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Co-overexpression of *AVP1* and *PP2A-C5* in Arabidopsis makes plants tolerant to multiple abiotic stresses

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ABSTRACT

Abiotic stresses are major threats to agricultural production. Drought and salinity as two of the major abiotic stresses cause billions of losses in agricultural productivity worldwide each year. Thus, it is imperative to make crops more tolerant. Overexpression of *AVP1* or *PP2A-C5* was previously shown to increase drought and salt stress tolerance, respectively, in transgenic plants. In this study, the hypothesis that co-overexpression of *AVP1* and *PP2A-C5* would combine their respective benefits and further improve salt tolerance was tested. The two genes were inserted into the same T-DNA region of the binary vector and then introduced into the *Arabidopsis* genome through Agrobacterium-mediated transformation. Transgenic *Arabidopsis* plants expressing both *AVP1* and *PP2A-C5* at relatively high levels were identified and analyzed. These plants displayed enhanced tolerance to NaCl compared to either *AVP1* or *PP2A-C5* overexpressing plants. They also showed tolerance to other stresses such as KNO_3 and LiCl at harmful concentrations, drought, and phosphorus deficiency at comparable levels with either *AVP1* or *PP2A-C5* overexpressing plants. This study demonstrates that introducing multiple genes in single T-DNA region is an effective approach to create transgenic plants with enhanced tolerance to multiple stresses.

1. Introduction

Abiotic stresses such as water deficit, flooding (anoxia), salinity, suboptimal temperatures, UV light and more, are real threats to agricultural productivity [1]. High salinity is one of the major stresses [2]. Only 0.5–1% of all angiosperm species can survive and reproduce under saline conditions and none of major crops nowadays falls into this category [3]. Unfortunately, major crops currently under cultivation have limited genetic diversity and potential for self-evolving salt tolerance due to long time selective breeding aimed at traits other than salt tolerance [4]. Thus, salt stress has become an increasingly severe problem compromising agricultural productivity, especially in arid and semi-arid regions [5,6]. Salts of high concentrations can cause hypertonic water efflux and thereafter loss of turgor pressure, which is essential for plant growth, development, and movement [7]. Lethal dehydration would occur when salt concentration reaches certain levels [8–10]. Moreover, many salt ions are toxic to plants at high concentration [11]. For example, protein translation was shown to be adversely affected by concentrated K^+, Na^+ and especially Cl^- [12,13]. Mitochondria are also negatively affected by high monovalent ions such as Na^+ and K^+, possibly through limiting substrate uptake and electron transport [14,15]. In chloroplast, both KCl and NaCl can uncouple CO_2 fixation and oxygen generation [16]. Malate dehydrogenase and phosphoenolpyruvate carboxylase are also inhibited by NaCl [17–19]. In general, salt ions at higher than optimal concentrations will adversely affect plants and therefore undermine agricultural productions [7]. Drought (water deficit) is another major environmental stress. Drought can affect many aspects of plant growth, such as photosynthesis, respiration, development [20]. Catastrophic yield losses were witnessed multiple times across the world due to severe episodes of drought [21,22]. In the foreseeable future, climate change will exacerbate this situation through making extreme weather patterns more frequent, which will be devastating to crop production [23]. With limited fresh water accessibility, strategies to help plants withstand drought stress is urgently needed [24].

Arabidopsis thaliana vacuolar H^+-pyrophosphatase 1 (AVP1) is partly responsible for establishing proton gradient across tonoplast membrane by using the energy from hydrolyzing pyrophosphates [25].

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Proton gradient across the membrane is thought as the main transducer of light energy based on the chemiosmosis hypothesis [26]. Thus, AVP1 is pivotal to many essential processes, such as energy metabolism, ions and pH homeostasis in plant cells. Overexpression of *AVP1* in *Arabidopsis* leads to increased drought and salt tolerance [27]. This was firstly attributed to the higher Na^+ sequestration into vacuole by secondary transporter such as Na^+/H^+ antiporter that is energized by AVP1. This leads to lower toxicity of Na^+ in the cytoplasm and lower water potential in plant cells [28]. Later, AVP1 was also found to facilitate auxin polar transportation and mediate auxin-stimulated root development [29]. Besides the increased salt and drought tolerance, overexpression of *AVP1* also induces P type ATPase to acidify the extracellular space, resulting in better nutrients, such as phosphorus, uptake [30]. Interestingly, AVP1 was also showed to scavenge the intracellular pyrophosphate, which can impair gluconeogenesis at high level [31]. This multifunctional and beneficial gene has been transformed into different species such as tobacco, creeping bentgrass, and cotton, to improve their stress tolerance [32–34].

The catalytic subunit 5 of protein phosphatase type 2A (i.e. PP2A-C5) is one of the five catalytic subunits of PP2A in *Arabidopsis* [35]. PP2A, as one subgroup of the Ser/Thr protein phosphatase family, was shown to be involved in many pivotal cellular processes [36], including auxin response and transportation [37,38], nitrate assimilation [39], blue light response [40], ABA signaling [41,42], ethylene biosynthesis [43], brassinosteroid signaling [44], and responses to biotic and abiotic stresses [45]. The early evidence about the involvement of PP2A in abiotic stress response came from pharmacological and gene expression studies [46,47]. Later gain and loss of function studies further demonstrated the indispensable roles of PP2A in this process [48]. In particular, PP2A-C5 was recently shown to mediate salt stress response in plants, probably through regulating the activities of vacuolar membrane chloride channel proteins (CLCs) to increase the sequestration of Cl^- and NO_3^- into vacuole under salt stress conditions [49].

Thus far, most studies on abiotic stress responses focused on single stress factors. However, in real world, stresses tend to come together or in various combinations [20,50]. Quantitative studies by regression modeling focusing on major crops, such as rice, wheat, and maize, have also demonstrated additive negative effects of concurrent high temperature and water deficient stresses on biomass accumulation and photosynthesis [51]. Among the different stresses, drought and salt are closely related with overlapping physiological consequences (osmotic stress). Transcriptome analysis showed that over half of the genes regulated by each stress were also similarly regulated by the other stress in poplar [52]. Detailed analyses of salt and drought stress responses indicated that the very early response to high salinity is dominated by water retention. Salt specific effects would rise in days and weeks later when salt accumulates in and surrounding leaf cells [53]. Drought and salinity tend to occur at the same time in many places in the world due to the fact that drought facilitates salinization of agricultural land. Thus most arid or semiarid lands are also affected by high salinity [54]. Negative physiological consequences of both stresses could be additive or even worse due to the significantly overlapped effects. In this case, overexpression of single gene in transgenic plants would not be sufficient [55]. Multiple gene transformation is a promising solution [56,57]. Among the available methods delivering multiple genes into plants, linked genes (constructing genes with their own promoter and terminator sequences into one T-DNA region) is deemed the most efficient one [58,59].

This study was to test the hypothesis that co-overexpressing *AVP1* and *PP2A-C5* in Arabidopsis might further improve salt tolerance compared to plants over-expressing either *AVP1* or *PP2A-C5* and confer other beneficial traits such as drought tolerance at the same time. Our results showed that transgenic plants overexpressing both genes indeed outperformed wild-type plants under various stress conditions, and outperformed single gene overexpressing plants under NaCl stress conditions.

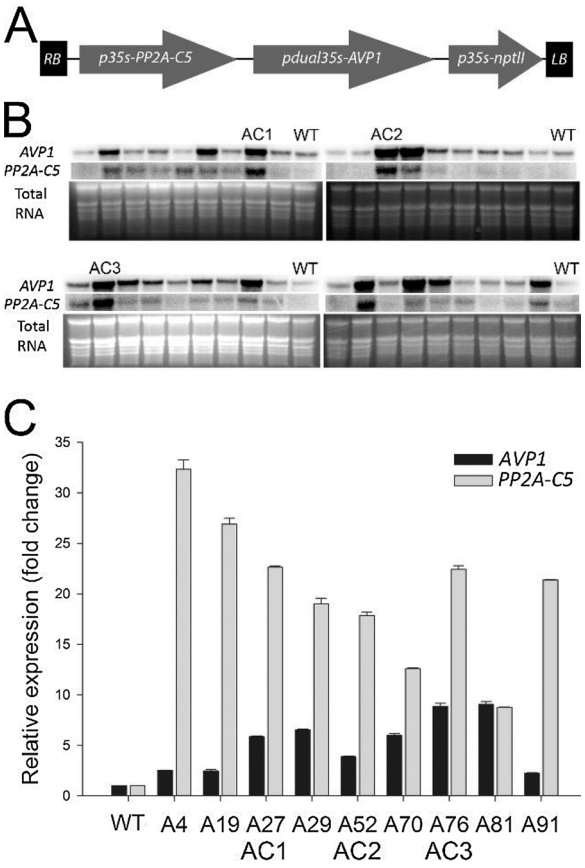

Fig. 1. Creation and characterization of *AVP1/PP2A-C5* co-overexpressing plants. A. Diagram of the T-DNA region of the transforming vector of p212-C5-AVP. p35s, 35S promoter; pdual35s, double 35S promoter; *nptII*, neomycin phosphotransferase gene. RB and LB, right and left border sequences of T-DNA. B. RNA blot analysis of *AVP1/PP2A-C5* co-overexpressing plants. AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants; WT, wild-type plants. C. Quantitative PCR analysis of *AVP1/PP2A-C5* co-overexpressing plants. A4 to A91, nine independent *AVP1/PP2A-C5* co-overexpressing plants with relatively high transcript levels for *AVP1* and *PP2A-C5*, respectively.

2. Results

2.1. Creation and characterization of AVP1/PP2A-C5 co-overexpressing plants

The binary vector p212-C5-AVP1 (Fig. 1A) that contains *AVP1* and *PP2A-C5* was made and transformed into wild-type *Arabidopsis* (Col-0) plants by using the Agrobacterium-mediated floral dip method [60]. There were 177 independent transgenic lines obtained in the T_1 generation. All T_1 putative transgenic plants were grown for T_2 seeds, which were plated on MS medium with 30 mg/l kanamycin to check T_2 segregation ratios. There were 109 putative single T-DNA insertion lines based on the segregation analysis of T_2 seeds. To obtain homozygous plants, 5 or more T_2 plants from each line were grown for seeds which were screened for kanamycin resistance again. A total of 52 putative homozygous lines were obtained. To test transgene expression, total RNAs from all 52 putative homozygous lines were extracted and subjected to RNA blot analysis with probes for *PP2A-C5* and *AVP1* (Fig. 1B). Over 40% of these lines showed higher transcript levels of either one or both genes compared to wild-type plants. To further confirm and quantify the transcript levels of *AVP1* and *PP2C-C5*, real-time PCR was employed to analyze the relative gene expression in nine transgenic lines (Fig. 1C). The results were consistent with RNA blot data. Three lines that showed co-overexpression of both genes from this population, indicated as AC1, AC2 and AC3 in Fig. 1B and C, were

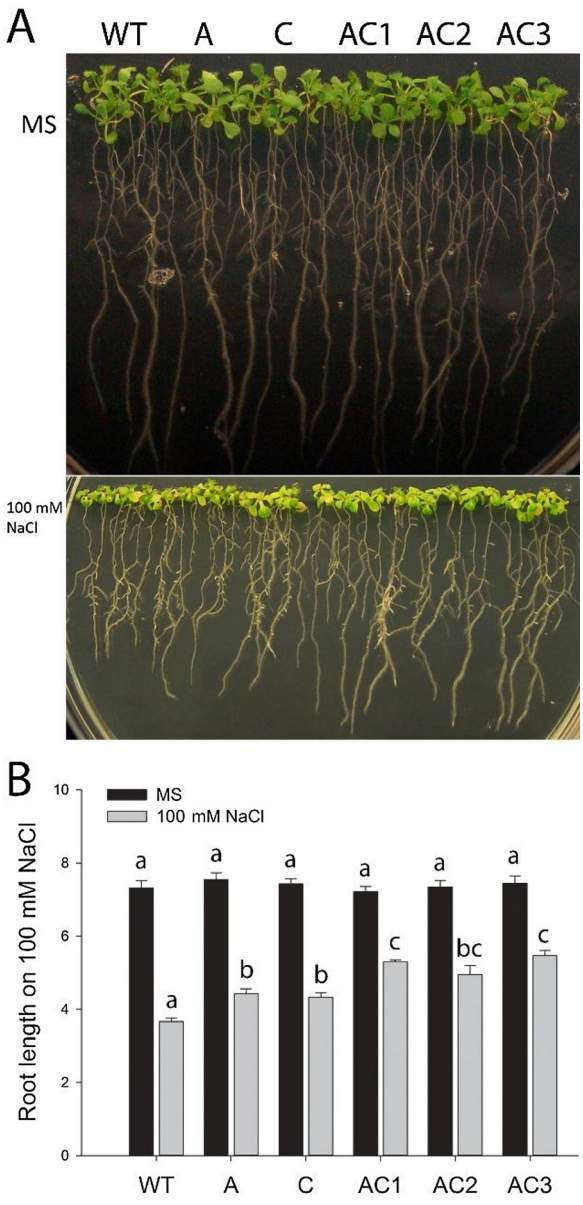

Fig. 2. Analysis of plants growth in the absence or presence of NaCl. A. Phenotypes of controls and *AVP1/PP2A-C5* co-overexpressing plants in the absence (MS) or presence of NaCl (MS + 100 mM NaCl) on day 10 after transferring to salt media. WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5* overexpressing plants; AC1, AC2, AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. MS, Murashige and Skoog medium. B. Quantitative analysis of the root lengths shown in A. The results shown are the means ± SE, n = 6 plants.

chosen for further molecular and physiological analyses. Meanwhile, homozygous *AVP1*-overexpressing line was obtained by transferring pPZP212-AVP into *Arabidopsis* (Supp. Fig. 1). Homozygous *PP2A-C5* overexpressing plants were provided by Dr. Hu [49]. Both *AVP1*-overexpressing plants (indicated as A) and *PP2A-C5* overexpressing plants (indicated as C) were used as reference lines in the following experiments.

2.2. *AVP1/PP2A-C5co-overexpression further increases salt tolerance*

Considering that both *AVP1* and *PP2A-C5* would increase salt tolerance when overexpressed in plants, we tested if transgenic plants overexpressing both *AVP1* and *PP2A-C5* would show even higher salt tolerance. Three *AVP1/PP2A-C5* co-overexpressing lines along with control lines were grown on MS plates vertically without salt for 3 days, then transferred onto plates containing different concentrations of NaCl (100 mM, 125 mM, and 150 mM, respectively) to continue to grow vertically. Under 100 mM of NaCl, seedlings from all lines could grow to some extents, but after 10 days, the leaves of seedlings from all 6 lines turned yellow at a comparable level (Fig. 2A). However, wild-type plants grew the slowest and produced the smallest shoots and the shortest roots. *AVP1*-overexpressing plants produced similar shoots but longer roots in comparison to wild-type plants. *PP2A-C5* overexpressing plants produced larger shoots and longer roots in comparison to wild-type plants. All three *AVP1/PP2A-C5* co-overexpressing lines produced larger shoots than wild-type plants and *AVP1*-overexpressing plants, and longer roots than wild-type and both single gene overexpressing plants (Fig. 2A and B). Similarly, under 125 mM of NaCl, the growth of all seedlings was arrested to a greater extent. But *AVP1/PP2A-C5* co-overexpressing plants again produced longest roots and most true leaves after 5 days of growth (Supp. Fig. 2A, middle panel and 2B). Under 150 mM of NaCl, all seedlings stopped growing after transferring onto the salt plates, however, all wild-type plants and roughly half of both single overexpressing plants became bleached by the 4th day after transferring, while most cotyledons from *AVP1/PP2A-C5* co-overexpressing plants were still green (Supp. Fig. 2A, lower panel). All seedlings became chlorotic and dead after 5 days on 150 mM of NaCl (data not shown). Evidently, *AVP1/PP2A-C5* co-overexpressing plants outperformed not only wild-type plants, but also *AVP1*-overexpressing plants and *PP2A-C5* overexpressing plants under NaCl stress. Plants growing on plates with 300 mM mannitol did not show any difference (Supp. Fig. 3), indicating that the observed differences on NaCl plates were not due to differential responses to osmotic stress. These experiments were conducted three times and similar results were observed.

To test if *AVP1/PP2A-C5* co-overexpression would further improve salt tolerance in soil, salt stress experiments in soil were conducted by watering plants with incremental NaCl solutions from 50 mM to 200 mM. Before salt treatment, there were no differences among these lines (Fig. 3A). Under normal condition, no observable differences were detected among different lines (Fig. 3B). After salt stress treatment, a large portion of wild-type plants were dying and only a small portion of *AVP1*-overexpressing and *PP2A-C5* overexpressing plants were dying. The *AVP1/PP2A-C5* co-overexpressing plants maintained the highest survival rates (close to 100%), which is significantly higher than wild-type plants (Fig. 3C–E). On average, most rosette leaves of wild-type plants showed a severe chlorotic phenotype, while less leaves from *AVP1*- overexpressing and *PP2A-C5* overexpressing plants were chlorotic, and the *AVP1/PP2A-C5* co-overexpressing plants showed the healthiest leaves that were still mostly green after NaCl treatment (Fig. 3C and D). Regarding to plant height, there were no significant differences among all plants, but the trend showed that the average heights of transgenic lines overexpressing either a single gene or both genes were taller than that of wild-type plants (Fig. 3F). Silique number and seed weight per plant were measured at harvest to compare the yields among the different lines. *AVP1*-overexpressing and *PP2A-C5* overexpressing plants produced more siliques and higher seed yields than wild-type plants on average, while *AVP1/PP2A-C5* co-overexpressing plants showed higher average silique numbers and yields than wild-type, *AVP1*-overexpressing, and *PP2A-C5* overexpressing plants (Fig. 3G & H). Line No. 2 of *AVP1/PP2A-C5* co-overexpressing plants (AC2) showed the highest silique number and yield that were significantly higher than that of wild-type plants. Line No. 3 of *AVP1/PP2A-C5* co-overexpressing plants (AC3) showed significantly higher yield than wild-type plants as well (Fig. 3G & H). Even most comparisons were not significant at 5% level, all transgenic lines displayed better performance than wild-type plants on average under this salt treatment condition in soil, and *AVP1/PP2A-C5* co-overexpressing lines were more tolerant on average than *AVP1*-overexpressing and *PP2A-C5* overexpressing plants consistently. This experiment was conducted three times and similar results were observed.

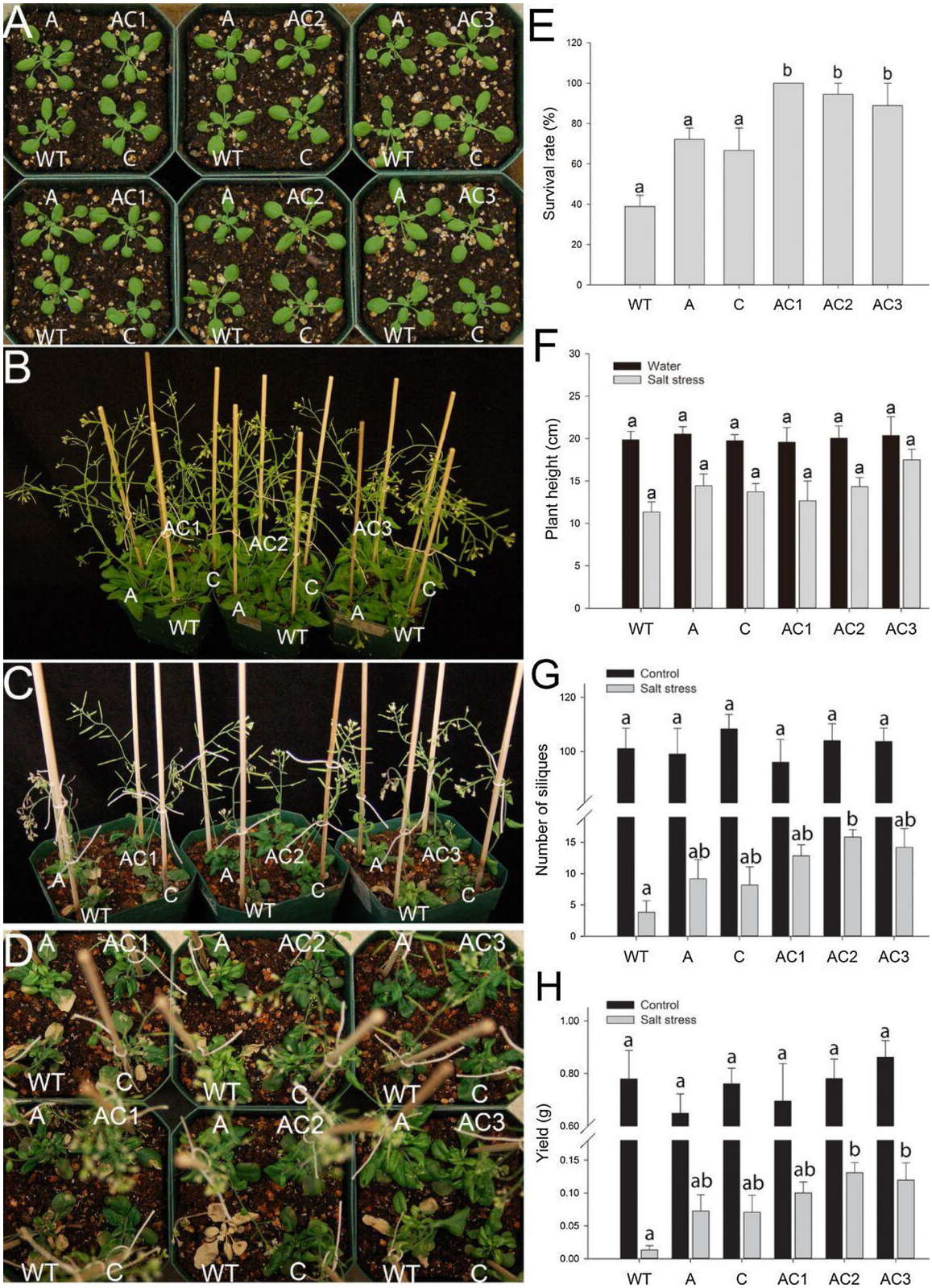

Fig. 3. Analysis of *AVP1/PP2A-C5* co-overexpressing plants in soil under salt stress conditions. A. Phenotype of three-week-old plants just before salt treatment. WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5* overexpressing plants; AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. B. Phenotypes of plants under normal growth condition for 32 days. C and D. Phenotypes of plants under incremental NaCl treatment for 32 days. E. Survival rates (plants with most leaves bleached and wilted shoots are considered dead) of plants after salt treatment. F. Plant heights after salt treatment. G. Silique number per plant at harvesting after salt treatment. H. Seed yield per plant after salt treatment.

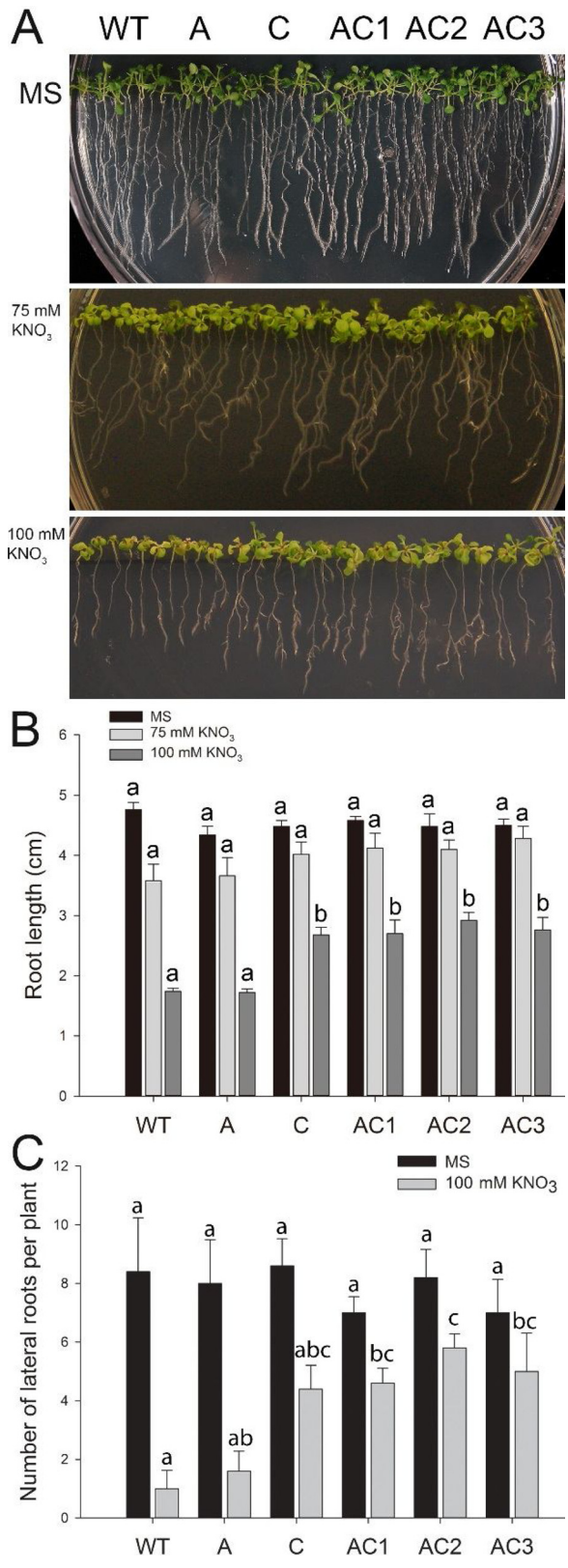

Fig. 4. Analysis of plant growth in the absence or presence of KNO_3. A. Phenotypes of controls and *AVP1/PP2A-C5* co-overexpressing plants in the absence of salt (MS plate, upper panel), presence of 75 mM KNO_3 (middle panel), and presence of 100 mM KNO_3 for 7 days (lower panel). WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5* overexpressing plants; AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. MS, Murashige and Skoog medium. B. Root length analyses of controls and *AVP1/PP2A-C5* co-overexpressing plants in the absence or presence of KNO_3. C. Lateral root numbers of controls and *AVP1/PP2A-C5* co-overexpressing seedlings on MS or MS with 100 mM of KNO_3. The results shown are the means ± SE, n = 5 plants.

2.3. AVP1/PP2A-C5 co-overexpression increases tolerance to other salts

We also studied how *AVP1/PP2A-C5* co-overexpressing plants would respond to salts other than NaCl. PP2A-C5 overexpressing plants were shown to be more tolerant to KNO_3 at high concentrations [49]. We tested if double overexpressing plants are also tolerant to KNO_3. Under 75 mM of KNO_3, all plants showed curled and yellowish leaves to similar extent (Fig. 4A middle panel). When the concentration of KNO_3 was raised to 100 mM, the shoots of seedlings from wild-type and *AVP1*-overexpressing plants were more yellowish and smaller, and the roots were shorter than those of *PP2A-C5* overexpressing and *AVP1/PP2A-C5* co-overexpressing plants (Fig. 4A, lower panel and B). Similarly, the means of lateral root numbers were larger in *PP2A-C5* overexpressing and *AVP1/PP2A-C5* co-overexpressing plants compared to wild-type and *AVP1*-overexpressing lines, however, not all comparisons are significant due to large variation (Fig. 4C). Under higher concentrations of KNO_3 (i.e. 125 mM and 150 mM), the same trend was observed that *PP2A-C5* overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants consistently outperformed others by showing longer roots and maintaining more green cotyledons (Supp. Fig. 4). Lithium is very toxic to plants even at very low concentration. Nowadays, Lithium problem in agriculture is getting more and more attentions [61]. Thus, the performances of wild-type and transgenic plants on plates supplemented with 15 mM LiCl were also analyzed. Root growth was arrested, and the leaves were bleached under low concentration of LiCl. *PP2A-C5* overexpressing and *AVP1/PP2A-C5* co-overexpressing plants had more green cotyledons and the true leaves were emerging, while most and approximately 50% of the cotyledons from wild-type and *AVP1*-overexpressing plants, respectively, were chlorotic (Fig. 5A, lower panel). All transgenic plants had longer roots on average in comparison to that of wild-type plants (Fig. 5B). These results indicated that *AVP1/PP2A-C5* co-overexpressing plants perform better under stresses of different types of salts such as KNO_3 and LiCl than wild-type plants, but similar to *PP2A-C5* overexpressing plants. These experiments were conducted three times and similar results were observed.

2.4. AVP1/PP2A-C5 co-overexpressing plants are drought tolerant

Overexpression of *AVP1* could lead to increased drought tolerance in transgenic plants [27,34,62]. Thus, we tested the performance of *AVP1/PP2A-C5* co-overexpressing plants under osmotic stress. Plant germination was tested on MS plates supplemented with PEG. No difference was observed regarding germination rates among all lines (Supp. Fig. 5). We further tested drought tolerance of these plants in soil. Drought condition was applied by withholding water. Before the drought stress was started, seedlings from all lines looked similar to each other (Fig. 6A). After two rounds of drought and re-watering treatments, all plants in the control group (normal watering) grew normally and no differences were observed (Fig. 6B). But in the treated group, *AVP1*-overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants showed bigger and greener rosette leaves (Fig. 6C) and taller and bushier shoots (Fig. 6D, F) than *PP2A-C5* overexpressing plants and wild-type plants. However, the differences in plant height were not significant. No differences were observed between wild-type and *PP2A-C5* overexpressing plants. After another 10 days of growth without stress, *AVP1*-overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants fully recovered and grew much more robustly than wild-type and *PP2A-C5* overexpressing plants (Fig. 6E). At harvest, silique number of each plant was counted, and seed weight was

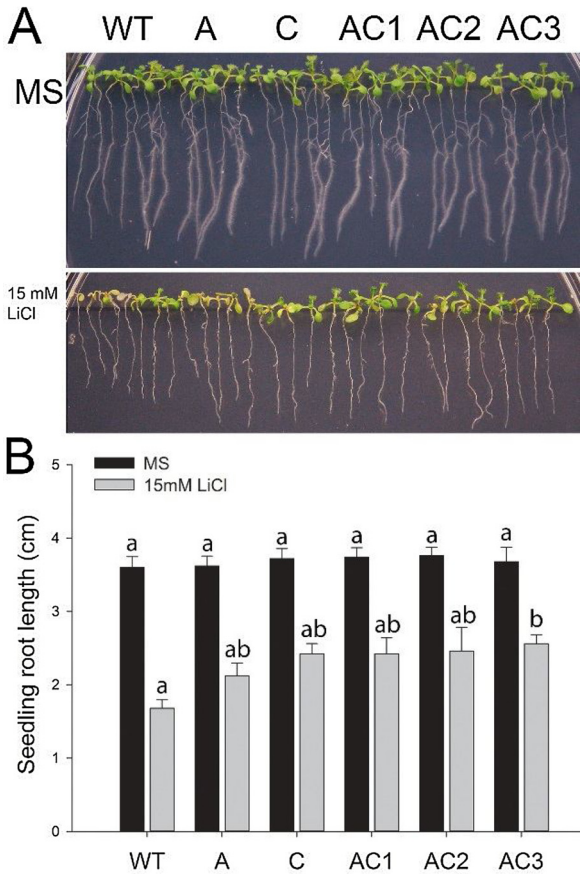

Fig. 5. Phenotypes and analyses of *AVP1/PP2A-C5* co-overexpressing plants in the absence or presence of LiCl. A. Phenotypes of controls and *AVP1/PP2A-C5* co-overexpressing plants in the absence (MS) or presence of LiCl (MS + 15 mM LiCl) on day 6. WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5* overexpressing plants; AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. MS, Murashige and Skoog medium. B. Root length analyses of controls and *AVP1/PP2A-C5* co-overexpressing plants shown in A. The results shown are the means ± SE, n = 5 plants.

measured. As expected, *AVP1*-overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants produced more siliques and more seeds than those of wild-type and *PP2A-C5* overexpressing plants (Fig. 6G & H). This experiment was conducted three times and similar results were observed. To explore whether the stomatal regulation is partly responsible for the increased drought tolerance, water loss from detached leaves was analyzed. No significant difference in the rate of water loss was observed among these different lines (Supp. Fig. 6). These results showed that *AVP1*-overexpression indeed make plants more tolerant to water deficit after seed germination and this trait is maintained in the *AVP1/PP2A-C5* co-overexpressing plants.

2.5. AVP1/PP2A-C5 co-overexpressing plants showed better performance under limited phosphorus

Overexpression of *AVP1* was shown to increase the uptake of phosphorus under low phosphorus conditions [30]. Thus, *AVP1/PP2A-C5* co-overexpressing plants were tested under phosphorus deficient conditions. Three days old seedlings from the six different lines were allowed to grow for 8 more days after they were transferred onto synthetic plant growth medium with (SM) or without phosphorus (SM-Pi) [63]. On phosphorus deficient plates, the most obvious phenotype was yellowish and small leaves, however, all 6 lines had similar shoots. Differences were observed in their roots. Wild-type and *PP2A-C5* overexpressing plants produced significantly shorter roots, about 20% shorter in comparison to those of *AVP1*-overexpressing and *AVP1/PP2A-C5* co-overexpressing plants (Fig. 7A, lower panel and B). This indicates that *AVP1*-overexpressing and *AVP1/PP2A-C5* co-overexpressing plants could elongate their roots better under phosphorus deficient condition, which is consistent with previous studies. This experiment was conducted three times and similar results were observed.

2.6. AVP1/PP2A-C5 co-overexpressing plants are sensitive to ABA but not to ethylene

Hormones play important roles in plant response to abiotic stresses. ABA is a well-known stress hormone that helps plants to deal with drought and salt stresses [48,64]. Germination test on MS plates supplemented with ABA was used to study how different lines would respond to ABA. Germination rates were monitored for 7 days after stratification at 4 °C for 3 days. Seeds from all six lines germinated well on MS plates, reached approximate 100% on day 3 (Fig. 8A), indicating that there were no differences in germination rates among these lines under normal conditions. However, in the presence of 1 μM of ABA, germination rates of all lines were slowed, but to different extents (Fig. 8B). Wild-type and *AVP1*-overexpressing plants germinated faster and reached 100% on day 7, while *PP2A-C5* overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants germinated slightly slower with the line AC1 and AC3 reaching only 90% germination on day 7. The differences at day 4 were most significant. Multiple pairwise comparisons indicated that the *PP2A-C5* overexpressing plants and the three *AVP1/PP2A-C5* co-overexpressing plants germinated significantly slower in comparison to wild-type and *AVP1*-overexpressing plants on plates supplemented with 1 μM ABA. This indicates that *PP2A-C5* overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants are more sensitive to ABA during germination.

Similar to ABA, ethylene was also shown to be involved in salt stress response [65], and PP2A was known to regulate ethylene biosynthesis [43]. All six lines were put on MS plates containing 10 μM 1-Aminocyclopropane-1-carboxylic acid (ACC) and grown in darkness at 4 °C for 8 days after stratification. In the presence of ACC, hypocotyl growth and root growth were both arrested (Supp. Fig. 7A). The quantitative data showed no differences in the length of hypocotyls among all six lines (Supp. Fig. 7B). This indicates that overexpression of *AVP1* alone, *PP2A-C5* alone, or both genes do not affect plant response to ethylene at germination and early seedling development stages.

2.7. AVP1/PP2A-C5 co-overexpression plants accumulate more Na^+, Cl^-, and K^+

Ion homeostasis is the essential component of salt stress tolerance in plant cells [66]. To better understand the mechanism underlying salt stress tolerance, content of three important ions, Na^+, Cl^-, K^+, were measured under both normal and stress conditions, by atomic absorption spectrometer. No obvious differences were detected under normal growth condition, except slightly higher K^+ in transgenic plants (Fig. 9C). Under salt stress condition, *AVP1*-overexpressing and *AVP1/PP2A-C5* co-overexpressing plants accumulate the most Na^+, around 30% more than that of *PP2A-C5* overexpressing and wild-type plants (Fig. 9A). This could be due to the fact that AVP1 can energize secondary transporters on vacuolar membranes like AtNHX1 to increase Na^+ sequestration into vacuole. Interestingly, *AVP1* overexpression seems to prevent Cl^- accumulation in plant cells while PP2A-C5 promotes it. *AVP1/PP2A-C5* co-overexpressing plants accumulate more Cl^- than wild-type but less than *PP2A-C5* overexpressing plants (Fig. 9B). This indicates that the two genes are possibly counteracting each other. Salt stress reduces K^+ contents most severely in wild-type and *PP2A-C5* overexpressing plants. *AVP1*-overexpressing plants showed less reduction and maintained higher K^+ content after salt stress. *AVP1/PP2A-C5* co-overexpressing plants did not show any reduction at all (Fig. 9C). This implies a potential synergic action between

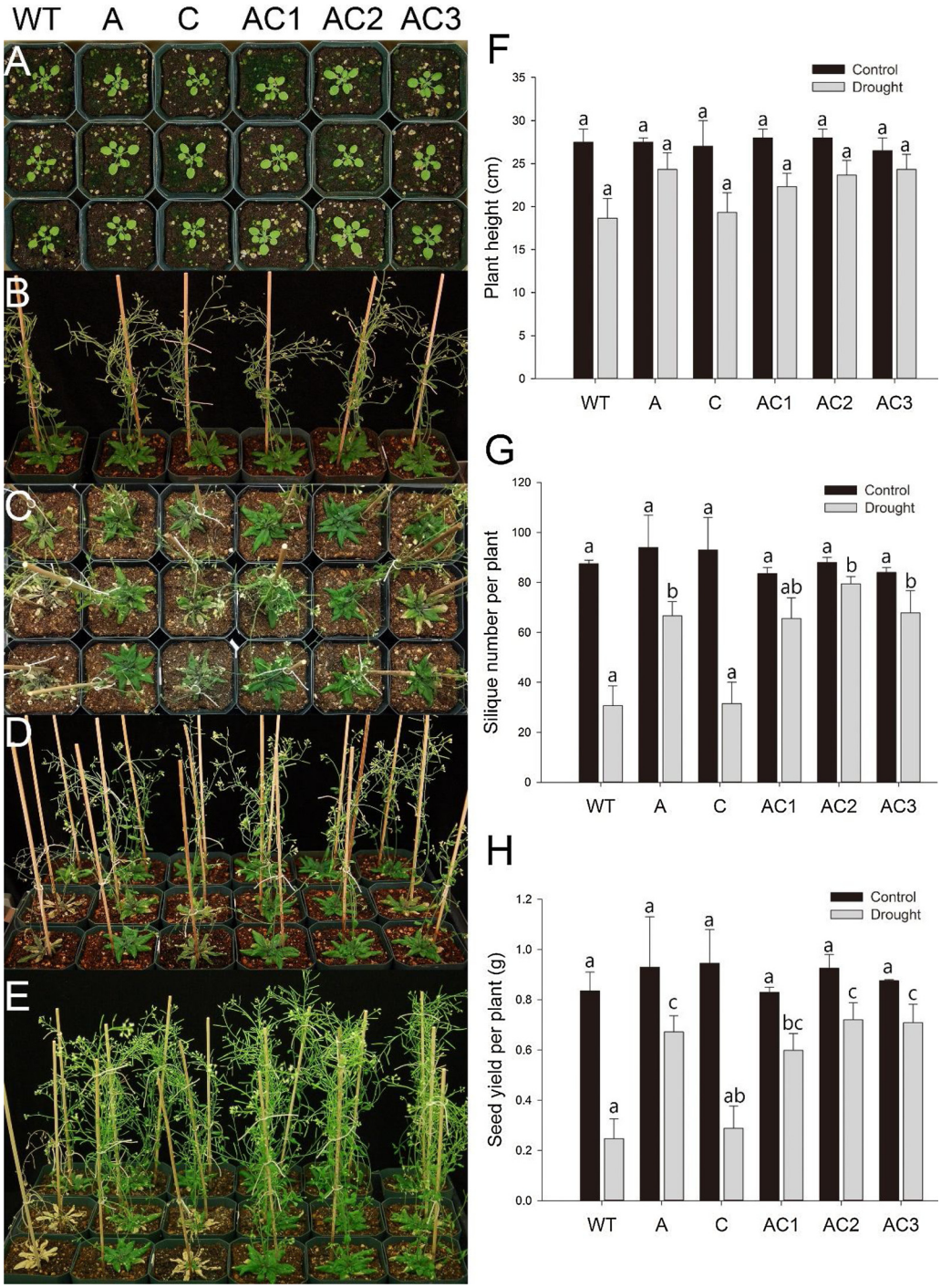

Fig. 6. Phenotypes and analysis of *AVP1/PP2A-C5* co-overexpressing plants in soil under drought stress conditions. A. Phenotype of three-week-old plants just before water withholding. WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5* overexpressing plants; AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. B. Phenotypes of plants under normal growth condition. C and D. Phenotypes of plants right after drought treatment. E. Phenotype of plants 10 days after the end of drought treatment. F. Plant heights of controls and *AVP1/PP2A-C5* co-overexpressing plants after drought treatment. G and H. Silique number and seed yield per plant of controls and *AVP1/PP2A-C5* co-overexpressing plants at harvesting.

AVP1 and PP2A-C5 on maintaining intracellular K^+ level under salt stress conditions. Above all, *AVP1/PP2A-C5* co-overexpressing plants seem to accumulate more K^+, Na^+, and Cl^- in cells under salt stress conditions.

3. Discussion

In this study, we created transgenic *Arabidopsis* plants over-expressing *AVP1* and *PP2A-C5* simultaneously (Fig. 1). Our main goal was to generate stronger multiple stress tolerant plants by 'stacking' these two genes. This approach has been proved successful in some cases [55,67,68], and unsuccessful in others [69]. It appears that combining genes from the same pathway might not work as effective as combining genes from different pathways. Both *AVP1* and *PP2A-C5* have been overexpressed separately in different plant species including crops to increase the salt tolerance [27,34,49,62]. AVP1 enhances salt tolerance mainly by energizing secondary transporters such as AtNHX1 on tonoplast membrane to sequester more Na^+ into vacuoles, while PP2A-C5 might work through activating vacuolar membrane chloride channel proteins CLCa and CLCc [49,70]. Therefore, co-overexpressing *AVP1* and *PP2A-C5* might be an effective strategy to further improve salt tolerance because they are not upstream or downstream components of each other in the salt response pathway. Indeed, *AVP1/PP2A-C5* co-overexpressing plants performed significantly better than plants over-expressing either gene under high NaCl concentration conditions on MS plates and in soil (Figs. 2, 3 and Supp. Fig. 1). *AVP1/PP2A-C5* co-

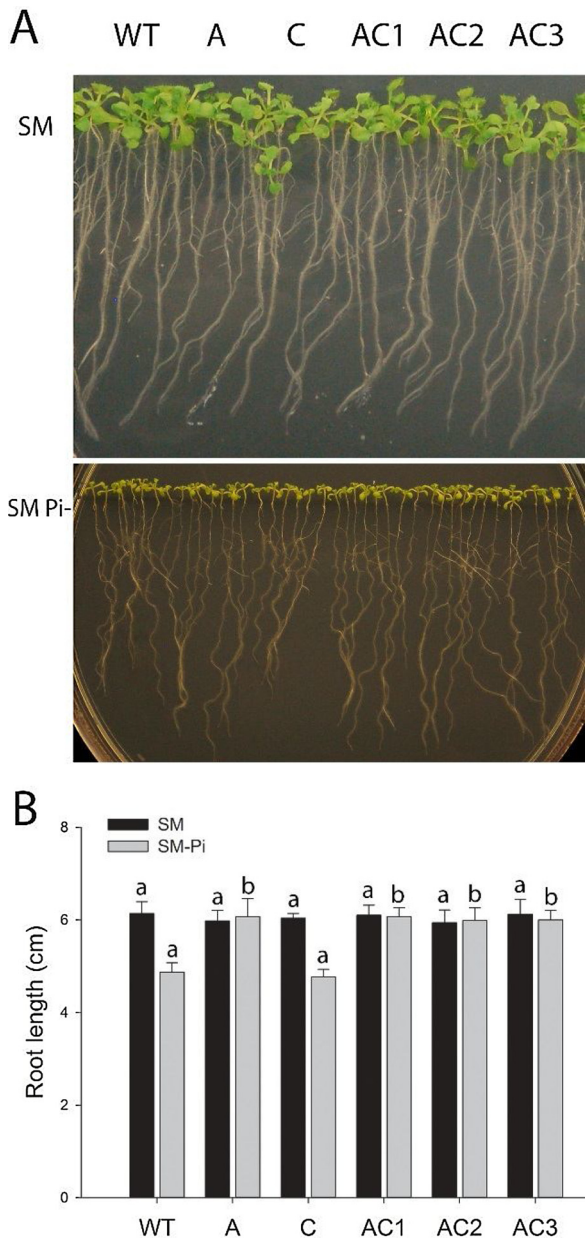

Fig. 7. Analysis of *AVP1/PP2A-C5* co-overexpressing plants in the presence or absence of phosphorus. A. Phenotypes of transgenic plants in normal (SM) or phosphorus-deficient media (SM Pi-) on day 8. WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5*-overexpressing plants; AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. SM, synthetic medium. B. Root length analysis of plants shown in A. The results shown are the means ± SE, n = 7 plants.

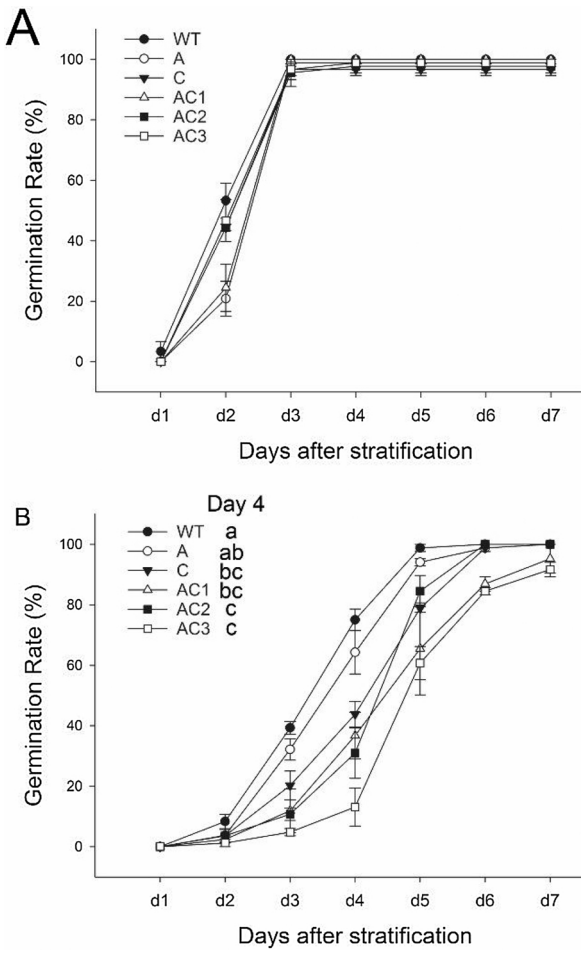

Fig. 8. Germination of controls and *AVP1/PP2A-C5* co-overexpressing plants in the absence or presence of abscisic acid. A. Germination rates of controls and *AVP1/PP2A-C5* co-overexpressing plants on day 1 to day 7 in the absence of abscisic acid (MS) after stratification. WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5* overexpressing plants; AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. MS, Murashige and Skoog medium. ABA, abscisic acid. B. Germination rates of controls and *AVP1/PP2A-C5* co-overexpressing plants on day 1 to day 7 in the presence of 1 μM of abscisic acid after stratification. Three repeats were conducted with 35 seeds per line each time. Pairwise comparison was done based on data of day 4 using Tukey's MSD at 5% level.

overexpressing plants could tolerate up to 200 mM NaCl in soil, which is slightly better than single gene overexpressing plants. This might be partly attributed to better ion homeostasis. Our data showed that more K^+ was accumulated in *AVP1/PP2A-C5* co-overexpressing plants under salt stress condition. Increased K^+ content under salt condition was also observed in *AtNHX/SOS1* co-overexpressing plants [55]. In our case, elevated K^+ level was observed in *AVP1*-overexpressing plants, but not in *PP2A-C5* overexpressing plants, and the *AVP1/PP2A-C5* co-overexpressing plants showed the highest K^+ level (Fig. 9C). AVP1 seems to help retain more K^+, and PP2A-C5 appears to add more to this process. Stronger K^+ retention has been associated with high salinity stress by analyzing 82 natural *Arabidopsis* ecotypes [71]. This supported the superior performance of *AVP1/PP2A-C5* co-overexpressing plants under salt stress. These plants also contain more Na^+ and Cl^- than wild-type plants, but similar to *AVP1* and *PP2A-C5* single overexpressing plants, respectively (Fig. 9). Even though the subcellular localizations of these ions are not determined, we speculate that the accumulation of harmful Na^+ and Cl^- are mainly in the vacuole. Because AVP1 could energize AtNHX1 to transport Na^+ into vacuole, and PP2A-C5 might activate CLCc or its closely related homolog CLCa on vacuolar membrane, which could transport Cl^- and NO_3^- into vacuole. Our real-time PCR data showed no change in the transcript level of CLCc, which indicates that the activation might be post-transcriptional if there is any (Supp. Fig. 8H).

Gene expression analysis provides more insights into mechanisms of high salt tolerance of *AVP1/PP2A-C5* co-overexpressing plants. Lower ROS accumulation under stress conditions might be one potential contributor, because *AVP1/PP2A-C5* co-overexpressing plants showed higher transcript level of antioxidant gene *AtAOX1* (Supp. Fig. 8F) [72]. *RD22*, a drought and salt stress responsive gene, is down-regulated in *PP2A-C5* overexpressing and *AVP1/PP2A-C5* co-overexpressing lines, but up-regulated in all transgenic lines under salt stress (Supp. Fig. 8I). This stronger induction (higher fold change upon salt stress) of *RD22*

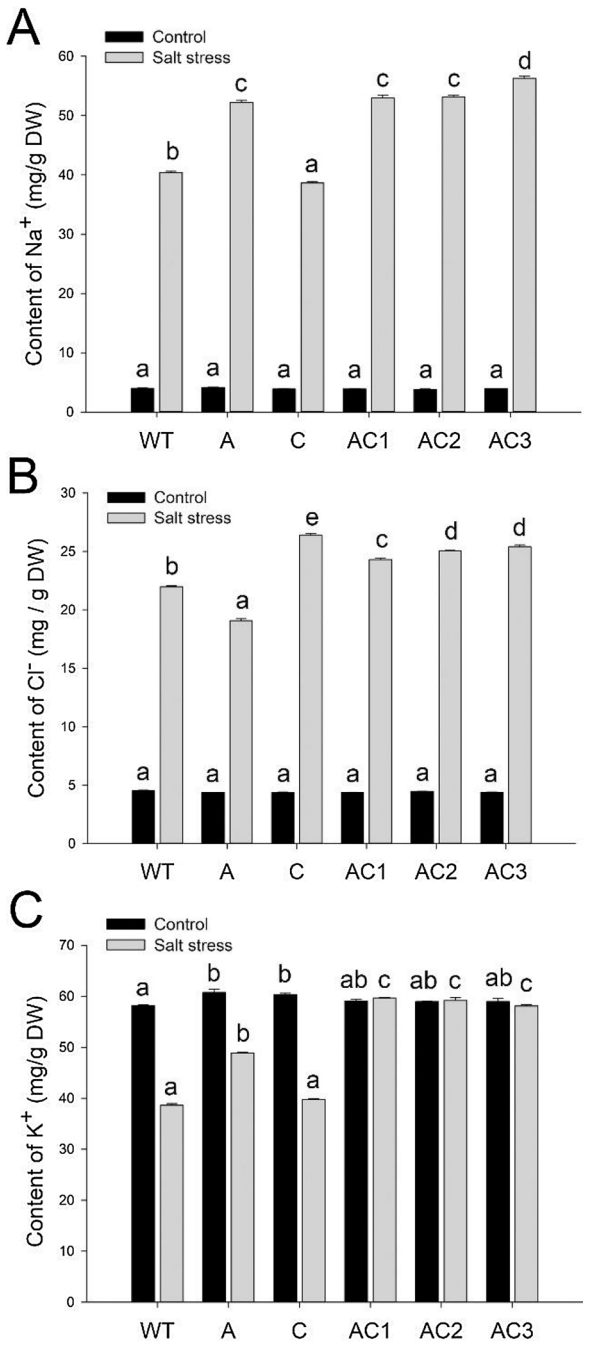

Fig. 9. Ion content analysis of controls and *AVP1/PP2A-C5* co-overexpressing plants under normal and salt stress conditions. A. Na^+ content; B. Cl^- content; C. K^+ content. WT, wild-type plants; A, *AVP1*-overexpressing plants; C, *PP2A-C5* overexpressing plants; AC1, AC2, and AC3, three independent *AVP1/PP2A-C5* co-overexpressing plants. The results shown are the means ± SE.

might lead to better salt tolerance [73]. *SOS1*, a gene encoding sodium/proton antiporter on plasma membrane, is slightly up-regulated at transcript level under normal condition in all transgenic lines but to different degrees. Interestingly, under salt stress condition, *SOS1* transcript was strongly up regulated in WT and *AVP1* overexpressing plants, but less so in other plants (Supp. Fig. 8G). This indicates a possible negative role of PP2A-C5 in regulating the *SOS1* transcript level under salt stress condition. Moreover, transcripts of *PP2A-C1*, *C2*, and *C5* were up regulated by salt, while *PP2A-C3* transcript was down regulated by salt stress, implying different or even opposite functions of PP2A catalytic subunits in salt stress response (Supp. Fig. 8A–C, and E).

Besides higher tolerance to NaCl, *AVP1/PP2A-C5* co-overexpressing plants also showed higher tolerance to KNO_3 and LiCl at comparable level with *PP2A-C5* overexpressing plants. The potassium salt KNO_3 as a major component of fertilizer is beneficial to plant growth. KNO_3 was even used to antagonize NaCl effects and as priming agent to ameliorate forthcoming salt stress at concentrations from 5 mM to 20 mM [74]. However, at high concentrations, such as that higher than 75 mM, KNO_3 becomes detrimental to plants. Overexpression of *PP2A-C5* in *Arabidopsis* increases tolerance to KNO_3 at high concentrations [49]. *PP2A-C5* overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants showed similar phenotype as expected (Fig. 4). Interestingly, *AVP1*-overexpressing plants performed better than wild-type plants by maintaining greener leaves at 125 mM and 150 mM of KNO_3 (Supp. Fig. 4A, middle and lower panel). This effect appears to be additive to the effects brought by overexpressing *PP2A-C5*, because *AVP1/PP2A-C5* co-overexpressing plants performed on average better than *PP2A-C5* overexpressing plants under higher concentrations (Supp. Fig. 4A and B). Increased tolerance to KNO_3 was attributed to more sequestrated anions (NO_3^-) into vacuole, possibly due to that PP2A-C5 activates anion channels CLC-c and CLC-a [49]. Ion sequestration depends on proton gradient across tonoplast membrane established by proton pumps such as AVP1. Thus, under higher concentrations, proton gradient might become insufficient to transport excessive ions. Hence overexpressed AVP1 becomes beneficial only under higher concentrations. It is also possible that PP2A-C5 could regulate some other channels (e.g. SLAC) under lower salt concentrations, while CLCc plus AVP1 start to work only when the NO_3^- concentration rises above 125 mM [75].

LiCl is a different type of salt compared to NaCl or KNO_3, which is very toxic to plants and cause real problems in agricultural production. Two major targets of lithium have been found, inositol-1-phosphatase [76] and glycogen synthase kinase 3 (GSK3) [77]. The enzyme FRY1 (FAR-RED ELONGATED HYPOCOTYL 1) in plants is inhibited in vitro strongly by lithium [78]. In our study, *AVP1/PP2A-C5* overexpressing plants showed slightly higher resistance to LiCl in comparison to wild-type plants. This might be related to slightly elevated K^+ content even under normal condition in all transgenic plants (Fig. 9). Because Li^+ resistance has been associated with K^+ uptake and pH homeostasis in yeast [79]. To further elucidate the LiCl stress tolerance mechanism, further analysis of Li^- contents, subcellular localization, and other biochemical attributes of transgenic plants are required. Higher tolerance to KNO_3 and LiCl might have less practical implication, but these results did prove the concept that multiple stress tolerance could be achieved by over-expressing multiple genes simultaneously.

Drought tolerance was improved in different species by over-expressing *AVP1* [27,34,62]. In this study on PEG plates during seed germination, no change in germination rate was observed in any transgenic plants compared to wild-type seeds (Supp. Fig. 5). This might be due to the fact that there is no well-established vacuolar space in seeds yet, which limits the function of AVP1. After germination, differences in drought tolerance were observed in the water withholding and re-watering experiment. *AVP1*-overexpressing and *AVP1/PP2A-C5* co-overexpressing plants showed more robust growth and produced more seeds (Fig. 6). This is attributed to AVP1's functions of lowering plant cell's water potential through accumulating solutes in vacuole, regulating plant root growth through facilitating auxin polar transport, and helping relocation of sucrose to sink tissues [80]. These drought tolerant plants did not show any change in water loss rate from detached leaves (Supp. Fig. 6), indicating that ABA mediated stomatal response to drought stress might not be affected in *AVP1/PP2A-C5* co-overexpressing plants. *PP2A-C5* overexpression does not help improve drought tolerance.

Slightly enhanced response to ABA of *PP2A-C5* overexpressing and *AVP1/PP2A-C5* co-overexpressing plants was observed during germination (Fig. 8). PP2A was shown to be a negative regulator in ABA signaling pathway [42], yet the results in this study seem to be

contradictory. However, the previous study could not identify which catalytic subunit of PP2A is involved in ABA signaling pathway, because immunoprecipitation could not distinguish the five catalytic subunits. Real-time PCR analysis of the five PP2A catalytic subunit transcripts showed that the transcript of *PP2A-C3*, which belongs to a different subfamily, was significantly reduced in *PP2A-C5* overexpressing plants and the two *AVP1/PP2A-C5* co-overexpressing plants (Supp. Fig. 8C). This indicates possible different or opposite functions of PP2A-C5 and PP2A-C3. Moreover, we found that transcripts of PP2A catalytic subunit genes *PP2A-C1* and *PP2A-C4* were increased in *PP2A-C5* overexpressing lines, while the transcript of *PP2A-C2* was not significantly changed (Supp. Fig. 8A, B and D). This implies that transcript levels of different PP2A catalytic subunit genes are affected by each other.

AVP1 was shown to be involved in improving plant performance under phosphorus limiting condition in *Arabidopsis*, rice, maize and tomato [30,81]. Indeed, in this study, *AVP1*-overexpressing plants and all three *AVP1/PP2A-C5* co-overexpressing plants showed more robust root growth under phosphorus deficient condition (Fig. 7). PP2A-C5 does not seem to help under this condition. Phosphorus deficiency has been shown to stimulate the expression of *AVP1*, which could stabilize plasma membrane localized ATPase [29]. The resultant stronger acidification of apoplastic space facilitates phosphorus uptake by increasing proportion of $H_2PO_4^-$ and amount of protons which are co-transported [82]. In this study, the better root systems of *AVP1*-overexpressing plants and *AVP1/PP2A-C5* co-overexpressing plants could be attributed to better phosphorus uptake. Considering that phosphorus is the second most important factor limiting the optimal growth of plants, this is a very desirable trait that will allow superior growth of crops under low phosphorus conditions.

This study shows that co-overexpression of *AVP1* and *PP2A-C5* indeed further improves plant tolerance to NaCl, and combines other beneficial traits brought about by *AVP1* or *PP2A-C5*, such as tolerance to highly concentrated KNO_3, drought and low phosphorus conditions. These traits combined could potentially make crops grown in saline and arid area better with less phosphorus fertilizer, which will in turn help reduce runoff pollution and clean up the soil more efficiently. This could be very valuable to make agriculture not only more productive but also more environmental friendly [20]. The study also proved the concept that introducing beneficial genes in the same T-DNA into plants could be an effective strategy to help plants deal with multiple stresses.

4. Materials and methods

4.1. Vector construction

The *AVP1* gene was amplified from an *Arabidopsis* cDNA library with primers avpf1 and avpr1, and inserted into the *Sma* I digested pRT103 vector. The 35S promoter, the *AVP1* coding sequence, and the polyadenylation sequence as a whole was then released with *Hind* III and inserted into the vector pPZP212 to form pPZP212-AVP. The pFGC5941-C5 vector [49] was linearized using *Hind* III, and then a *Hind* III fragment (340 bp) from the vector pJG4-5 was ligated to the linearized pFGC5941-C5 to form the pFGC5941-EC5E. The PP2A-C5 expression cassette was then released by partial digestion with *Eco* RI and then inserted into pPZP212-AVP to form the p212-C5-AVP transforming vector. PCR primers are listed in Table 1.

4.2. Plant materials

The *Arabidopsis* ecotype Columbia-0 (Col-0) was used in this study. Homozygous *PP2A-C5* overexpressing plants were provided by Dr. Hu [49]. The *AVP1*-overexpressing and *AVP1/PP2A-C5* co-overexpressing transgenic plants were generated by transferring pPZP212-AVP and p212-C5-AVP vectors, respectively, into *Arabidopsis* plants using the "floral dip" transformation method [60]. Homozygous plants were selected on MS plates with 30 mg/l kanamycin in T_3 generation.

Table 1
Primers used for vector constructions.

Name	Sequences
Gyp	AATTGATCGGCTAAATGGTATGG
gyp-xho-hin5	AGTCCTCGAGAAGCTTTCACGTAATAAGTGTGCGTTGAATT
gyp-sac-eco5	AGTCGAGCTCGAATTCTCACGTAATAAGTGTGCGTTGAATT
35s-820-R	CATCTGTGGGTTAGCATTCTTTCTG
35s-415-R	AGAGTGTCGTGCTCCACCATGT
35S	CCCACGAGGAGCATCGTGGAAAAAGAAGACGT
35S-1	GGGATGACGCACAATCCCACT
35S-2	CAAGACCCTTCCTCTATATAAGG
C5-568-F1	TTAGATCGAATTCAAGAGGTTCCA
SL-AVP-RTF1	TCATGCTCACACCTCTCATTG
SL-AVP-RTF2	GGTGGAGCTAACAATGGGAAG
SL-AVP-RTF3	GTGGGATCTACACTAAGGCTG
SL-AVP-RTR1	GTATTTCTTGGCGTTGTCCC
SL-AVP-RTR2	CCGTGAATAGGAATGAAGTTGC
SL-AVP-RTR3	TCCCATACCAGCAATGTCAC
RT-AVP1-1857-F1	CCCTGGACTTATGGAAGGAACC
RT-AVP1-3UTR-R2	CCTTATCTGGGAACTACTCACACATTA
RT-AVP1-512-R1	CGTATGTAGCAATCTTCATCCCAA
RT-AVP1-3UTR-R1	GAGAGACTGGTGATTTGCGGAC
RT-AVP1-5UTR-F1	ATGGGCGAGCTCGGTACC
AVP1-F1	ATGGTGGCGCCTGCTTTGT
AVP1-B1	TTAGAAGTACTTGAAAAGGATAC
C5fullF	ATGTACCCATACGATGTTCCAGATTAC
C5fullR	TTACAAAAAAATAATCTGGAGTCTTGC
act2fullF	ATGGCTGAGGCTGATGATATTC
act2fullR	TTAGAAACATTTTCTGTGAACGATTC

4.3. Plant growth conditions

Arabidopsis seeds were surface sterilized with 15% household bleach, followed by washing with sterile distilled water three times. Seeds were then stratified for 4 days in a refrigerator (4 °C) and plated on MS plates containing Murashige and Skoog salts (MS, Murashige and Skoog 1962), 1% sucrose and 0.7% agar, with pH at 5.8. Plants on MS plates were grown under continuous light at 22 °C. For treatments on plates, different components (e.g. NaCl, mannitol, KNO_3, LiCl, ABA, and ACC) were added to MS plates as specified at noted concentrations. *Arabidopsis* plants grown in soil were kept in growth chamber (Enconair AC-60, Ecological Chamber Inc., Canada) with 16 h light and 8 h dark photoperiod (light intensity at 120 mmol $s^{-1}m^{-2}$) at 22 °C and 50% relative humidity.

4.4. Phosphorus deficiency treatment

Low phosphorus medium was prepared as described by Estelle and Somerville [63]. The components of this medium were 5 mM KNO_3, 0.1 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 μM EDTA, 70 μM H_3BO_3, 14 μM $MnCI_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaC1, 0.01 μM $CoCl_2$. This synthetic medium supplemented with 1% sucrose and 0.7% agar was used as phosphorus deficient medium, with pH at 5.8. Seedlings were grown on MS plates for 3 days, then transferred to the low phosphorus synthetic plate to grow for another 8 days. This experiment was repeated three times.

4.5. Water deficit treatment on MS plates with polyethyleneglycol (PEG)

Solid and liquid MS media were prepared without sucrose, with or without 1.5% agar respectively. After both media were autoclaved, 20 ml of solid medium were poured into 10 cm diameter plates under room temperature for solidification. The correct amount of PEG8000 was then added to the cooled liquid MS medium to make a final

concentration of 40%, and stirred to dissolve it. Then 30 ml MS-PEG (40%) solution was poured on top of the solidified MS medium and kept for one day to homogenize (water potential around −0.7 MPa) before use. This experiment was repeated three times.

4.6. RNA isolation

Plant materials were grounded in liquid nitrogen into fine powders, which was then re-suspended into 1 ml Trizol reagent (38% acid phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate with pH equal to 5.0, 5% glycerol). It was then homogenized by votexing gently and left in room temperature for 2 min. Chloroform (Fisher Scientific, US) was added to separate phases. The upper aqueous phase was transferred into a new tube. Isopropanol was then added to precipitate RNA for 10 min at room temperature, followed by centrifugation. Pellet was then washed with 70% ethanol once and dissolved into 30–50 μl DEPC (diethylpyrocarbonate) treated water. RNA concentration was thereafter determined by using NanoDrop ND-1000 (Thermo Scientific, US) according to manufacturer's instructions.

4.7. RNA blot analysis

RNA electrophoresis gel contains 1.2% agarose (Amresco, Ohio, US) supplemented with 0.04 M MOPS and 1.1% formaldehyde. About 10 to 15 μg of total RNAs were mixed with RNase free sample premix (final concentration: 0.04 M MOPS, 5.4% formaldehyde, 42% Formamide, 1X loading buffer. Forty μl of RNA samples were loaded into each wells in the gel after heating and rapid cooling. Electrophoresis was carried out at 100 V for 3 h in RNase free buffer (1.1% formaldehyde, 0.4 M MOPS). After electrophoresis, the gel was carefully removed from the gel tank to clean tray containing DEPC treated water and washed twice to remove extra formaldehyde. Then the gel was soaked in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate) and shaken gently for 1 h. The gel was then placed on a salt bridge upside down and sandwiched between 3 MM filter paper and BrightStar-Plus positively charged nylon membrane (Ambion, US). One more layer of 3 MM filter paper, a stack of towel papers, and 1.5 kg weight were placed on top of the membrane to transfer RNA overnight. After transfer was complete, the membrane was crosslinked in a UV-crosslinker (Fisher Scientific, US) at optimal condition twice. The membrane was then air dried before use.

The membrane was wetted first by DEPC treated water and pre-hybridized in the hybridization solution [1% BSA, 1 mM EDTA pH8, 0.5 M $NaHPO_4$ (pH7.2), 7% SDS (sodium dodecyl sulfate)] for 60 min at 63 °C. Meanwhile, the probe was made by random priming using DECAprime II DNA labeling kit (Ambion, US) according to manufacturer's instructions. Template DNA was obtained by PCR reaction with primer pairs C5fullF and C5fullR, avpf1 and avpr1, act2fullF and act2fullR for gene *PP2A-C5*, *AVP1,* and *Actin2*, respectively. Purified probes were hybridized with membrane overnight at 63 °C. After hybridization, the membrane was washed in the concentrated wash solution [0.5% BSA, 1 mM EDTA, 0.04 M $NaHPO_4$ (PH7.2), 5% SDS] once at room temperature, followed by washing twice with the diluted washing buffer [1 mM EDTA, 0.04 M $NaHPO_4$ (pH7.2), 1% SDS] at 64 °C. The washed membrane was exposed to a phosphorimager screen (Amersham Biosciences, US) overnight and then scanned with the Personal Molecular Imager (Bio-Rad, US).

4.8. Reverse-transcription and RT-PCR

One μg of total RNAs was used for reverse transcription to make cDNAs using PrimeScript RT reagent Kit with gDNA eraser (Takara, Japan) according to manufacturer's instructions. A mixture of primers containing oligo dT and random hexamers provided in the reverse transcription kit was used for the first strand cDNA synthesis. The resultant cDNAs (20 μl) were diluted in RNase free water to 400 μl and 4 μl of that were used for the real-time PCR reaction. The real-time PCR reaction was set up by using SYBR Premix Ex Taq II (Tli RNaseH free) kit (Takara, Japan) according to manufacturer's instructions. The primers were used are listed in Table 2. Real-time PCR reaction was carried out on Applied Biosystems 7500 real-time PCR system (Thermo Scientific, US) with initial denaturation at 95 °C for 30 s, followed by 40 cycles of 2 step reaction; 95 °C denaturation for 5 s, 60 °C annealing and elongation for 34 s. Real-time PCR data were analyzed by using the Delta-Delta Ct method.

Table 2
Primers used for real-time PCR experiments.

Name	Sequences
ACT-QF	CAGCATGAAGATTAAGGTCGTTG
ACT-QR	TTCTGTGAACGATTCCTGGAC
AVP-QF	GGTGGAGCTAACAATGGGAAG
AVP-QR	CCGTGAATAGGAATGAAGTTGC
SOS1-QF	CGACCGATTCGTCTTCTTCTT
SOS1-QR	GAAGCAATACCGAGTACCAGAG
CLC-QF	GCATTATAAACGAGAAAGGTCCGA
CLC-QR	TCATTGGTGTTGAGAAGTAGGG
C1-QF	ATACAAGAGGTTCCACACGA
C1-QR	CTGAGTAGCAATGTCCTGTC
C2-QF	AGGTGCTGGTTATACATTTGG
C2-QR	CACCACATTCTTATCCTGACAC
C3-QF	TTTATGGGAGACTATGTGGAC
C3-QR	AAACCTGAGTAATCTGACGAC
C4-QF	CTTGAAGGAAAGAAGACGAC
C4-QR	TTCTCACATAATGCTGACCT
C5-QF	TTAGATCGAATTCAAGAGGTTCCA
C5-QR	TTGTCCGAAAGTGTAGCCTG
AOX-QF	ACGATTGGAGGTATGAGATTCGC
AOX-QR	GTTCAACACCCCAATAGCTCGCG
RD22-QF	TTCGCGGTGTTCTACTGCC
RD22-QR	CGGAACCGCGTAGACGG

4.9. Abiotic stress treatment in soil

Seeds were sown on MS plates to germinate and grown for one week. Seedlings were then transferred into soil mix (SunGro Horticulture, Vancouver, Canada) and the humidity was maintained at a saturated level for 2 days after transferring before the humidity was gradually decreased to 50%. The plants were grown under normal condition for 2 more weeks without stresses. For salt stress, the plants were watered with NaCl solution at 50 mM, 100 mM, 150 mM, 200 mM, every 3 days, twice at each concentration. At the end of treatment, pictures were taken, and plant heights were measured. Plain water was then used to water plants until harvesting. Plants were individually harvested, and seed weight was measured. For drought stress, after 3 weeks of growth under normal condition as mentioned above, soil was saturated with water and no more water was given for 2 weeks. Then the soil was saturated, and water was withheld again for another 2 weeks. After that, plants were re-watered and phenotypes were recorded. Plants were let to grow under normal condition until harvest, and yields were measured. Both salt stress and drought stress experiments were repeated three times.

4.10. Water loss assays from detached leaves

Plants were grown under normal condition for 6 weeks. Four fully expanded rosette leaves were collected from these plants and placed on bench. Weight was measured every 1 hour from detachment to the 7th hour. Three replicates were conducted. Water loss was expressed as percent loss of fresh weight.

4.11. Ion content analysis

Ion content was measured by Ion Chromatography Instrument 883

Basic IC plus, following the steps described by Pehlivan [55].

4.12. Statistical analysis

Tukey's method was used for pairwise comparison among different lines (wild-type, *AVP1*-overexpressing plants, *PP2A-C5* overexpressing plants, and *AVP1/PP2A-C5* co-overexpressing plants) at significant level $\alpha = 0.05$.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.05.026.

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