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Co-overexpression of *AVP1, PP2A-C5, and AtCLCc* in *Arabidopsis thaliana* greatly increases tolerance to salt and drought stresses

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ABSTRACT

Abiotic stresses such as salinity and drought impose a severe constraint on global food production, posing a serious challenge in agriculture. Stacking beneficial genes in transgenic crops will likely improve crop yield under abiotic stress conditions. Previous studies showed that individually overexpressing the Arabidopsis vacuolar H^+-pyrophosphatase gene *AVP1*, the protein phosphatase 2 A catalytic subunit gene *PP2A-C5,* and the chloride channel protein gene *AtCLCc* contributed to enhanced salt tolerance and overexpression of *AVP1* alone could also improve drought tolerance. We hypothesized that co-overexpressing *AVP1*, *PP2A-C5,* and *AtCLCc,* would combine the benefits of these three genes, leading to a further increase in salt tolerance in transgenic plants due to the potential synergism of these genes. Indeed, co-overexpression of these three genes in Arabidopsis significantly improved salt and drought tolerance under single as well as under combined salt and drought stresses. The *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants displayed robust growth and produced greater amount of biomass as well as viable seeds than wild-type and single gene overexpression plants under saline and drought conditions. This study demonstrates that successful co-overexpression of several well-chosen genes is an effective strategy to achieve greater abiotic stress tolerance and could potentially lead to higher crop yield in regions of the world with saline soil and low precipitation.

1. Introduction

The predicted climate changes pertaining to elevated temperatures, changes in precipitation patterns, salinity, and intensified drought and heatwaves worsen the unfavourable effects on crop growth and productivity. This unprecedented challenge could jeopardize global food production that is already in need to be maximized to keep pace with the increasing world population. Hence, it is imperative to ensure food security by creating affirmative changes in the current crop yield trends to keep on track in doubling the crop yield by 2050. Crops during their growth and development encounter a broad range of environmental disturbances, which restricts their yield potentials. Suboptimal environmental conditions such as biotic and abiotic stresses cause a yield gap due to the reduction of actual average yield (Liu et al., 2016).

Crop loss due to two foremost abiotic stresses, salinity and drought, challenges agriculture industry to ensure sustainable global food security (Pandey et al., 2015). Drought and salinity are the most widespread abiotic stress factors in the world and the severity of their occurrence is expected to increase in the future (Paul et al., 2019). Hence, there is an urgent need of increasing tolerance against salinity and drought in crops to cope with increasing food demand. Salinity stress is caused by a rapid osmotic stress or reduction of water availability and a long-term toxic effect of the ions, which in turn leads to severe damages to enzymes and membranes, oxidative stress in plant cells and eventually cell death (Ma et al., 2020). Salt accumulation in plants could cause many detrimental effects such as growth inhibition, abnormal development, and disturbances in cellular metabolisms. For an example, photosynthesis is impaired by salinity stress as a result of reduced leaf area, stomatal conductance, and chlorophyll content, consequently diminished crop yield (Hnilickova et al., 2021). Among several salinity causing salts, NaCl is the most common salt and critical for plant growth and development. Excessive NaCl in soil causes higher Na^+/K^+ and Na^+/Ca^{2+} ratios, which leads to loss of ion homeostasis in plant cells. The salinity tolerance phenotype is a multigenic trait, which involves many genes

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including those responsible for sequestering excess toxic ions into vacuole or exporting them from cytoplasm to the apoplast via plasma membrane (Ji et al., 2013).

Besides salinity stress, another major abiotic stress is drought that varies in intensity with regards to spatiotemporal pattern (Paixão et al., 2019). Salinity stress shares a common feature with drought stress, as salinity reduces water potential in soil, which leads to less water available to plant root cells, similar to prolonged scarcity of water in soil. Low water availability near the root zone hampers plant growth and development by reducing the nutrient uptake and subsequently leads to poor crop yield (Seleiman et al., 2021). Drought initially causes reduction in plant growth, and ultimately affects crop production (Martignago et al., 2020). Plants exhibit diverse responses to drought stress such as drought escape, dehydration avoidance, and desiccation tolerance in variable magnitudes (Basu et al., 2016). Despite the adverse effects, plants have evolved to have more flexible systems to cope with salinity and drought stress by modifying their physiological, biochemical, and molecular responses (Evelin et al., 2019).

Abiotic stresses tend to occur simultaneously as one often induces the other and concurrent occurrence of these stresses can be additive and more destructive (Mittler, 2006), therefore, it is very important to develop multi-stress tolerant crop plants. One of the approaches towards obtaining stress tolerance is to create transgenic crops by manipulating or boosting expression of key genes responsible for the signalling and regulatory pathways (Vinocur and Altman, 2005). Overexpressing single gene might not be sufficient to tolerate multiple stresses, therefore, choosing co-expression of multiple genes would be an ideal option to further increase abiotic stress tolerance. In this study, three genes, the Arabidopsis vacuolar H^+-pyrophosphatase 1 gene (*AVP1*), the catalytic subunit 5 gene of protein phosphatase 2 A (*PP2A-C5*), and a chloride channel protein gene *AtCLCc* are stacked together and co-transformed into Arabidopsis plants to achieve the desired trait of improved salt and drought stress tolerance.

The AVP1 enzyme is a tonoplast membrane localized protein that acidifies the vacuolar lumen using the energy derived from hydrolyzing inorganic pyrophosphate into orthophosphate (Schilling et al., 2017). The acidification establishes a difference in proton electrochemical gradient across the vacuolar membrane, which can be used by secondary transporters such as Na^+/H^+ antiporters to actively sequester excess Na^+ from cytosol into vacuole, therefore lessening the toxicity level of sodium ion in the cytosol and lowering the water potential in plant cells (Gaxiola et al., 2002; Duan et al., 2007). Thus, increased tolerance to salt stress can be obtained through overexpressing *AVP1* (Gaxiola et al., 2001). Overexpression of *AVP1* also facilitates auxin polar transport, which stimulates root development in plants, subsequently leading to greater water absorption and increased drought tolerance (Li et al., 2005). In addition, AVP1 can assist towards elevated rhizosphere acidification, enhancing plant response to phosphate starvation and exhibiting positive correlation between nitrate use and phosphate use efficiency (Pei et al., 2012; Paez-Valencia et al., 2013). Previous studies have shown that overexpressing *AVP1* increases drought and salt tolerance in various plants including Arabidopsis (Gaxiola et al., 2002; Wijewardene et al., 2020), creeping bentgrass (Li et al., 2010), cotton (Pasapula et al., 2011), peanut (Qin et al., 2013), barley (Schilling et al., 2014), tomato (Yang et al., 2014), rice (Kim et al., 2014), sugarcane (Kumar et al., 2014), and wheat (Regmi et al., 2020).

Protein phosphatase 2 A (PP2A) is a multi-subunit enzyme that belongs to the family of serine/threonine protein phosphatases in plants (DeLong, 2006). PP2A functions as a heterotrimeric complex that is composed of a scaffolding subunit A, a regulatory subunit B and a catalytic subunit C (Janssens and Goris, 2001). Thus far, various specific roles of PP2A have been recognized including responses to biotic and abiotic stresses (Pais et al., 2009). In Arabidopsis, among the five catalytic C subunits, PP2A-C1, PP2A-C2 and PP2A-C5 are grouped into the subfamily I and PP2A-C3 and PP2A-C4 are grouped into the subfamily II based on their sequence conservation (Farkas et al., 2007). When the PP2A-C subfamily I genes were suppressed by virus induced gene silencing in *Nicotiana benthamiana*, PP2A activity was greatly reduced and localized cell death was observed in stems and leaves, which suggested that catalytic subunits of the subfamily I act as negative regulators of plant defence responses (He et al., 2004). While two members of the subfamily II have redundant functions in plant development by contributing to the establishment of auxin gradients (Ballesteros et al., 2013) and are involved in cortical microtubule organization (Yoon et al., 2018). Our previous work showed that overexpression of *PP2A-C5* could enhance salt tolerance possibly by upregulating the activities of vacuolar membrane bound chloride channel proteins (CLC), primarily AtCLCc and AtCLCa, which sequester chloride and nitrate ions into vacuole (Hu et al., 2017a). Physical interaction and functional correlation of chloride channel proteins and PP2A-C5 have been demonstrated by using a yeast 2-hybrid system, which suggests a positive interaction between PP2A-C5 and AtCLCc or AtCLCa (Hu et al., 2017a). These two chloride channel proteins are likely substrates of PP2A-C5 and they are more active when they are in dephosphorylated form, therefore overexpression of *PP2A-C5* might activate AtCLCa and AtCLCc, leading to increased sequestration of chloride and nitrate ions into vacuoles and improved salt tolerance (Hu et al., 2017b).

Chloride is considered as an essential macronutrient and is involved in plant growth and development, photosynthesis, stomatal movement, and water relations (Colmenero-Flores et al., 2019; Jossier et al., 2010). Chloride channels are anion channels omnipresent in prokaryotes and eukaryotes (Poroca et al., 2017). Thus far, there are seven CLC proteins, i.e. AtCLCa to AtCLCg, identified in Arabidopsis, and they are predominantly mediating Cl^- and NO_3^- flux across the vacuolar membranes (Jossier et al., 2010). Subcellular localization studies showed that AtCLCa, AtCLCb, AtCLCc and AtCLCg are localized to the vacuolar membrane, whereas AtCLCd and AtCLCf are found in Golgi vesicles, and AtCLCe is found on chloroplast membranes (De Angeli et al., 2006; Von der Fecht-Bartenbach et al., 2007). AtCLCc acts as a H^+/Cl^- antiporter and it sequesters excess Cl^- ion into vacuole using the H^+ gradient difference generated by H^+-ATPase or H^+ -pyrophosphatase, which leads to increased salt tolerance in plants (Jentsch, 2008; Wong et al., 2013; Wei et al., 2016). AtCLCc was shown to be actively expressed in guard cells and pollen and plays an important role in Cl^- homeostasis during stomatal movement (Jossier et al., 2010). Studies on overexpression of CLC genes in various plants reported a higher Cl^- accumulation in the vacuole and increased salt tolerance. For examples, overexpression of *AtCLCc* in Arabidopsis (Jossier et al., 2010), *AtCLCg* in Arabidopsis (Nguyen et al., 2016), *OsCLC-1* in rice (Nakamura et al., 2006), *GmCLC1* in soybean (Wei et al., 2016), *GsCLC-c2* in soybean (Wei et al., 2019), and *PgCLCs* in pomegranate (Liu et al., 2020), all support the positive role of CLC genes in plant salt tolerance. Hu et al. (2017a) overexpressed *AtCLCc* in the *pp2a-c5–1* mutant background and found that *AtCLCc* functions downstream of *PP2A-C5* as *AtCLCc*-overexpressing plants were just as sensitive as *pp2a-c5–1* mutant plants.

In this study, we tested the hypothesis that Arabidopsis plants co-overexpressing *AVP1*, *PP2A-C5,* and *AtCLCc* would show significantly increased tolerance to salt stress, drought stress, and combined salt and drought stresses. Our results indicate that *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants perform far better than wild-type plants and plants overexpressing either *AVP1,* or *PP2A-C5,* or *AtCLCc* alone under salt and drought conditions. *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants also accumulated the highest amount of Na^+, K^+, and Cl^- in comparing to wild-type and single gene overexpression plants. The increased salt tolerance in Arabidopsis is the highest ever achieved in our laboratory and to our knowledge, the highest in the literature today. This study validates the effectiveness of pyramiding beneficial genes in the hope to significantly improve abiotic stress tolerance in plant so that crop yield loss due to environmental stresses could be minimized in agriculture.

2. Results

2.1. Creation and molecular analysis of AVP1/PP2A-C5/AtCLCc co-overexpressing plants

The DNA construct harboring the expression cassettes of *AVP1*, *PP2A-C5* and *AtCLCc* (Fig. 1A) was created and used to transform wild-type Arabidopsis plants (Col-0) using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998). There were 144 putative, independent transgenic plants generated in the T_1 generation by screening with 30 mg/l kanamycin on MS plates. The T_2 seeds obtained from T_1 plants were analyzed for segregation ratio of kanamycin resistant vs sensitive phenotype from which 80 putative single T-DNA insertion lines were obtained. Those 80 T_2 seeds were further screened with 30 mg/l kanamycin to obtain homozygous T_3 seeds, and a total of 75 putative homozygous T_3 plants were obtained. All the putative homozygous lines were used to extract total RNAs and RNA blot analysis was conducted using the *AVP1* cDNA probe and *PP2A-C5* cDNA probe (Fig. 1B). Because the coding sequences of *PP2A-C5* and the *AtCLCc* were fused together with the self-cleavage sequence 1 (SCE1) and transcribed as a single transcript, we only used *PP2A-C5* cDNA as a probe to detect the fusion transcript in the RNA blot. Fifteen of the homozygous lines exhibited higher transcript levels of *AVP1* and *PP2A-C5* compared to wild-type plants, and they were subjected to preliminary physiological experiments on Murashige and Skoog (MS) plates containing 100 mM NaCl. Three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing lines, designated as APC1, APC2, and APC3 in Fig. 1B, chosen from the RNA blot analysis and the preliminary salt stress experiment, were used for further molecular and physiological experiments. Single gene overexpression lines for *AVP1* (A), *PP2A-C5* (P) and *AtCLCc* (C) were also used as reference lines. Reverse transcription polymerase chain reaction (RT-PCR) was carried out with total RNAs isolated from APC1, APC2 and APC3 to ascertain the presence of transgene transcript as well as to compare their relative transcript level in wild-type plants (Fig. 1C). The three *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants did show higher levels of transcripts for *AVP1, PP2A-C5,* and *AtCLCc.* We then analyzed the protein levels of the catalytic subunit of PP2A in the three *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants by performing Western blot analysis using the anti-PP2A-1/2 catalytic subunit antibody from *Arabidopsis thaliana*. Compared to the wild-type plants, *PP2A--C5*-overexpressing plants (P) and the three *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants displayed a band with more intensity at the anticipated size of 35 kDa that presumably is PP2A-C5 (Fig. 1D). This indicates that the overexpressed PP2A-C5/AtCLCc fusion protein is processed into separate proteins of PP2A-C5 and AtCLCc at the SCE1 site.

2.2. AVP1/PP2A-C5/AtCLCc co-overexpressing plants are the most tolerant to salt stress

A series of experiments on MS plates and in potting soil were conducted with wild-type plants (WT), *AVP1*-overexpressing plants (A), *PP2A-C5* overexpressing plants (P), *AtCLCc*-overexpressing plants (C), and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (APC1 to APC3) to examine how they would respond to salt stress. Sterilized seeds from each genotype were sowed on MS plates containing 100 mM NaCl and 150 mM NaCl, respectively, and grown vertically for seven days. Seedlings from *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants produced the longest root lengths under 100 mM NaCl (Fig. 2A & B) and their growth and appearance were better in comparing to WT and single gene overexpression plants. WT plants produced the shortest root length. Seedlings from single gene overexpression plants were able to grow better than WT plants, but their root length and shoot size were shorter and smaller than *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Under 150 mM of NaCl, all seedlings exhibited slow growth and chlorotic phenotype (Supp. Fig. 1). However, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants still generated the longest roots and produced true leaves at the 7th day of growth (Supp. Fig. 1). Plants grown on MS plates containing 300 mM mannitol did not exhibit much differences in their germination rate and general phenotype (Supp. Fig. 2), indicating that the differential responses on salt media were solely due to the toxic effect of NaCl and not due to the osmotic stress that came from the lower water potential in the MS medium. Experiments were repeated three times and consistent results showed that *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants performed better than single gene overexpression plants and wild-type plants under NaCl stress conditions.

To test the salinity tolerance of the *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants in soil, two-week-old plants were irrigated with saline water, starting with 50 mM of NaCl, incrementally increased to 100 mM, 150 mM, 200 mM, 250 mM, and finally to 300 mM, every three days. No observable phenotypic differences were found in plants before exposure to salt stress (Fig. 3A) and in plants that were grown under normal growth condition for two months with no salt stress treatment (Fig. 3B). The phenotypic difference among these genotypes

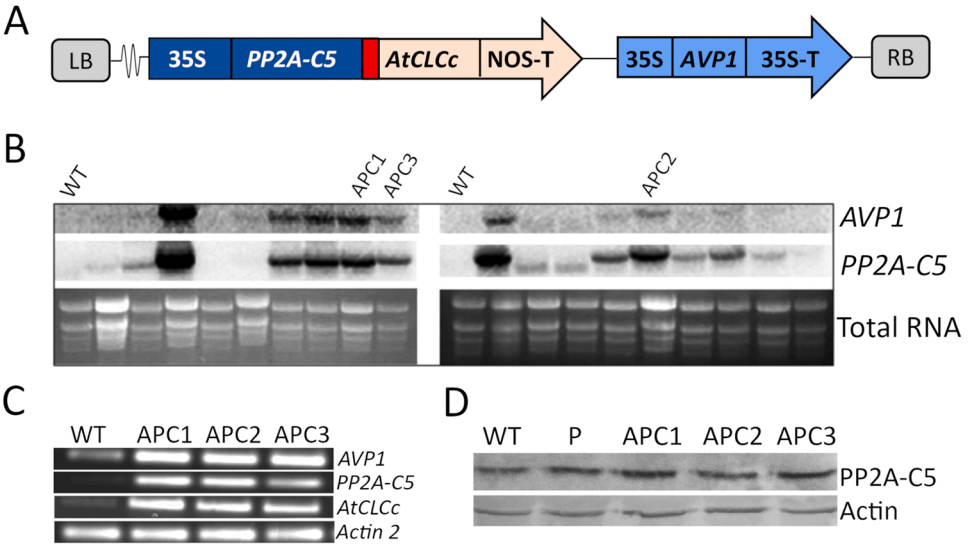

Fig. 1. Creation and molecular analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. A. Schematic diagram of the T-DNA region of the transforming vector. 35 S, 35 S promoter from tobacco mosaic virus; the red segment is the self-cleavage sequence 1; NOS-T, nopaline synthase gene terminator; 35S-T, 35 S terminator from tobacco mosaic virus; LB and RB, left and right border sequences of T-DNA, respectively. B. RNA blot analysis of selected *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Genes *AVP1* and *PP2A-C5* used as probes are listed on the right. C. Transcript analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants by using reverse transcription PCR. Amplified gene fragments are marked on the right and *Actin 2* was used as the internal control. **D.** Western blot analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. The proteins recognized by antibodies are marked on the right. WT, wild-type plants; P, *PP2A-C5*-overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants.

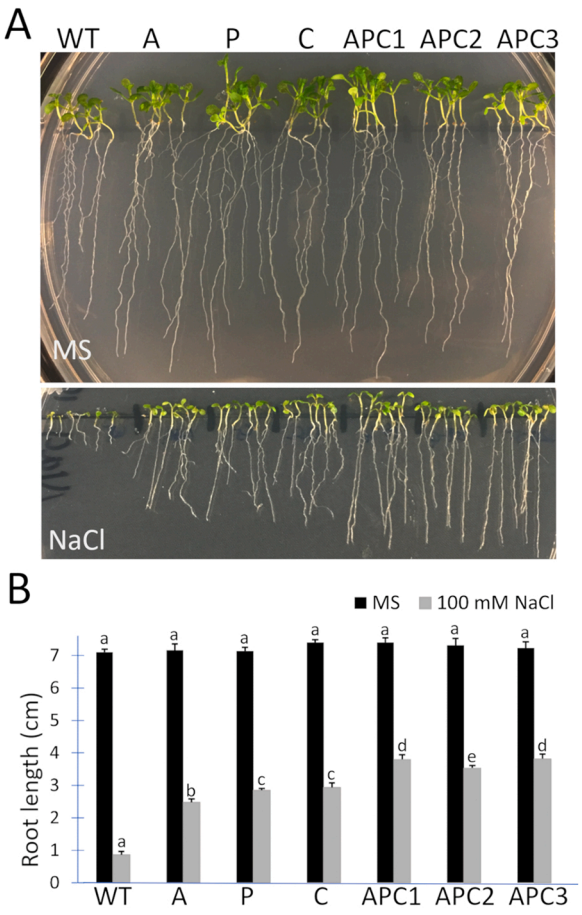

Fig. 2. Analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants in the absence and presence of salt on MS plates. **A.** Phenotypes of wild-type, single gene overexpression plants and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants in the absence and presence of salt. MS, Murashige and Skoog medium; NaCl, MS + 100 mM of NaCl. **B.** Root length analysis of wild-type, single gene overexpression and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants in the absence and presence of salt. WT, wild-type plants; A, *AVP1*-overexpressing plants; P, *PP2A-C5*-overexpressing plants; C, *AtCLCc*-overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Black bar, no salt in the MS media; grey bar, MS media + 100 mM NaCl. Different letters represent the statistical significance between samples according to Student's *t*-test. The results shown are the means ± SE, n = 6 plants.

became apparent when the salinity level reached 250 mM of NaCl. Wild-type plants showed chlorotic rosette leaves and retarded growth while single gene overexpression plants (i.e., A, P, and C plants) were still green. However, the *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants showed the largest rosette leaves and continued growth (Fig. 3C). When these plants were treated with 300 mM NaCl, wild-type plants died completely, and the growth of single gene overexpression plants was severely inhibited. The *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants displayed better appearance with rosette leaves still green at 300 mM NaCl (Figs. 3D and E). Average plant heights among all genotypes measured at 250 mM and 300 mM of NaCl indicated that *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants displayed taller height than all other plants (Fig. 3F). Silique number and seed weight produced by *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants were significantly greater than those of WT and single gene overexpression plants (Fig. 3G & H). Based on these results, we conclude that *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants display the highest salt tolerance, and the tolerance level of these plants are APC plants > C plants > or = P plants > A plants > WT plants.

2.3. *AVP1/ PP2A-C5/AtCLCc co-overexpressing plants are very tolerant to other salts as well*

Based on previous studies, transgenic Arabidopsis plants over-expressing *PP2A-C5* (Sun et al., 2018; Hu et al., 2017a) or *AtCLCc* (Jossier et al., 2010) exhibited increased tolerance not only to NaCl but also to other salts such as KCl and KNO_3. We anticipated that Arabidopsis plants co-overexpressing *AVP1, PP2A-C5,* and *AtCLCc* would also show enhanced tolerance to other salts. Therefore, the performance of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants in the presence of 150 mM KCl and 200 mM KNO_3 was tested. We found that the growth of wild-type plants was severely inhibited by KCl and KNO_3, while the single gene overexpression plants performed slightly better by developing slightly larger but yellowish leaves (Fig. 4A). In contrast, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants developed greener and larger leaves with the longest root lengths (Fig. 4A, middle panel and lower panel). These results indicate that *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants performed much better than wild-type and single gene overexpression plants under other types of salt treatment.

2.4. *AVP1/PP2A-C5/AtCLCc co-overexpressing plants are the most tolerant to osmotic and drought stresses*

Early studies showed that overexpression of *AVP1* leads to increased tolerance to drought stress (Gaxiola et al., 2001; Pasapula et al., 2011; Wijewardene et al., 2020), thus, we hypothesized that *AVP1/PP2A--C5/AtCLCc* co-overexpressing plants would also be more drought tolerant than wild-type plants. Polyethylene glycol-8000 (PEG-8000) is known to mimic water-deficit stress by acting as an osmotic agent to reduce the water potential in the media, therefore we used PEG-8000 in the water deficit experiments. To test how Arabidopsis seedlings would respond to the low water potential in the MS media, sterilized and stratified Arabidopsis seeds were sown directly on MS media supplemented with 40% PEG-8000. *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants displayed the longest roots (100% longer than WT plants), followed by *AVP1*-overexpressing plants, then the other two single gene overexpression plants, finally the wild-type plants (Supp. Fig. 3). Even though one of the *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants, APC2, did not exhibit significant difference that could be differentiated from *AVP1*-overexpressing plants, it did show healthier and greener leaves as other *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Supp. Fig. 3A). Although the increased tolerance to water deficit stress is attributed mainly to *AVP1*-overexpression, it appears that co-overexpression of *AVP1* with *PP2A-C5* and *AtCLCc* further increased water deficit stress tolerance in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants.

To validate these results in soil, similar experiments were conducted in pot under drought stress conditions. Plants from each genotype did not show any noticeable phenotypic differences before the drought stress was applied (Fig. 5A) and plants grown under normal growth condition did not show much differences in phenotype either (Fig. 5B). After the drought stress treatment, plants showed dramatic differences in phenotypes with a significant difference observed in plant height (Fig. 5C & Fig. 5E). Among the single gene overexpression plants, *AVP1*-overexpressing plants exhibited green and large rosette leaves although the height of these plants was comparatively shorter when compared with other plants. The *AVP1*-overexpressing plants grew relatively slow at early stage of drought treatment, but caught up at later stages, and eventually produced considerably greater number of siliques and seed yield in comparing with other single gene overexpression plants. Overall *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants performed the best in terms of plant height, silique number, and seed yield (Figs. 5E, 5 F, & 5 G). While the increased drought tolerance trait should be mainly contributed by *AVP1*-overexpression, like the water-deficit experiment with PEG treatment, it appears that the co-overexpression of *AVP1* with *PP2A-C5* and *AtCLCc* also contributed to the much higher tolerance

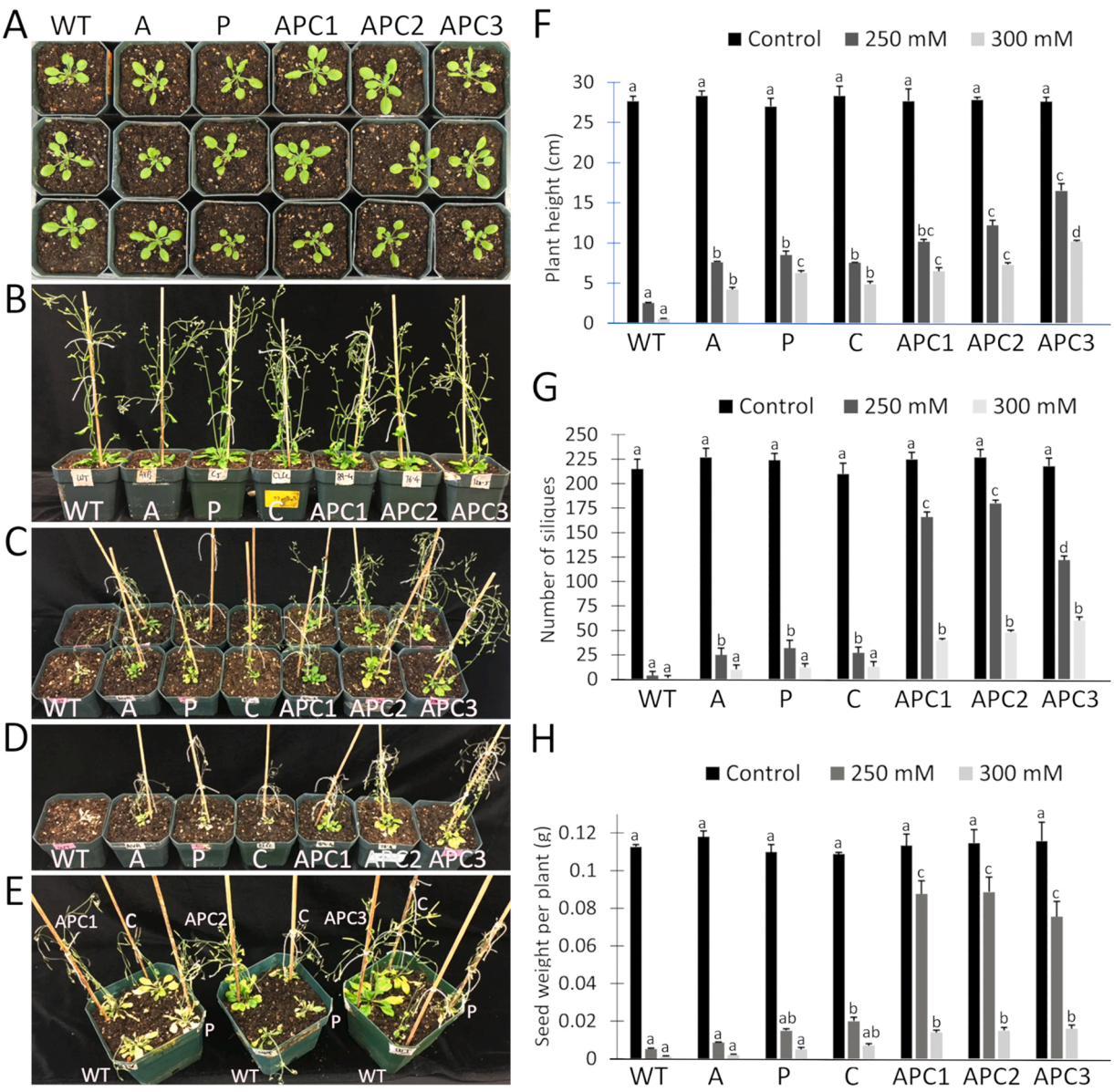

Fig. 3. Analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants in the absence and presence of salt in soil. A. Phenotype of two-week-old plants before salt treatment. B. Phenotype of plants grown under normal growth condition. C. Phenotype of plants after treatment with 250 mM of NaCl. D. Phenotype of plants after treatment with 300 mM of NaCl. E. Phenotype of plants in the same pot after treatment with 250 mM of NaCl. F. Plant heights after treatment with 250 mM and 300 mM of NaCl. G. Silique numbers per plant after treatment with 250 mM and 300 mM of NaCl. **H.** Seed yield per plant after treatment with 250 mM and 300 mM of NaCl. WT, wild-type plants; A, *AVP1*-overexpressing plants; P, *PP2A-C5*-overexpressing plants; C, *AtCLCc*-overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Different letters represent the statistical significance between samples according to Student's *t*-test. The results shown are the means ± SE, n = 6 plants.

displayed by *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under drought stress condition in soil. The drought tolerance level of these plants are APC plants > A plants > C plants > P and WT plants.

2.5. AVP1/PP2A-C5/AtCLCc co-overexpressing plants are the most tolerant to combined salt and drought stresses

As we hypothesized that co-overexpression of these three genes might add additional benefits that could lead to even higher tolerance to single stress of salt or drought, as well as combined salt and drought stresses, we did the following experiments to test this hypothesis. We sowed Arabidopsis seeds on MS plates supplemented with 100 mM NaCl and 40% PEG-8000, and we found that *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants exhibited the best growth with most true leaves appearing and produced the longest root lengths than all other plants (Fig. 6A and 6B). To test how these plants would respond to combined salt and drought stresses in soil, two-week-old plants were treated with half volume of increasing concentration of NaCl solution up to 200 mM. Plants of all genotypes before the combined stresses were applied or under normal growth conditions for two months did not show major phenotypic differences (Fig. 7A & B), but after the treatment of combined salt and drought stresses, these plants exhibited dramatic phenotypical differences (Fig. 7C). The *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants were bushier and greener, while the wild-type plants showed severe wilting and yellow phenotype. The single gene overexpression plants grew to some extent but were not as healthy as *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Interestingly, among the single gene overexpression plants, *AtCLCc*-overexpressing plants were taller than *AVP1*-overexpressing and *PP2A-C5* overexpressing plants (but not statistically significant), the *AVP1/PP2A-C5/AtCLCc* co-

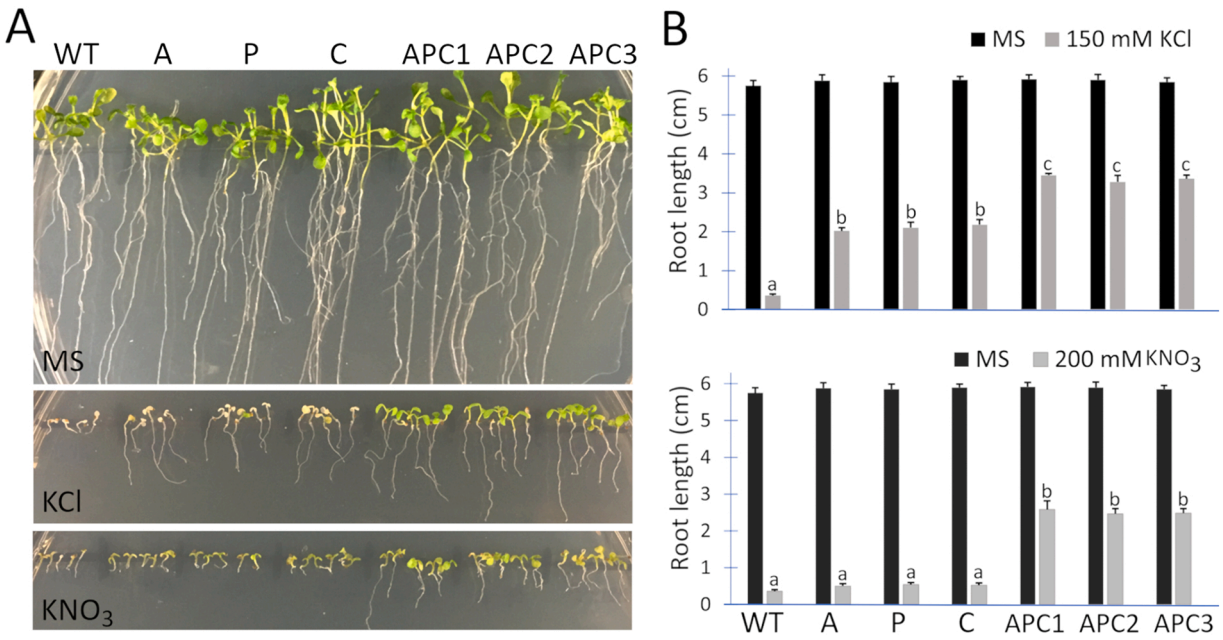

Fig. 4. Analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants in the absence and presence of KCl and KNO_3. A. Phenotypes of wild-type and various transgenic plants in the absence of salt, presence of KCl, and presence of KNO_3. MS plate, upper panel; MS + 150 mM KCl, middle panel; MS + 200 mM KNO_3, lower panel. WT, wild-type plants; A, *AVP1*-overexpressing plants; P, *PP2A-C5*-overexpressing plants; C, *AtCLCc*- overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpression plants. B. Root length analysis of wild-type and various transgenic plants on MS plate and MS plate supplemented with salts. Black bar, MS media; grey bar in upper panel, MS + 150 mM of KCl; grey bar in lower panel, MS + 200 mM KNO_3. Different letters represent the statistical significance between samples according to Student's *t*-test. The results shown are the means ± SE, n = 6 plants.

overexpressing plants were still the tallest (Fig. 7E). Although all plants managed to survive until the harvesting stage, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants produced the greatest number of siliques and the highest seed yield (Fig. 7F & G). It must be noted that there were no statistical differences in the number of siliques and seed yield between wild-type and single gene overexpression plants. It appears that the single gene overexpression plants could not tolerate the severity of the combined salt and drought stresses, but co-overexpression of the three genes clearly elevated the tolerance to the combined drought and salt stresses, leading to the significantly increased seed yield under this severe stressful condition (Fig. 7 G).

2.6. *AVP1/PP2A-C5/AtCLCc co-overexpressing plants accumulate the highest amount of chloride, sodium, and potassium ions*

Overexpression of *AtCLCc* in Arabidopsis leads to increased accumulation of chloride ion in transgenic plants (Hu et al., 2017a), as AtCLCc is a chloride channel or H^+/Cl^- antiporter on the vacuolar membrane. We analyzed the ion contents of chloride, sodium, and potassium in these plants. As we expected, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants accumulated significantly higher amounts of Cl^- and Na^+ than *AVP1*-overexpressing plants, and *AVP1*-overexpressing plants accumulated higher amounts of Cl^- and Na^+ than wild-type plants (Fig. 8). Overexpression of *AVP1* leads to increased H^+ concentration in vacuoles, which could energize the vacuolar membrane bound chloride channel proteins such as H^+/Cl^- antiporter (AtCLCc) and H^+/Na^+ antiporter (NHX1), leading to increased sequestration of Cl^- and Na^+ into vacuoles. It is likely that co-overexpression of *AVP1* with *AtCLCc* and *PP2A-C5* further increases AtCLCc and NHX1's activities in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants, which explains why the contents of both chloride and sodium ions were significantly higher in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Interestingly, the potassium content was also significantly increased in *AVP1/PP2A--C5/AtCLCc* co-overexpressing plants, which is likely due to the electric balance for the increased anion content such as Cl^- in plant cells and using K^+ to neutralize Cl^- is a solution in plant cells.

2.7. *AVP1/PP2A-C5/AtCLCc co-overexpressing plants have greater capacity of acidifying the media than AVP1-overexpressing plants*

Rhizosphere acidification is a key mechanism in nutrient acquisition by plants since the acidic nature of proton helps the solubility of nutrients. As stated in previous studies appertaining to *AVP1*-overexpression (Pizzio et al., 2015a, 2015b; Khadilkar et al., 2016; Wijewardene et al., 2020), we hypothesized that *AVP1/PP2A-C5/AtCLCc* co-overexpression plants might have higher rhizosphere acidification capacity, and therefore higher ability to absorb nutrients. To test this hypothesis, we conducted the rhizosphere acidification experiment by following the protocol described by Pizzio et al. (2015). Our result indicated that two of the three *AVP1/PP2A-C5/AtCLCc* co-overexpression plants (APC1 and APC2) acidified the rhizosphere more effectively than all other genotypes (Supp. Fig. 4). Interestingly, the one *AVP1/PP2A-C5/AtCLCc* co-overexpressing (APC3) line displayed a similar capacity as *AVP1*-overexpressing plants (Supp. Fig. 4). Indeed, transgenic plants overexpressing *AVP1* released more protons into the medium than wild-type plants and *PP2A-C5*-overexpressing plants. Surprisingly, *AtCLCc*-overexpressing plants appeared releasing similar amounts of protons as *AVP1*-overexpressing plants. Therefore, these two genes might have attributed to the greater release of proton in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants, which might be the determination factor for better plant growth of the *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants.

2.8. *Transcripts of many abiotic stress defence genes are highly upregulated in AVP1/PP2A-C5/AtCLCc co-overexpressing plants under combined drought and salt stresses*

To further study why *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants would perform so much better than every other plants under salt, drought, and combined salt and drought stresses, we examined the

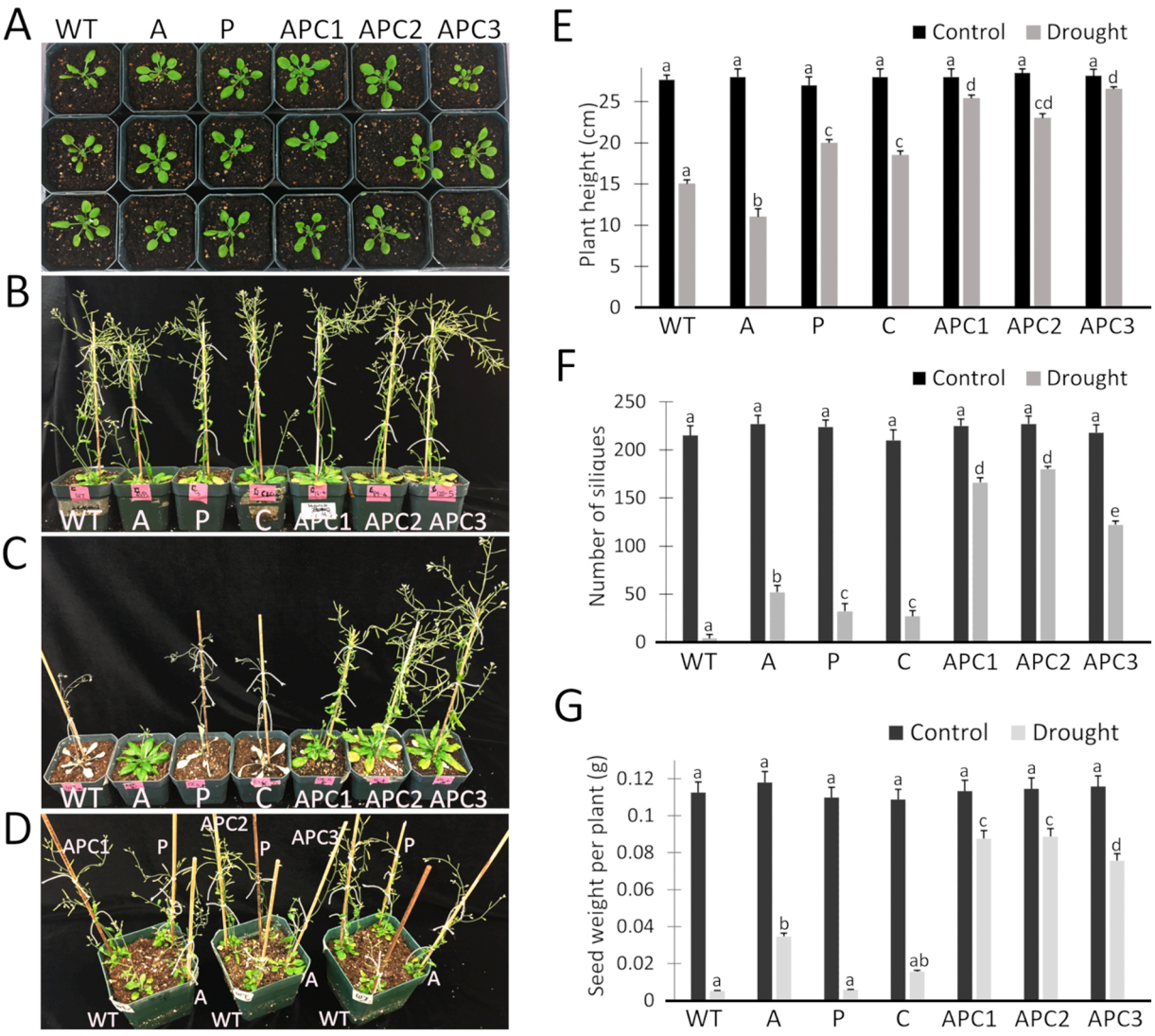

Fig. 5. Analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under drought stress conditions in soil. A. Phenotype of two-week-old plants before drought stress treatment. B. Phenotype of plants grown under normal growth condition. C. Phenotype of plants after drought stress treatment. D. Phenotype of plants in the same pot after drought stress treatment. E. Plant heights after drought treatment. F. Silique numbers per plant after drought treatment. **G.** Seed yield per plant after drought treatment. WT, wild-type plants; A, *AVP1*-overexpressing plants; P, *PP2A-C5*-overexpressing plants; C, *AtCLCc*- overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Different letters represent the statistical significance between samples according to Student's *t*-test. The results shown are the means ± SE, n = 6 plants.

transcript levels of 12 defence genes that play important roles in plant tolerance to salt and drought stresses. For examples, for salt tolerance genes we selected *SOS1, HAK5, HKT1, NHX1*; for drought tolerance genes, we selected *LEA4–5, LEA14, RD29A, RD29B*; for multi-stress transcriptional factor genes, we selected *DREB26, CBF2, NAC2, and MYB87*. Our qRT-PCR analysis of the selected genes revealed that except for the *SOS1* transcript, the transcript levels of the other 11 genes were the highest in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Fig. 9). The transcript levels of 10 genes (other than *SOS1* and *LEA14*) were clearly higher in *AVP1*-overexpressing plants and *PP2A-C5* overexpressing plants than wild-type plants. These data provide strong evidence that the superior performance of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants is likely due to higher expression of these beneficial genes under the combined salt and drought stresses because of the reduced levels of Na^+ and Cl^- ions in cytosol.

3. Discussion

In this study, we demonstrated that co-overexpression of *AVP1*, *PP2A-C5,* and *AtCLCc* in Arabidopsis could greatly increase tolerance to salt, drought, and combined salt and drought stresses. The key for the success of this research is that the three proteins overexpressed in transgenic plants would function synergistically, not antagonistically. Here, AVP1 builds a proton gradient across the tonoplast membrane, which can be utilized by chloride channel proteins such as H^+/Cl^- antiporter (AtCLCc) to move a chloride into vacuole in exchange for a proton. Therefore, AVP1 and AtCLCc work synergistically on the tonoplast membrane. We previously showed that PP2A-C5 physically interacts with AtCLCc and AtCLCa, and overexpression of *PP2A-C5* increases salt tolerance in Arabidopsis (Hu et al., 2017a). We assumed that the function of PP2A-C5 is to activate AtCLCc and AtCLCa on the tonoplast membrane (Hu et al., 2017b) and their relationship is also synergistic. Consequently, we hypothesized that co-overexpression of *AVP1*, *PP2A-C5,* and *AtCLCc* in Arabidopsis would lead to much higher tolerance to salt and drought stresses due to the synergism of these three proteins working together. We proved that our hypothesis is correct, because we created Arabidopsis plants that are the most salt tolerant Arabidopsis plants ever created in our laboratory, and these plants could

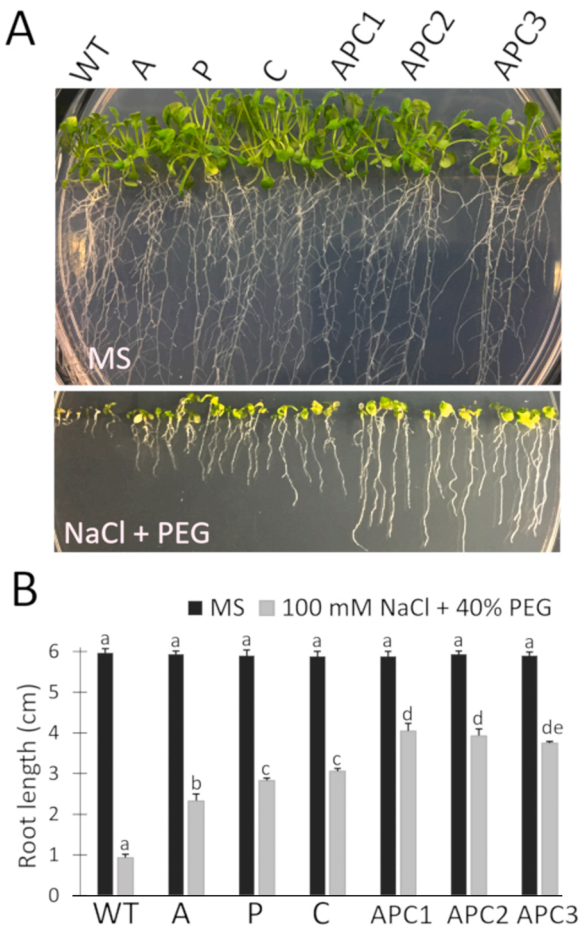

Fig. 6. Analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under the condition of combined salinity and water deficit stresses. A. Phenotypes of wild-type and various transgenic plants in the absence and presence of combined salinity and water deficit stresses. MS, Murashige and Skoog medium (upper panel); NaCl + PEG, MS medium with 100 mM NaCl and 40% polyethylene glycol-8000 (PEG-8000) (lower panel). B. Root length analysis of wild-type and various transgenic plants on MS plate and MS plate supplemented with 100 mM NaCl and PEG-8000. WT, wild-type plants; A, *AVP1*-overexpressing plants; P, *PP2A-C5*-overexpressing plants; C, *AtCLCc*-overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Different letters represent the statistical significance between samples according to Student's *t*-test. The results shown are the means ± SE, n = 6 plants.

produce functional seeds in the presence of 300 mM NaCl, which is an unprecedented attempt.

AVP1/PP2A-C5/AtCLCc co-overexpressing plants exhibited significantly greater salt tolerance than single gene overexpression plants on MS plates and in soil (Figs. 2 and 3). They produced longer roots, larger biomass, and substantially more seeds than any other Arabidopsis plants tested here. This study is a big step further in creating salt tolerant plant when compared with previous studies that overexpressed one or two relevant genes such as *AVP1* (Park et al., 2005), *AtCLCc* (Jossier et al., 2010; Hu et al., 2017a), *PP2A-C5* (Hu et al., 2017a), *AtNHX1* and *SOS1* (Pehlivan et al., 2016), or *AVP1* and *PP2A-C5* (Sun et al., 2018), *AVP1* and *RCA* (Wijewardene et al., 2020). We had never achieved a salt tolerance reaching 300 mM of NaCl before, and the closest study in our laboratory would be the *AtNHX1/SOS1* co-overexpressing Arabidopsis that could tolerate up to 250 mM of NaCl (Pehlivan et al., 2016). All other transgenic plants, made by us or by others, could tolerate NaCl between 100 and 200 mM at most.

Like *PP2A-C5* overexpressing plants, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants could withstand the salt stress imposed by other salts such as KCl and KNO_3 (Fig. 4). However, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants are much more tolerant to these salts than *PP2A-C5* overexpressing plants. Potassium is an important ion in plant physiological processes including osmotic regulation, enzyme activities and stomatal movement (Wang et al., 2013). KNO_3, a key component of fertilizer in agriculture, is usually a beneficial salt, and at adequate concentration KNO_3 prevents the accumulation of toxic salts and counteracts the negative effects of sodium ion. However, at higher concentrations KNO_3 has adverse effects on plants (Zheng et al., 2008). Enhanced tolerance to 200 mM KNO_3 obtained in this study was party contributed by overexpression of *PP2A-C5* and partly by overexpression of *AVP1*. The fact that *AVP1/PP2A-C5/AtCLCc* co-overexpression plants exhibited enhanced tolerance to KNO_3 than *PP2A-C5*-overexpression plants, might be due to the additive effect of co-overexpression of *AVP1* and *AtCLCc,* which likely assists salt tolerance when the NO_3^- concentration is higher than 125 mM (Krapp et al., 2014).

Chloride is a dominant anion, and it becomes toxic at high concentration to glycophytes such as Arabidopsis (Geilfus, 2018). When plants experience incessant flow of Cl^- into cells, chloride must be prevented from being transported to shoots and kept away from dividing cells and photosynthetic cells (Tavakkoli et al., 2010). Intracellular compartmentalization of excessive chloride ions from cytosol into the vacuole is achieved by AtCLCc. The increased tolerance to 150 mM KCl is also due to co-overexpression of *AVP1* and *PP2A-C5* for the same reason as above. Both *PP2A-C5*-overexpressing and *AtCLCc*-overexpressing plants exhibited greener and broader leaves than *AVP1*-overexpressing plants under KCl, which supports this interpretation (Fig. 4). Regardless of the selective preference for nitrate ions, AtCLCa might also help in the compartmentalization of Cl^- in the cytosol due to the abundant concentration of cytosolic Cl^- concentration over NO_3^- (Lorenzen et al., 2004). The results obtained in our study are consistent with the studies from others (Wong et al., 2013; Wei et al., 2016; Jossier et al., 2010).

We also observed that *AVP1/PP2A-C5/AtCLCc* co-overexpression plants displayed much enhanced drought tolerance, which we believe is the trait mainly attributed by overexpression of *AVP1* (Fig. 5). Robust growth, broader rosette leaves, and greater seed yield of *AVP1*-overexpressing plants and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under low water availability is due to the activity of *AVP1* in accumulating more solutes into vacuole (schilling et al., 2017). Both *AVP1*-overexpressing plants and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants displayed similar water-deficit tolerance on MS plate supplemented with PEG (Supp. Fig. 3) and in soil under drought stress treatment (Fig. 5). *PP2A-C5* overexpressing plants and *AtCLCc*-overexpressing plants also showed better plant growth than wild-type plants, however, they were not performing as good as *AVP1*-overexpressing plants. Interestingly, *AVP1/PP2A-C5/AtCLCc* co-overexpression plants performed much better than *AVP1*-overexpressing plants with respect to plant height, siliques number, and seed yield. We assume that overexpression of *PP2A-C5* and *AtCLCc* might help improving the drought tolerance, even though the exact mechanism is not clear. When the drought tolerance phenotype appeared, *AVP1*-overexpressing plants displayed a better visual appearance than *PP2A-C5* and *AtCLCc*-overexpressing plants, despite that they did not grow much taller (Fig. 5 C). This perhaps could be explained as such: *PP2A-C5* overexpressing plants and *AtCLCc*-overexpressing plants experienced water deficit stress earlier and then began transitioning into reproductive phase for survival, while *AVP1*-overexpressing plants were still acclimatizing to drought stress. Enhanced tolerance to drought by overexpressing *AVP1* has been demonstrated by many people in diverse plant species (Wijewardene et al., 2020, 2011; Kim et al., 2014; Yang et al., 2014).

In this study, the increased tolerances to combined water deficit and salt stresses were so impressive (Fig. 6 & 7) that we believe there might be synergistic effect among these three genes overexpressed. It appears that the increased yield with *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants is larger than the sum of the three genes individually overexpressed under combined drought and salt stresses (Fig. 7). The responses

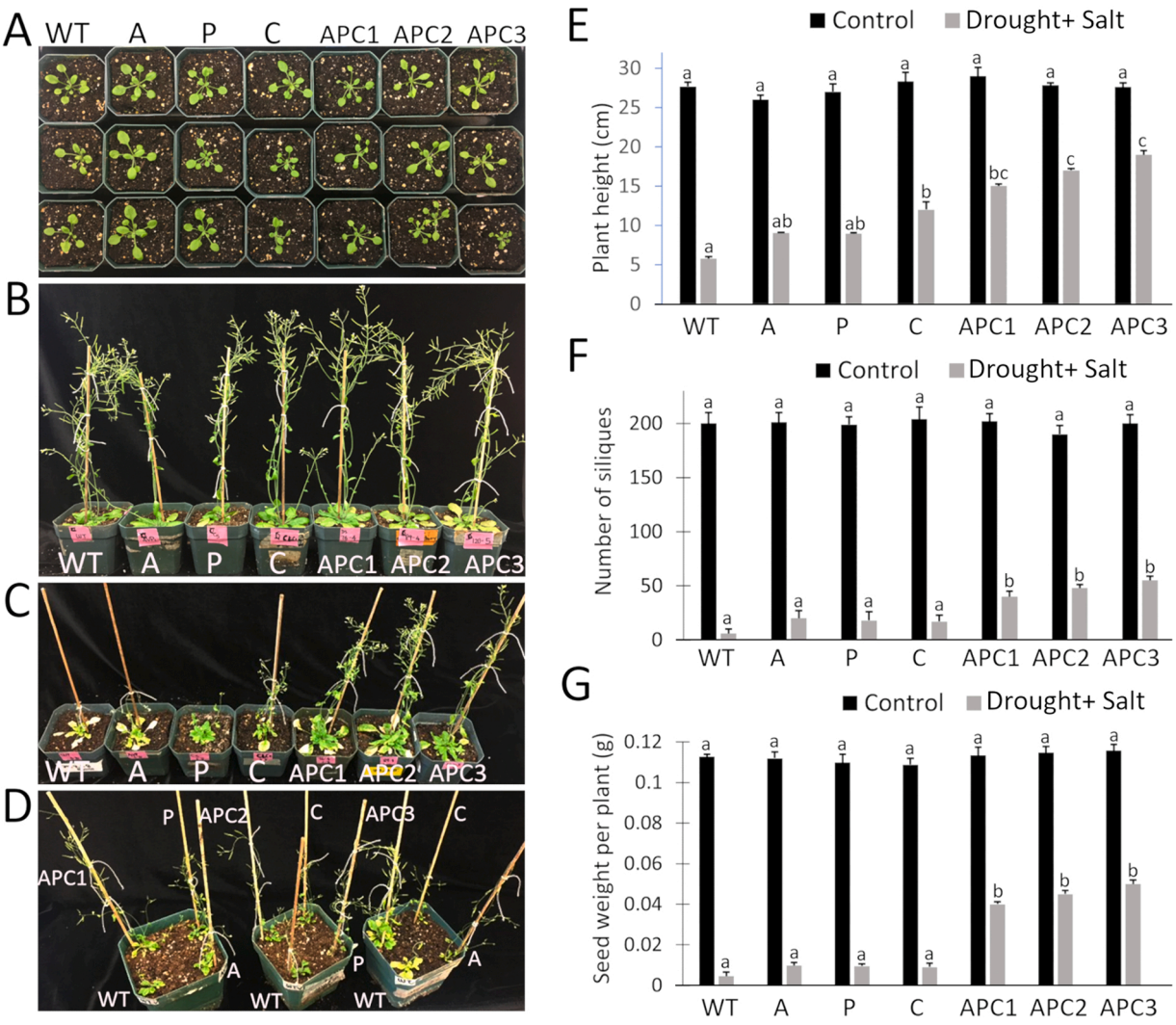

Fig. 7. Analysis of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under combined drought and salt stresses in soil. A. Phenotype of two-week-old plants before the combined drought and salt stress treatment. B. Phenotype of plants grown under normal growth condition. C. Phenotype of plants after the treatment of combined drought and salt stresses. D. Phenotype of plants in the same pot after the treatment of combined drought and salt stresses. E. Plant heights after the treatment of combined drought and salt stresses. F. Silique numbers per plant after the treatment with combined drought and salt stresses. G. Seed yield per plant after the treatment of combined drought and salt stresses. WT, wild-type plants; A, *AVP1*-overexpressing plants; P, *PP2A-C5*-overexpressing plants; C, *AtCLCc*-overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Different letters represent the statistical significance between samples according to Student's *t*-test. The results shown are the means ± SE, n = 6 plants.

to drought stress and salinity stress are related, as they both induce osmotic stress and reduce water uptake, which would affect many aspects of plant growth and development (Ma et al., 2020). Reducing sodium toxicity by sequestering sodium and chloride ions into vacuole would also help water absorption as they further reduce water potential of vacuoles in root cells, especially in conjunction with the robust root system due to overexpression of *AVP1* in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Indeed, analysis of chloride, sodium, and potassium contents in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Fig. 8) supports this notion.

Overexpression of *AVP1* could enhance rhizosphere acidification, which facilitates better uptake of soluble nutrients (Wijewardene et al., 2020; Esmaeili et al., 2019). Hence, we analysed the rhizosphere acidification of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. We measured the H^+ released to the medium relative to average number of H^+ moles per root fresh weight (Supp. Fig. 4). As expected, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants and *AVP1*-overexpressing plants displayed greater release of protons into the media. Surprisingly, *AtCLCc*-overexpressing plants also exhibited similar amount of protons released to the media, for which we do not have an explanation as there is no information on the relationship between the function of AtCLCc and the rhizosphere acidification. Nevertheless, we believe that there might be some synergistic interactions between *AVP1* and *AtCLCc*, which leads to better nutrient acquisition and consequently larger biomass in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Supp. Fig. 4). This is also the reason why *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants performed the best under salt, drought, and combined drought and salt stresses.

Salt Overly Sensitive 1 (*SOS1*), a salt tolerance gene in Arabidopsis, encodes a plasma membrane-bound Na^+/H^+ antiporter that is crucial for Na^+ and K^+ homeostasis in plants (Shi et al., 2000). The transcript level of *SOS1* was upregulated in *AVP1*-overexpressing, *PP2A-C5* overexpressing, and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plant even in the absence of any stress (Fig. 9). Interestingly, under combined drought and salt stresses, the *SOS1* transcript was increased in wild-type and *AVP1*-overexpresing plants, but no further increase in *PP2A-C5* overexpressing and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Fig. 9). This was the only gene whose transcript was not further

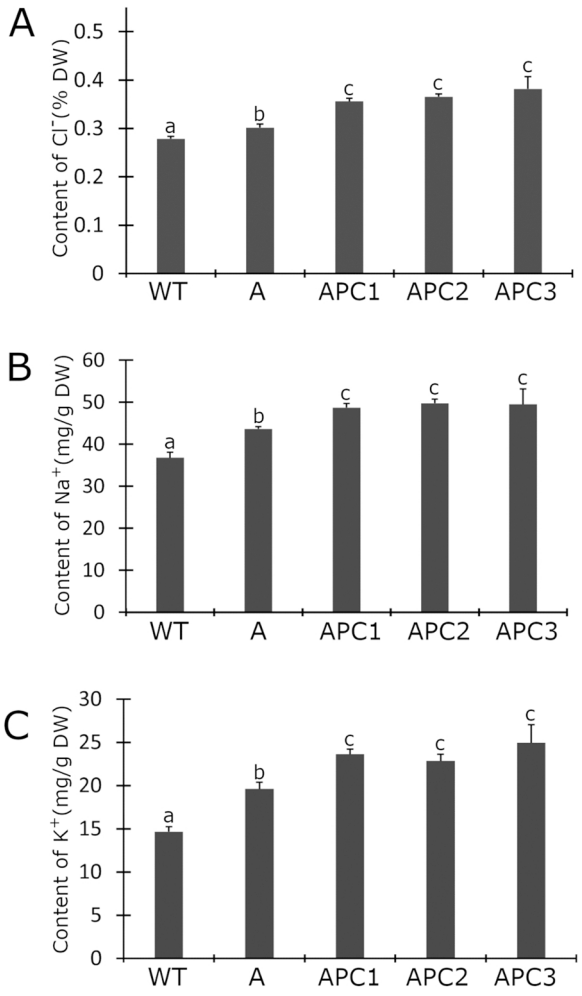

Fig. 8. Analysis of chloride, sodium, and potassium content in wild-type, *AVP1*-overexpressing, and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants after salt stress treatment. **A.** Cl^- content; **B.** Na^+ content; **C.** K^+ content. WT, wild-type plants; A, *AVP1*-overexpressing plants; APC1, APC2 and APC3, three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants. Different letters represent the statistical significance between samples according to Student's *t*-test. The results shown are the means ± SE, n = 6 plants.

increased under combined drought and salt stresses. The HAK5 is one of the high affinity transporter proteins for the uptake of K^+ to maintain lower Na^+/K^+ ratio in Arabidopsis and the transcript level of *HAK5* was found to be upregulated when plants were under salinity stress (Nieves-Cordones et al., 2010). We found the *HAK5* transcript was up-regulated under combined drought and salt stresses, and the biggest increase was found in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants, followed by *PP2A-C5* overexpressing plants, then *AVP1*-overexpressing plants, and finally wild-type plants (Fig. 9). This helps explain why K^+ content is the highest in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Fig. 8). The high affinity K^+ transporter 1 (HKT1) inhibits the transport of Na^+ from root to shoot and facilitates Na^+ transport from shoot to root by unloading Na^+ from the xylem into xylem parenchyma cells and loading Na^+ into phloem (Almeida et al., 2017). Therefore, HKT1 performs a critical role in maintaining low Na^+ concentration in leaf tissues. Our qRT-PCR analysis showed a strong upregulation of *HKT1* transcript in all transgenic plants, especially the biggest increase was found *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Fig. 9). The Na^+/H^+ antiporter 1 (NHX1) plays a key role in salinity tolerance by sequestering Na^+ into vacuole (Apse et. al, 1999). Like *HKT1*, the *NHX1* transcript was highly up regulated in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under combined drought and salt stresses (Fig. 9), which is also consistent with the ion analysis data in Fig. 8.

Late embryogenesis-abundant proteins (LEA) are a group of proteins that play protective roles under water deficit conditions. We found that the transcript levels of two LEA genes, *LEA4–5* and *LEA14* were up-regulated under combined drought and salt stresses, and the biggest increase was also found in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Fig. 9). Desiccation responsive genes *RD29A* and *RD29B* are important genes in plant response to several abiotic stresses including salt and water deficit stresses (Msanne et al., 2011). We found that transcripts of *RD29A* and *RD29B* were greatly up-regulated under combined salt and drought stresses, and the highest increase was found in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants as well (Fig. 9). Transcription factors are responsible for expression of a large number of stress inducible genes that confer abiotic stress tolerance, especially the transcription factor families such as the dehydration-responsive element binding proteins (DREBs)/C-repeat binding factors (CBFs), the NAM-ATAF1/2 and CUC transcription factors (NACs), and the MYB transcription factors. We analysed the transcripts of four transcriptional factor genes *DREB26, CBF2, NAC2 and MYB87*, and found that all of them were highly up-regulated in *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (Fig. 9). We believe that the superior performance of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under combined drought and salt stresses are also due to the higher expression of these transcriptional factor genes and their downstream genes.

In summary, we showed that co-overexpressing *AVP1*, *PP2A-C5*, and *AtCLCc* could significantly enhance tolerance to salt stress, drought stress, and combined salt and drought stresses. Additionally, we demonstrated the substantially enhanced tolerance to other salts such as KCl and KNO_3. Co-overexpression of beneficial genes in transgenic plants might be an ideal technique that could be applied to agriculture. Exploring the potential of overexpression of *AVP1* in various crops have been conducted to a great extent while the research on *PP2A-C5* and *AtCLCc* are still in their early stages. It would be interesting to investigate the molecular mechanism behind the greatly enhanced tolerance to salinity and water deficit of *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants, especially the potential synergistic interactions of these three enzymes biochemically, which may lead to fascinating discoveries. This study provides a proof-of-concept for future work with agronomically important crops. If a major crop could tolerate salinity level up to 300 mM of NaCl, one cannot imagine how many lands lost to salinity could be reclaimed and how much more food and fibre could be produced in the future.

4. Materials and methods

4.1. Construction of the transforming vector

The coding sequences of *PP2A-C5* (AT1G69960), *AtCLCc* (AT5G49890), and *AVP1* (AT1G15690) were amplified from cDNAs that were synthesized from Arabidopsis total RNAs using the Q5 high-fidelity DNA *Taq* polymerase according to manufacturer's instructions (New England Bio Labs, MA). The primer sets used for PCR amplifications were listed in Supp. Table 1. For creation of the transforming plasmid, the coding sequences (CDs) of *PP2A-C5* and *AtCLCc* were first assembled into a binary vector pBI121 using the approach of Gibson Assembly (New England Bio Labs, MA). The self-cleavage element 1 or SCE1 was inserted between *PP2A-C5* and *AtCLCc* coding sequences to minimize plasmid size as demonstrated by Kim et al. (2011). The SCE1 sequence was also listed in Supp. Table 1. Secondly, for co-expression of *AVP1* in the same vector, another set of 35 S promoter and terminator sequences were amplified from the plasmid of pFGC5941 and assembled into the plasmid previously made, i.e. PP2A-C5-AtCLCc-pBI121 plasmid with the *AVP1* coding sequence through Gibson Assembly (New England Bio Labs, MA). To avoid the interference between these two expression cassettes, an exogenous insulator, GYPSY, was introduced into the plasmid as described by Carballar-Lejarazú et al. (2013). All sequences

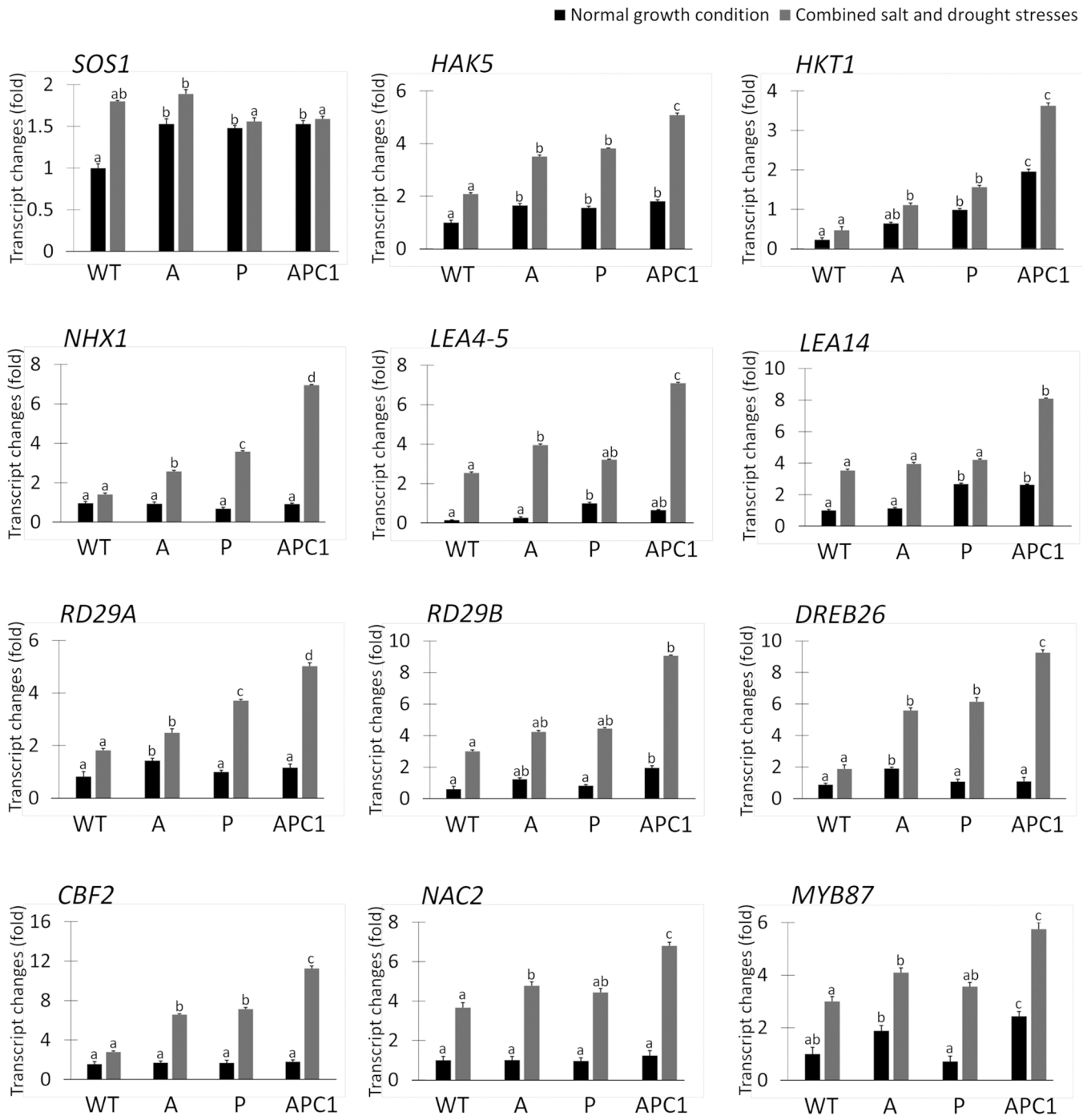

Fig. 9. Quantitative real-time PCR analysis of transcript levels of selected stress responsive genes and transcription factor genes in wild-type, *AVP1*-overexpressing, *PP2A-C5*-overexpressing, and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants under combined drought and salt stresses. Black bar, under normal growth condition; grey bar, under combined drought and salt stresses. WT, wild-type plants; A, *AVP1*-overexpressing plants; P, *PP2A-C5*-overexpressing plants; APC1, *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants (line 1). Different letters represent the statistical significance between samples according to Student's *t*-test. Data are means ± SE (n = 3).

were confirmed through Sanger sequencing.

4.2. Arabidopsis transformation

Transforming vector harboring *AVP1*, *PP2A-C5,* and *AtCLCc* was introduced into the Agrobacterium strain GV3101 using the heat shock method (Höfgen and Willmitzer, 1988). Transgenic plants co-overexpressing *AVP1, PP2A-C5,* and *AtCLCc* were generated by transforming the Agrobacterium cells containing the gene construct using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected using 30 mg/l kanamycin.

4.3. Plant materials and growth conditions

Wild-type Arabidopsis (ecotype Columbia-0 or Col-0), homozygous plants overexpressing single gene *AVP1*, *PP2A-C5,* or *AtCLCc* (generated in our laboratory previously) and three independent *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants, APC1, APC2, APC3 were used in this

study. Arabidopsis seeds were surface sterilized with 70% ethanol for one min and 20% of bleach for 20 min and then washed four times with sterile distilled water. Sterilized seeds were kept at 4 °C for stratification for 3 days, sowed on petri dish plates containing Murashige and Skoog salts (MS) (Murashige and Skoog, 1962), 1% sucrose, 0.8% agar with pH at 5.7. Seedlings were grown under continuous light at 22 °C until transferred into soil. Different substances such as NaCl, KCl, mannitol, or KNO_3 were added to MS plates at specified concentrations on plates. Soil (SunGro Horticulture, Vancouver, Canada) grown plants were placed in a growth chamber (Enconair AC-60, Ecological Chamber Inc., Canada) with 16 h light and 8 h dark photoperiod with the light intensity of 150 μE m^{-2} s^{-1} at 22 °C and 50% relative humidity.

4.4. Total RNA isolation

The selected plants from each genotype were ground into fine powder in liquid nitrogen. Approximately 100 mg of chilled powder was re-suspended in 1 ml Trizol reagent from Invitrogen (Carlsbad, CA). Re-suspended plant materials were homogenized by vortexing and incubated at room temperature for two min. Phase separation was achieved by adding 0.2 ml of chloroform per 1 ml of Trizol reagent. Samples were centrifuged at 12,000 rpm for 15 min at 4 °C and supernatant was then transferred into a new tube. RNA from aqueous phase was precipitated by mixing with 0.5 ml ice-cold isopropanol at room temperature for 10 min and followed by centrifugation at 12,000 rpm for 10 min at 4 °C. Resulted RNA pellet was washed using 1 ml of 75% ethanol and centrifuged at 7500 rpm for 5 min at 4 °C. This step was repeated twice, and leftover ethanol was removed. RNA was dissolved into 30 μl diethylpyrocarbonate (DEPC) treated water. Concentration of RNA was measured at A_{260}/A_{280} ratio using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, US).

4.5. RNA blot analysis

Extracted total RNA (about 15 μg per lane) was used for agarose gel electrophoresis. The 1.2% denaturing gel supplemented with 37% formaldehyde and 10X MOPS buffer was used in the electrophoresis. RNA sample was mixed with freshly prepared 2X loading buffer and DEPC water and a total of 30 μl of the mixture was added into each well after a rapid heating and cooling. Gel electrophoresis was performed at 60 V for 2 h in the running buffer. Next, RNAs in agarose gel were transferred onto a positively charged BioTrans (+) ™ nylon membrane (MP Biomedicals, US). Capillary blotting was assisted using 20X saline sodium citrate (SSC) solution and RNAs were cross-linked to the nylon membrane with UV light for 2 min. Then the membrane was subjected to overnight hybridization using ^{32}P-labelled probes of *AVP1 and PP2A-C5* cDNA fragments. The sequence information of primers used in probe preparation is provided in Supp. Table 2. Radiolabelling of probes was made using DECAprime™ II DNA labelling kit (Thermo Fisher Scientific, US) according to manufacturer's instruction. Membrane was exposed overnight to a phosphorimager screen (Amersham Biosciences, US) and image was obtained using Personal Molecular Imager (Bio-Rad, US).

4.6. Reverse transcription and quantitative real-time PCR analysis

For analysing transgene transcripts, complementary DNAs (cDNAs) were synthesized using TaKaRa PrimeScript™ reagent Kit (TaKaRa, Japan) according to manufacturer's instruction. One μg of total RNAs was used for reverse transcription in a 20 μl of reaction mixture to prepare the first strand cDNAs, then RNase free water was added to dilute the cDNAs solution to 100 μl and 5 μl of that was taken for RT-PCR. The gene *Actin 2* in the RT-PCR experiments was the internal control for transcript analysis. The reaction for RT-PCR was created by using QuantiFast® SYBR® Green PCR kit according to manufacturer's instruction (Qiagen, Germany). Details of the primers used in the RT-PCR reaction were listed in Supp. Table 3. The RT-PCR reaction was carried out on Bio-Rad CFX96 Touch Deep Well Real-Time PCR system (Bio-Rad, US) with initial activation at 95 °C for 5 min, followed by 39 cycles of two step cycling reaction: 95 °C for 10 s, 60 °C combined annealing and extension for 30 s. The resulting data were analysed using the Delta-Delta Ct ($2^{-\Delta\Delta Ct}$) method. For analysing transcripts of abiotic stress inducible genes, three-week-old plants from each genotype were subjected to combined salt and drought treatment (200 mM NaCl and irrigation was withheld) for one week, then leaf materials were collected for total RNA isolation. Three biological and three technical replicates were conducted, and relative transcript level in comparing with wild-type plants was compared using the Delta-Delta Ct ($2^{-\Delta\Delta Ct}$) method. The Arabidopsis gene *Actin 2* was used as the internal reference gene and the primers used for stress inducible genes are listed in Supp. Table. 4.

4.7. Protein extraction and SDS-polyacrylamide gel electrophoresis

Arabidopsis seeds of WT, *PP2A-C5* overexpressing and *AVP1/PP2A-C5/AtCLCc* co-overexpressing plants were sterilized, stratified, and sown on MS plates. Two-week-old seedlings from each genotype were used for protein extraction. Seedlings were ground into fine powder in liquid nitrogen, then protein extraction buffer [10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium lauryl sarcosine, 40 mM sodium phosphate buffer, pH 7.0, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride] and protein inhibitor cocktail were added, and the mixture was incubated on ice for 30 min. Centrifugation at 15,000 rpm for 15 min at 4 °C was conducted to separate supernatant that contains soluble protein. The protein assay from Bio-Rad (Hercules, California) was used to measure protein concentration. Fifteen μl of 2X SDS loading buffer [50 mM tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 100 mM dithiothreitol, and 0.01% bromophenol blue] was added to 15 μl of protein sample (20 μg) of each genotype, boiled for 5 min and then loaded into a 12% SDS-polyacrylamide gel for electrophoresis.

4.8. Western blot analysis

Once SDS-polyacrylamide gel electrophoresis was completed, proteins were transferred overnight onto a PVDF membrane (Bio-Rad, Hercules, California) with the incubation of Towbin transfer buffer [25 mM Tris, 192 mM glycine and 20% methanol (v/v), pH 8.3]. Membrane was initially washed with TBS buffer and followed by incubating in blocking solution [TBST with 5% BSA (W/V)] for one h at room temperature with constant shaking. Membrane was rinsed three times with TBST [TBS buffer with 0.1% Tween (v/v)] before incubating with primary antibody solution (anti-serine/threonine-protein phosphatase PP2A-1/2 catalytic subunit antibody from PhytoAB Inc. of California) for two h at room temperature. TBST rinsed membrane was incubated with alkaline phosphatase (AP) conjugated-anti rabbit antibody from Bio-Rad for one h. Detection through a colorimetry was done with AP conjugate substrate kit of Bio-Rad and visualization was done with a chemiluminescent imaging system, ChemiDoc MP, from Bio-Rad. Actin antibody from PhytoAB Inc. was used as the loading control to normalize protein loading of each genotype in the Western blot analysis.

4.9. Stress experiments

Water deficit treatment on plants grown on MS plates supplemented with PEG-8000: MS plates with 40% PEG-8000 were prepared for seed germination assay as described by Sun et al. (2018). Seeds were directly sown onto the MS-PEG plates and allowed to germinate under room temperature for 10 days. Seed germination was examined daily for 10 days, and root length of seedlings was measured.

Salt stress treatment on plants grown on MS plates supplemented with different concentration of NaCl: Arabidopsis plants were grown vertically on MS plates containing different concentrations of NaCl ranging from 100 mM to 150 mM. After 10 days, root length of plants

was measured.

Salt stress treatment on plants grown in soil irrigated with different concentration of NaCl: Plants were grown in a growth chamber for two weeks under normal growth condition (i.e., with SunGro horticulture soil with 16 h/8 h light/dark photoperiod and 50% relative humidity at 22 °C and watered every 3 days). Then, plants were irrigated with saline solution of increasing concentration of NaCl from 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, and 300 mM, once every 3 days. Plants were irrigated with regular water after the final irrigation of 300 mM NaCl until harvest. Pictures were taken during the salt stress treatment. Plant height, number of siliques, and seed weight per plant were measured at the end of the experiment. Experiment was repeated 4 times.

Drought stress treatment on plants grown in soil: Arabidopsis plants were grown for two weeks under normal growth conditions. Then plants were withheld from irrigation for 7 days. Re-irrigation was started after plants displayed wilted phenotype. Irrigation was withheld for another 7 days, then regular irrigation resumed until harvest. Phenotypic differences were documented, plant height, number of siliques, and seed weight per plant were measured at the end of the experiment. Experiment was repeated 4 times.

Combined salt and drought stresses on plants grown in soil: Plants were grown for two weeks under normal growth condition and withheld from irrigation for 5–7 days (or till wilted phenotype appeared), then irrigated with half the volume of saline solution but with the incremental increase of salinity from 50 mM, 100 mM, 150 mM, and 200 mM of NaCl. Plants were then irrigated with normal water until harvest. Phenotypical differences were documented, and plant height, number of siliques and seed yield were measured at the end of the experiment. Experiment was repeated 4 times.

4.10. Ion content analysis

Plants were grown in a growth chamber that was set with 16 h of light (150 μmolm^{-2} s^{-1}) and 8 h of darkness at 22 ℃. Plants were first grown under normal condition for one week, then saline solution was used to irrigate plants incrementally from 50 mM, 100 mM, 150 mM, and 200 mM of NaCl every 3 days. The above-ground portion of plants were harvested 5 days after the last irrigation with saline solution, dried in a 70 ℃ oven for 24 h, and then ground into powder for ion analysis. The contents of chloride, sodium, and potassium were measured by Ion Chromatography Instrument 883 Basic IC plus (Metrohm) as described by Pehlivan et al. (2016).

4.11. Rhizosphere acidification

Quantification of protons released by the root system of plants to MS media was performed according to the protocol by Pizzio et al. (2015).

Statistical analysis

Student's *t*-test was performed to analyse data from transgenic plants and wild-type plants, then Tukey's method was used in the pairwise comparison among different plant lines at significant level of $\alpha = 0.05$.

CRediT authorship contribution statement

TB, IW, RH, and GS conducted experiments; TB and IW wrote the manuscript; JZ and HZ edited and revised the manuscript; GS and HZ supervised experiments and funded the research project.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2022.104934.

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