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Research article

Overexpression of *PeHA1* enhances hydrogen peroxide signaling in salt-stressed *Arabidopsis*

Meijuan Wang [a,1], Yang Wang [a,1], Jian Sun [a,b], Mingquan Ding [a,c], Shurong Deng [a], Peichen Hou [a,d], Xujun Ma [a], Yuhong Zhang [a], Feifei Wang [a], Gang Sa [a], Yeqing Tan [a], Tao Lang [a], Jinke Li [a], Xin Shen [a], Shaoliang Chen [a,*]

[a] College of Biological Sciences and Technology, Box 162, Beijing Forestry University, Beijing 100083, PR China
[b] College of Life Science, Jiangsu Normal University, Xuzhou, Jiangsu 221116, PR China
[c] College of Agricultural and Food Science, Zhejiang Agricultural and Forestry University, Hangzhou, Zhejiang 311300, PR China
[d] National Engineering Research Center for Information Technology in Agriculture, Beijing 100097, PR China

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ABSTRACT

The plant plasma membrane (PM) H^+-ATPase plays a crucial role in controlling K^+/Na^+ homeostasis under salt stress. Our previous microarray analysis indicated that *Populus euphratica* retained a higher abundance of PM H^+-ATPase transcript versus a salt-sensitive poplar. To clarify the roles of the PM H^+-ATPase in salt sensing and adaptation, we isolated the PM H^+-ATPase gene *PeHA1* from *P. euphratica* and introduced it into *Arabidopsis thaliana*. Compared to wild-type, *PeHA1*-transgenic *Arabidopsis* had a greater germination rate, root length, and biomass under NaCl stress (50–150 mM). Ectopic expression of *PeHA1* remarkably enhanced the capacity to control the homeostasis of ions and reactive oxygen species in salinized *Arabidopsis*. Flux data from salinized roots showed that transgenic plants exhibited a more pronounced Na^+/H^+ antiport and less reduction of K^+ influx versus wild-type. Enhanced PM ATP hydrolytic activity, proton pumping, and Na^+/H^+ antiport in *PeHA1*-transgenic plants, were consistent to those observed *in vivo*, i.e., H^+ extrusion, external acidification, and Na^+ efflux. Activities of the antioxidant enzymes ascorbate peroxidase and catalase were typically higher in transgenic seedlings irrespective of salt concentration. In transgenic *Arabidopsis* roots, H_2O_2 production was higher under control conditions and increased more rapidly than wild-type when plants were subjected to NaCl treatment. Interestingly, transgenic plants were unable to control K^+/Na^+ homeostasis when salt-induced H_2O_2 production was inhibited by diphenylene iodonium, an inhibitor of NADPH oxidase. These observations suggest that *PeHA1* accelerates salt tolerance partially through rapid H_2O_2 production upon salt treatment, which triggers adjustments in K^+/Na^+ homeostasis and antioxidant defense in *Arabidopsis*.

1. Introduction

The plasma membrane (PM) H^+-ATPase establishes an electrochemical gradient of protons to drive secondary transport of ions and metabolites [1]. The proton pump has a central function in the regulation of a variety of key physiological processes, such as stomata opening, phloem loading, root ion uptake, and salt tolerance [1]. The H^+-ATPase is encoded by a multigene family, and the expression of isogenes is differentially regulated according to tissue type and developmental stage [2]. Gene expression of the PM H^+-ATPase was previously shown to be altered by various environmental stimuli, including salinity [3], heavy metals [4], mechanical stress [5], and externally applied hormones [6]. In addition to genetic regulation, the activity of the PM H^+-ATPase may be modulated at the post-translational level, mainly via reversible phosphorylation [7].

Active Na^+ extrusion to the apoplast or the external environment is essential for sustaining intracellular Na^+ homeostasis in salt-treated plants [8]. This process depends on electrochemical H^+ gradients generated by the PM H^+-ATPase in various plant species [2,3,7–9]. In addition to controlling Na^+ homeostasis, the PM H^+-ATPase also plays an important role in the reduction of salt-induced K^+ loss [9,10]. This contribution is mainly due to the up-regulation of the H^+ pumps, which preserves a less-depolarized membrane potential and thus restricts K^+ efflux through depolarization-

* Corresponding author. Tel.: +86 10 62338129; fax: +86 10 62336164.
E-mail address: Lschen@bjfu.edu.cn (S. Chen).
[1] These authors contributed equally to this work.

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activated K^+ outward rectified channels and/or depolarization-activated non-selective cation channels [9,10]. Recent studies revealed that NaCl-induced H_2O_2 production contributes to K^+/Na^+ homeostasis via the PM H^+-ATPase [11–14]. However, the interaction between the PM H^+-ATPase and H_2O_2 signaling in salt adaptation in plants is still poorly understood.

H_2O_2 is a reactive oxygen species (ROS) that functions as an important regulator in various plant physiological processes such as root hair growth, ion transport, and stress response [15–17]. H^+ translocation derived by the PM H^+-ATPase is implicated in H_2O_2 signaling [12,17,18]. Increased PM H^+-ATPase activity in barley mesophyll cells was previously shown to correspond to exposure to powdery mildew [18]; the enhanced H^+ pumps caused apoplast acidification, which elevated H_2O_2 generation and led to a subsequent hypersensitive response in epidermal cells [18]. In *Arabidopsis thaliana* suspension cells, the PM proton pumps have also been implicated in hypo- or hyper-osmotic stress-induced production of an H_2O_2 wave [17]. Interestingly, H_2O_2 may be involved in the regulation of PM H^+-ATPase activity in herbaceous and woody species [11,19]. In *Vicia faba* guard cell protoplasts, abscisic acid inhibition of blue light-stimulated stomatal opening depends on the H_2O_2-induced dephosphorylation of the PM H^+-ATPase [19]. Therefore, the interaction between the PM H^+-ATPase and H_2O_2 signaling is crucial for plant growth, development, and stress adaptation.

Populus euphratica has been widely used as a model plant for the elucidation of the physiological and molecular mechanisms of salt tolerance in woody species [12,13,20–24]. Compared to salt-sensitive poplars, *P. euphratica* usually maintains a higher K^+/Na^+ ratio at both the tissue and cellular levels, which is mainly due to PM H^+-ATPase-dependent Na^+ extrusion and K^+ preservation [12,13,20–24]. Our previous microarray analysis revealed that *P. euphratica* leaves have a higher expression of PM H^+-ATPase than the leaves of the salt-sensitive poplar *Populus popularis* under both normal growth conditions and NaCl stress [3]. More rapid H_2O_2 production in response to NaCl salinity was observed in *P. euphratica* cells versus *P. popularis* [13]. A pharmacological investigation revealed that the PM H^+-ATPase may function as an ionic sensor to induce an early H_2O_2 burst, which contributes to the regulation of K^+/Na^+ homeostasis in *P. euphratica* cells [11–13]. However, there is no genetic evidence for this signaling cascade in *P. euphratica*.

The objective of the present study was to investigate the role of the H^+-ATPase in salt stress signaling and the control of ionic homeostasis. We cloned a putative PM H^+-ATPase gene, *PeHA1*, from *P. euphratica* and introduced it into the model plant *Arabidopsis*. *PeHA1* overexpression enabled *Arabidopsis* to retain K^+/Na^+ and ROS homeostasis under prolonged NaCl salinity. Our data reveal that H^+ pump-dependent ionic homeostasis control in transgenic *Arabidopsis* relies on a rapid burst of H_2O_2 after the onset of salt treatment. Our observations suggest that the PM H^+-ATPase functions as an ionic sensor and contributes to H_2O_2 signaling in higher plants.

2. Results

The gene *PeHA1*, encoding a putative PM H^+-ATPase, was cloned from the salt-resistant tree species *P. euphratica*. *PeHA1* contains the complete 2848-bp open reading frame encoding a polypeptide with 955 amino acids, which is predicted to be 104.8 kDa in size (Fig. 1A). Alignment of H^+-ATPases from several species indicates that PeHA1 harbors similar regulatory domains that are important for enzyme activity. Regions I and II in PeHA1 are two conserved auto-inhibitory domains, confirming the prediction that PeHA1 is an auto-inhibitory H^+-ATPase (Fig. 1A). The presence of a 14-3-3 binding domain suggests that the 14-3-3 protein interact with PeHA1 to regulate the enzyme's activity (Fig. 1A).

Phylogenetically, the amino acid sequence of PeHA1 exhibits the most similarity (98.7%) to the homologous sequence in *Populus trichocarpa* (NCBI Reference Sequence: XM_002330768.1; protein_id = XP_002330804.1; Fig. 1B). PeHA1 has a relatively high degree of similarity with AHA5 (NP_180028.1) in *Arabidopsis* (Fig. 1B). Evolutionary divergence is evident between PeHA1, *Arabidopsis* AHA7, and the PM H^+-ATPase genes from monocotyledon plants such as *Oryza sativa* and *Zea mays* (Fig. 1B).

The cellular localization of PeHA1 was determined by colocalization of the chimeric YFP::PeHA1 protein and the PM marker plasmid CFP::AtPIP2, which was transiently expressed in *Arabidopsis* mesophyll protoplasts under the control of a double CaMV 35S promoter. Fluorescence of YFP::PeHA1 was restricted to the PM of *Arabidopsis* protoplasts, without any detectable fluorescence in other parts of the cells (Fig. 2A). This observation was consistent with the fluorescence distribution of the PM marker, indicating the PM localization of PeHA1 (Fig. 2A).

We transformed *PeHA1* into WT *Arabidopsis* under the control of the CaMV 35S promoter. *PeHA1* expression and hydrolytic activity of the plasma membrane H^+-ATPase were examined in WT *Arabidopsis* and transgenic lines. Genomic DNA PCR and RT-PCR revealed *PeHA1* expression in the T3 lines of transgenic *Arabidopsis* (H1, H3, H8, and H9; Fig. 2B, C). Real-time PCR showed that the mRNA abundance of *PeHA1* was significantly higher in transgenic lines (especially H1 and H3) than in the wild-type (Fig. 2C). Using purified plasma membrane vesicles, hydrolytic activity of H^+-ATPase was measured in transgenic and wild-type *Arabidopsis*. Result showed that H1 and H3 exhibited a higher activity of ATP hydrolysis than H8, H9 and the wild-type (Fig. 2D).

Salt tolerance of wild-type and *PeHA1*-transgenic *Arabidopsis* were compared in this study. H1 and H3 showed higher germination rates at 100 and 150 mM NaCl versus WT (Fig. 3A, B). Therefore, H1 and H3 transgenic *Arabidopsis* lines were used for further analysis. Root elongation in H1 and H3 *Arabidopsis* was greater than in WT plants under both control and salinity conditions (Fig. 3C, D). Moreover, the dry weight of transgenic *Arabidopsis* plants was significantly higher than that of WT plants after 10 days of NaCl treatment (50 and 100 mM, Fig. 3E). Overall, our results indicate that ectopic expression of *PeHA1* improves salt tolerance in *Arabidopsis*. In our study, we found that the wild-type *Arabidopsis* and vector controls had not significant difference in either germination or root growth, irrespective of control or salt treatment (Supplemental Fig. S1).

We examined ion levels in wild-type *Arabidopsis* and *PeHA1*-transgenic lines under control and saline conditions. Long-term (21 days) exposure to 100 mM NaCl caused a significant rise in Na^+ levels in WT and transgenic *Arabidopsis*; however, a more pronounced effect occurred in WT seedlings (Fig. 4). NMT flux data revealed that Na^+ efflux in the apical regions of the roots was significantly increased in all genotypes under salinity conditions (50 and 100 mM NaCl; Fig. 5A). Notably, *PeHA1*-transgenic plants displayed 79–282% higher flux than WT plants (Fig. 5A). A net H^+ efflux was detected in the control roots of all tested genotypes (Fig. 5C). The H^+ extrusion was greater in H1 and H3 roots, resulting in a more acidic pH than wild-type (Fig. 5C, D). Salt treatment (50 or 100 mM NaCl) caused a net H^+ influx in WT plants (Fig. 5C), implying that H^+ efflux was consumed by Na^+ extrusion via an antiporter. This is consistent to Jayakannan et al., who found that NaCl-induced a H^+ influx from the mature root zone [25]. H1 and H3 roots retained a net H^+ efflux although the flux rate was reduced by exposure to 50 or 100 mM NaCl (Fig. 5C), suggesting that transgenic plants had created an increased proton motive force for Na^+/H^+ antiport. Proton transport activity and Na^+/H^+ antiport activity in membranes isolated from WT and transgenic plants were

A

At_AHA3	845	WAGVIWLYSI	VFYFPLDIMK	FAIRYILAGT	AWKNIIDNRT	AFTTKQNYGI EEREAQWAHA QRTLHGLQNT	905
Pe_HA1	845	WAGVIWLFSV	VTYVPLDILK	FAIRYILSGK	AWDNLLENKT	AFTTKKDYGK EEREAQWATA QRTLHGLQPP	905
Pt_HA	845	WAGVIWLFSV	VTYVPLDILK	FAIRYILSGK	AWDNLLENKT	AFTTKKDYGK EEREAQWATA QRTLHGLQPP	905
LHA	845	WAGVIWIFSI	VTYFPLDIMK	FAIRYILSGK	AWNNLLDNKT	AFTTKKDYGK EEREAQWALA QRTLHGLQPP	905
Zm_PMA2	845	WAGVVWLYSI	VFYFPLDLLK	FFIRFVLSGR	AWDNLLDTRI	AFTRKKDLRK GEREAQWATA QRTLHGLQPP	905
Os_PMA	845	WAGVIWLYSI	VTFLPLDIFK	FAVRYALSGR	AWDTLIEHKI	AFTSKKDYGR GEREAQWATA QRTLHGLQTP	905
At_AHA5	845	WAGVIWLYSF	LTYIPLDLLK	FGIRYVLSGK	AWLNLLENKT	AFTTKKDYGK EEREAQWAAA QRTLHGLQPA	905
Nt_HA	845	WAAVIWIYTI	ITYIPLDILK	FIIRFALSGR	AWDSMIQNKT	AFTTKKDYGR GEREAQWALA QRTLHGLQTP	905

Region I

At_AHA3	915	ET---ANVVP	ERGGYRELSE IANQAKRRAE IARLRELHTL	KGHVESVVKL	KGLDIETAG-	HYTV	975
Pe_HA1	915	ET--SHNMFS	EKNSYRELSE IAEQAKRRAE MARLRELNTL	KGHVESVVKL	KGLDIDTIQQ	HYTV	975
Pt_HA	915	ET--SHNMFS	EKNSYRELSE IAEQAKRRAE MARLRELNTL	KGHVESVVKL	KGHVESVVKL	HYTV	975
LHA	915	EA---SNLFN	EKNSYRELSE IAEQAKRRAE MARLRELHTL	KGHVESVVKL	KGLDIETIQQ	HYTV	975
Zm_PMA2	915	ES---NTLFN	DKSSYRELSE IAEQAKRRAE IARLRELNTL	KGHVESVAKL	KGLDIDTIQQ	NYTV	975
Os_PMA	915	EM-----GTT	SAASYRELSE IAEQAKRRAE VARLRELSTL	KGQMESTVRL	KGLDMDNVQH	HYTV	975
At_AHA5	915	EK---NNIFN	EKNSYSELSQ IAEQAKRRAE VVRLREINTL	KGHVESVVKL	KGLDIDTIQQ	HYTV	975
Nt_HA	915	EN---TGLFN	DKN-YRELSE IAEQAKRRAE VARLRELHTL	KGHVESVVKL	KGLDIETIQQ	HYTV	975

Region II 14-3-3

B

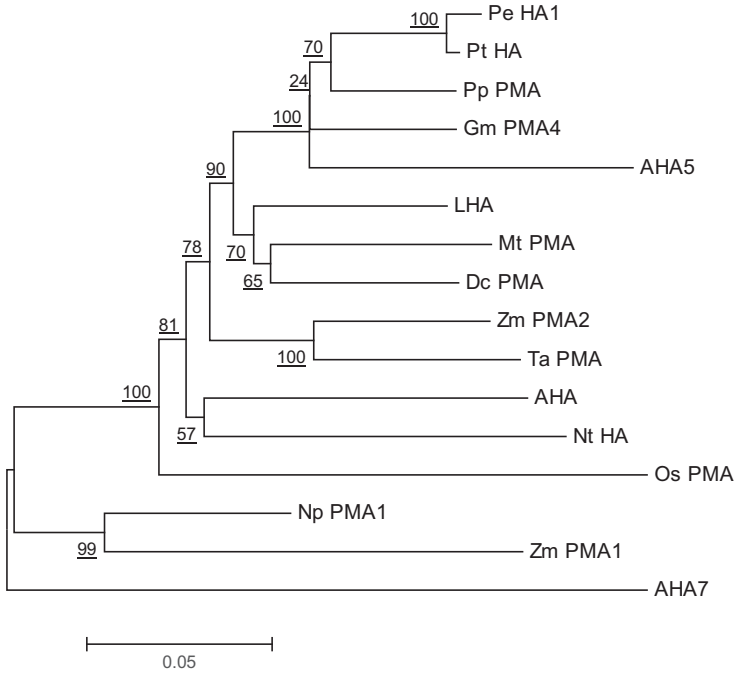

Fig. 1. Sequence analysis of PeHA1. (A) Amino acid sequences of the H^+-ATPase from several species were aligned using ClustalW. The conserved domain for regulation at the C terminus is boxed in gray. Region I and Region II, regulatory domains for enzyme activity; 14-3-3, 14-3-3 protein-binding site. (B) The neighbor-joined phylogenetic tree for the H^+-ATPase protein sequence in various plant species was constructed with Mega 5 using a bootstrap value of 1000. The H^+-ATPases are as follows: *P. euphratica* H^+-ATPase 1 (PeHA1), *P. trichocarpa* autoinhibited H^+-ATPase (PtHA, XP_002330804.1), *Prunus persica* PM H^+-ATPase (PpPMA, CAB69823.1), *Glycine max* PM ATPase 4-like isoform 1 (GmPMA4, XP_003544641.1), *Arabidopsis thaliana* H^+-ATPases (AHA3, NP_001190559.1; AHA5 NP_180028.1, AHA7 NP_191592.5), *Solanum lycopersicum* PM H^+-ATPase (LHA, AAB17186.1), *Medicago truncatula* PM H^+-ATPase (MtPMA, XP_003610080.1), *Daucus carota* PM H^+-ATPase (DcPMA, BAD16688.1), *Zea mays* PM H^+-ATPases (ZmPMA1, NP_001105360.1; ZmPMA2, NP_001105470.1), *Triticum aestivum* PM H^+-ATPase (TaPMA, AAV71150.1), *Nicotiana tabacum* proton P-ATPase (NtHA, AAR32129.2), *Oryza sativa* PM H^+-ATPase (Os PMA, CAD29311.1).

examined in this study. NaCl (150 mM) and H_2O_2 (10 mM) markedly increased PM H^+-transport activity, although a higher activity was observed in transgenic lines (Fig. 6A, B). Na^+/H^+ antiport activity of isolated membranes was dependent on Na^+ concentration in the reaction buffer, and a high value was observed at 60–80 mM NaCl (Fig. 6C). H1 and H3 exhibited typically a higher Na^+/H^+ antiporter activity than wild-type especially under high Na^+ (Fig. 6). These *in vitro* data in *PeHA1*-transgenic plants were consistent to those observed *in vivo*, i.e., Na^+ efflux, H^+ extrusion and external acidification (Fig. 5A, C, D).

The K^+ and Ca^{2+} levels were 31–86% higher in transgenic *Arabidopsis* than in WT seedlings under salt stress (Fig. 4). Although

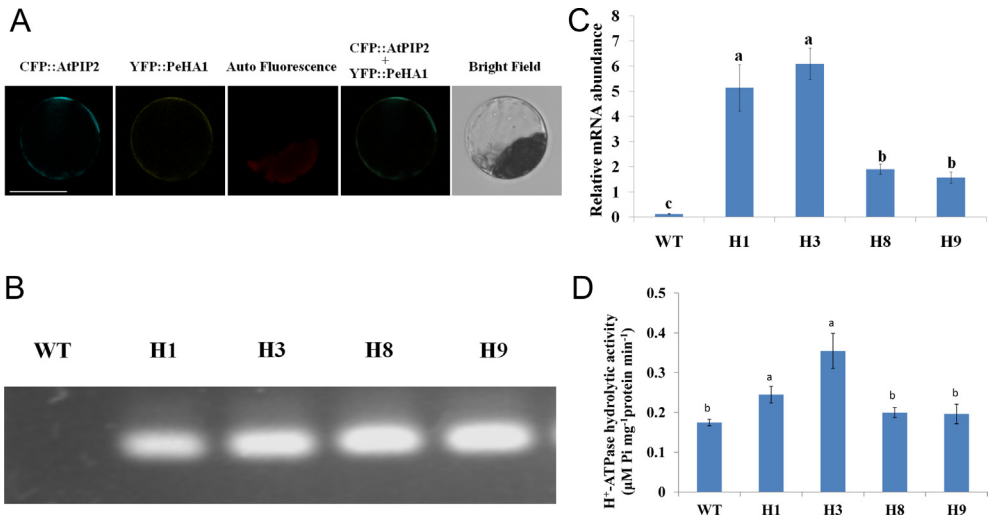

Fig. 2. PeHA1 localization, transformation, expression, and PM H^+-ATPase hydrolytic activity in WT *Arabidopsis* and transgenic lines (H1, H3, H8, and H9). (A) Cellular localization of PeHA1 determined by transient expression of YFP::PeHA1 and the PM marker CFP::AtPIP2 in *Arabidopsis* mesophyll protoplasts. Images from representative cells are presented. Scale bars = 50 μm. (B) PCR confirmation of transgenic *Arabidopsis* using DNA as the template. (C) Relative mRNA abundance of *PeHA1* in WT *Arabidopsis* and transgenic lines (H1, H3, H8, and H9). Data were obtained by real-time RT-PCR normalized against *AtActin*. (D) Plasma membrane H^+-ATPase hydrolysis activity of WT *Arabidopsis* and transgenic lines (H1, H3, H8, and H9). (C and D) Each column is the mean of three biologically independent samples, and bars represent the standard error of the mean. Columns labeled with different letters (a, b, c) indicate significant differences between WT *Arabidopsis* and transgenic lines at $P < 0.05$.

salt treatment lowered the K^+ and Ca^{2+} levels in transgenic and WT seedlings, a greater reduction in K^+ and Ca^{2+} levels was observed in WT plants versus the H1 and H3 plants (Fig. 4). Salinity reduced the K^+ influx (50 mM NaCl) or shifted the rectification toward an efflux in WT roots (100 mM NaCl), while a steady K^+ influx was usually observed in the roots of transgenic plants (Fig. 5B).

To determine the relationship between the PM H^+-ATPase and H_2O_2 signaling, we measured endogenous H_2O_2 levels in salinized roots from WT and transgenic *Arabidopsis*. We used H_2DCF-DA, a specific fluorescence probe, to monitor H_2O_2 levels within root cells. Transgenic *Arabidopsis* exhibited higher levels of H_2O_2 than WT seedlings under no-salt conditions (Fig. 7A–C). NaCl shock (100 mM) caused a rapid increase in H_2DCF-DA-dependent fluorescence in the roots of transgenic *Arabidopsis*, but salt-induced H_2O_2 was not evident in WT plants (Fig. 7A–C).

The effect of salinity on H_2O_2 in *Arabidopsis* roots was also examined over a prolonged period of stress. As shown in Fig. 8A, WT seedlings exhibited a gradual increase in H_2O_2 levels over a 15-

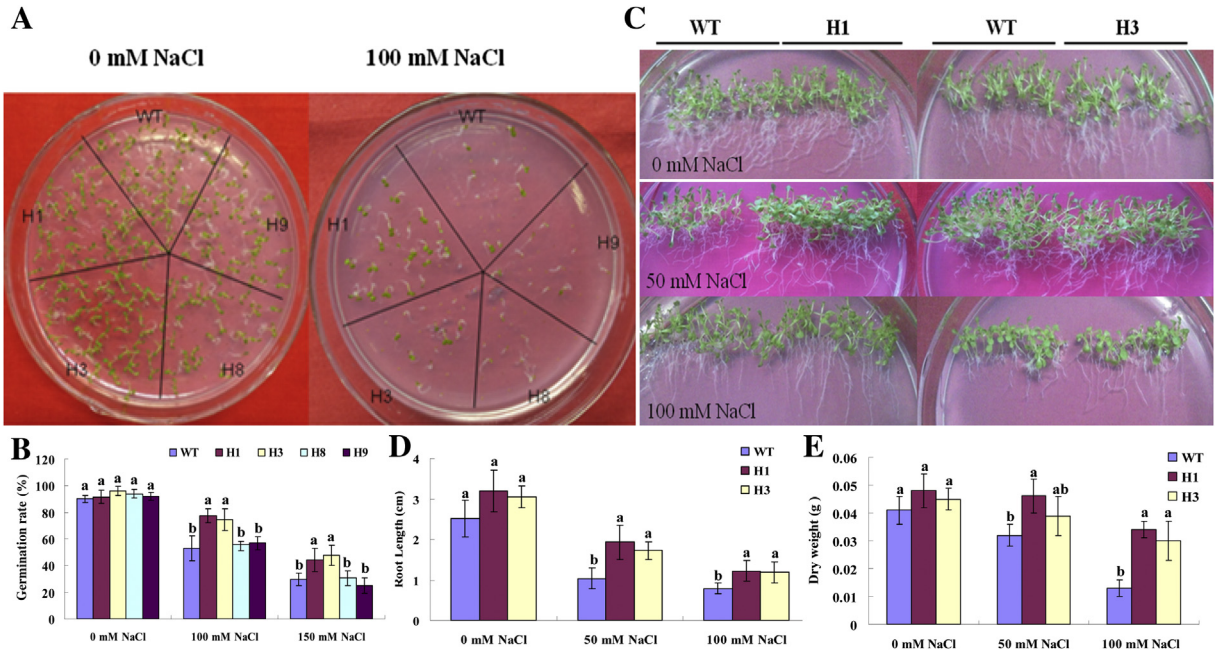

Fig. 3. Salt tolerance of WT and *PeHA1*-transgenic *Arabidopsis* (H1, H3, H8, and H9). (A) Representative images showing seed germination on culture media with or without NaCl. (B) Germination rate. Seeds from the *PeHA1*-expression and WT lines were sterilized, then germinated on solid 1/2MS containing 0, 100, or 150 mM NaCl. Germination rates were measured after 5 days of salt treatment. (C) Phenotypes of seedlings under control and salinity conditions. Five-day-old seedlings were transferred to 1/2MS with or without NaCl for 10 days. (D) Root lengths of WT and *PeHA1*-transgenic *Arabidopsis*. (E) Dry weights of WT and *PeHA1*-transgenic *Arabidopsis*. In panels B, D, and E, each column is the mean of three biologically independent samples, and bars represent the standard error of the mean. Columns labeled with different letters (a, b) show significant differences between WT and transgenic lines at $P < 0.05$.

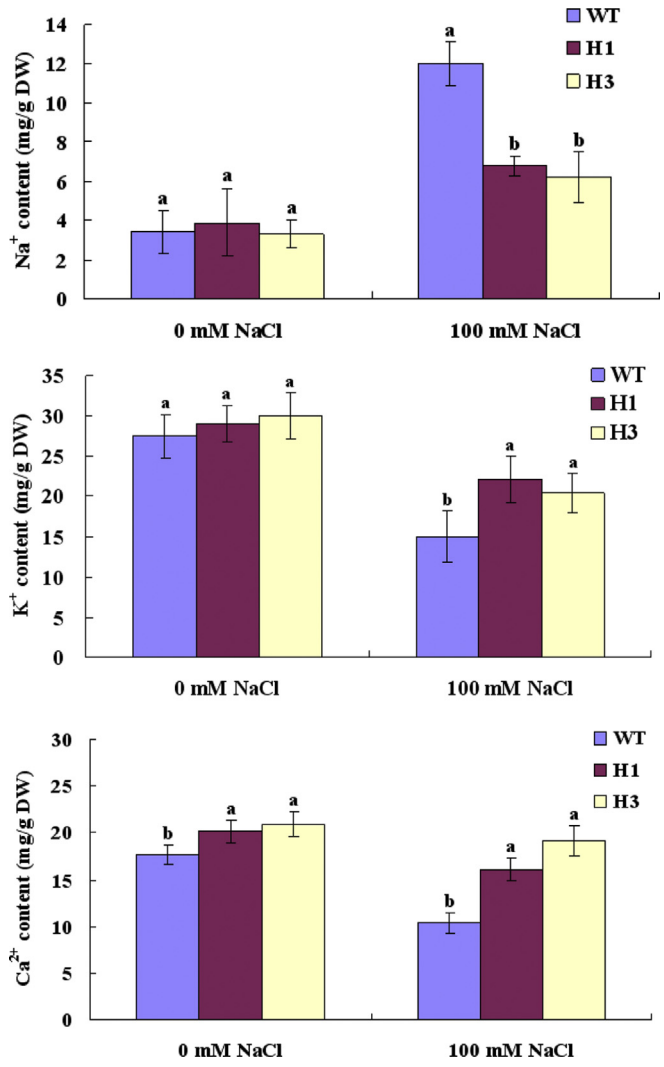

Fig. 4. Na^+, K^+, and Ca^{2+} content in WT *Arabidopsis* and transgenic lines (H1 and H3). Three week-old seedlings were grown on 1/2MS medium with or without 100 mM NaCl. Each column is the mean of three biologically independent samples, and bars represent the standard error of the mean. Columns labeled with different letters (a, b) exhibit significant differences between WT and transgenic lines at $P < 0.05$. DW, dry weight.

day exposure to 100 mM NaCl; the highest H_2O_2 level occurred at the end of the experiment. In contrast, in transgenic plants, H_2O_2 levels in the roots were markedly enhanced after the onset of salt treatment and remained constant through the remainder of the experiment (Fig. 8A).

APX and CAT are the two main antioxidant enzymes that scavenge H_2O_2. Therefore, we measured the activities of these enzymes in salt-stressed plants from WT and transgenic lines. CAT activity was elevated in WT plants during the period of salt treatment (Fig. 8B). APX activity in WT plants showed a trend similar to that of CAT, despite a 25% decline at day 15 (Fig. 8C). Compared to WT, transgenic plants exhibited higher APX and CAT activities throughout the experiments (Fig. 8B, C).

Pharmacological experiments were carried out to determine the contribution of H_2O_2 to ionic homeostasis in transgenic *Arabidopsis*. DPI, a PM NADPH oxidase inhibitor, was used to limit the production of salt-induced H_2O_2. The inhibitory effect of DPI on PM NADPH oxidase has been shown in *Arabidopsis* seedlings [15] and poplar [12,13]. DPI treatment had no effect on root Na^+ flux in control plants, but significantly inhibited Na^+ efflux in salt-treated WT and transgenic plants (Fig. 9A). Our results indicate that the enhanced Na^+ extrusion observed in *PeHA1*-transgenic *Arabidopsis* is dependent on H_2O_2 signaling under NaCl stress. DPI reduced the influx of K^+ in control plants from WT and transgenic lines (Fig. 9B). Under NaCl stress, DPI increased K^+ efflux in WT plants and caused a drastic shift in K^+ influx toward an efflux in H1 and H3 seedlings (Fig. 9B). These data highlight the involvement of H_2O_2 in the control of K^+ homeostasis under salt stress.

3. Discussion

In salty environments, the PM H^+-ATPase plays a key role in regulating K^+/Na^+ homeostasis in both herbaceous and woody plants [8,9,23,24,26]. Root cells of *P. euphratica* retained K^+/Na^+ homeostasis after exposure to NaCl stress, mainly due to the high activity of the PM H^+-ATPase [23,24]. Microarray data show that *P. euphratica* leaves have a higher abundance of the PM H^+-ATPase transcript under control and saline conditions than the salt-sensitive poplar *P. popularis* [3]. In this study, ectopic expression of *PeHA1*, a gene encoding a PM H^+-ATPase in *P. euphratica* (Fig. 1), significantly increased ATP hydrolysis and proton pumping activity of the PM H^+-ATPase in *Arabidopsis* (Figs. 2 and 6). This led to an enhanced salt tolerance in transgenic lines (H1 and H3) in terms of germination rate, root growth, and biomass (Fig. 3). Gévaudant et al. found that tobacco plants expressing a constitutively activated plasma membrane H^+-ATPase increased salt tolerance but displayed several developmental abnormalities [27]. However, no negative effect was observed on plant development in plants expressing wild-type PM H^+-ATPase4 [27]. This is similar to our findings in *PeHA1*-expressed *Arabidopsis*. In this study, overexpression of *PeHA1* did not cause developmental abnormalities in *Arabidopsis*, except that transgenic plants had approximately 20% higher root length and dry mass under no-salt conditions (Fig. 3). Gévaudant et al. showed that there is basically no effect of expressing WT ATPase, as most probably its *in vivo* activity is downregulated [27]. In our study, the *in vitro* data in *PeHA1*-transgenic plants, such as ATP hydrolytic activity, proton pumping, and the corresponding Na^+/H^+ antiport, are consistent to those observed *in vivo*, i.e., H^+ extrusion, external acidification, and Na^+ efflux (Figs. 5 and 6). This implies that H^+-ATPase from salt-resistant poplar species, *P. euphratica*, displayed a greater capacity to tolerate salinity than that from salt-sensitive plant species. Moreover, the rapid increase of H_2O_2 that elicited by salinity may in turn enhance ATPase activity in *PeHA1*-transgenic plants (Figs. 6 and 7). Salinity induced a rapid H_2O_2 production in the salt-resistant *P. euphratica* [12,13]. The salt-elicited H_2O_2 is shown to mediate ATPase activity and Na^+/H^+ antiport in *P. euphratica* cells [12,13]. However, the salt-induced H_2O_2 was not seen in wild-type *Arabidopsis* (Fig. 7) and salt-sensitive poplar, *P. popularis* [13]. Therefore, the mediation of H_2O_2 on ATPase seems to be absent in salt-sensitive species.

The H^+ pump provides a driving force for Na^+ extrusion and prevents further depolarization of membrane potential under salt stress, which leads to K^+ efflux through depolarization-activated channels [9,12,24,28]. An increase in H^+ extrusion and corresponding apoplast acidification may directly reflect the *in vivo* activity of the PM H^+-ATPase in transgenic *Arabidopsis* (Fig. 5). In the presence of NaCl, H^+ efflux was consumed by Na^+ extrusion via an antiporter (Fig. 5). K^+ influx in the transgenic plants was reduced correspondingly, presumably due to the decreased membrane hyperpolarization (Fig. 5). Taking our observations together, we conclude that overexpression of *PeHA1* in *Arabidopsis* enhances the proton motive force that favors Na^+ extrusion and K^+ maintenance.

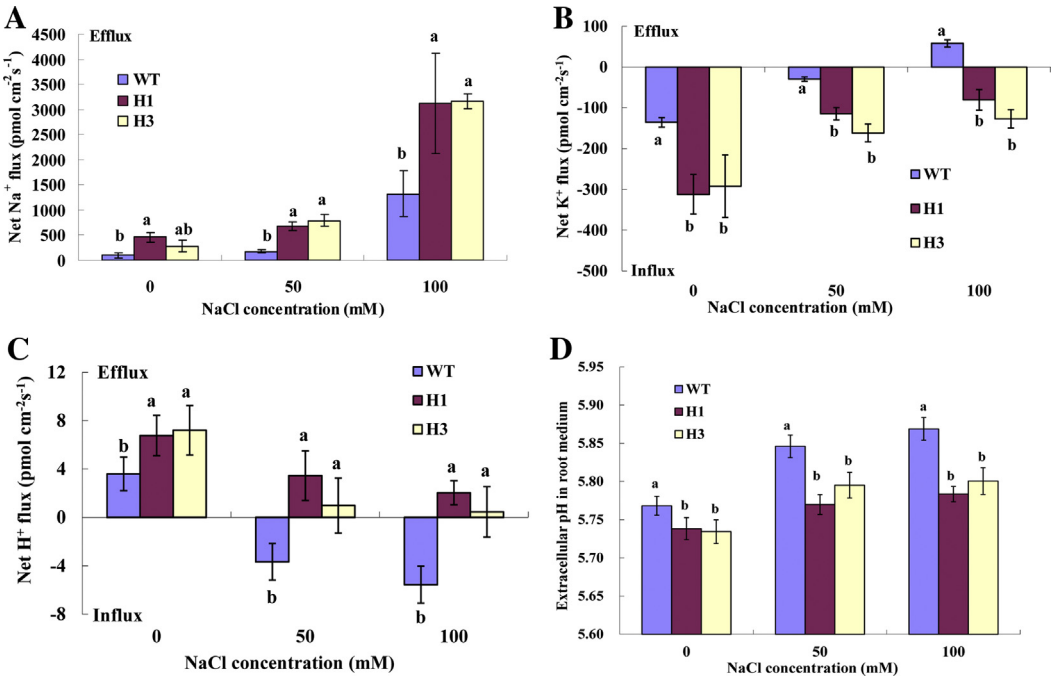

Fig. 5. Steady-state Na^+, H^+, K^+ fluxes and external pH in roots of WT *Arabidopsis* and transgenic lines (H1 and H3). Seven-day-old seedlings were transferred to 1/2MS with or without NaCl (50 or 100 mM) for 7 days, after which the seedlings were collected for NMT measurements. External pH values were calculated according to the established curve between pH values and original potentials acquired by NMT from H^+ flux measurements. Each column is the mean of six independent seedlings, and bars represent the standard error of the mean. Columns labeled with different letters (a, b) show significant differences between WT and transgenic lines at $P < 0.05$.

In our study, transgenic *Arabidopsis* roots exhibited a greater elongation rate than WT seedlings under normal growth condition (Fig. 3). This scenario is presumably due to the high H_2O_2 levels in the root cells of *PeHA1*-transgenic plants (Fig. 7). H_2O_2 may stimulate growth in various cell types [15,29,30]; additionally, our pharmacological experiments show that H_2O_2 is involved in the regulation of K^+/Na^+ homeostasis in salt-treated *Arabidopsis* seedlings (Fig. 9). Inhibition of H_2O_2 production by DPI increased K^+ efflux and simultaneously reduced Na^+ extrusion across the PM under saline conditions (Fig. 9). The enhanced K^+ efflux in transgenic *Arabidopsis* is due to a reduction of K^+ influx through inward K^+ channels or to an enhancement of K^+ efflux via outward K^+ channels, which is mediated by the activity of the PM H^+-ATPase [9,12,14]. In *P. euphratica* cells, salt-induced H_2O_2 plays a crucial role in up-regulating the abundance of the PM H^+-ATPase [11]; the absence of H_2O_2 impairs cytosolic Ca^{2+} signaling, which is critical for activation of the PM Na^+/H^+ antiport via the SOS signaling pathway [12,14,26]. H_2O_2 was found to increase H^+-pumping activity of isolated membranes in WT and *PeHA1*-transgenic plants (Fig. 6). Furthermore, H_2O_2 critically contributes to the maintenance of the stability of the SOS1 mRNA in *Arabidopsis* [31]. Thus, we speculate that salt-induced H_2O_2 production is indispensable for the regulation of the PM H^+-ATPase at the translational level in both *P. euphratica* and *Arabidopsis*.

The proton pump-derived translocation of H^+ across the PM triggers H_2O_2 signaling in salinized plants [12,17,18]. Our previous studies revealed that NaCl induces a H^+ influx that contributes to an early H_2O_2 burst in *P. euphratica* cells [12]. In this study, transgenic *Arabidopsis* plants with enhanced H^+ extrusion caused a high H_2O_2 production under control and NaCl treatment (Figs. 6 and 7). Similarly, proton extrusion is an essential signaling component to trigger H_2O_2 in the hypersensitive response of barley to powdery mildew [18]. Moreover, an acidic medium increased H_2O_2 production in *Arabidopsis* roots (Supplemental Fig. S2). We conclude that the apoplast acidification caused by enhanced H^+ extrusion contributes to the activation of the PM NADPH oxidase, thus leading to a greater accumulation of H_2O_2 in the root cells of transgenic *Arabidopsis*. *PeHA1*-transgenic plants typically have higher Ca^{2+} levels than WT seedlings irrespective of salt treatment (Fig. 4). It has also been suggested that the PM NADPH oxidase has a cytosolic N-terminal region with two EF-hand motifs that bind Ca^{2+}; the activation of this enzyme is Ca^{2+}-dependent [32]. Therefore, we cannot exclude the possibility that the higher Ca^{2+} level in transgenic seedlings activates the PM NADPH oxidase under control and salinity conditions.

In this study, *PeHA1*-trangenic plants exhibited more rapid H_2O_2 production after the onset of salt treatment than WT seedlings (Figs. 7 and 8). An early H_2O_2 burst is considered to be an inducer of the antioxidant defense system under various stress conditions [3,16]. Accordingly, transgenic plants displayed higher APX and CAT activities under both normal and salinity conditions (Fig. 8). These activated antioxidant enzymes are favorable to the maintenance of H_2O_2 levels during prolonged salinity stress (Fig. 8). Our previous studies demonstrated that salinized *P. euphratica* plants control ROS homeostasis through two pathways: (1) maintaining cellular ionic homeostasis and thereby limiting the NaCl-induced enhancement of ROS production under long-term saline conditions; and (2) rapidly up-regulating the antioxidant defense system to prevent oxidative damage [22]. Our observations suggest that high expression of the PM H^+-ATPase in *Arabidopsis* causes a rapid burst of H_2O_2, which induces the antioxidant defense system to avoid oxidative injury after long-term exposure to salinity. This hypothesis is in agreement with Ding et al., who found that *P. euphratica* plants exhibited a higher abundance of PM H^+-ATPase mRNA, which enables salt-treated plants to retain ionic and ROS homeostasis [3].

In conclusion, our data demonstrate that ectopic expression of *PeHA1* in *Arabidopsis* improves salt tolerance in transgenic seedlings. The increased apoplast acidification due to the enhanced H^+ extrusion, causes increased H_2O_2 production, which would in turn

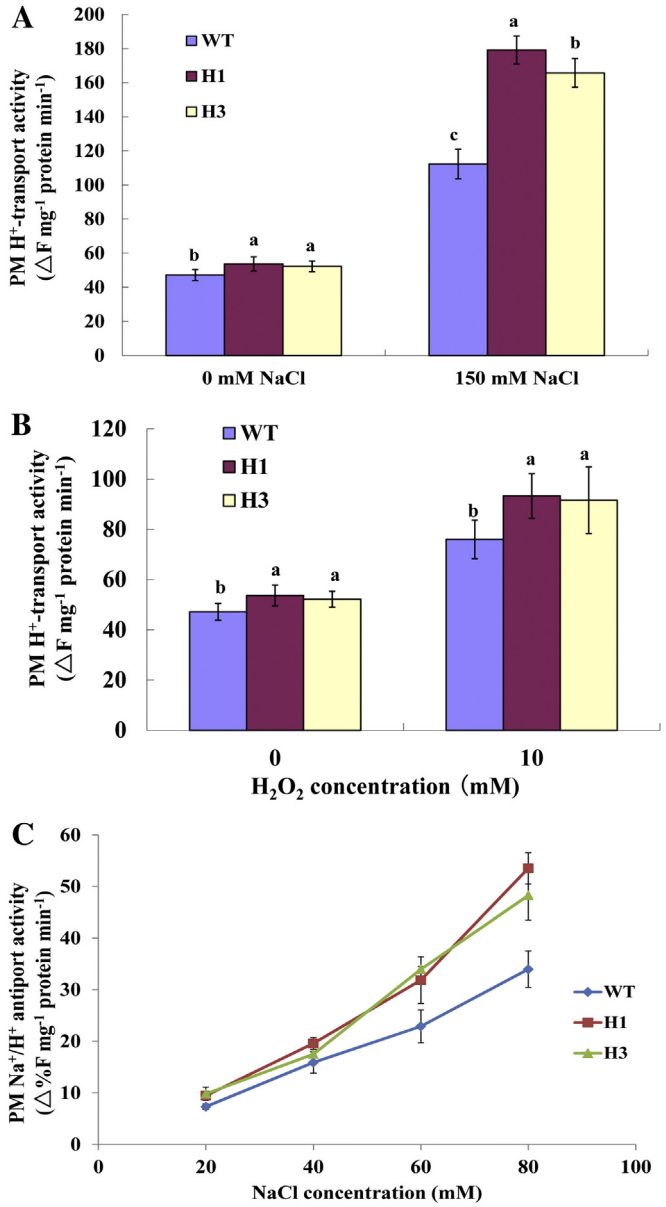

Fig. 6. PM H^+-transport activity and Na^+/H^+ antiport activity in WT *Arabidopsis* and transgenic lines (H1 and H3). (A) PM H^+-transport activity in response to NaCl. Four week-old seedlings were treated with 0 or 150 mM NaCl for three days prior to H^+-transport activity assay. The initial rate of fluorescence quenching (percent quenching min^{-1}) was used as a relative estimate of the rate of H^+ translocation. (B) PM H^+-transport activity in response to H_2O_2. Four week-old seedlings were treated with 0 or 10 mM H_2O_2 for 12 h prior to H^+-transport assay. In A and B, each column is the mean of five biologically independent samples, and bars represent the standard error of the mean. Columns labeled with different letters (a, b, c) indicate significant differences between WT *Arabidopsis* and transgenic lines at $P < 0.05$. (C) PM Na^+/H^+ antiport activity. Seedlings of wild-type *Arabidopsis thaliana* and transgenic lines (H1 and H3) were grown under a short photoperiod for four weeks. PM Na^+/H^+ antiport activity was detected as a Na^+-induced dissipation of the pH gradient. These initial rates were taken within the first 3 min after the addition of 20–80 mM NaCl.

stimulate Na^+/H^+ antiport in transgenic plants (Fig. 10). Moreover, the rapid increase of H_2O_2 that elicited by salinity may also upregulate ROS scavenging capacity in *PeHA1*-transgenic plants (Fig. 10). Therefore, the positive effects were mainly due to stimulation of H_2O_2 signaling pathway involving NADPH oxidase, which contributes to controlling K^+/Na^+ homeostasis and inducing the antioxidant defense system over prolonged salinity stress (Fig. 10). Taken together, our observations supply genetic evidence to confirm the role of PeHA1 in salt-stress signaling; the PM H^+-ATPase may function as an ionic stress receptor for the induction of H_2O_2 signaling pathway under salinity conditions.

4. Materials and methods

4.1. Plant materials and growth conditions

Two-year-old seedlings of *P. euphratica*, obtained from the Xinjiang Uygur Autonomous Region of China, were planted in individual pots (10 L) containing loam soil and placed in a greenhouse at Beijing Forestry University. Potted plants were well-irrigated according to evaporation demand and watered with 1 L full-strength Hoagland nutrient solution every 2 weeks. The temperature in the greenhouse ranged from 20 to 25 °C with a 16-h photoperiod (7:00–23:00) consisting of 150–1000 μmol m^{-2}–s^{-1} of photosynthetically active radiation. In order to reduce the differences between individual seedlings, leaves from three different plants were collected per harvest and were immediately frozen in liquid nitrogen for later use.

4.2. PeHA1 isolation

Total RNA was extracted from *P. euphratica* leaves with the TRIzol reagent (Cat. 15596-026; Invitrogen; Carlsbad, California, USA) according to the manufacturer's protocol. cDNA synthesis was performed as described in Ding et al. (2010) [3]. Given the sequence of an autoinhibited H^+-ATPase (GenBank accession no. XM_002330768.1) in *P. trichocarpa*, the primers 5′-ATGTCCAGCAAGGGCGGCATCAGTC-3′ and 5′-CTTAAAGGGTGTAATGTTGTTG-3′ were used to identify the full-length cDNA sequence of the H^+-ATPase homolog from *P. euphratica* (*PeHA1*). Multiple protein sequence alignments and a phylogenetic tree were made using the MEGA version 5 (MEGA5.0 software, http://www.megasoftware.net/index.php). Amino acid sequences of the H^+-ATPase from several species were aligned using ClustalW (http://www.genome.jp/tools/clustalw/).

4.3. Binary expression vector construction and Arabidopsis transformation

Invitrogen's Gateway technology was used to create an over-expression vector from an intermediate vector harboring *PeHA1* and a plant expression vector carrying EGFP-pK7WG2D [33]. The coding sequence of *PeHA1* plus CACC sites at the 5′ ends was obtained via PCR with primers 5′-CACCATGTCTAGTAAGGGCGGGAT-3′ and 5′-CTTAAAGGGTGTAATGTTGTTG-3′. The constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 and transformed into wild-type (WT) *A. thaliana* (Columbia-0 ecotype) by the floral dip method. Genomic DNA PCR and reverse transcription PCR (RT-PCR) confirmed the presence of the heterologous gene in transgenic *Arabidopsis*. T2 seeds were germinated on half-strength MS plates containing kanamycin (50 mg/L), and the resistant plants were transferred to nursery soil to obtain homozygous T3 seeds. T3 homozygous lines were used for phenotypic analyses and further physiological analyses.

4.4. Subcellular localization of PeHA1

Subcellular localization of PeHA1 was determined via the transient expression of chimeric YFP::PeHA1 and the PM marker plasmid CFP::AtPIP2 in *Arabidopsis* mesophyll protoplasts, which were obtained as described in Yoo et al. [34] with minor modifications (we used polyethylene glycol in the presence of $CaCl_2$). In brief, *Arabidopsis* protoplasts were isolated from leaves of 3–4-

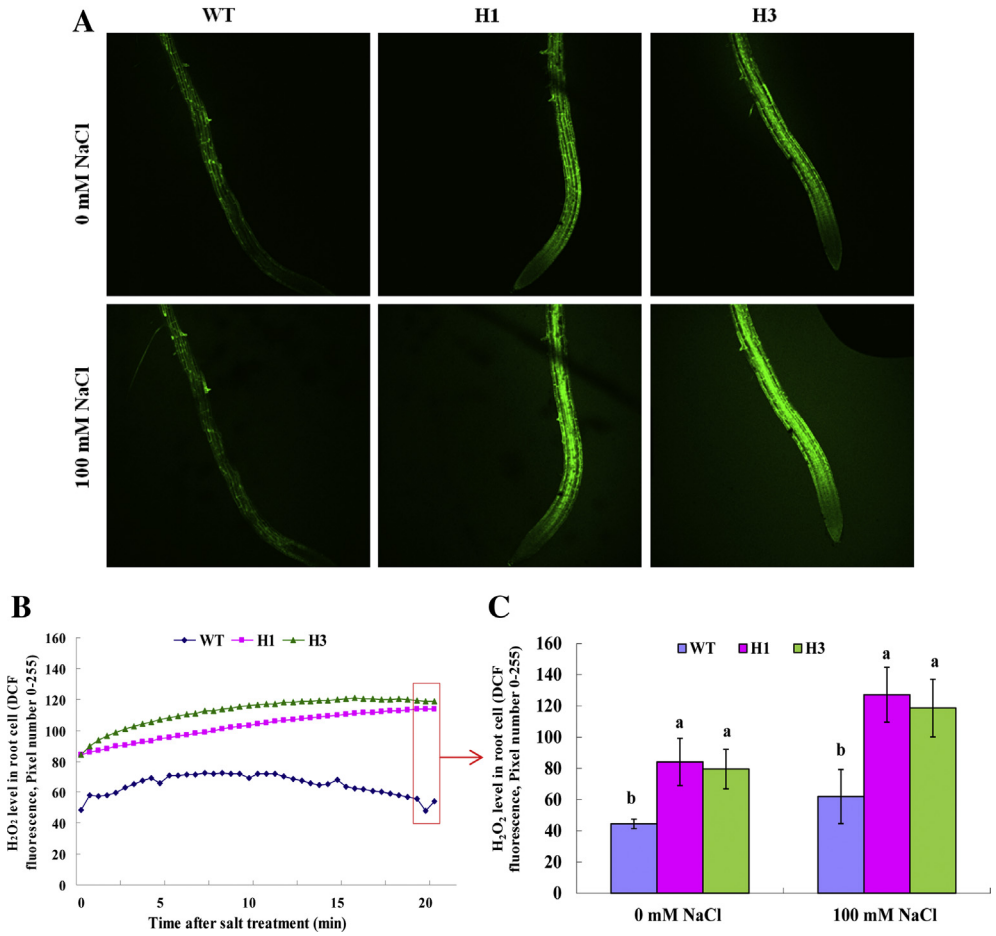

Fig. 7. NaCl-induced early H_2O_2 production in roots of WT *Arabidopsis* and transgenic lines (H1 and H3). (A) Representative confocal images of 100 mM NaCl-induced H_2O_2 production (20 min) in WT *Arabidopsis* and transgenic lines. (B) Time course of H_2DCF-DA fluorescence intensity. (C) Mean H_2DCF-DA fluorescence intensity after 20 min of NaCl treatment. Each column is the mean of 5–10 independent seedlings, and bars represent the standard error of the mean. Columns labeled with different letters (a, b) exhibit significant differences between WT and transgenic lines at $P < 0.05$.

week-old plants. Leaf strips were digested in digestion buffer containing cellulase R-10 and macerozyme R-10 (Yakult). Protoplasts were transfected with 5 μg of recombined plasmid in a 40% polyethylene glycol solution containing 100 mM $CaCl_2$ and incubated for 14–18 h prior to imaging. Documentation of YFP and CFP fluorescence was carried out with SP5 Leica laser scanning confocal microscopy (Leica Microsystems GmbH, Wetzlar, Germany) at 514 nm and 453 nm excitation, respectively, and emission bandwidths of 525–535 nm (YFP), 470–500 nm (CFP), and 677–753 nm (chlorophyll).

4.5. PeHA1 expression in wild-type Arabidopsis and transgenic plants

Real-time RT-PCR was used to analyze expression levels of *PeHA1* in wild-type *Arabidopsis* and transgenic lines (H1, H3, H8, and H9). Total RNA of the leaves was extracted with TRIzol (Invitrogen) according to the instructions from the manufacturer. The first-strand cDNA was synthesized from 2 μg of total RNA using oligo(dT) and SuperScriptII (Invitrogen) according to the manufacturer's instructions. Primers for the PCR reactions were designed by Primer5 software with amplicon lengths between 200 and 300 bp. The housekeeping gene *AtActin* was used as the reference gene. Quantitative real-time PCR was performed on a MJ option2 instrument (Bio-Rad Corp.) using ABI SYBR Green PCR Master Mix. The reaction procedures were as follows: denatured at 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s. The data obtained were analyzed with MJ Opticon Monitor Software Version 3.1.

4.6. Determination of salt tolerance in transgenic Arabidopsis

A. thaliana [ecotype Columbia-0 (Col-0)] plants were grown in nursery soil under a short photoperiod (9 h of light; 100–200 μmol m^{-2} s^{-1} at 22 °C; 70% relative humidity) for 3–4 weeks, followed by growth under a long photoperiod (16 h of light; 100–200 μmol m^{-2} s^{-1} at 22 °C; 70% relative humidity). After surface disinfection, seeds of WT *Arabidopsis* and T3 homozygous lines (H1, H3, H8, and H9) were planted in half-strength MS medium (1/2MS) containing different concentrations of NaCl (0, 100, or 150 mM) and placed in a disinfection chamber with 25/20 °C day/night temperature and 16/8 h photoperiod (light/dark). The germination rates were counted after 5 days. Seedlings from WT, H1, and H3 plants were then transferred to 1/2MS with or without 100 mM NaCl. Root length and dry weight were measured after 10 days of salt treatment.

4.7. Purification of plasma membrane and H^+-ATPase assay

Seedlings of wild-type *A. thaliana* and transgenic lines (H1, H3, H8, and H9) were grown in nursery soil under a short photoperiod for four-weeks. Then plants were harvested for H^+-ATPase

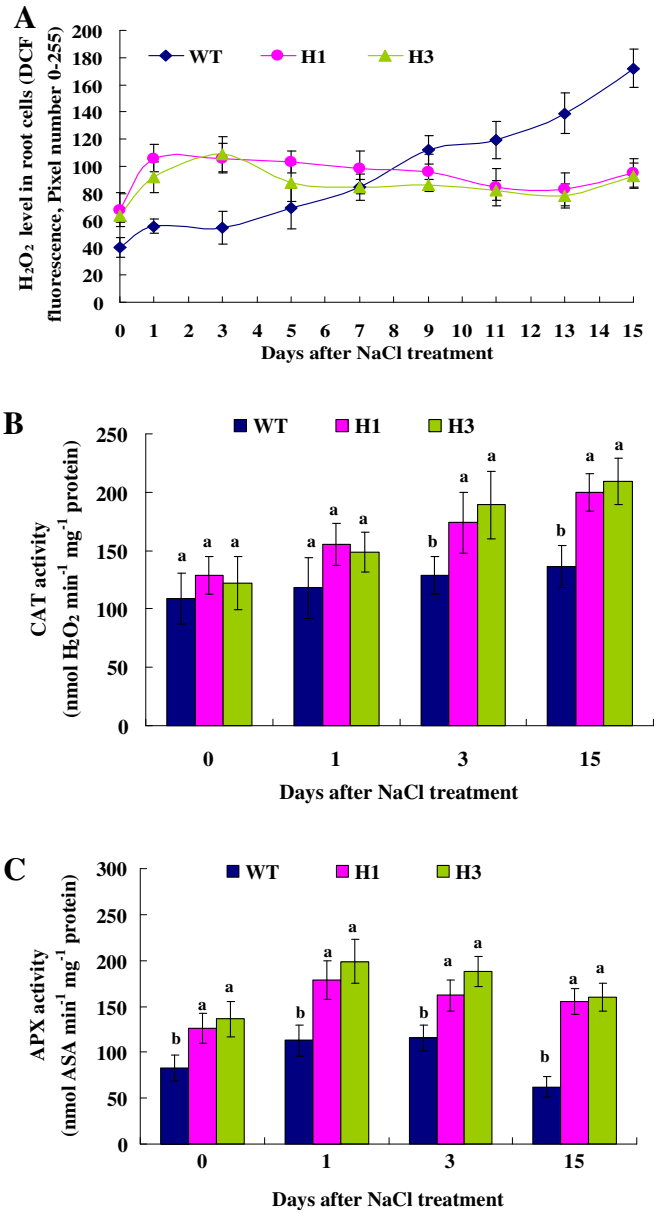

Fig. 8. H_2O_2 production and activity of antioxidant enzymes during prolonged NaCl salinity in WT *Arabidopsis* and transgenic lines (H1 and H3). Seven-day-old seedlings were transferred to 1/2MS with or without 100 mM NaCl for 15 days, after which seedlings were collected at the indicated time points for measurements of H_2O_2 levels and antioxidant enzymes activities. (A) NaCl-induced H_2O_2 production in root cells. Each point is the mean of 8–10 individual seedlings, and bars represent the standard error of the mean. (B and C) Effects of NaCl on the total activities of CAT and APX in WT *Arabidopsis* and transgenic lines. Each column is the mean of three biologically independent samples, and bars represent the standard error of the mean. Columns labeled with different letters (a, b) show significant differences between WT and transgenic lines at $P < 0.05$.

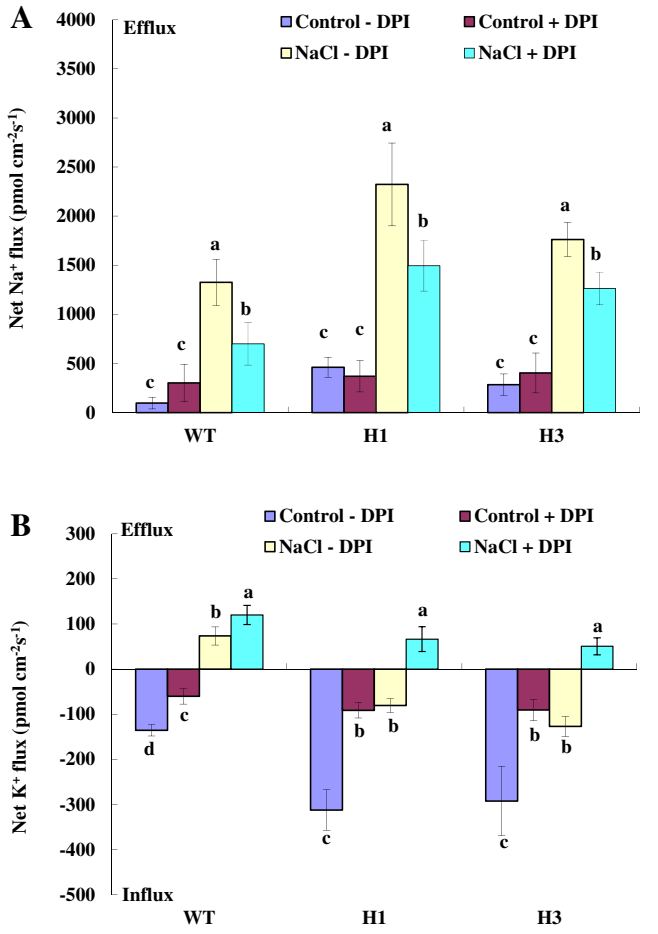

Fig. 9. Effects of DPI on NaCl-induced fluxes of Na^+ and K^+ in root tips of WT and transgenic lines (H1 and H3). Seven-day-old seedlings were transferred to 1/2MS with or without 100 mM NaCl and with or without 100 μM DPI for 7 days, after which seedlings were collected for NMT measurements. Each column is the mean of six independent seedlings, and bars represent the standard error of the mean. Columns labeled with different letters (a, b, c, d) exhibit significant differences between treatments at $P < 0.05$.

hydrolysis activity assay. Plasma membrane vesicles were prepared using aqueous two-phase partitioning [35]. In brief, plants were homogenized in cold extraction lysis containing 0.33 M sucrose, 0.2% (w/v) BSA, 5 mM EDTA, 5 mM DTT, 5 mM ascorbate, 1% (w/v) PVP-40, 1 mM PMSF, and 25 mM HEPES–KOH, pH 7.5. Four milliliters of homogenization buffer were used per gram of tissue. The homogenate was filtered and centrifuged at 13,000 *g* for 10 min. The supernatant then was centrifuged for 1 h at 100,000 *g* to obtain a microsomal pellet. The pellet was resuspended in a buffer containing 0.33 M sucrose, 1 mM PMSF, and 5 mM potassium phosphate, pH 7.8. The suspension was added to a phase mixture to obtain a final phase system consisting of 6.3% (w/w) Dextran T-500 and 6.3% (w/w) polyethylene glycol 3350 in 5 mM potassium phosphate (pH 7.8), 0.33 M sucrose and 3 mM KCl. The final upper phases were collected after portioned for three times, and then diluted with resuspension buffer [0.33 M sucrose, 1 mM PMSF, 2 mM DTT, and 10 mM MOPS–KOH, pH 7.5], then centrifuged for 30 min at 110,000 *g*. The resulting pellet was collected and resuspended to measure H^+-ATPase activity.

H^+-ATPase hydrolysis activity was measured by determining the Pi released from ATP [36]. The reaction mixture (200 μL) contained 10 mM MOPS–KOH, pH 6.5, 3 mM $MgCl_2$, 50 mM KNO_3, 1 mM NaN_3, 0.5 mM Na_2MoO_4, 50 mM KCl, 20 μg protein, in the presence or absence of 400 μM Na_3VO_4. The reaction was started by adding 1 μL 200 mM ATP to the mixture that mentioned above. After 30 min of incubation (37 °C), the reaction was stopped by adding 10% (w/v) trichloroacetic acid. The H^+-ATPase activity was determined by measuring the release of Pi through molybdenum-blue colorimetry at 820 nm.

PM H^+-transport activity was measured by inside-acid pH gradient (ΔpH) which was formed in the vesicles by H^+ pump [37]. The initial rate of fluorescence quenching (percent quenching min^{-1}) was used as a relative estimate of the rate of H^+ translocation

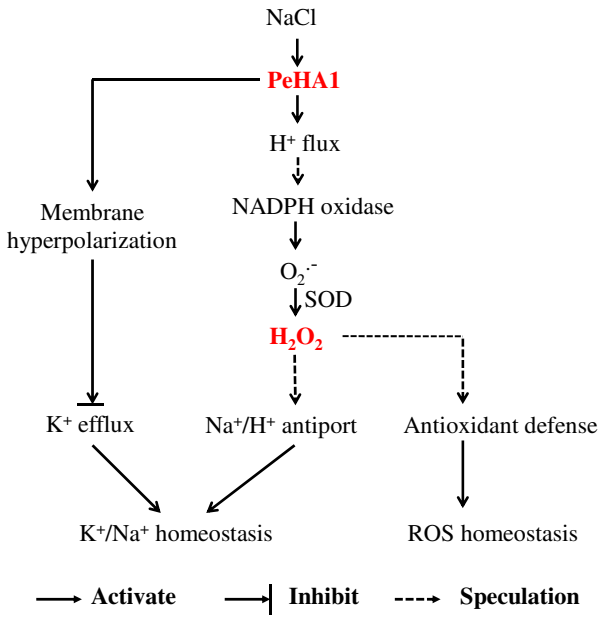

Fig. 10. Schematic model showing mediation of PeHA1 in K^+/Na^+ homeostasis via H_2O_2 signaling pathway involving NADPH oxidase.

[37]. PM Na^+/H^+ antiport activity was detected as a Na^+-induced dissipation of the pH gradient as described [37]. These initial rates were taken within the first 3 min after the addition of 20–80 mM NaCl. In this series, seedlings of wild-type *A. thaliana* and transgenic lines (H1 and H3) were grown in nursery soil under a short photoperiod for four weeks. Then plants were treated with 150 mM NaCl for three days or 10 mM H_2O_2 for 12 h prior to H^+-transport and Na^+/H^+ antiport activity assay. Control plants were treated without NaCl or H_2O_2. In addition, effect of DPI on the H^+-transport activity was examined in WT *Arabidopsis* and transgenic lines (H1 and H3). Our data show that DPI (100 μM) did not change H^+ translocation of the isolated membrane vesicles (Supplemental Fig. S3).

4.8. Ion content determination

Three-week-old seedlings from WT, H1, and H3 plants grown on 1/2MS with or without 100 mM NaCl were used for the determination of ion content. Seedlings were collected and flushed with distilled water 3–4 times, heated to 120 °C for 30 min to inactivate enzymes, and dried at 80 °C for at least 48 h. Dry, powered plant materials were pressure-digested in HNO_3, and the extracts were used for elemental (K^+, Na^+, and Ca^{2+}) analysis in a Varian Atomic Absorption Spectrometer (AA240FS, Varian, Inc., California, US) [20,22].

4.9. Steady-state ion flux measurements

Steady-state fluxes of Na^+, H^+, and K^+ were measured non-invasively using the Non-invasive Micro-test Technique (NMT-YG-100, YoungerUSA LLC, Amherst, MA 01002, USA) with ASET 2.0 (Sciencewares, Falmouth, MA 02540, USA) and iFluxes 1.0 (YoungerUSA, LLC, Amherst, MA 01002, USA) Software, which is capable of integrating and coordinating differential voltage signal collection, motion control and image capture simultaneously [12,13,23,24].

Prior to flux measurements, seven-day-old seedlings grown on 1/2MS were transferred to fresh medium containing 0 mM (control), 50 mM, and 100 mM NaCl for another seven days. The seedlings were then washed 4–5 times with redistilled water and transferred to the measuring chamber containing 10–15 mL measuring solution [0.5 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM NaCl, and 2.5% sucrose, pH 5.5 (adjusted with KOH and HCl)]. After the roots were immobilized on the bottom of the chamber, ion fluxes were measured at the meristematic zone of the root apex, where ion flux rates are significantly larger than in mature zones [23,24]. Two to three minutes of continuous recording were performed at each measurement point in the apical region. The steady-state Na^+, H^+, and K^+ flux rates were expressed as the mean of all the measured points within one seedling. External pH values were calculated according to the established curve between pH values and original potentials acquired by NMT from H^+ flux measurements [24].

For diphenylene iodonium (DPI) treatments, seven-day-old seedlings grown on 1/2MS were transferred to fresh medium containing 0 mM (control) or 100 mM NaCl. DPI was prepared with 1/2MS without agar and added to the surface of solid 1/2MS after filter-sterilization. Steady-state ion fluxes were measured as described above after treatment for seven days.

4.10. H_2O_2 detection

A specific fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR, USA), was used for H_2O_2 detection in the roots of WT and transgenic *Arabidopsis* plants [12,13]. Transient and long-term responses of H_2O_2 to NaCl exposure were examined in this study.

For the transient salt response, seven-day-old seedlings (WT, H1, and H3) grown on 1/2MS were transferred to liquid 1/2MS for a 1-h adaptation, then incubated with 50 μM H_2DCF-DA (prepared in liquid 1/2MS, pH 5.7) for 10 min at room temperature in the dark. The H_2DCF-DA-loaded seedlings were washed 3–4 times with liquid 1/2MS and exposed to a 100-mM NaCl shock. Confocal measurements were taken every 30 s for 20 min of NaCl stress via xyt mode. H_2DCF-DA fluorescence intensities in root cells and apoplast spaces were measured with Image-Pro Plus 6.0 (Image-Pro Plus version 6.0 software, http://www.mediacy.com/index.aspx?page=IPP).

To investigate the longer-term response to salt, seven-day-old seedlings (WT, H1, and H3) grown on 1/2MS were transferred to fresh medium with 0 mM (control) or 100 mM NaCl for another 15 days. Seedlings were collected and loaded with 50 μM H_2DCF-DA (prepared in NaCl solution) for 10 min at the indicated time points. H_2DCF-DA-dependent fluorescence in the roots was detected with confocal microscopy and quantitatively analyzed with Image-Pro Plus 6.0. The confocal settings were excitation 488 nm and emission 510–530 nm.

In addition to NaCl stress, effect of external pH on H_2O_2 production in roots of WT *Arabidopsis* and transgenic lines (H1 and H3) was examined in this study. Seven days old seedlings grown on solid 1/2MS were transferred to liquid 1/2MS with pH values at 5.0, 6.0, 7.0, or 8.0. H_2DCF-DA fluorescence intensity was measured after 1 h of treatment as described above.

4.11. Antioxidant enzyme assays

Seven-day-old seedlings (WT, H1, and H3) grown on 1/2MS were transferred to fresh medium with 0 mM (control) or 100 mM NaCl for another 15 days. Seedlings were collected at 0, 1, 3, and 15 days. *Arabidopsis* seedlings (0.2000 g) were ground to a fine powder in liquid N_2 and then homogenized in 2 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid and 1% polyvinylpyrrolidone [16]. The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C, and the supernatant was used to determine catalase (CAT) activity. For ascorbate peroxidase (APX) measurements, 1 mM ascorbic acid was

added to the enzyme extraction buffer. Protein concentration was determined by the method of Bradford (1976) [38], with bovine serum albumin as the standard.

Total CAT activity was determined as the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) measured at 240 nm for 3 min at 25 °C [39]. The 3-mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2% H_2O_2, and 30 μL of cell enzyme extract. Immediately after the enzyme extract was added to the reaction mixture, the initial linear rate of decrease in A_{240} was recorded, and CAT activity was calculated.

Total APX activity was determined as the decrease in A_{290} (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 2 min at 25 °C in a 3-mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 15 mM ascorbate, 30 mM H_2O_2, and 30 μL of enzyme extract. The reaction was started by the addition of H_2O_2, and a correction was made for the low, non-enzymatic conversion of ascorbate by H_2O_2 [40].

5. Data analysis

Ionic fluxes were obtained using MageFlux developed by Yue Xu (http://www.youngerusa.com). All mean data were subjected to analysis of variance. Significant differences between means were determined by Duncan's multiple range test. Unless otherwise stated, differences were considered statistically significant when $P < 0.05$.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.06.020.

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