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Improving drought-, salinity-, and heat-tolerance in transgenic plants by co-overexpressing Arabidopsis vacuolar pyrophosphatase gene *AVP1* and Larrea Rubisco activase gene *RCA*

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ABSTRACT

The severity and frequency of many abiotic stresses such as drought, salinity and heat, cause substantial crop losses worldwide, which poses a serious challenge in food security. To increase crop production, new approaches are needed. Previous research has shown that overexpression of the tonoplast H^+ pyrophosphatase gene *AVP1* leads to improved drought and salt tolerance in transgenic plants. Other research showed that overexpression of thermotolerant ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase gene could maintain photosynthesis at higher temperatures, which contributes to higher heat tolerance in transgenic plants. In nature, abiotic stresses rarely come alone, instead these stresses often occur in various combinations. Therefore, it is desirable to make crops more tolerant to multiple stresses, which will likely lead to higher crop yield under various stress conditions. It is shown here that co-overexpression of the Arabidopsis gene *AVP1* and the Larrea Rubisco activase gene *RCA* significantly increases drought, salinity and heat tolerance, resulting in higher biomass and seed yield than wild-type plants. *AVP1/RCA* co-overexpressing plants are as more drought- and salt-tolerant as *AVP1*-overexpressing plants, and as more heat-tolerant as *RCA*-overexpressing plants. More importantly, they produce higher seed yields than *AVP1*-overexpressing, *RCA*-overexpressing, and wild-type plants under combined drought and heat conditions.

1. Introduction

Despite the increase in the global agricultural productivity during the past century, the world still faces several challenges such as meeting the demand for food for the growing population [1] and improving agricultural productivity sustainably by the use of limited freshwater, nutrient resources, and available or shrinking arable land. Current world population of 7.7 billion is expected to exceed 9 billion by the middle of this century if the existing trend of the growth rate continues [2,3]. Correspondingly, food production should increase by 70–100 % in order to meet the needs of the rising population and its dynamic consumption patterns [2,4]. Thus, novel methods and approaches should be adopted to increase agricultural productivity by utilizing the same or less land area and fewer water resources [5]. Furthermore, the abiotic stresses imposed on plants including heat, drought and salinity are more potent than in the past, and countless research has shown that these stresses, drought and salinity in particular [6], affect plant photosynthetic process, vegetative and reproductive development, which ultimately translates into poor yield [2,7–9]. Therefore, to maintain and increase the productivity, plants would be required to adjust and have higher tolerance to a variety of abiotic stresses [10].

Considering these facts, numerous studies have reported successful cases where plant productivity was improved and tolerance to single abiotic stresses was enhanced by introducing a diverse collection of transgenes into a variety of plant species, such as Arabidopsis [11], tobacco [12], peanut [13], and barley [14]. The abiotic stresses that genetic engineering has focused on over the last decades include heat [15,16], drought [17], and salt [18]. Among the many transgenes that were used to improve drought and salinity tolerance, *AVP1* is one of the best characterized [19,20], which encodes a type I vacuolar H^+-

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pyrophosphatase located on the tonoplast membrane in Arabidopsis [21]. Similar to vacuolar ATPase (V-ATPase), AVP1 acidifies the vacuole lumen and assists in maintaining a proton electrochemical gradient across the vacuolar membrane, which subsequently contributes towards ion and solute accumulation in the vacuoles [22]. The H^+ pumped into the vacuole lumen by AVP1 is used as energy by the Na^+/H^+ antiporter to sequester Na^+ into the vacuole, thereby reducing the sodium concentration in the cytosol from reaching toxic levels [23]. Additionally, several researches provide evidences for AVP1 playing a role in auxin polar transport and auxin mediated root development [21], ensuring longer roots and larger root system, as well as higher shoot biomass and flower development [14,24]. Over the years, *AVP1* has been overexpressed in many crops such as peanut [25], cotton [26], barley [14], rice [27], and sugar cane [28], where tolerance to drought and salinity was significantly improved when compared to wild-type plants.

Heat stress is another factor that limits plant productivity, affecting numerous metabolic and developmental activities in plants including respiration, evaporation, vegetative and reproductive development and photosynthesis [7]. Of these, the latter was found to be highly sensitive to moderate to high heat stress in many crop plants [9]. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), located in the stroma of chloroplasts, is the principle enzyme that fixes CO_2 [29]. To carry out photosynthesis, CO_2 is bound to the active site of Rubisco (carbamylation) followed by the binding of the divalent cation Mg^{2+} [30]. Next, the substrate of Rubisco, ribulose-1,5-bisphosphate or RuBP, binds with Rubisco. Apart from RuBP, several sugar-phosphates could also bind to Rubisco [31]. Interestingly, these sugar phosphates including RuBP bind to the uncarbamylated Rubisco to form a closed conformation or an inactive form of Rubisco, causing a decline in photosynthesis [32]. This closed complex is converted to an open complex or active form by the soluble enzyme Rubisco activase (RCA), a member of AAA^+ family of proteins (ATPases associated with a variety of cellular activities), by clearing off the Rubisco active sites of the inhibitory sugar phosphates, utilizing ATP [33,34], thereby maintaining Rubisco in its active state and continuing RuBP regeneration. However, under moderate to high heat stress conditions, the closed complex formation increases, causing the rate of Rubisco deactivation to increase [35]. Although under optimal temperature conditions RCA can keep Rubisco in its active state, as the temperature increases RCA is unable to compete and offset the rate of misfire product formation and maintain Rubisco activation rate [36]. Moreover, RCA tends to form insoluble protein aggregates even at moderately high temperatures due to its low temperature optimum, which further contributes to the increase of Rubisco deactivation [16,37]. Thus, Rubisco activation by RCA becomes a critical factor in photosynthesis for many temperate crops under heat stress conditions.

Rubisco in many crops is rather heat tolerant, in fact, many Rubisco are thermostable even at temperatures above 40 °C [38], whereas Rubisco activase is not, therefore it was proposed that improving RCA's heat tolerance or using a more thermostable RCA could improve photosynthesis under heat stress condition [33,39], thereby leading to higher productivity. This hypothesis proved to be correct, as Kurek et al. [15] demonstrated that increasing the thermostability of Arabidopsis' RCA was possible by introducing a more thermostable RCA variant into the Arabidopsis *rca* mutant, leading to improved photosynthesis and growth rate of transgenic plants grown at moderate heat stress conditions. Later, Kumar et al. [40] confirmed this hypothesis by introducing a chimeric RCA, i.e. a hybrid RCA where a Rubisco recognition domain in the more thermostable tobacco activase was replaced with that from Arabidopsis, into the Arabidopsis *rca* mutant, and the transgenic plants could produce higher biomass and seed yield under moderately elevated temperatures. In both cases, the increased heat tolerance was limited to moderate temperatures around 30 °C and the increased seed yields were also limited. In this context, to further improve heat tolerance for photosynthesis, creating transgenic plants using a naturally more thermostable RCA would prove to be an effective strategy in enhancing heat tolerance in Arabidopsis. For this purpose, using the *RCA* gene of a desert shrub creosote, *Larrea tridantata*, would assist in maintaining higher photosynthetic rates under higher temperature conditions due to creosote RCA's higher thermostability, while remaining as a soluble enzyme instead of forming insoluble protein aggregates under moderate to high temperatures, thereby contributing to Rubisco activation under high temperature conditions.

In spite of the vast amount of research carried out in relation to improving abiotic stress tolerance in plants, relatively limited information on enhancing tolerance to multiple abiotic stresses is available [25,41–43]. In particular, improving plant tolerance to heat has been a less explored avenue when compared to drought and salinity. Further, in the context of natural environmental conditions, enhancing plant tolerance to multiple abiotic stresses appears to be the more practical approach than conferring tolerance towards a single stress like heat or drought, since in nature plants are more often exposed to a combination of stresses instead of one. In this study, we overexpressed the Arabidopsis *AVP1* and Larrea *RCA* in transgenic Arabidopsis and investigated how *AVP1/RCA* co-overexpressing plants would respond to drought, salinity and heat stresses that came alone or in various combinations. We found that *AVP1/RCA* co-overexpressing plants are more tolerant than wild-type plants under drought, salinity and heat stress conditions, indicating that *AVP1/RCA* co-overexpressing plants possess the beneficial traits of *AVP1*- overexpression as well as *RCA*-overexpression. Furthermore, *AVP1/RCA* co-overexpressing plants produced more seeds than *AVP1*-overexpressing and *RCA*-overexpressing plants under combined drought and heat conditions, indicating that there might be positive interaction between *AVP1*-overexpression and *RCA*-overexpression, which leads to higher tolerance and higher seed yield under combined drought and heat conditions. This research provides the proof-of-concept that co-overexpression of *AVP1* and *RCA* can be utilized as a practical approach to enhance the abiotic stress tolerance of agronomically important crops.

2. Results

2.1. Creation and molecular analysis of AVP1/RCA co-overexpressing plants

To make plants more tolerant to drought, heat and salt, one approach would be co-overexpressing two or more genes that can confer increased tolerance towards different stresses. The Arabidopsis *AVP1* can confer increased tolerance to drought and salt when overexpressed, but not to heat; the tobacco RCA [40] and the Arabidopsis RCA variant [15] can confer increased heat, but not drought and salt. *AVP1* has been widely used to improve drought and salt tolerance, especially when it was co-overexpressed with the rice SUMO E3 ligase gene *OsSIZ1*, *AVP1* proves to be exceedingly effective in conferring drought and salt tolerance in transgenic plants. The RCAs used by Kumar et al. [40] and Kurek et al. [15] are inadequate in conferring tolerance to high temperatures (e.g. > 30 °C), indicating that a different RCA gene would have to be used. A preliminary experiment by our collaborator Dr. Michael Salvucci suggested that the RCA from *Larrea tridentata* might be a good source for improving heat tolerance of photosynthesis. We therefore decided to overexpress the Arabidopsis *AVP1* and the Larrea *RCA* in our effort to improve plant tolerance to drought, heat and salinity. The pPZP212 vectors containing the *AVP1/RCA* expression cassettes were constructed in our laboratory [44], and then used to transform wild-type Arabidopsis plants via Agrobacterium mediated floral dip method [45]. The putative T_1 transgenic plants identified on MS media containing 30 μg/mL kanamycin were subsequently grown for two generations to obtain single T-DNA insertion homozygous plants. A total of 129 T_1 independent lines, among which 73 lines in tandem orientation (i.e. expression of the two transgenes is in the same direction) and 56 lines in divergent orientation (i.e. expression of the two transgenes is in opposite directions), were screened on MS media

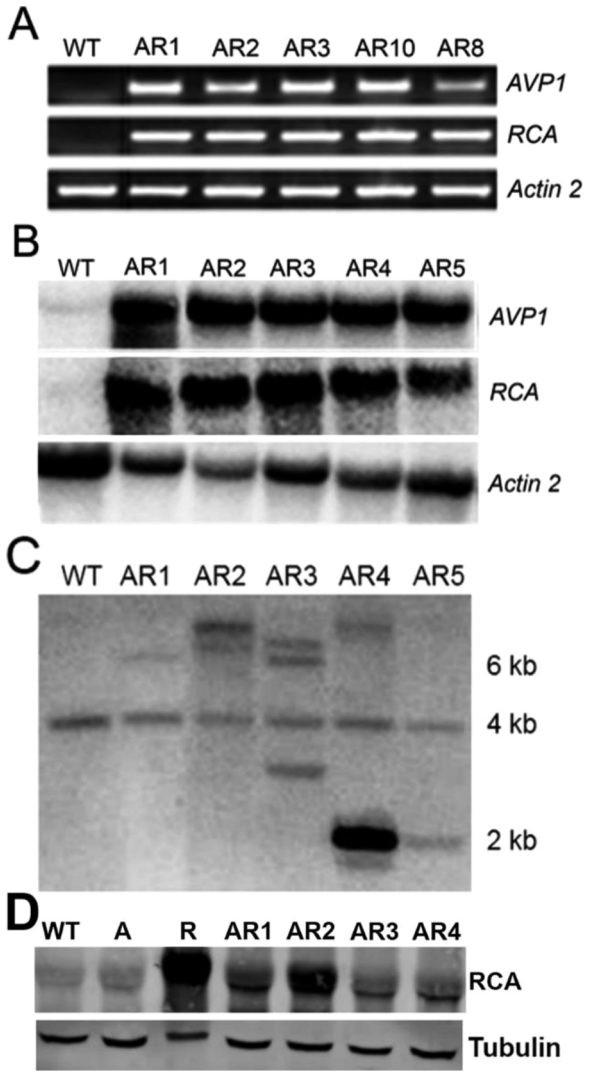

Fig. 1. Molecular characterization of *AVP1/RCA* co-overexpressing plants. **A**. Analysis of wild-type and five independent *AVP1/RCA* co-overexpressing plants by PCR. **B**. RNA blot analysis of wild-type and *AVP1/RCA* co-overexpressing plants. **C**. DNA blot analysis of wild-type and *AVP1/RCA* co-overexpressing plants. **D**. Western blot analysis of wild-type, *AVP1*-overexpressing, *RCA*-overexpressing, and *AVP1/RCA* co-overexpressing plants. The genes used for PCR and RNA blot analysis are listed on the right. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant, AR1to AR5, AR8 and AR10, seven independent *AVP1/RCA* co-overexpressing plants.

containing 30 µg/mL kanamycin. A total of 105 lines exhibited 3:1 for kanamycin resistance vs sensitive phenotype, and these lines were likely single T-DNA insertion lines.

Reverse transcription-polymerase chain reaction (RT-PCR) experiment was carried out for randomly selected T_1 lines to confirm the successful transformation and presence of transgene transcripts (Fig. 1A). Among the 105 likely single T-DNA insertion lines, 50 were homozygous at T_3 stage (24 of tandem and 26 of divergent) and were used for RNA blot analysis. We selected five high expression lines based on the RNA blot analysis (Fig. 1B) for further physiological experiments. DNA blot analysis was also carried out to confirm the stable integration of the transgene construct in the 5 independent transgenic Arabidopsis lines used for RNA blot analysis above. An *AVP1* cDNA fragment was used as the probe to identify the copy number of the transgene. Based on DNA blot analysis (Fig. 1C), it appears that the line AR4 displays an intense band possibly as a result of inverted repeats being present. AR1 and AR5 contain a single copy of the transgene construct while AR2 and AR3 contain two and three copies of T-DNA insertions, respectively. With this DNA blot result, the AR4 line was dropped and the AR5 was renamed as AR4, and the independent transgenic lines AR1, AR2, AR3 and AR4 were used for the subsequent experiments.

To test if the RCA protein levels in these transgenic plants were increased, immunoblot analysis was performed using the Arabidopsis RCA polyclonal antibodies. When compared to WT and *AVP*-overexpressing line (A), the Western blot analysis indicated that the *RCA*-overexpressing plants (R) and the four *AVP1/RCA* co-overexpressing plants (AR1 to AR4) showed higher levels of RCA protein by giving a more intense band at the expected value of 48 kDa. It appears that the single-gene overexpressing line R and the two-gene overexpressing line AR1 had the highest protein level in terms of band intensity (Fig. 1D). This result confirmed the higher protein levels of RCA in the transgenic lines overexpressing the *RCA* gene, indicating that the *RCA* gene was overexpressed in both single-gene and two-gene overexpressing plants.

2.2. AVP1/RCA co-overexpressing plants are more drought tolerant

To test if *AVP1/RCA* co-overexpressing plants were more drought tolerant than wild-type plants, we conducted drought stress experiments using three-week-old *AVP1/RCA* co-overexpressing plants (4 independent lines named AR1, AR2, AR3 and AR4), wild-type plants (WT), *AVP1*-overexpressing plants (A), and *RCA*-overexpressing plants (R). Under normal growth condition, no obvious differences in phenotype were found between *AVP1/RCA* co-overexpressing plants and other plants (Fig. 2A). However, *AVP1/RCA* co-overexpressing and *AVP1*-overexpressing plants showed better phenotypes than wild-type and *RCA*-overexpressing plants under drought stress (Fig. 2B), for example, with roughly 1.5-fold increase in plant height (Supp. Fig. 1) and over 50-fold increase in seed yield compared to wild-type and *RCA*-overexpressing plants (Fig. 2C). It appears that co-overexpression of *AVP1* and *RCA* does not lead to increased drought stress tolerance in comparison with *AVP1*-overexpressing plants (Fig. 2C). In fact, only one out of the four *AVP1/RCA* co-overexpressing plants displayed similar seed yield as *AVP1*-overexpressing plants under drought stress conditions.

2.3. AVP1/RCA co-overexpressing plants are more salt tolerant

To test if *AVP1/RCA* co-overexpressing plants were more salt tolerant than wild-type plants, we conducted salt stress experiments on MS media and in soil. Under control conditions (i.e. no additional salt added in MS medium), no significant differences in appearance were found among the four different genotypes on MS plate (Fig. 3A). However, *AVP1/RCA* co-overexpressing plants and *AVP1*-overexpressing plants displayed a significantly longer root length when compared to wild-type and *RCA*-overexpressing plants on MS plates supplemented with 100 (Fig. 3B) and 125 mM NaCl (Supp. Fig. 2), with an average of 100 % and 68 % increase in root length, respectively (Fig. 3C). However, there does not appear a difference in root length between *AVP1*-overexpressing plants and most *AVP1/RCA* co-overexpressing plants (Fig. 3C).

In the soil experiment, there were no obvious differences in phenotypes before exposure to salt treatment or under normal growth conditions among the four genotypes (data not shown), but it was clear that *AVP1/RCA* co-overexpressing and *AVP1*-overexpressing plants were more salt tolerant than wild-type and *RCA*-overexpressing plants, as they were taller by approximately 23 % (Fig. 3D and Supp. Fig. 3) and produced 58 % more seeds (Fig. 3E) than wild-type and *RCA*-overexpressing plants. Yet, no difference was apparent between *AVP1/RCA* co-overexpressing and *AVP1*-overexpressing plants in seed production under salt stress condition.

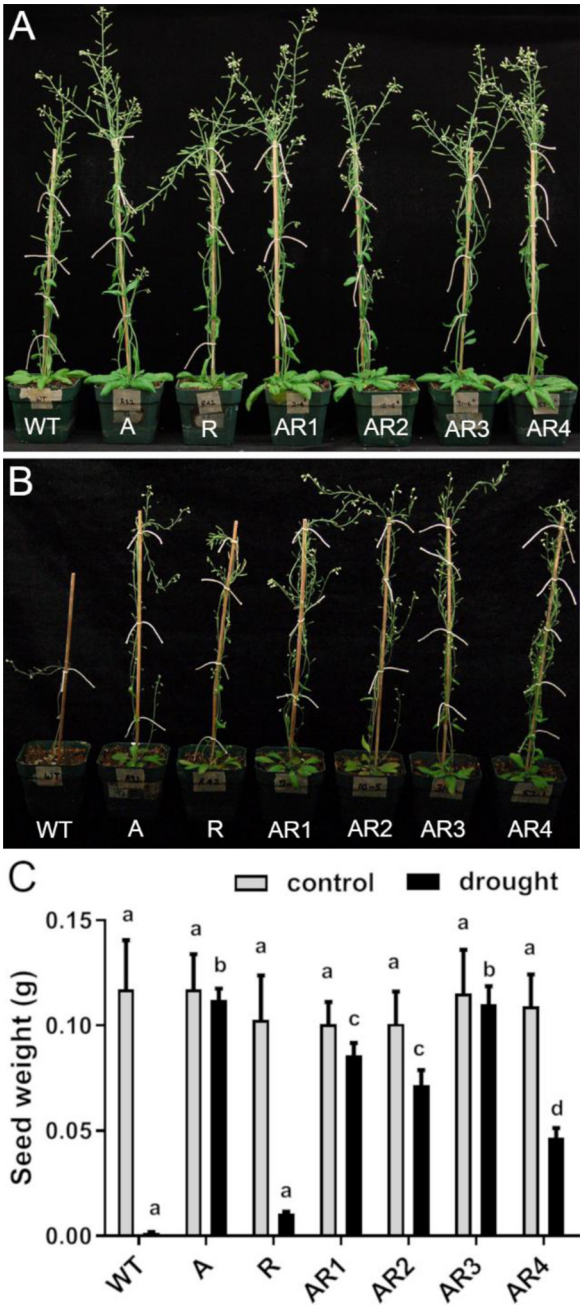

Fig. 2. Phenotype and seed yield analysis of *AVP1/RCA* co-overexpressing plants under normal growth condition and after drought stress treatment. **A**. Phenotype of wild-type and *AVP1/RCA* co-overexpressing plants under normal growth condition. **B**. Phenotype of wild-type and *AVP1/RCA* co-overexpressing plants under water deficit conditions. **C**. Seed yield analysis of wild-type and *AVP1/RCA* co-overexpressing plants after water deficit treatment. Grey bars, under normal growth condition; black bars, under water deficit conditions; n = 6 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

2.4. AVP1/RCA co-overexpressing plants are more heat tolerant

To test if *AVP1/RCA* co-overexpressing plants were more heat tolerant than wild-type plants, we conducted heat stress experiments with plants grown on MS plate and plants grown in soil under heat stress conditions. On MS media, *AVP1/RCA* co-overexpressing plants and *RCA*-overexpressing plants displayed roots that were roughly 2-fold longer than *AVP1*-overexpressing plants and wild-type plants (Fig. 4A and B). Interestingly, *AVP1*-overexpressing plants showed longer root length than wild-type plants, while the root lengths of *AVP1/RCA* co-overexpressing plants and *RCA*-overexpressing plants were about the same, indicating that there is no addition of the increased root length of overexpression of *AVP1* and the increased root length of overexpression of *RCA* in *AVP1/RCA* co-overexpressing plants (Fig. 4B). In the heat stress experiment with soil grown plants, we found that transgenic plants were slightly taller and bushier than wild-type plants (Fig. 4C), and they all outperformed wild-type plants by producing around 3-fold higher seed yields under heat stress condition (Fig. 4D). Although overexpression of *RCA* led to an increased seed yield, there was no statistical difference in seed yield between *AVP1/RCA* co-overexpressing plants and *RCA*-overexpressing plants. *AVP1*-overexpressing plants also produced a higher seed yield than wild-type plants, indicating that *AVP1*-overexpressing plants have higher tolerance to heat as well. Overexpression of *AVP1* also contributes to a higher heat tolerance, and this might be due to *AVP1*-overexpressing plants being healthier, which leads to better growth under heat stress condition.

2.5. AVP1/RCA co-overexpressing plants display enhanced tolerance to reduced water potential in growth media

To test how *AVP1/RCA* co-overexpressing plants would respond to reduced water potential in growth media, we analyzed the performance of *AVP1/RCA* co-overexpressing plants on MS plates supplemented with polyethylene glycol-8000 (PEG-8000). This high molecular weight molecule is an inert non-ionic polymer and relatively impermeable to cells, which affects the osmotic pressure, leading to reduced water potential in the media. The PEG-8000 was used to reduce the water potential in MS media to approximately −0.7 MPa where it was applied as an overlay on MS agar in Petri plates. When surface sterilized and stratified Arabidopsis seeds were either directly sown or three-day-old seedlings were transferred to MS plates containing PEG, it was observed that *AVP1/RCA* co-overexpressing plants and *AVP1*-overexpressing plants had the longest roots (80 % longer than WT), followed by *RCA*-overexpressing plants and then wild-type plants (Fig. 5A and B). Even under normal growth conditions, *AVP1/RCA* co-overexpressing plants and *AVP1*-overexpressing plants had statistically longer root length than *RCA*-overexpressing plants and wild-type plants. Yet in the presence of PEG, the root length difference become much larger (Fig. 5B). It is clear that the increased root length is mainly due to *AVP1*-overexpression, not *RCA*-overexpression.

However, when another stress was applied to these plants in addition to PEG, the results became complex. For instance, when 75 mM NaCl was applied together with PEG, *RCA*-overexpressing plants produced 62 % longer root length than wild-type plants, *AVP1*-overexpressing plants produced 18 % longer roots than *RCA*-overexpressing plants, and most *AVP1/RCA* co-overexpressing plants produced an average of 10 % longer root length than *AVP1*-overexpressing plants (Fig. 5C and D), suggesting that there might be an addition of the increased root length by *AVP1*- and *RCA*-overexpression in *AVP1/RCA* co-overexpressing plants. Interestingly, when heat stress was applied with PEG, all transgenic plants produced approximately 43 % longer roots than wild-type plants including *RCA*-overexpressing plants (Fig. 5E and F and Supp. Fig. 4). However, there is was no difference between the root lengths of *AVP1/RCA* co-overexpressing and *AVP1*-overexpressing plants, indicating that there was no addition of increased root length by *AVP1*-overexpression and *RCA*-overexpression in *AVP1/RCA* co-overexpressing plants.

2.6. AVP1/RCA co-overexpressing plants are more tolerant under multiple stress conditions

In nature, plants are often exposed to a combination of stresses instead of a single stress condition. Therefore, we tested the performance of *AVP1/RCA* co-overexpressing plants under a variety of drought, salt

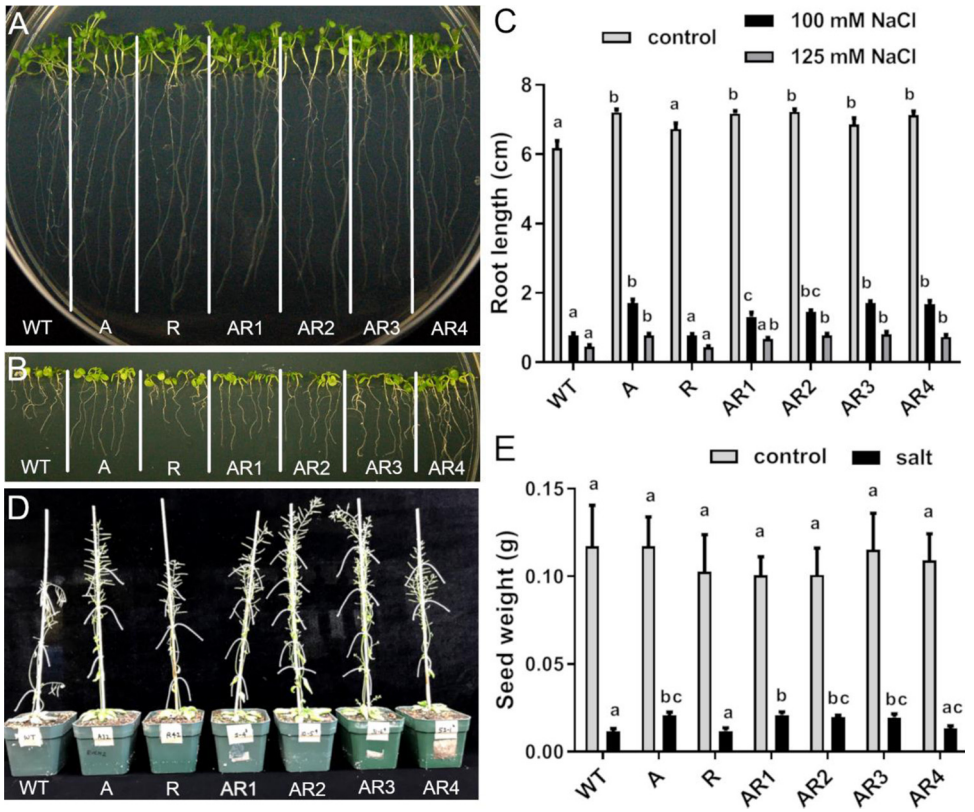

Fig. 3. Analysis of *AVP1/RCA* co-overexpressing plants under normal growth condition and salt stress condition. **A**. Phenotype of wild-type and transgenic plants under normal growth condition on MS plate. **B**. Phenotype of wild-type and transgenic plants on MS plate supplemented with 100 mM NaCl. **C**. Root length analysis of wild-type and transgenic plants after salt stress treatments on MS plates. Grey bars, MS plate; black bars, MS plate supplemented with 100 mM NaCl; dark grey bars, MS plate supplemented with 125 mM NaCl. n = 10 plants. **D**. Phenotypes of wild-type and transgenic plants in soil after salt stress treatment. **E**. Seed yield analysis of wild-type and transgenic plants under normal and salt stress conditions. Grey bars, normal growth condition; black bars, after salt stress treatment in soil. n = 6 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

and heat stress combinations. Under normal growth conditions, *RCA*-overexpressing plants displayed shorter plant height, while the other three genotypes showed no significant difference in plant height (Supp. Fig. 5). Under combined drought and salt stresses, *AVP1/RCA* co-overexpressing and *AVP1*-overexpressing plants displayed better phenotypes, e.g. 28 % taller and 150 % higher in seed yield, than *RCA*-overexpressing and wild-type plants (Fig. 6A and B). However, it must be noted that there is no statistical difference in seed yield between *AVP1/RCA* co-overexpressing plants and *AVP1*-overexpressing, indicating that the increased seed yield is due to the contribution of *AVP1* overexpression, not *RCA* overexpression.

Under combined heat and salt stresses, *AVP1/RCA* co-overexpressing plants performed differently at different salt concentrations on MS plates. At 32 °C with 100 mM NaCl treatment, all transgenic plants produced around 45 % longer root length than wild-type plants and no statistical difference was found among transgenic plants (Fig. 7A and B), whereas at 37 °C with 100 mM NaCl treatment, none of the transgenic plants could survive (data not shown). In the soil experiment, under normal growth conditions there was no significant phenotypic difference between transgenic plants and wild-type plants (data not shown), whereas all transgenic plants displayed better phenotype, i.e. 12 % taller than wild-type plants after the treatment of combined heat and salt stresses (Fig. 7C). However, *AVP1/RCA* co-overexpressing plants and *AVP1*-overexpressing plants produced significantly more seeds (123 % higher) than wild-type plants and *RCA*-overexpressing plants under combined heat and salt stresses (Fig. 7D). Although *RCA*-overexpressing plants did produce slightly more seeds than wild-type plants, the difference was not significant.

Under combined drought and heat stresses, three out of four *AVP1/RCA* co-overexpressing plants outperformed all other plants (Fig. 8A and B). *AVP1/RCA* co-overexpressing plants displayed the best phenotype and wild-type plants were largely dead at the end of treatment. *RCA*-overexpressing plants were clearly much better than wild-type plants, but not as good as *AVP1*-overexpressing plants. *AVP1/RCA* co-overexpressing plants produced the highest seed yields and on average they produced 588 % more seeds than wild-type plants; *AVP1*-overexpressing plants produced the second highest seed yield, which was 352 % more seeds than wild-type plants; *RCA*-overexpressing plants ranked No. 3 in seed yield, which was 305 % more seeds than wild-type plants (Fig. 8B). It appears that there might be an addition in the increased seed yield due to *AVP1*-overexpression and *RCA*-overexpression in *AVP1/RCA* co-overexpressing plants (Fig. 8A and B).

Finally, we analyzed how *AVP1/RCA* co-overexpressing plants would respond to three stresses, drought, heat and salt that come simultaneously. When exposed to three stresses, plant phenotype, height, and seed yield were all affected, with the biggest seed reduction found in wild-type plants, followed by *RCA*-overexpressing plants (Fig. 9). Similar to combined drought and heat stresses, *AVP1/RCA* co-overexpressing plants, on average, produced 317 % more seeds than wild-type plants, *AVP1*-overexpressing plants produced 253 % more seeds and *RCA*-overexpressing plants produced 77 % more seeds than wild-type plants (Fig. 9). Again, there appears to be some positive interaction between *AVP1*-overexpression and *RCA*-overexpression, which leads to the highest seed production in *AVP1/RCA* co-overexpressing plants.

2.7. AVP1/RCA co-overexpressing plants release more proton into media

In accordance with previous research work pertaining to *AVP1* overexpression [46,47], in this study, it was observed that transgenic plants overexpressing *AVP1* were healthier looking and had longer roots, suggesting that *AVP1/RCA* co-overexpressing plants might have a higher capacity in absorbing nutrients from the media. To test this hypothesis, rhizosphere acidification assay was carried out as described in the literature [48]. The results showed that *AVP1/RCA* co-overexpressing plants released more protons into media than the other three genotypes (Fig. 10). There is no difference in the amount of proton released from *RCA*-overexpressing plants and wild-type plants, as this trait is conferred by *AVP1*-overexpression. However, it appears that *RCA*-overexpression helps *AVP1/RCA* co-overexpressing plants release more proton into media, which might attribute to *AVP1/RCA* co-

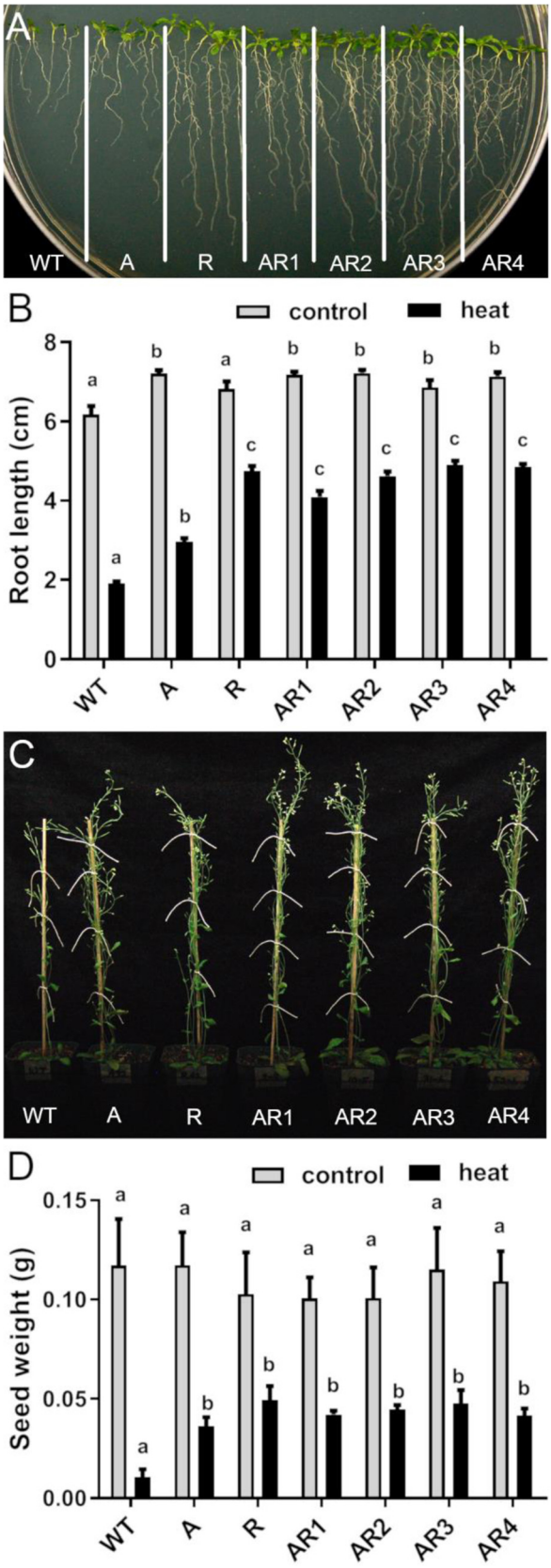

Fig. 4. Analysis of *AVP1/RCA* co-overexpressing plants under normal growth condition and under heat stress condition. **A**. Phenotype of wild-type and *AVP1/RCA* co-overexpressing plants on MS plate after treatment at 37 °C for 10 days. **B**. Root length analysis of wild-type and *AVP1/RCA* co-overexpressing plants on MS plate after heat treatment. Grey bars, at 22 °C; black bars, at 37 °C. n = 10 plants **C**. Phenotype of wild-type and *AVP1/RCA* co-overexpressing plants in soil after treatment at 37 °C for 5.5 h per day for 60 days. **D**. Seed yield analysis of wild-type and *AVP1/RCA* co-overexpressing plants in soil after heat treatment. Grey bars, normal growth condition; black bars, after treatment at 37 °C for 5.5 h per day for 60 days. n = 6 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

overexpressing plants' capacity to acidify rhizosphere so that more nutrients could be solubilized by the extra protons for absorption into plant cells, yet the acidification is lowest in *RCA*-overexpressing plants.

3. Discussion

In this study, we investigated the possibility of simultaneously increasing tolerance towards drought, salt and heat as single stress as well as their various combinations by co-overexpressing *AVP1* and *RCA* in *Arabidopsis thaliana*. Among all abiotic stress factors, drought, salinity and heat are the most detrimental towards plant development and productivity [2]. More often, when plants are exposed to more than one stress, a greater penalty is paid in terms of plant biomass and/or yield, due to various developmental and physiological activities being affected by these harsh environmental conditions [49]. Therefore, it is imperative to create plants that can withstand multiple stresses. Our results showed that *AVP1/RCA* co-overexpressing plants are indeed more tolerant to drought, heat, and salt under single stress and multi-stress conditions. More importantly, *AVP1/RCA* co-overexpressing plants also outperformed *AVP1*-overexpressing and *RCA*-overexpressing plants under combined drought and heat stress conditions. This is of great importance because, at present only a handful of research has provided successful results in enhancing plant tolerance to multiple stresses and improving the multiple abiotic stress tolerance of agronomically important crops is critical in increasing productivity.

AVP1 is a tonoplast located type I pyrophosphatase, which largely contributes towards maintaining a proton electrochemical gradient across tonoplast membrane by pumping H^+ into the vacuole [50,51]. This activity in turn aids in higher Na^+ as well as other ion and sugar sequestration into vacuole in exchange for H^+, which reduces the vacuolar water potential, leading to improved drought and salt tolerance [52]. Our work shows that co-overexpression of *AVP1* and *RCA* confers enhanced drought and salt tolerance in transgenic Arabidopsis compared to the non-transgenic plants. This is in accordance with previous reports that have shown enhanced tolerance to drought and salt stresses in transgenic cotton, tomato and rice plants overexpressing *AVP1* [17,27,53]. Specifically, we found that *AVP1/RCA* co-overexpressing plants produced significantly more seeds than wild-type plants under drought stress condition (Fig. 2C). *RCA*-overexpressing plants also produced slightly more seeds than wild-type plants under drought condition, which might be attributed to the relatively higher photosynthetic capacity due to *RCA*-overexpression. In the availability of optimum irradiance, as plants adapt to environments with limited water availability, *RCA*-overexpression might provide the transgenic plant the ability to better adapt to water deficit conditions and maintain higher photosynthetic rates, thereby resulting in better drought tolerance.

Under salt stress conditions, *AVP1/RCA* co-overexpressing plants produced longer roots on MS plate (Fig. 3C), and higher seed yield in soil conditions (Fig. 3E), which is consistent with previous research involving overexpression of *AVP1*. Under heat stress conditions, *AVP1/RCA* co-overexpressing plants showed increased heat tolerance by producing 300 % higher seed yield than wild-type plant (Fig. 4D), which is likely due to the contribution of *RCA*-overexpression, as *RCA*-overexpressing plants produced the highest amount of seeds under heat stress condition. We also analyzed the RCA protein levels in the single *RCA* as well as *AVP1/RCA* co-overexpression plants through Western

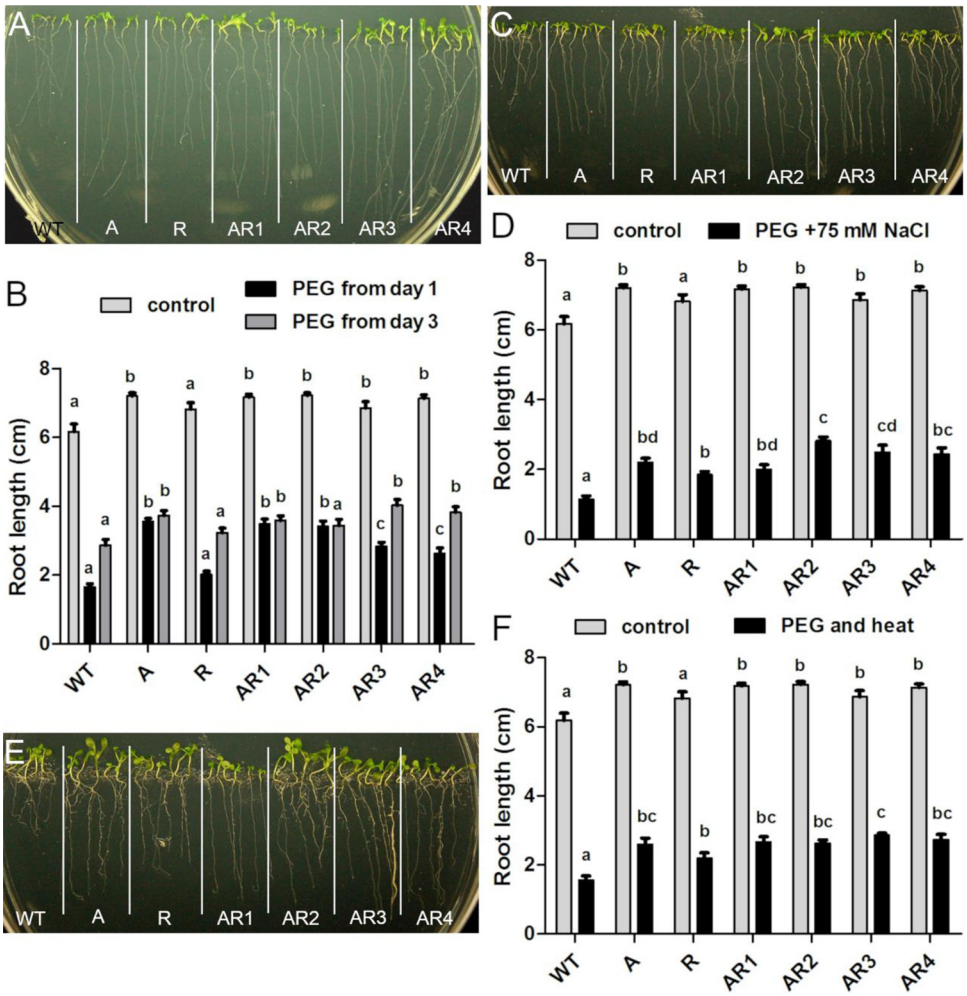

Fig. 5. Analysis of *AVP1/RCA* co-overexpressing plants on MS plates supplemented with PEG. **A**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants on MS plates containing 40 % PEG. **B**. Root length analysis of wild-type and AVP1/RCA co-overexpressing plants on MS plates with PEG. Grey bars, MS plate; black bars, MS plate supplemented with PEG from day 1; dark grey bars, MS plate supplemented with PEG from day 3. n = 10 plants. **C**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants on MS plates containing 40 % PEG and 75 mM NaCl. **D**. Root length analysis of wild-type and AVP1/RCA co-overexpressing plants on MS plates supplemented with 40 % PEG and 75 mM NaCl. Grey bars, MS plate; black bars, MS plate containing PEG and 75 mM NaCl; n = 10 plants. **E**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants on MS plates containing 40 % PEG after heat treatment. **F**. Root length analysis of wild-type and AVP1/RCA co-overexpressing plants on MS plates supplemented with 40 % PEG after heat treatment. Grey bars, MS plate; black bars, MS plate containing PEG and heat treatment; n = 10 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

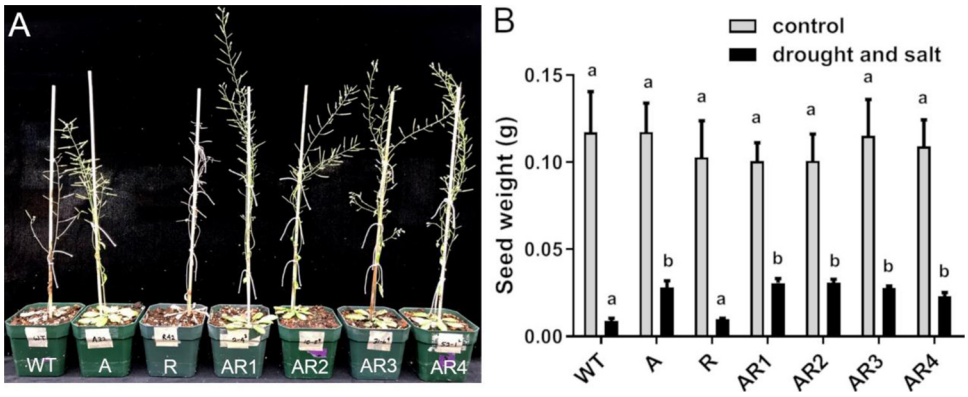

Fig. 6. Performance of *AVP1/RCA* co-overexpressing plants under combined drought and salt stresses. **A**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants under combined drought and salt stresses. **B**. Seed yield analysis of wild-type and *AVP1/RCA* co-overexpressing plants after treatment with combined drought and salt stresses. Grey bars, normal growth condition; black bars, after treatment with combined drought and salt stresses. n = 6 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

blot analysis. Our results showed that there were significantly higher levels of RCA proteins in the *RCA*-overexpressing and *AVP1/RCA* co-overexpressing plants when compared to the WT plants (Fig. 1D), indicating that this may be the reason for better heat tolerance in photosynthesis of these plants, which leads to higher yields in single and multiple stress conditions involving heat stress. Our data show that the creosote RCA might interact with the Arabidopsis Rubisco, thereby leading to higher thermotolerance when compared to their wild-type counterparts. However, it is interesting to note that *AVP1*-overexpressing plants also displayed significantly improved heat tolerance by showing significantly increased seed yield under heat stress conditions. *AVP1*-overexpression appears to be beneficial to plant, as *AVP1* plays an important role in polar auxin transport, which leads to increased biomass and a robust root development [24,54]. Additionally, with the finding of AVP1 on the plasma membrane of companion cells, it was suggested that in contrast to its function on the tonoplast, AVP1 on the plasma membrane might synthesize inorganic pyrophosphate (PPi) using the proton electrochemical gradient [46,55]. This PPi is in turn utilized by the P-type ATPases located on the plasma membrane, causing a higher phloem loading of sucrose. Consequently, plants overexpressing *AVP1* in companion cells are bigger and have a more robust root system. Conversely, stunted seedlings were produced when phloem-specific AVP1was knocked down, showing its importance in regulating pyrophosphate homeostasis [47]. This helps explain why *AVP1*-overexpressing and *AVP1/RCA* co-overexpressing plants in this study showed better phenotype under both non-stressed as well as heat

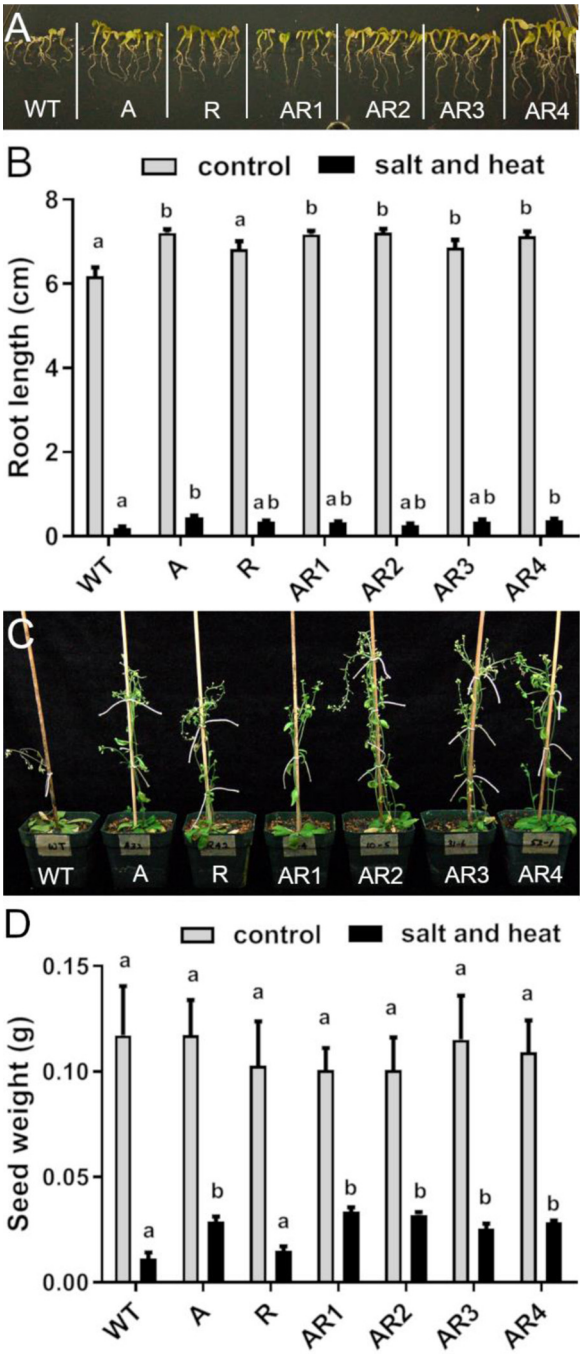

Fig. 7. Performance of *AVP1/RCA* co-overexpressing plants under combined heat and salt stresses. **A**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants on MS plate under combined moderate heat and salt stresses. **B**. Root length analysis of wild-type and *AVP1/RCA* co-overexpressing plants after treatment with combined moderate heat and salt stresses. Grey bars, normal growth condition; black bars, after treatment with combined heat and salt stresses. n = 10 plants. **C**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants in soil under combined heat and salt stresses. **D**. Seed yield analysis of wild-type and *AVP1/RCA* co-overexpressing plants after treatment with combined heat and salt stresses. Grey bars, normal growth condition; black bars, after treatment with combined heat and salt stresses. n = 6 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

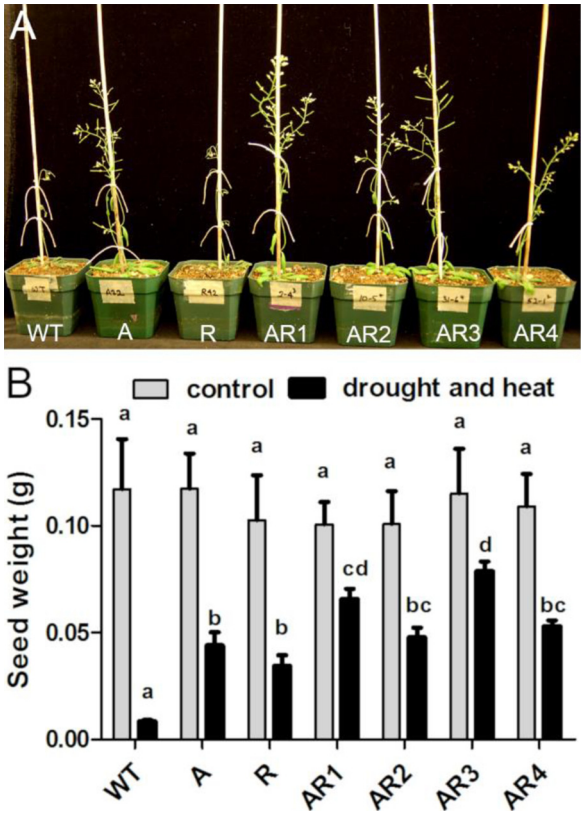

Fig. 8. Performance of *AVP1/RCA* co-overexpressing plants under combined heat and drought stresses. **A**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants in soil under combined heat and drought stresses. **B**. Seed yield analysis of wild-type and *AVP1/RCA* co-overexpressing plants after treatment with combined heat and drought stresses. Grey bars, normal growth condition; black bars, after treatment with combined heat and drought stresses. n = 6 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

stress conditions. Previous research on heat stress tolerance in Arabidopsis have shown that plants were able to withstand a variety of temperatures from 30 to 35 °C for a few hours to a few days [55,56]. In our study, *AVP1/RCA* co-overexpressing plants displayed not only tolerance to heat stress but remained healthy at 37 °C for more than 10 days on MS plate (Fig. 4A) and around two months in soil (Fig. 4B). The research with the chimeric RCA [40] and the more thermotolerant mutant RCA [15] improved heat tolerance in transgenic Arabidopsis only up to 27 °C for 8 h or 38 °C for 2 h, and 30 °C for 4 h daily, respectively. In contrast, we show here that it is possible to substantially increase plant tolerance to heat stress with a considerably higher seed yield than wild-type plants by introducing a naturally more thermotolerant RCA from creosote bush *Larrea tridentata* (Fig. 4D).

Under water deficit stress caused by PEG treatment, we found different results. First, if PEG was the only stress, *AVP1/RCA* co-overexpressing plants behaved just like *AVP1*-overexpressing plants, both produced similarly longer root length on MS plates; *RCA*-overexpressing plants behaved like wild-type plants, both produced short root length (Fig. 5B). This suggests that *RCA*-overexpression does not contribute to the root length difference between *AVP1/RCA* co-overexpressing plants and wild-type plants. However, if salt stress is applied in addition to PEG treatment, the *RCA*-overexpression does make a difference in the root length of *AVP1/RCA* co-overexpressing plants. We found that the root length of *RCA*-overexpressing plants was longer than wild-type plants, although still shorter than the root length of *AVP1*-overexpressing plants, which shows the contribution of *RCA*-

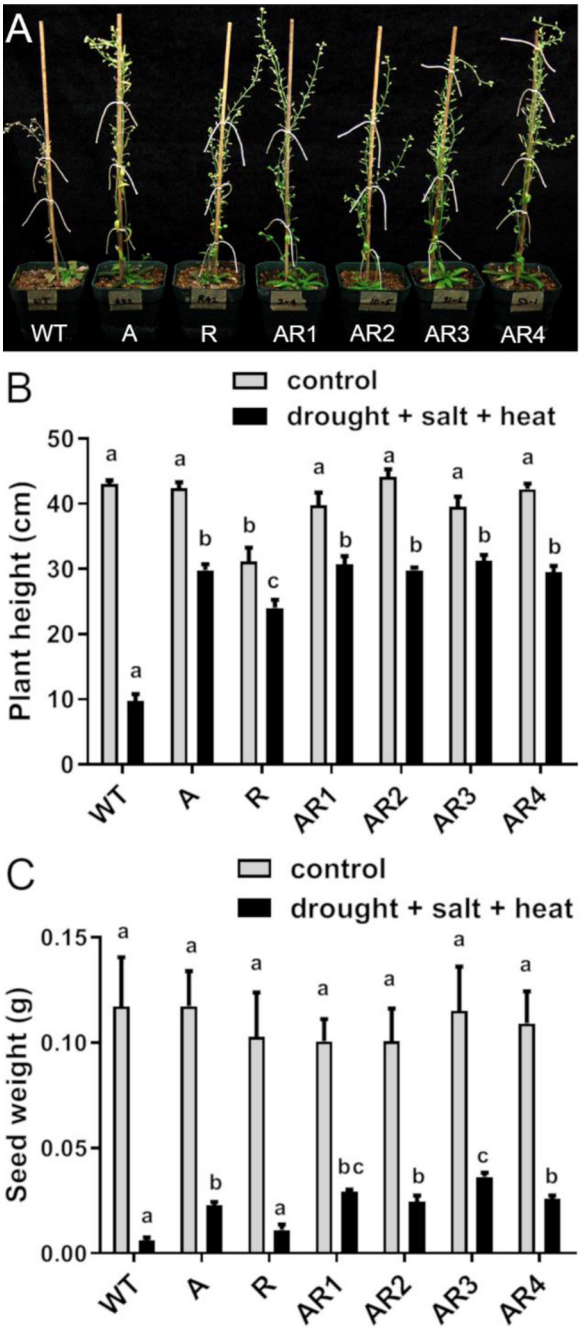

Fig. 9. Performance of *AVP1/RCA* co-overexpressing plants under combined drought, heat, and salt stresses. **A**. Phenotypes of wild-type and *AVP1/RCA* co-overexpressing plants under combined drought, heat, and salt stresses. **B**. Plant height analysis of wild-type and *AVP1/RCA* co-overexpressing plants after treatment with combined drought, heat, and salt stresses. Grey bars, normal growth condition; black bars, after treatment with combined drought, heat, and salt stresses. **C**. Seed yield analysis of wild-type and *AVP1/RCA* co-overexpressing plants after treatment with combined, drought, heat, and salt stresses. Grey bars, normal growth condition; black bars, after treatment with combined drought, heat, and salt stresses. n = 6 plants. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

overexpression in the root length under combined PEG and salt stress conditions. *AVP1/RCA* co-overexpressing plants showed slightly longer roots than *AVP1*-overexpressing plants, again supporting the notion that *RCA*-overexpression contributes to the longer root length observed in *AVP1/RCA* co-overexpressing plants (Fig. 5D). Interestingly, when

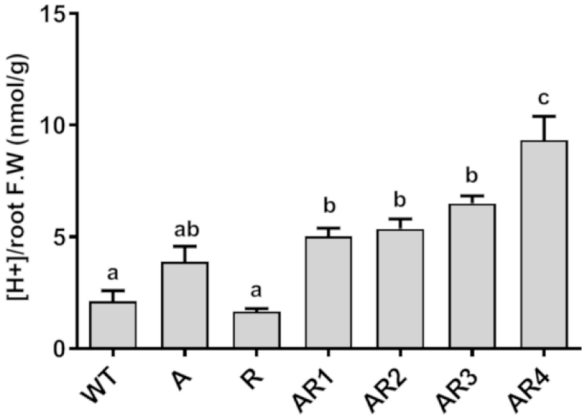

Fig. 10. Rhizosphere acidification assay of *AVP1/RCA* co-overexpressing plants. Data are mean ± SE (n = 5), where each replicate (n) consisted of 10 plants from each genotype; three technical trials were conducted. WT, wild-type plant; A, *AVP1*-overexpressing plant; R, *RCA*-overexpressing plant; AR1 to AR4, four independent *AVP1/RCA* co-overexpressing plants.

the heat stress was applied in addition to PEG treatment, the *RCA*-overexpression's contribution to the longer root length observed in *AVP1/RCA* co-overexpressing plants was lost, despite that *RCA*-overexpression's contribution to the longer root length was still observed in *RCA*-overexpressing plants (Fig. 5F). We do not have a sound explanation for the RCA's role in the longer root length observed in *RCA*-overexpressing and *AVP1/RCA* co-overexpressing plants under combined PEG and salt stress conditions.

This study also examined how *AVP1/RCA* co-overexpressing plants would perform under multiple stress conditions. In the semiarid regions, the overuse of fertilizer has led to abandonment of many croplands that used to be fertile, as the combination of drought and salt stresses is detrimental to plant growth and development. We tested the performance of *AVP1/ RCA* co-overexpressing plants under combined drought and salt stresses. *AVP1/RCA* co-overexpressing plants produced 323 % more seeds than wild-type plants (Fig. 6). Under this stressful condition, *RCA*-overexpression did not make any contribution to the seed yield increase in *AVP1/RCA* co-overexpressing plants, as *AVP1/RCA* co-overexpressing plants produced similar amount of seeds as *AVP1*-overexpressing plants (Fig. 6). Under salt (100 mM NaCl) and moderate heat (32 °C) conditions, all transgenic plants produced slightly longer root length than wild-type plants on MS plates and there were no statistical differences in root length among all transgenic plants (Fig. 7A & B). In soil experiment, however, *AVP1/RCA* co-overexpressing and *AVP1*-overexpression plants produced higher seed yields than *RCA*-overexpressing plants and wild-type plants, where *RCA*-overexpression did not contribute to the seed yield increase in *AVP1/RCA* co-overexpressing plants (Fig. 7C & D). Under combined drought and heat stresses, *AVP1/RCA* co-overexpressing plants produced higher seed yields than *AVP1*-overexpressing, *RCA*-overexpressing, and wild-type plants (Fig. 8). This result suggests that *RCA*-overexpression contributed to the seed yield increase in *AVP1/RCA* co-overexpressing plants, as *AVP1/RCA* co-overexpressing plants produced more seeds than *AVP1*-overexpressing plants (Fig. 8). We also examined how *AVP1/RCA* co-overexpressing plants would perform under combined drought, heat, and salt stresses, and we obtained similar results: *AVP1/RCA* co-overexpressing plants produced the highest amount of seeds (Fig. 9). It appears that there is a positive interaction between *AVP1*-overexpression and *RCA*-overexpression under combined drought and heat stresses, which makes *AVP1/RCA* co-overexpressing plants produce the highest seed yield and *AVP1*-overexpressing plants the second highest seed yield under combined drought and heat stresses. This discovery could be applicable to agricultural production, as drought and heat are two stresses that often come together in the semiarid

regions of the world such as India, Pakistan, China, and United States, causing significant crop losses. Making crops more tolerant to combined drought and heat stresses could potentially improve crop yields substantially.

Earlier research shows that overexpression of *AVP1* leads to better root development and better nutrient uptake by releasing more protons into the surrounding media [46–48,57]. Thus, in this study, we tested if these *AVP1/RCA* co-overexpressing plants have this ability as well, through the rhizosphere acidification assay, where the protons released by the roots were measured as a function of the pH value in the media. The *AVP1/RCA* co-overexpressing plants acidified media more extensively by releasing more protons (Fig. 10). This could also explain why *AVP1/RCA* co-overexpressing plants display a healthier phenotype under normal growth as well as under stress conditions. Due to the better root system as a consequence of more efficient polar auxin transport and the improved translocation of reduced carbon from source to sinks, *AVP1/RCA* co-overexpressing plants are able to release more protons into the surrounding rhizosphere, thereby absorbing nutrients more efficiently from soil.

In our study, we were able to demonstrate that *AVP1/RCA* co-overexpressing plants outperformed wild-type plants under every single stress conditions yet could outperform neither *AVP1*-overexpressing plants under single stresses of either drought or salt, nor *RCA*-overexpressing plants under heat stress conditions. Nevertheless, under multiple stress conditions, *AVP1/RCA* co-overexpressing plants clearly perform better than all other plants under combined drought and heat conditions. The tolerance capacity that is observed in this case is more likely due to the additive effect rather than a synergistic effect. That is, the tolerant phenotype is equal to the sum of effects contributed by *AVP1*-overexpression and *RCA*-overexpression, and not greater than the sum of effects of *AVP1*-overexpression and *RCA*-overexpression when considered independently. Overall, our results suggest that co-overexpression of *AVP1* and *RCA* increases plant tolerance to drought, salinity and heat as single as well as multiple stresses. While *AVP1* is a gene that has been extensively researched on, *RCA* is a lesser known territory which we believe holds great promise in enhancing the heat tolerance in plants. It would be interesting to see if RCA from creosote bush would interact with or increase the activity of the Arabidopsis Rubisco *in vitro,* which would validate our discovery biochemically. In conclusion, our work shows that co-overexpression of *AVP1* and *RCA* in Arabidopsis substantially increases drought-, salt- and heat-tolerance in transgenic plants, signifying the impact of this proof-of-concept research as a potential approach that could be applied in improving global agricultural productivity.

4. Materials and methods

4.1. Vector construction and Arabidopsis transformation

The coding DNA sequence (CDS) of full-length cDNAs of *AVP1* and *RCA* were used for vector construction. The 5'-untranslated region (UTR) and 3'-UTR of the transgene *AVP1* were designed to be different from those of the endogenous *AVP1*, so transcripts from the transgene *AVP1* and the endogenous *AVP1* can be distinguished using different oligonucleotide primers in the PCR experiments. *AVP1* and *RCA* sequences were inserted in tandem and divergent orientations as two separate constructs. The plant binary vector pPZP212 containing the p35S::AVP1/pCab3::RCA construct (either tandem or divergent) was introduced into the Agrobacterium GV3101 strain using the freeze thaw method [58]. Wild-type Arabidopsis (ecotype Columbia or Col-0) was transformed with the Agrobacterial strain GV3101 harboring the p35S::AVP1/pCab3::RCA construct using floral dip technique [45]. The 35S driven neomycin phosphotransferase II gene (*NPTII*) conferring kanamycin resistance was used as the plant selectable marker for transgenic seedling identification. A total of 129 independent T_1 lines were grown to obtain 50 independent homozygous lines, which were identified by plating the seeds on Murashige and Skoog (MS) media [59] containing 30 μg/mL kanamycin.

4.2. Plant growth conditions

Wild-type plants, *AVP1*-overexpressing plants and *RCA*-overexpressing plants (both generated in our laboratory), and four independent *AVP1/RCA* co-overexpressing plants, AR1, AR2, AR3, and AR4, were used in this study. For experiments conducted in soil, plants were grown in soil (SunGro Horticulture, Vancouver, Canada), in growth chambers with a 16 h/8 h light/dark photoperiod, 150 μE m^{-2} s^{-1} of light irradiance, 50 % relative humidity at 22 °C. For experiments conducted on MS plates, Arabidopsis seeds (> 100) were surface sterilized with 70 % ethanol (v/v) for one min, and 15 % commercial bleach (v/v) for 20 min, followed by washing with sterilized distilled water 3–4 times. Seeds were then kept at 4 °C for 4 days in darkness for stratification and subsequently plated on MS plates and used for various physiological experiments.

4.3. RNA blot and DNA blot analyses with radiolabeling of gene specific probes

Total RNA was extracted from two-week-old Arabidopsis plants seedlings using the TRIZOL reagent from Invitrogen (Carlsbad, CA), and quantified using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, www.fishersci.com/us/en/home.html). Ten μg of total RNA was electrophoresed on a 1.2 % (W/V) formaldehyde denaturing agarose gel in 1X MOPS buffer according to standard procedures [60]. Next, RNA was blotted onto a positively charged BioTrans (+)™ nylon membrane from Ambion (Austin, TX) and subjected to hybridization with [α-^{32}P] dATP labeled gene specific probes for *AVP1*, *RCA*, and *Actin 2* using the DECAprime™ II DNA Labeling Kit (Life Technology, NY) according to Church and Gilbert [61]. RT-PCR was carried out for a few randomly selected RNA samples using 2 μg of total RNA in a 25 μl system, using M-MLV reverse transcriptase from Promega (Madison, WI), followed by PCR using suitable primers (listed below). The final PCR products were separated by agarose gel electrophoresis. The *Actin 2* was used as the loading control in both PCR and RNA blot analysis. Genomic DNA extraction was carried out from Arabidopsis seedlings using CTAB method with minor modifications [62]. A total of 20 μg of genomic DNA was digested overnight and run on a 0.8 % agarose gel, followed by blotting onto a positively charged BioTrans (+)™ nylon membrane from Ambion (Austin, TX). Subsequently, DNA hybridization was performed as described by Hu et al. [63].

4.4. Primers used for RT-PCR, RNA and DNA blot analysis are listed below

RT-AVP1-1857-F1: CCCTGGACTTATGGAAGGAACC
RT-AVP1-3UTR-R1: GAGAGACTGGTGATTTGCGGAC
RT-RCA-1091-F3: TCAGTGAGGTCGGAGTCGCA
RT-RCA-1458-R3: GGATCCAACCACAAAAGCTTAAAAC
AVP1-5UTR-F: ATGGGCGAGCTCGGTACC
AVP1-3UTR-R: GAGAGACTGGTGATTTGCGGAC
RCANbF1: TTCTTGAGTCTCCCTAA
RCANbR1: ATCCTGGTTAGCATCA
Act2-F: TCACCACAACAGCAGAGCGGG
Act2-R: GGACCTGCCTCATGATACTCGG

4.5. Protein extraction and SDS-PAGE

Pre-stratified Arabidopsis seeds of WT, R, and AR1to AR4 were sown on MS media plates and grown for one week. These seedlings were transferred to liquid MS media and grown for another week on a shaker under controlled conditions. Next, total protein was extracted from the seedlings of each genotype by first grinding these into a powder using liquid nitrogen and adding 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 b-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail), followed by centrifugation at 15,000 rpm for 10 min at 4 °C to obtain soluble protein fraction in the supernatant. Protein concentration was determined by performing the Bio-Rad protein assay of Bio-Rad (Hercules, California) [64]. A total of 20 µg of each protein sample was mixed with 10 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 % bromphenol blue), boiled for 5 min followed by a brief spin down and separated on a 10 % SDS-polyacrylamide gel.

4.6. Western blot analysis

Proteins from the SDS-PAGE gel were transferred onto a PVDF membrane (Sequi-blot ™PVDF of Bio-Rad, Hercules, California) with Towbin buffer (25 mM Tris, 192 mM glycine, and 20 % methanol [v/v] pH 8.3) overnight and washed with TBST (TBS buffer with 0.1 % Tween [v/v]) thrice before incubating in TBST with 5% BSA [w/v] for one hour at room temperature to block non-specific sites on the membrane. Next, the membrane was rinsed thrice with TBST and incubated with the antibody raised against RCA from PhytoAB Inc. (San Jose, California) at room temperature for 1.5 h, followed by washing with TBST thrice and incubating with the alkaline phosphatase (AP) conjugated-goat anti rabbit antibodies from Bio-Rad (Hercules, California) for one hour. The membrane was once again washed thrice with TBST before color developing with AP conjugate substrate kit of Bio-Rad (Hercules, California) according to the manufacturer instruction and visualized by using the Chemidoc MP imaging system of Bio-Rad (Hercules, California). The band intensity of each genotype was calibrated with the loading control tubulin from Cell Signaling Technology (Danvers, Massachusetts), and compared to the band of wild-type plant.

4.7. Stress physiology experiments

Physiological experiments were carried out on MS plates and in soil using wild-type and homozygous transgenic plants. For experiments on MS plates, seeds were surface sterilized and stratified as described above. Normal growth conditions were 16 h/8 h light/dark photoperiod, 150 µE m^{-2} s^{-1} of light irradiance, and 50 % relative humidity at 22 °C. For experiments with soil grown plants, 6-day-old seedlings were transferred from MS plates to soil and grown for three weeks before the single stress or combined stresses were applied. Seed collectors were used to ensure minimum seed loss until all siliques were harvested. Control plants were watered with 1 L of regular water per tray every other day until harvest. All experiments were repeated at least three times with 6 or more biological replicates for each genotype.

4.8. Drought stress experiment

Three-week-old plants grown under normal growth conditions were subjected to water deficit stress treatment, i.e. watering was withheld for two weeks, then plants were watered every three days for three weeks until harvest. Plant height was measured after the recovery period, photographs were taken two weeks after water deficit treatment, followed by harvesting and measuring seed yield.

4.9. Salt stress experiments

For salt stress treatment on MS plate, seeds were either directly plated on MS plates containing 75, 100, or 125 mM NaCl, or three-day-old seedlings growing on MS plates were transferred to MS plates supplemented with 75, 100, or 125 mM NaCl, and grown vertically. Seedling phenotypes were documented, and root lengths were measured 7–10 days after salt stress treatment. In salt stress treatment in soil, plants were grown under normal growth conditions for three weeks, followed by watering with NaCl solutions every 3 days with incremental concentrations of NaCl of 50 mM, 100 mM and 125 mM twice with each concentration. After that, plants were irrigated with regular water until the end of experiment and seed yield and plant height were documented.

4.10. Heat stress experiments

Surface sterilized and stratified seeds were grown vertically on MS plates either at moderate heat (32 °C) or heat (37 °C) conditions, or three-day-old seedlings grown under normal growth condition were then exposed to the same heat stress conditions stated above. The root length of the stressed and control plants was measured 10 days after treatment. For heat stress experiment in soil, three-week-old plants grown under normal growth conditions were transferred to a growth chamber that was set at a 16 h/8 h photoperiod with 37 °C for 5.5 h and 22 °C for the remaining 18.5 h each day. Plants were kept in the heat chamber until harvest (approximately 2 months) and watered with regular water as control plants. Plant phenotype was documented during the heat treatment, and plant height and seed yield were measured at the end of heat stress experiment.

4.11. Osmotic stress experiment

The effect of reduced water potential in the growth media on seedling growth was evaluated as described by van der Weele et al. [65], by plating surface sterilized and stratified seeds either directly on MS plate containing 0.05 M (40 %) polyethylene glycol (PEG-8000) or transferring 3-day-old seedlings to plates containing 0.05 M (40 %) polyethylene glycol (PEG-8000). Root length was measured 7–10 days later.

4.12. Combined heat and drought/osmotic stress experiment

Three-day-old seedlings of WT, *AVP1*-overexpressing, *RCA*-overexpressing, and *AVP1/RCA* co-overexpressing plants were transferred to MS plates containing 40 % PEG-8000 and exposed to either 32 °C or 37 °C and kept for 7–10 days, followed by measuring the root length.

4.13. Combined salt and drought/osmotic stress experiment

Three-day-old seedlings were transferred to MS plate containing 40 % PEG-8000, supplemented with either 75 mM or 100 mM NaCl. Seedlings were grown for 7–10 days, after which root length was measured.

4.14. Combined drought and salt stress experiments

Plants were grown under normal growth conditions for 3 weeks. Then, these plants were irrigated with 500 ml each of 50 mM NaCl solution on the 1st and 4th day post stress initiation, and 100 mM NaCl solution on 7th, 10th, and 13th day post stress initiation. Plants were then irrigated with regular water every 3 days until the end of experiment. Phenotype, plant height, and seed yield were measured at the end of experiment.

4.15. Combined drought and heat stress experiments

Plants grown under normal growth conditions for three weeks, then moved into a growth chamber set at a photoperiod of 16 h light and 8 h night, with an elevated temperature of 37 °C for 5.5 h a day and 22 °C in the remaining hours. At the time of transfer to heat condition, water deficit stress was also initiated by watering the plants every 4 days with half the volume of water used for control plants. After three weeks under combined stress conditions, heat stress was removed while the water deficit conditions were maintained for two more weeks, after

which these plants were irrigated as the control group. Phenotype was recorded and photographs were taken at the time when both stresses were removed, and plant height and seed yield were documented at the time of harvest.

4.16. Combined salt and heat stress experiments

For conducting experiments involving combined heat and salt stresses on MS plates, different NaCl concentrations (i.e. 75 mM and 100 mM) and different heat conditions (32 °C and 37 °C) in various combinations were designed. In the first set of experiments, Arabidopsis seeds were directly exposed to either salt or heat stress first, and after three days, the other stress was applied. In the second set of experiment, three-day-old Arabidopsis seedlings grown under normal growth conditions were exposed to both salt and heat stresses simultaneously. After 7–10 days under combined stresses, phenotypes of plants were documented, and root lengths were measured. For soil experiments, three-week-old plants growing under normal growth conditions were transferred to the above-mentioned growth chamber set at 37 °C for 5.5 h a day. Then these plants were irrigated with 50 mM NaCl on 1st and 4th day, and 100 mM NaCl on 7th, 10th and 13th days after moving to the heat chamber. Next, plants were irrigated with regular water similar to control plants and, heat stress was also removed after a total of three weeks. Photographs of the plants were taken, and seed yield and plant height were measured at the end of experiments.

4.17. Combined drought, salt and heat stress experiment

Plants grown under normal growth condition for three weeks, were transferred to a growth chamber set at 16 h light and 8 h darkness photoperiod with 37 °C for 5.5 h and 22 °C for the remaining 18.5 h each day. Plants were irrigated with 500 ml of 50 mM NaCl on the 1st and 4th day after transferring to the heat chamber, followed by 500 ml of 100 mM NaCl on the 7th day. Next, plants were irrigated every 3 days with half the volume of regular water used for control plants. Heat stress was removed 3 weeks later, while the moderate water deficit condition continued until harvest. Photographs were taken upon the removal of the heat stress, while seed yield and plant height were measured at the time of harvest.

4.18. Rhizosphere acidification assay

The amount of protons released by plant root system per total fresh root weight was obtained based on the protocol described by Pizzio et al. [48].

4.19. Statistical analysis

Student's *t*-test was used to analyze data including plant yield, height, and root length between transgenic and wild-type plants. Tukey's method was utilized to study the pairwise comparison among wild-type, *AVP1*-overexpressing, *RCA*-overexpressing lines, and *AVP1/RCA* co-overexpressing plants (the significance of differences level $p < 0.05$).

Author contributions

I.W., L.S., and H.Z. conceived and designed the experiments; I.W., N.M., J.S., L.S., and X.Z. performed the experiments; I.W., G.S., P.P., and H.Z. analyzed the data and wrote the manuscript.

Declaration of Competing Interest

There is no Conflict of Interest in this submission.

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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.2020.110499.

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