the means ±SE from independently analysed parallel samples. This experiment was repeated twice with similar results. DAG, diacylglycerol.

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treated plants (Fig. 4A). The highest intensity of GUS staining in control plants was found in the apical meristem and partly in the region of elongation, whereas after incubation in 100 mM NaCl the staining had spread to the region of mature cells. The highest expression outside the root tips was observed in epidermal cells, vascular tissues, and lateral root primordium.

Hydroponically grown 5-week-old plants were analysed to see if a similar expression pattern would occur in adult plants of the same age used to observed *NPC* expression measured by quantitative PCR. Despite the differences in the age of plants, the GUS expression pattern remained the same (Fig. 4B).

Sensitivity of NPC4 *knockout* Arabidopsis *lines to salt*

Two lines of homozygous *Arabidopsis* T-DNA insertion mutants of *NPC4* were used as described in Wimalasekera *et al.* (2010): *npc4-1* (SALK_046713) and *npc4-2* (GK_571E10) (Supplementary Fig. S2 at *JXB* online). There is no obvious phenotype of either of these lines when grown in normal conditions in soil either in the greenhouse or in the growth chamber.

However, when mutant plants were grown in Petri dishes on agar MS medium supplemented with MES, inositol, and sucrose (see Materials and methods), there were slight but not significant differences in *npc4-1*, *npc4-2*, and the WT in fresh weight and root length. At the same time, significant decreases (*t*-test P <0.05) in both fresh weight and root length were revealed under salt stress conditions (Fig. 5A–C). The fresh weight and main root length phenotype of *npc4-2* was dose dependent. The fresh weight of *npc4-2* seedlings was reduced by 20% at 50 mM NaCl compared with the WT and by almost 25% at 100 mM NaCl (Fig. 5B). Similarly the main root length of *npc4-2* was reduced by 25% at 50 mM NaCl compared with the WT and by ~55% at 100 mM NaCl compared with the WT (Fig. 5C). Even though the fresh weight and the main root length of *npc4-1* were also reduced at 100 mM compared with the WT (Fig. 5B, C), these reductions were not significant (*t*-test P <0.05).

It is well known that salt inhibits seed germination. Therefore, germination of *npc4* seeds on control medium and on medium supplemented with 150 mM NaCl was determined. Germination of seeds of both *npc4* lines was indistinguishable from germination of WT seeds on control medium, and nearly 100% of WT and *npc4* seeds

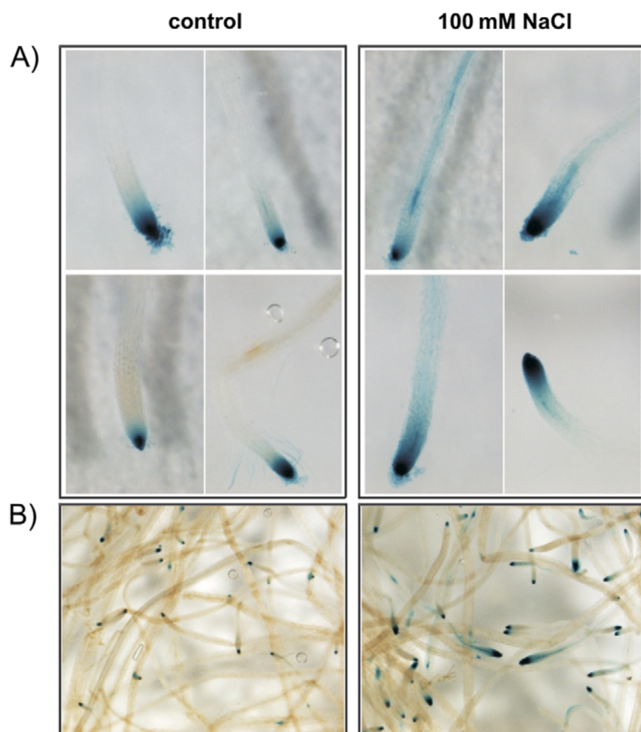

Fig. 4. Histochemical analysis of P_{NPC4}:*GUS* expression under salt stress in *Arabidopsis* plants. (A) Effect of salt on the P_{NPC4}:*GUS* expression pattern in the main root of 10-day-old seedlings. The plants were grown on agar and transferred to liquid nutrient solution with or without 100 mM NaCl for 24 h. (B) Effect of salt on the P_{NPC4}:*GUS* expression pattern in roots of 5-week-old *Arabidopsis* plants. The plants were grown hydroponically in modified half-strength Hoagland's solution and exposed to 100 mM NaCl for 4 h.

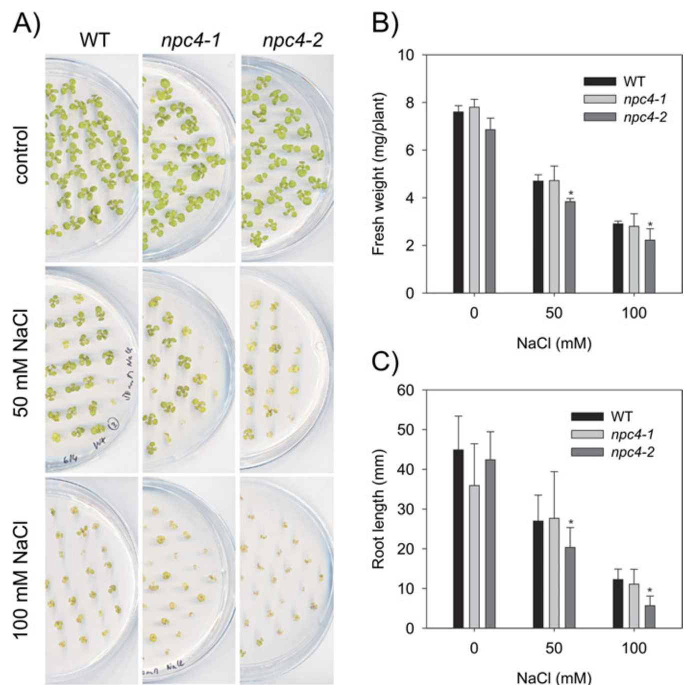

Fig. 5. Effect of NaCl on fresh weight and root length of *npc4-1* and *npc4-2* knockouts. (A) *Arabidopsis thaliana* Col wild-type (WT), *npc4-1*, and *npc4-2* were grown on agar plates supplemented with 0, 50, and 100 mM NaCl for 14 d. (B) Fresh weight of 14-day-old WT, *npc4-1*, and *npc4-2* seedlings. Forty-five seedlings from one agar plate were pooled and weighed. Data represent the means ±SE, n=4. This experiment was repeated three times with similar results. (C) Root length of 14-day-old WT, *npc4-1*, and *npc4-2* seedlings. Data represent the means ±SE, n=52. This experiment was repeated three times with similar results. Asterisks indicate a statistically significant (*t*-test P <0.05) difference in comparison with the WT.

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germinated within 24 h. However, on the medium with 150 mM NaCl, 82% of WT seeds, 76% of *npc4-1*, and only 27% of *npc4-2* seeds germinated after 24 h. Still nearly all seeds of the WT and *npc4* germinated within 42 h. Thus it was shown that the germination rate of *npc4* seeds decreased in comparison with WT seeds, but 150 mM NaCl does not inhibit germination completely (Fig. 6).

These results show a higher sensitivity of *npc4* knockout lines to NaCl and demonstrate that *NPC4* plays a role in response of *Arabidopsis* to salt stress.

Transcription pattern of salt and phospholipid signalling-related genes

The results mentioned above demonstrate that NPC4 is involved in the response of *Arabidopsis* plants to salt stress. Genes which are known to be related to salt stress as well as those related to phospholipid signalling were selected and their expression pattern was tested for alterations in *npc4-1*, *npc4-2*, and the WT under salt stress conditions. Expression of the genes was monitored in roots and leaves of 5-week-old hydroponically grown *Arabidopsis* plants treated with 100 mM NaCl. Samples were taken 3, 6, 9, and 12 h after addition of NaCl to the medium.

Differences between the gene expression pattern of salt-treated WT and knockout plants were found only in roots, and data from leaves are, therefore, not shown.

Expression of salt overly sensitive (SOS) signal transduction pathway genes: The SOS signalling pathway is probably the best characterized salt-specific signal transduction pathway (Zhu, 2002, 2003; Munns and Tester, 2008). The calcineurin B-like protein (CBL4/SOS3) interacts with CBL-interacting protein kinase (CIPK24/SOS2) (Gong *et al.*, 2004) and the CBL4–CIPK24 complex then activates the Na^+/H^+ antiporter SOS1 located at the plasma membrane (Qiu *et al.*, 2002). In roots of salt-treated WT plants, expression of all SOS genes doubled during 12 h. Expression of both SOS2 and SOS3 genes preceded expression of the SOS1 gene. This increase was distinctly smaller in *npc4* knockouts (Fig. 7). Such results might indicate a link between the SOS signalling pathway and NPC4.

Expression of ion transporters: During salt stress, Na^+ ions enter mainly passively across the plasma membrane into the cytoplasm of root cells. A low concentration of Na^+ in the cytoplasm has to be maintained and, therefore, vacuolar Na^+/H^+ antiporter (NHX) is activated during salt stress and Na^+ is sequestered into vacuoles (Pardo *et al.*, 2006; Munns and Tester, 2008). Expression of *NHX1* and *NHX8* was monitored. NHX1 is one of the predominant isoforms in *Arabidopsis* and is an important member of the salt tolerance machinery (Rodríguez-Rosales *et al.*, 2009). NHX8 also belongs to the monovalent cation:proton antiporter-1 family; however, it is more specific as a plasma membrane Li^+/H^+ antiporter (An *et al.*, 2007). No clear tendency in the *NHX* expression pattern in WT and *npc4*

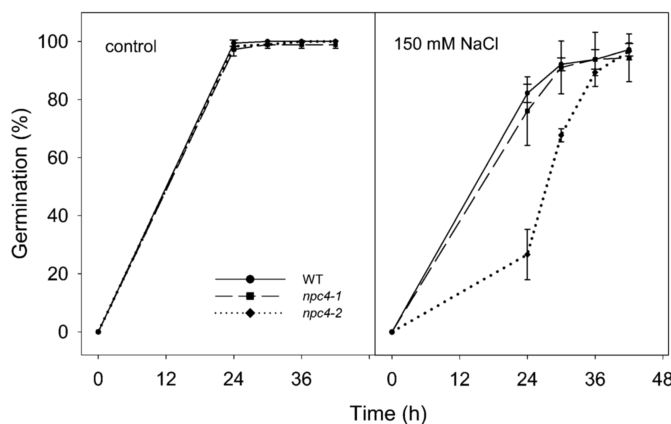

Fig. 6. Effect of NaCl on the seed germination phenotype of *npc4-1* and *npc4-2* knockouts. Forty-five seeds of the WT, *npc4-1*, or *npc4-2* were germinated on an agar plate with or without 150 mM NaCl. Germinated seeds were counted at 24, 30, 36, and 42 h after transferring seeds from 4 °C. Data represent the means ±SE, *n*=4. This experiment was repeated three times with similar results.

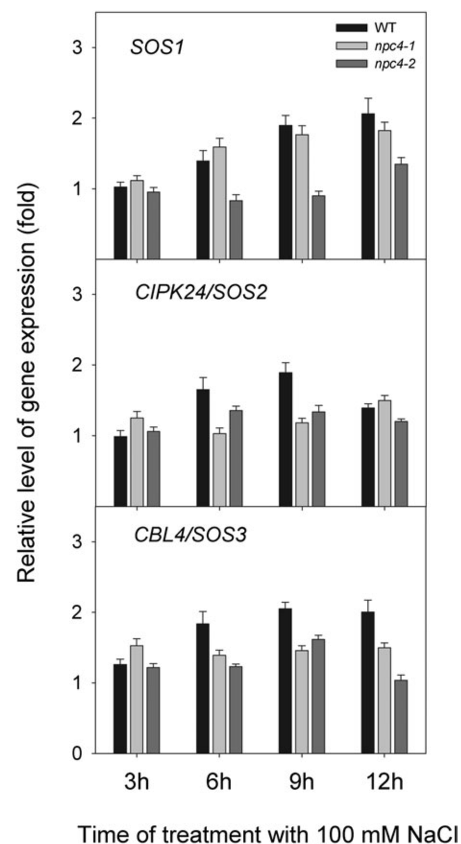

Fig. 7. Relative level of gene expression of SOS signal transduction genes in the WT, *npc4-1*, and *npc4-2* under 100 mM NaCl treatment. The transcript levels of *SOS1*, *CIPK24/SOS2*, and *CBL4/SOS3* were measured by quantitative real-time PCR in roots of non-treated plants and in plants treated with 100 mM NaCl for 3, 6, 9, and 12 h. *Actin2* and *UBQ10* were used as internal controls. The expression of the genes in non-treated controls at the respective times was set to 1. Data represent the means ±SE, *n*=2 discrete samples from one biological experiment. This experiment was repeated three times with similar results.

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plants under salt stress was observed (Supplementary Fig. S3A at *JXB* online).

Expression of phospholipid signalling-related genes: The role of DAG, the product of PC-PLC activity, in plants is not very clear. DAG is likely to act as a signalling molecule at least in some systems such as tobacco pollen tubes (Helling *et al.*, 2006). However, it is generally assumed that the most probable mechanism of DAG action is the conversion of DAG to PA by diacylglycerol kinase (DGK). It was shown that the PI-PLC/DGK pathway is activated during water stress. Therefore, expression of *DGK1*, the isoform which is induced by salt and drought according to the Genevestigator database (https://www.genevestigator.com) (Hruz *et al.*, 2008), was monitored. PA is a well documented signalling molecule that is also involved in responses to salt stress. One of the genes which is known to be activated by PA is *TOR* (Fang *et al.*, 2001). TOR is important in cell growth and development and also in hyperosmotic stress (Menand *et al.*, 2002; Mahfouz *et al.*, 2006). Thus, expression of *TOR* was monitored in WT and *npc4* plants. Expression of *DGK1* was somewhat higher after salt treatment at later times; however, there were not very clear differences between WT and *npc* plants. Expression of *TOR* in WT and knockouts remained unchanged after salt treatment (Supplementary Fig. S3B at *JXB* online).

Expression of ABA-related genes: Abscisic acid (ABA) is a hormone whose concentration is elevated during salt stress, and ABA-related genes are induced afterwards (Knight *et al.*, 1997; Kreps *et al.*, 2002; Zhu, 2002; Christmann *et al.*, 2006). Among them, expression of *ABI1*, *ABI2*, *RAB18*, *PP2CA*, *SOT12*, and *TSPO* was monitored. Additionally, expression of the stress-responsive gene *RD29/LTI78/COR78* that is supposed to be functional in both ABA-dependent and ABA-independent signalling pathways was also monitored (Agarwal and Jha, 2010). Expression of *ABI1*, *ABI2, PP2CA*, and *SOT12* in roots of salt-treated *npc4-1* and *npc4-2* plants clearly decreased in comparison with expression of these genes in salt-treated WT plants (Fig. 8). The decrease was detected at all monitored time points. However, the expression of *RAB18* in *npc4-1* and *npc4-2* roots was decreased only at early time points (3 h and 6 h). The expression pattern of *TSPO* (Fig. 8) as well as of *RD29/LTI78/COR78* (Supplementary Fig. S3C) did not differ distinctly in WT and *npc4* plants.

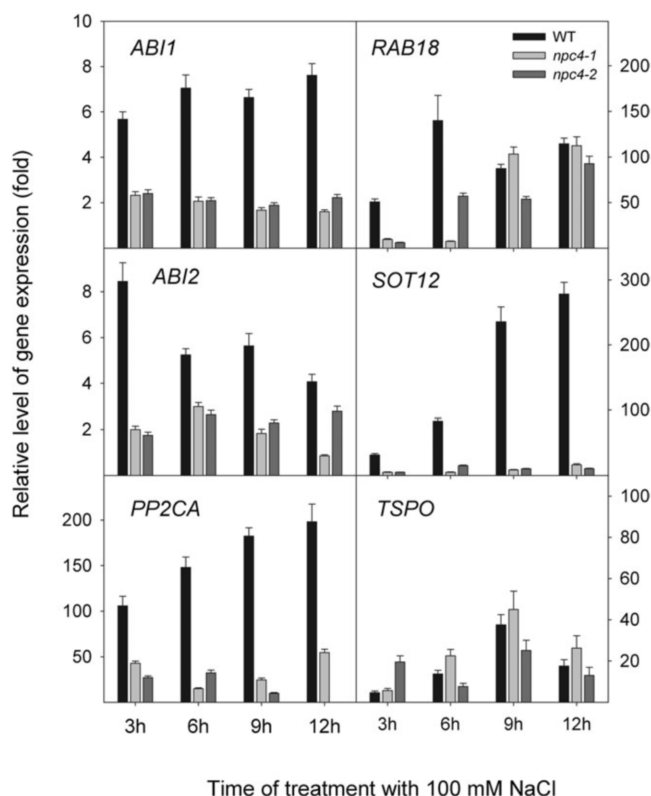

Fig. 8. Relative level of gene expression of ABA-related genes in the WT, *npc4-1*, and *npc4-2* under 100 mM NaCl treatment. The transcript levels of *ABI1*, *ABI2*, *RAB18*, *PP2CA*, and *SOT12* were measured by quantitative real-time PCR in roots of non-treated plants and in plants treated with 100 mM NaCl for 3, 6, 9, and 12 h. *Actin2* and *UBQ10* were used as internal controls. The expression of the genes in non-treated controls at the respective times was set to 1. Data represent the means ±SE, n=2 discrete samples from one biological experiment. This experiment was repeated three times with similar results.

Discussion

The level of NPC4 *transcript and enzyme activity increased rapidly in response to salt treatment*

Results of quantitative RT-PCR analysis demonstrated that salt stress induced expression of genes coding for PC-PLC, *NPC2*, *NPC4*, and *NPC6*. Among them, *NPC4* was the most highly induced gene. The expression was rapid (within 1 h), transient, and induced by a relatively low salt concentration (25 mM NaCl). A rapid reaction to low salt concentration was also observed at the level of NPC activity. The activity of NPC doubled during 30 min treatment with 100 mM NaCl, and even 10 mM NaCl treatment led to a 2-fold increase in activity within 90 min.

Enzymatic activity measurements were done using seedlings (Fig. 3) because lipid labelling can only be done with seedlings, whereas the time course of expression of NPC genes was carried out on 5-week-old plants (Fig. 2). However, in seedlings, an increase in promoter activity was found after 24 h NaCl treatment (Fig. 4) and when expression of *NPC4* was checked in experimental conditions similar to those used for enzymatic activity measurements a 2.5-fold increase of *NPC4* transcript was found in the WT seedlings. All these results suggest a signalling role for NPC4.

NPC4 *expression response to salt stress was localized to roots*

Quantitative RT-PCR and P_{NPC4}:*GUS* results localize salt-induced expression of *NPC4* to roots, more precisely to root tips. On the basis of previous results it was hypothesized that *NPC4* expression correlates with auxin abundance (Wimalasekera *et al.*, 2010). The present results support this assumption

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because the changes in the distribution of auxin under salt stress detected by Wang *et al.* (2009) using the *DR5:GUS* system and the changes observed in the localization of P_{NPC4}:*GUS* showed a similar pattern. Expression of P_{NPC3}:*GUS* after salt treatment was also analysed because it was shown earlier (Wimalasekera *et al.*, 2010) that *NPC3* and *NPC4* expression patterns are similar. Both control plants and plants under salt stress showed no change in P_{NPC3}:*GUS* expression (Supplementary Fig. S1 at *JXB* online). These results indicate a specific role for NPC4 in salt stress response.

Expression of SOS signalling and ABA response genes was down-regulated in NPC4 *knockout plants*

SOS1 together with SOS2 and SOS3 plays an important role in the initial phases of salt stress. These proteins in concert play a role in maintenance of cell sodium homeostasis in high salt conditions. Similarly to *NPC4* (this work), expression of *SOS1* and *SOS2* under salt stress was localized to roots or root tips, respectively (Shi *et al.*, 2000, 2002). In the *npc4* mutants, the expression of SOS genes was diminished. A possible explanation can be found in the reports of SOS1 regulation by PA via mitogen-activated protein kinase 6 (MPK6). Yu *et al.* (2010) showed direct PA stimulation of MPK6 phosphorylation of SOS1 under salt stress where PA was derived from PC by PLDα activity. However, the role of PA originating from DAG by DGK activity is not excluded. Rapid conversion of DAG to PA has already been reported (Bargmann *et al.*, 2009).

Expression of *ABI1*, *ABI2*, and *PP2CA* together with *SOT12* was strongly decreased in salt-treated *npc4* plants. *ABI1*, *ABI2*, and *PP2CA* are key players in ABA signalling, and products of these genes function as negative regulators of ABA response (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Kuhn *et al.*, 2006). These proteins control various ABA responses such as stomatal closure, seed dormancy, or plant growth (Leube *et al.*, 1998). ABI1, ABI2, and PP2CA belong to the large family of Mg^{2+}- and Mn^{2+}-dependent serine/threonine phosphatases type 2C (PP2Cs) and they interact with the ABA receptor RCARs/PYR1/PYLs (Raghavendra *et al.*, 2010).

The expression data presented here allow the hypothesis that NPC4 interacts with the RCARs/PP2Cs signalling pathway. This, together with rapid activation of NPC4 enzyme activity (Fig. 3), makes NPC4 a good candidate for a salt signalling protein. The signalling mechanism of NPC4 might be based on rapid conversion of DAG, the product of NPC4 activity, to PA by DGK. Similarly, it has been shown (Munnik, 2001; Meijer and Munnik, 2003; Bargmann *et al.*, 2009) that DAG as a product of PI-PLC hydrolysis of PIP_2 was rapidly phosphorylated by DGK to PA. PA is the product of PLD, and both molecules, PA and PLD, were shown to participate in ABA signalling. PLDα1 plays a role in ABA-mediated stomatal closure (Zhang *et al.*, 2004; Mishra *et al.*, 2006). ABI1 is a well characterized plant PA target. PA binding decreases the phosphatase activity of ABI1 and thus promotes ABA signalling (Zhang *et al.*, 2004). The role of PA–MPK6 interaction during salt stress was discussed earlier. PA also plays an important role in ABA signalling during seed germination (Katagiri *et al.*, 2005). However, further investigation is required to reveal whether the mode of action of NPC4 in ABA signalling is PA mediated.

RAB18 is a drought-, salt-, ABA-, sugar-, and phosphate starvation-inducible gene (Knight *et al.*, 1997; Ciereszko and Kleczkowski, 2002). The induction of its expression requires Ca^{2+} influx via specific plasmalemma channels (Ghelis *et al.*, 2000). Changes in the expression pattern of both *RAB18* and *ABI1* were similar in plants with overexpressed *PLDα1* (Peng *et al.*, 2010), which is in agreement with the interpretations of the present results. Hallouin *et al.* (2002) showed that stimulation of PLD and not PI-PLC activity is necessary for ABA-induced *RAB18* expression. The present observation suggests that NPC4 may be essential for salt-induced *RAB18* expression. Whether signalling steps of salt induction and ABA induction leading to *RAB18* expression are the same or different is not clear.

SOT12 is a member of sulphotransferase protein family that seems to play an important role in plant growth, development, and adaptation to stress (Klein and Papenbrock, 2004). It was shown that *SOT12* in *Arabidopsis* was highly expressed under salt and osmotic stress and that the *sot12* mutant was hypersensitive to salt stress and ABA in seed germination (Kreps *et al.*, 2002; Baek *et al.*, 2010). Taking into account the observation that expression of *SOT12* was greatly decreased in *npc4* plants, it is possible to conclude that PP2C-mediated ABA signalling and SOT12 are on the identical branch of responses to salt stress. TSPO is a membrane-bound ABA-regulated protein that is proposed to amplify ABA signalling (Guillaumot *et al.*, 2009). In contrast to the above-mentioned genes, regulation by ABA of this gene is nearly identical in magnitude and time course in the mutants and WT plants.

Conclusion

In conclusion, the NPC4 gene, the member of the novel PLC gene family hydrolysing PC, is highly and specifically expressed in roots in salt-treated *Arabidopsis*. *NPC4* loss-of-function mutant plants revealed higher sensitivity to salt stress when compared with WT plants, which may be explained by putative partial disruption of the ABA signalling network. Expression of SOS signalling and ABA response genes in *npc4* plants suggests a positive regulatory function for NPC4 in ABA salt signalling processes. Further experiments are now required to determine precisely the location of NPC4 in this network and to clarify the molecular mechanism of NPC4 function in response to salt stress.

Supplementary data

Supplementary data are available at *JXB* online.

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Figure S1. Histochemical analysis of P_{NPC3}:*GUS* expression under salt stress in *Arabidopsis* plants.

Figure S2. The position of T-DNA insertions in two *Arabidopsis npc4* mutant lines.

Figure S3. Relative level of gene expression of ion transporter genes, phospholipid signalling-related genes, and of *RD29*/*LTI78*/*COR78* in WT, *npc4-1*, and *npc4-2* under 100 mM NaCl treatment.

Table S1. List of UPL (Universal Probe Library) probe numbers and corresponding quantitative RT-PCR primers

Table S2. List of quantitative RT-PCR primers

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