

Cloning and Characterization of a Plasma Membrane H⁺-ATPase (PMA) Gene from a Salt-tolerant Plant *Chloris virgata*

Xinxin Zhang¹✉, Hui Wang¹✉, Shenkui Liu¹✉, Tetsuo Takano²✉

1. Alkali Soil Natural Environmental Science Center (ASNEC), Stress Molecular Biology Laboratory, Northeast Forestry University, Harbin, 150040, P. R. China

2. Asian Natural Environment Science Center (ANESC), the University of Tokyo, Midori Cho 1-1-1, Nishitokyo City, Tokyo 188-0002, Japan

✉ Corresponding Author email: xxzhang@nefu.edu.cn; ✉ Author

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Abstract A plasma membrane H⁺-ATPase (PMA) gene (*ChvPMA*) was isolated from a wild salt-tolerant plant *Chloris virgata*. The expression of *ChvPMA* gene in leaves and roots of *Chloris virgata* seedlings under salt stress (NaCl, NaHCO₃) was examined. The results showed the *ChvPMA* gene expression was induced by salt stress. The *ChvPMA* gene was fused to the N-terminus of green fluorescence protein (GFP) gene, and transferred into onion epidermal cells for analyses of intracellular localization. The result showed that the *ChvPMA* protein was found to be in the plasma membrane of ion epiderm. Because the H⁺-ATPase activity is regulated by a C-terminal auto-inhibitory domain that can be displaced by phosphorylation, we analyzed transgenic yeast expressing either wild-type PMA (*ChvPMA*) or truncated *ChvPMA* lacking the C-terminal auto-inhibitory domain (*ChvPMAAC*) under high salt and pH conditions. The results showed that over-expression of *ChvPMA* and *ChvPMAAC* in transgenic yeasts increased the resistance to salt and lower pH conditions, especially, the yeast over-expressing *ChvPMAAC* showed better growth than *ChvPMA* at an external pH 4.0 in the presence NaCl. Transgenic Arabidopsis over-expressing *ChvPMAAC* also showed the better root growth than that of *ChvPMA* at an external pH 4.0 in the presence NaCl.

Keywords *Chloris virgata*; *ChvPMA*; Plasma membrane H⁺-ATPase; Salt and pH tolerance

Introduction

The H⁺-ATPase is the major electrogenic pump in the plasma membrane (PM) of the plant cells. By pumping protons from the cytoplasm to the apoplast it generates an electrochemical proton gradient, which drives the transport of mineral ions and organic solutes, and plays a crucial role in cytoplasmic and apoplastic pH homeostasis. The PM-H⁺-ATPase participates in a variety of physiological processes such as phloem loading, stomata opening, mineral nutrition, growth of root hairs and pollen tubes, salt and osmotolerance, leaf movements, and acid growth (Arango et al., 2003; Sondegaard et al., 2004).

The PM H⁺-ATPases are encoded by a multigene family, and at least 10 isoforms of the H⁺-ATPase in plants (Arango et al., 2003; Baxter et al., 2003). The

expression of several members in various species has been examined and shown to have different cell type or tissue specificities, development stage, environmental factors, some times with important overlap (Harper et al., 1994; Michelet et al., 1994; Moriau et al., 1999; Oufattole et al., 2000). Although much progress has been made towards gene cloning and expression analysis of the H⁺-ATPase genes, less information is available concerning the regulation of gene expression by environmental factors. Several studies have been reported on the response of PM-H⁺-ATPase to salt stress on the activity, mRNA expression level and translation level (Niu et al., 1993; Yang et al., 2004; Sibole et al., 2005). Niu et al. (1993) reported that the PM-H⁺-ATPase gene expression was induced by NaCl in roots and expanded leaves but not in stems or .

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expanding leaves. Moreover, the PM-H⁺-ATPase gene expression in the halophyte *Atriplex nummularia* was more responsive to NaCl than in the glycophyte tobacco. Yang et al. (2004) found that the salt stress increased the PM-H⁺-ATPase activity in roots of salt-tolerant wheat L-Ch20, but reduced the PM-H⁺-ATPase activity in salt-sensitive wheat Y-J24. Similarly, Sibole et al. (2005) used two *Medicago* species to examine the relation between changes in leaf PM-H⁺-ATPase and ion partitioning differing in their response to NaCl. The increased expression of the PM-H⁺-ATPase was found in salt-stressed species. Transgenic approaches have recently been used to obtain a clearer understanding of the roles played by PM-H⁺-ATPase in the plant (Zhao et al., 2000; Vitart et al., 2001; Gévaudant et al., 2007). Over-expression of a plasma membrane H⁺-ATPase4 gene (PMA4) in tobacco yielded transformants that showed little difference in development under normal growth conditions. On the other hand, plants in which the endogenous and transgenic PMA4 were co-suppressed showed a severe phenotype (Zhao et al., 2000). A T-DNA insertion mutant in AHA4 (subfamily I) was characterized by a slight reduction in root and leaf growth and a high sensitivity to salt stress (Vitart et al., 2001). As to post-translational regulation of the PM-H⁺-ATPase, the best known mechanism described to date involves the auto-inhibitory action of the C-terminal domain (Palmgren et al., 1991; Johansson et al., 1993). To better understand the physiological consequence of this activation, Gévaudant et al. (2007) analyzed transgenic tobacco (*Nicotiana tabacum*) plants expressing either wild-type plasma membrane H⁺-ATPase4 (wtPMA4) or a PMA4 mutant lacking the auto-inhibitory domain (PMA4), generating a constitutively activated enzyme. Over-expression of the PMA4 resulted in greater in vivo proton pumping activity, increasing salt tolerance, and altered plant development, possibly related to cell expansion.

In our study, we isolated a plasma membrane H⁺-ATPase gene (named as *ChvPMA*) from a wild salt-tolerant plant *Chloris virgata*. *C. virgata*, also known as feather finger grass. It is one of the few species that can grow naturally on the alkali soils in Northeast Plain of China. Here, we reported the

ChvPMA gene expression, intracellular localization, and the analyses of yeast and *Arabidopsis thaliana* plants that over-expressed either *ChvPMA* or *ChvPMA* lacking the last 103 residues (*ChvPMAΔC*), corresponding to the C-terminal autoinhibitory region.

1 Results

1.1 Sequence analyses and intracellular localization of *ChvPMA*

The full-length *ChvPMA* cDNA was directly obtained from the full-length cDNA library of *Chloris virgata* of our laboratory. The *ChvPMA* cDNA was 3 380 bp in length, including a complete open reading frame of 2 856 bp encoding a putative protein of 952 amino acids with a predicted protein molecular weight of 10 460 Da. The TMpred method predicted the *ChvPMA* protein has 10 transmembrane domains.

To investigate the intracellular localization of *ChvPMA*, we performed an in vivo targeting experiment that employed a *ChvPMA*-fused green fluorescent protein (GFP) as a fluorescent marker in a transient transfection assay using onion cells. As shown in Figure 1, control GFP was uniformly distributed throughout the onion epidermal cell, whereas the *ChvPMA*-GFP fusion protein was exclusively localized to the plasma membrane.

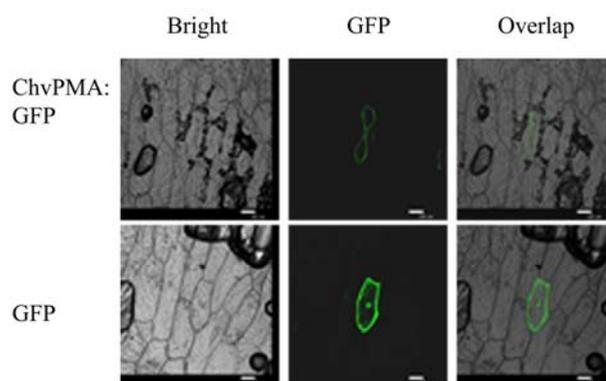


Figure 1 Intracellular localization of the *ChvPMA* protein in onion (*Allium cepa* L.) epidermal cells

Note: The expression of the introduced genes was viewed after 12 h through a fluorescence microscope

1.2 Expression of *ChvPMA* gene is induced by salt stress

ChvPMA gene expression was induced by NaCl in both leaves and roots (Figure 2). In the presence of

200 mM NaCl, the expression of *ChvPMA* gene in root was strongly than that of in leaves. These data indicate that the expression of *ChvPMA* gene was in response to salt stress.

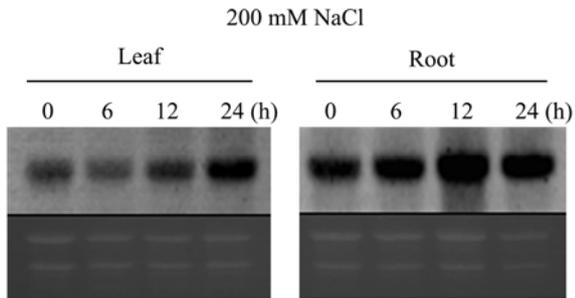


Figure 2 Expression of *ChvPMA* gene expression in leaf and root of *Chloris virgata* exposed to salt stress from NaCl

Note: 8 μ g of total RNA was isolated from leaves and roots, respectively. Hybridization analysis was performed using 3'-UTR region of *ChvPMA* cDNA labeled by digoxigenin (DIG) as a probe

1.3 Expression of *ChvPMA* and *ChvPMA* Δ C gene in yeast improves the tolerance to pH and salt stresses

Three transformed yeast lines were constructed. One was transformed with empty vector pYES2 as a

control; the other two were transformed with vector containing either *ChvPMA* or *ChvPMA* Δ C, respectively. A RNA gel blot analysis confirmed that *ChvPMA* and *ChvPMA* Δ C were expressed in yeast, respectively (data not shown). In the presence of 2% glucose (control condition in Figure 3), as result of the gene expression was not induced, the growth of yeast cells transformed with *ChvPMA* or *ChvPMA* Δ C was the same as that of the empty vector transformant. Under the pH condition (pH 4.0, pH 5.8, and pH 8.0), both *ChvPMA* and *ChvPMA* Δ C yeast transformants showed much better growth than vector transformant along with the reducing pH (Figure 3). In the presence of NaCl at an external pH (pH 5.8 and pH 8.0), both *ChvPMA* and *ChvPMA* Δ C yeast transformants were able to sustain the growth, whereas, the vector transformant grew not well or did not grow at all. In the presence of NaCl at an external pH 4.0, *ChvPMA* Δ C transformant allowed faster growth than *ChvPMA* transformant (Figure 3).

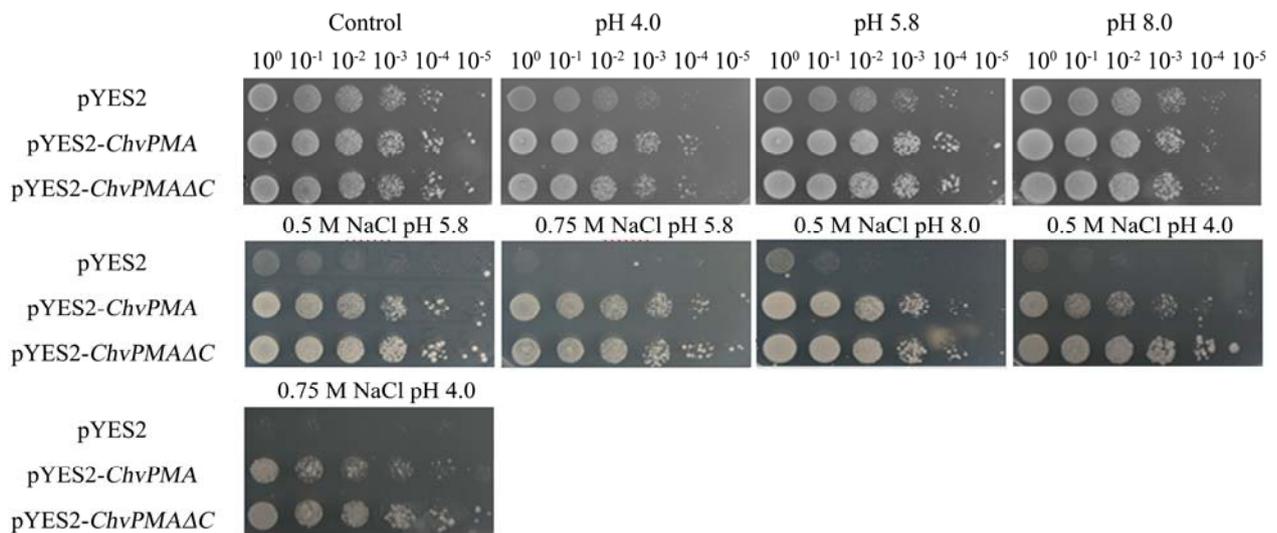


Figure 3 The growth of transgenic yeasts under high salt and pH conditions

Note: The overnight yeast cultures were adjusted to OD₆₀₀=0.3. Serial dilutions were spotted onto plates supplemented with NaCl at the concentrations indicated. Control plates: SD-Uracil medium. Other plates: SC-Uracil medium

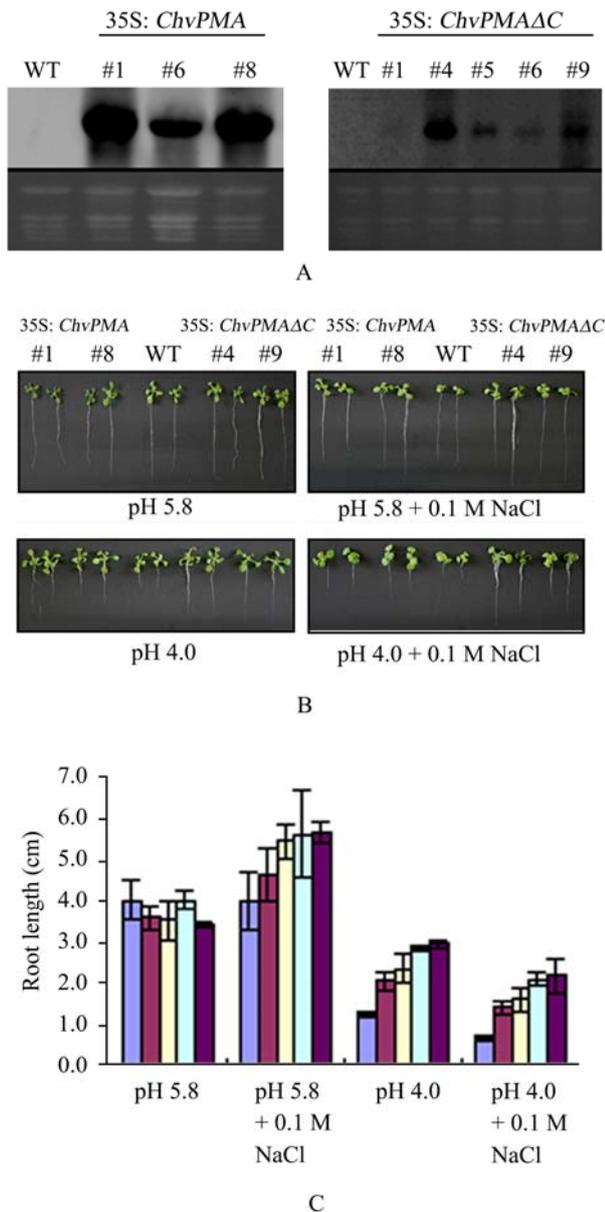


Figure 4 (A) RNA gel blot analysis of T₃ generation transgenic plants of *ChvPMA* and *ChvPMAΔC*. (B)-(C) Effects of salts on root lengths of wild-type (WT) and the T₃ generation transgenic plants over-expressing the *ChvPMA* or *ChvPMAΔC*. Note: (B) WT and transgenic seeds germinated on MS solid medium agar plates under different conditions as indicated. The pictures were taken after 14 d. (C) Root length of WT and transgenic plants under each condition as indicated. Each value represents the in (C) mean ±SE of five plants

1.4 Over-expression of *ChvPMA* and *ChvPMAΔC* gene in transgenic Arabidopsis improves the tolerance to pH and salt stresses

We generated Arabidopsis transgenic plants which over-expressed *ChvPMA* or *ChvPMAΔC* gene under the control of the strong constitutive *CaMV35S* promoter. Three independent T₃ generation lines transgenic lines of each gene were identified by RNA gel blot analysis. The results show that each of these lines expressed *ChvPMA* or *ChvPMAΔC* in plants.

Under pH 5.8 condition, the root length was not noticeably different between WT and two T₃ generation transgenic lines (#1 and #8) over-expressing *ChvPMA* or between WT and two T₃ generation transgenic lines (#4 and #9) over-expressing *ChvPMAΔC* (Figure 4B, C). In the presence of 100 mM NaCl at a pH of 5.8, the growth of roots was little stimulated in both of the *ChvPMA* and *ChvPMAΔC* transgenic lines. Under lower pH condition, the root growth of WT plants was significantly inhibited at external pH of 4.0 in presence of 100 mM NaCl or not (Figure 4B). Between the *ChvPMA* and *ChvPMAΔC* transgenic lines, the *ChvPMAΔC* transgenic lines exhibited a better tolerance at an external pH of 4.0, even on the MS media containing 100 mM NaCl at a pH of 4.0 (Figure 4B). Measurements confirmed that the root lengths of the *ChvPMAΔC* transgenic lines were a little higher than those of *ChvPMA* transgenic lines under lower pH condition (Figure 4C).

2 Discussion

Plasma membrane (PM) H⁺-ATPase plays a key role in the establishment and maintenance of cellular ion homeostasis. The proton and electrical gradient produced by PM H⁺-ATPase is the driving force for active secondary transport and the regulation of Na⁺ and Cl⁻ uptake (Arango et al., 2003). Multiple genes encoding PM H⁺-ATPase have been cloned in many plants such as tomato (Mito et al., 1996), *Arabidopsis thaliana* (Harper et al., 1994), rice (Zhang et al., 1999) and *Nicotiana plumbaginifolia* (Moriau et al., 1993).

In our study, we cloned a PM H⁺-ATPase gene (*ChvPMA*) from a wild salt-tolerant plant *Chloris virgata*. The expression of *ChvPMA* gene was investigated in the leaves and roots of *C. virgata* under salt stresses from NaCl (Figure 2). Our results showed that NaCl induced higher *ChvPMA* mRNA accumulation in the roots of *C. virgata*. In line with

our current results, message accumulation of the PM H⁺-ATPase gene in roots was also greater in the halophyte *Atriplex nummularia* than that in glycophyte tobacco (Niu et al., 1993). In *A. nummularia*, the H⁺-pump message was mainly localized in the epidermis and endodermis of the root, and the bundle-sheath cells in expanded leaves (Niu et al., 1996). A rice PM H⁺-ATPase gene (*OSA3*) was also expressed at a high level in the root of a salt-tolerant mutant rice M-20 (Zhang et al., 1999). Therefore, the higher expression of the PM H⁺-ATPase gene in root indicated that a requirement for this pump in these organs during salt adaptation.

Enzymatic regulation of PM H⁺-ATPase has been well documented following the identification of an auto-inhibitory domain in its C-terminal region that keeps enzyme activity at a low level (Palmgren et al., 1991). The PM H⁺-ATPase can be stimulated by displacement of the auto-inhibitory domain upon phosphorylation of the penultimate residue, a Thr, and the subsequent binding of 14-3-3 regulatory proteins (Camoni et al., 2000). We designed a constitutively activated *ChvPMA* isoform (*ChvPMAΔC*) by deleting the entire C-terminal region. Expression in yeast showed that the *ChvPMAΔC* conferred better growth to yeast cells than the full-length form in the presence of NaCl at an external pH 4.0 (Figure 3). Similar results were also obtained from the transgenic plants over-expressing *ChvPMAΔC* which showed better root growth than the full-length form at an external pH 4.0 condition (Figure 4). In line with our current results, a previous report showed that the Arabidopsis transgenic plants expressing the AHA3 isoform activated by deletion within its inhibitory C-terminal region showed reduced growth inhibition when seedlings were grown in vitro at a pH below 5.0 (Young et al., 1998). Another report also showed the transgenic tobacco over-expressing a truncated PMA4 (Δ PMA4) displayed increased salt tolerance during germination and seedling growth compared with WT plants and transgenic tobacco over-expressing wtPMA4 (Gévaudant et al., 2007). Taken together, our results suggest that over-expressing a constitutively activated PM H⁺-ATPase in transgenic plants can improve the low pH (pH 4.0) and salt tolerance, and

the extracellular H⁺ promotes the salt tolerance of the transgenic plants overexpressing an activated PM H⁺-ATPase (Wang et al., 2014). Therefore, we thought that expression of an activated H⁺-ATPase is an interesting solution for improving salt tolerance of plants. In the future study, how the H⁺-ATPase activity was regulated by the *ChvPMA* and *ChvPMAΔC* in transgenic plants will be investigated, which is helpful to explain the why the transgenic plants over-expressing *ChvPMAΔC* exhibited the better salt tolerance.

3 Materials and methods

3.1 Plant materials, growth condition and stress treatments

Seeds of *Chloris virgata* Swartz (*C. virgata*) were collected in the alkaline soil area located in northeast China. The seed was sterilized with 1% (v/v) Plant Preservative Mixture (Plant Cell Technology, USA) for 24 h. Plants were grown in hydroponic culture at 28°C with a 16 h photoperiod as described by Nishiuchi et al. (2007). 14-day-old seedlings were transferred to the hydroponic solution containing 200 mM NaCl, incubated at 28°C and sampled at 0 h, 6 h, 12 h, or 24 h. When leaves and roots were harvested, dropped immediately into liquid nitrogen and stored at -80°C for RNA extraction.

3.2 RNA isolation and gel blot analysis

Plant total RNA was isolated from leaves and roots using Trizol reagent (Invitrogen) according to manufacturer's instructions. 8 μg plant total RNA was separated on denaturing formaldehyde 1.0% agarose gel, and blotted onto a nylon membrane. Hybridization analysis was performed using 3'-UTR region of *ChvPMA* cDNA labeled by digoxigenin (DIG) as a probe. Hybridization signals were detected with CDP-Star (GE Healthcare) using the FujiFilm LAS-1000plus.

3.3 Intracellular localization of *ChvPMA*

Green fluorescent protein (GFP) cDNA was fused in-frame to the 3'-end of the full-length *ChvPMA* coding region. Transient expressions of GFP and GFP fusion constructs were performed by introducing the resultant DNAs into onion (*Allium cepa* L.) epidermal cells using the particle bombardment method,

according to the manufacturer's protocol (Bio-Rad). Fluorescent photographs of onion cells were captured through a fluorescence microscope (Nikon, Japan).

3.4 Media, yeast strain, transformation and stress treatment

The pYES2 vector (Clontech) driven by the GAL1 promoter was introduced for inducible expression of recombinant proteins in yeast. High level expression of the fusion protein was induced by galactose and repressed by glucose. The full-length *ChvPMA* and truncated *ChvPMAΔC* cDNA was ligated into the *Bam*HI/*Sph*I sites in pYES2 to construct the plasmid pYES2-*ChvPMA* and pYES2-*ChvPMAΔC*, respectively. The plasmid pYES2-*ChvPMA*, pYES2-*ChvPMAΔC* and pYES2 empty vector were introduced into *Saccharomyces cerevisiae* strain BY22740 (Osaka University, Japan), in which the original plasma membrane ATPase (PMA1) was deleted. Yeast cells were grown in two types of medium based on basic medium (6.7 g/L yeast nitrogen base+67 mg/L -Uracil Do Supplement). Yeast cells containing pYES2-*ChvPMA*, pYES2-*ChvPMAΔC* and pYES2 vector were incubated in SD-Uracil medium (basic medium+2% glucose, pH 5.8) at 30°C. The overnight cultures were adjusted to OD600 at 0.3, and diluted to 10-, 100-, 1000-, 10000-fold with appropriate basic medium, and then 10 μL of each dilution series were spotted on SC-Uracil solid medium (basic medium+1.94% galactose+0.06% glucose+2% agar, pH 4.0, 5.8 or 8.0), supplemented with the different concentrations of NaCl indicated in each case. Yeast growth was monitored for 5 d.

3.5 Transformation of Arabidopsis and stress tolerance assays of the transgenic plants

The full-length of *ChvPMA* and truncated *ChvPMAΔC* were subcloned into the plant transformation binary vector pBI121 (Clontech) under the control of a *CaMV35S* promoter with *nptII* as the selectable marker, respectively. The construct was introduced into *Agrobacterium tumefaciens* strain EHA105. Arabidopsis was transformed by the floral dip method (Clough and Bent 1998). The expression of *ChvPMA* and *ChvPMAΔC* gene in transgenic Arabidopsis plants was evaluated by RNA gel blot, respectively.

For abiotic stress treatment, the wild type (WT) Arabidopsis and transgenic Arabidopsis seeds of *ChvPMA* or *ChvPMAΔC* gene was surfaced-sterilized with 70% ethanol for 1 min and 1% NaClO solution for 30 min. The seeds were then rinsed three times in sterile water, and sown on agar plates containing MS medium, 1% (w/v) sucrose, and 0.8% (w/v) agar for 12 d, and the seedlings were transferred to fresh media containing the indicated compounds. The plates were positioned vertically on shelves to compare root growth. Seedlings were allowed to growth for 21 d.

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