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Co-overexpression of *RCA* and *AVP1* in cotton substantially improves fiber yield for cotton under drought, moderate heat, and salt stress conditions

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ABSTRACT

Abiotic stresses such as drought, heat, and salt are major causes of crop failure and are the main challenges that we face in agriculture. Genetic engineering has been successful in controlling harmful insects and conferring herbicide resistance, but has yet to produce similar results in reducing damages caused by abiotic stresses. It was previously shown that overexpression of *AVP1* that encodes a vascular H^+-pyrophosphatase in Arabidopsis could increase drought and salt tolerance and overexpression of *RCA* that encodes Rubisco activase in *Larrea tridentata* could increase heat tolerance in transgenic plants. It was therefore hypothesized that co-overexpression of *AVP1* and *RCA* would make transgenic plants more tolerant to all three stresses simultaneously. Indeed, this hypothesis was confirmed in Arabidopsis. To test if this result could be duplicated in an actual crop, *AVP1* and *RCA* were co-overexpressed in cotton. The results from this study indicated that *RCA/AVP1* co-overexpressing cotton plants produced 50% and 96% higher seed fiber yield than wild-type cotton under combined drought and salt stresses and combined drought and heat stresses, respectively. Furthermore, *RCA/AVP1* co-overexpressing cotton plants showed a 6.5-fold increase in net photosynthetic rates under heat stress as well as having much higher Vcmax rates under multiple stress conditions. Results from two field studies showed that *RCA/AVP1* co-overexpressing cotton plants had 90% and 66–75% increase in seed fiber yield in comparing to wild-type cotton under dryland conditions. This study proves that co-overexpression of *AVP1* and *RCA* can improve cotton's fiber yield in a dryland agricultural region, and this approach could increase other crops' yield in arid and semiarid regions of the world.

Introduction

There are critical challenges in agriculture to meet the food, fuel, and fiber demand with the world population predicted to increase to 9 billion by the year 2050 (UN, 2007). These challenges include abiotic stresses such as drought, salinity, high or low temperatures, pests, diseases, and climate changes (Boyer, 1982; IPCC 2007; Edgerton, 2009; Ainsworth and Ort, 2010; Rivero et al. 2021). Abiotic stresses contribute to significant declines in crop yields worldwide each year, particularly in the arid and semiarid regions of the world. Drought has always been the number one factor that limits crop production in the world, but heat stress has become a serious problem due to climate change (Keer, 2007; Long and Ort, 2010; Rivero et al. 2021). Temperature stress affects crop's cellular metabolism, growth, and development, and causes yield decline (Kim and Portis 2005; Kurek et al. 2007; Kumar et al. 2009; Ainsworth and Ort, 2010). The combined drought and heat stresses cause increased damages to crops, resulting in even bigger losses in agricultural productivity (Savage and Jacobson, 1935; Craufurd and Peacock, 1993; Savin and Nicolas, 1996). Due to the overuse of fertilizers in agricultural lands, it accumulates in soils and its harmful consequences intensify when there is a lack of precipitation, leading to combined drought and salt stresses, which imposes a detrimental effect on crop growth and productivity. Therefore, there is an urgent need to develop new crop varieties that would be tolerant to combined stresses of drought, heat, and salinity (Mittler and Blumwald, 2010; Rivero et al. 2021). Recent research has shown that the traditional breeding appears to have reached a plateau in terms of increasing crop yield (Mittler and

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Blumwald 2010), and there are virtually no crops on the market that are highly tolerant to abiotic stresses, consequently, crops suffer a severe yield penalty in the presence of combined abiotic stresses. Genetic engineering appears to be a promising approach to create new crop varieties that would be more tolerant to multiple environmental stresses.

In the last 30 years, selective breeding and recombinant DNA technology have provided the tools to enhance crop performance and produced successful products on the market. Some examples include the herbicide tolerance trait (Funke et al. 2006), insect resistance trait (Abbas 2018), virus resistant trait (Kreuze and Valkonen, 2017), and modified fatty acid composition (Phillips 2008), which are the results of the application of biotechnology in agriculture. However, there is still a gap in the development of genetically engineered crops that show enhanced performance under abiotic stress conditions. Because climate change negatively affects ecosystems and communities worldwide, it makes abiotic stress conditions even worse for crop production, which is a major threat for food security in many countries (NOAA, 2023). Therefore, it is imperative to develop new crop varieties that would produce higher yields under elevated temperature and water deficit stress conditions or under rain fed conditions in the arid and semiarid regions of the world.

Most transgenic studies in the past focused on using single genes to improve stress tolerance, which was largely successful based on laboratory studies (Lemaux, 2008, 2009; Mittler and Blumwald, 2010). However, few of those studies were successfully applied in crops in the field conditions (Hu and Xiong, 2014). One of the reasons for the failure in translating laboratory success into real gains in the field was that in nature, abiotic stresses rarely come alone; instead, they often come together or in various combinations. For example, drought and heat appear to come at the same time more often than not, causing much bigger damages than when these stresses appear alone (Mittler, 2006; Rivero et al. 2021). The beneficial trait conferred by overexpressing a single gene could easily be cancelled by the complex stress conditions in the field. Therefore, it might be necessary to simultaneously express two or more genes in transgenic plants in order to achieve higher tolerance to multiple stresses. Based on this hypothesis, scientists started stacking genes together in order to achieve higher stress tolerance or multi-stress tolerance (Zhao et al. 2006; Wijewardene et al., 2020; Shen et al., 2015; Pehlivan et al., 2016; and Sun et al., 2018). For example, co-overexpression of *AtNHX1* and *SOS1* led to increased salt tolerance in Arabidopsis (Pehlivan et al., 2016), co-overexpression *AVP1* and *AtNHX1* in cotton led to higher tolerance to both drought and salt stresses (Shen et al., 2015), and co-overexpression of *AVP1* and *PP2A-C5* led to improved tolerance to different salts (Sun et al., 2018). These studies indicated that co-overexpression of genes that function either cooperatively or synergistically in plant cells would be an effective approach in achieving higher salt stress tolerance or higher tolerance to multiple stresses such as combined drought and salt stresses. However, there were few studies in the literature that involved stacking genes for increased tolerance to multi-stress conditions including a heat stress component.

Our research focuses on developing drought-, heat-, and salt-tolerant plants as around 40% of the world's cropland are in arid and semiarid regions including America's Southwest, where drought, heat and salt stresses are the major limiting factors for agricultural production (Maestre et al., 2021). To create transgenic plants that have higher tolerance to drought, heat, and salt stresses, one of the two genes that we chose for this purpose was *AVP1*. The *AVP1* gene encodes a vascular H^+-pyrophosphatase in Arabidopsis and overexpression of *AVP1* leads to increased proton gradient across the vacuolar membranes, which energizes the activity of the vacuolar membrane bound sodium/proton antiporter AtNHX1, thereby sequestering more sodium ions into vacuoles, leading to higher salt tolerance in transgenic plants (Gaxiola et al. 2001; Pasapula et al., 2011; Qin et al., 2013). In addition to activating AtNHX1′s activity, overexpression of *AVP1* also leads to increased auxin polar transport, which stimulates lateral root development and consequently creates larger root systems, leading to increased drought tolerance due to its higher ability in absorbing water into root systems in transgenic plants (Park et al. 2005; Li et al. 2005; Pasapula et al. 2011).

The carbon fixation in photosynthesis is highly sensitive to temperatures due to the enzyme ribulose-1, 5-bisphophate carboxylase/oxygenase (Rubisco) (Salvucci and Crafts-Brandner, 2004). The optimal temperatures for carbon fixation are between 20 and 35 ℃ for crops in tropic, sub-tropic, and temperate regions, while temperatures above 35 ℃ usually substantially reduce carbon fixation (Salvucci and Crafts-Brandner, 2004). The main reason for the reduced carbon fixation above the optimal temperatures is the inactivation of Rubisco under heat stress conditions. For example, the net photosynthetic rate is inhibited at temperatures above 30 ℃ in cotton (Salvucci and Crafts-Brandner, 2004). With moderate heat stress (35 – 42 ℃), the reduction in carbon fixation is readily reversible, but at higher temperatures, e.g. above 42 ℃, this reversal requires longer exposure to optimal temperatures for recovery to occur (Salvucci and Crafts-Brandner 2004; Kurek et al. 2007). The activation state of Rubisco is the reason for a decline in net photosynthesis under elevated temperatures over the electron transport or other factors (Salvucci and Crafts-Brandner, 2004). Rubisco activase (RCA) is required to maintain Rubisco in its active state, opening the active site when it becomes blocked by certain inhibitory sugar-phosphates (Kumar et al. 2009). The RCA is a chloroplast-localized ATPase and part of the AAA^+ family of ATPases (Portis, 2003; Kurek et al., 2007). RCA requires ATP to loosen the binding of Rubisco for sugar phosphates (Portis, 2003). As the temperature increases, dead-end product formation that blocks the active site of Rubisco also increases while the catalytic competency of activase decreases, reducing its ability to keep Rubisco catalytically competent (Crafts-Brandner and Salvucci, 2000). To keep Rubisco active at high temperatures, RCA needs to be active. Unfortunately, RCAs in most crops are not heat tolerant (Crafts-Brandner and Salvucci 2000; Salvucci and Crafts-Brandner 2004; Sage et al., 2008; Carmo-Silva et al., 2015).

One strategy for increasing photosynthesis is to enhance the efficiencies of (Rubisco) by enhancing the catalytic turnover with genetic engineering (Parry et al. 2013). Understanding that Rubisco can also react with oxygen, leading to photorespiration, a reduction of photosynthesis by 30% even under optimal conditions (Sharwood et al. 2016; Zelitch, 1973); environmental stresses such as heat and drought add to the inefficiency of the catalytic turnover of Rubisco. Therefore, it is possible to improve carbon fixation under elevated temperatures by providing a heat tolerant RCA for crops, which could lead to improved yield under heat stress conditions. Therefore, we chose a *RCA* gene from *Larrea tridentate* that is a plant adapted to the hot deserts in southern Arizona and northern Mexico. We co-overexpressed this *RCA* gene in Arabidopsis and showed that indeed we could increase heat tolerance in transgenic plants (Wijewardene et al., 2020, 2021). Here, we provide evidence that co-overexpression of *AVP1* and *RCA* in cotton would confer increased tolerance simultaneously to drought, heat and salt stresses, leading to significantly higher fiber yield in field conditions. Our research demonstrates that the positive results of co-overexpressing *AVP1* and *RCA* in Arabidopsis could be duplicated in cotton, leading to a significant gain in fiber yield in field conditions, which can serve as a good model for improving yields of other major crops in the world.

Results

Creation of RCA/AVP1 co-overexpressing cotton

The *RCA/AVP1* co-overexpression construct used by Wijewardene et al. (2020) was introduced into the wild-type cotton (*Gossypium hir-*

sutum Coker 312) using the *Agrobacterium*-mediated transformation method (Bayley et al.,1992). A total of 24 putative transgenic lines were obtained, among which 14 lines were found to contain both *RCA* and *AVP1* transcripts and these lines were called RA plants, based on RT-PCR analysis of genomic DNAs isolated from the leaf tissues of the T_1 generation (first transgenic generation) plants (data not shown). A preliminary RNA blot analysis was conducted for those 14 lines and four lines, RA1, RA5, RA8 and RA9, were found to express transcripts for both *AVP1* and *RCA* (data not shown). These four lines, together with wild-type and single *RCA*-overexpressing plants were again analyzed using RT-PCR and RNA blot analyses. The results showed that indeed the four *RCA/AVP1* co-overexpressing plants did express both *AVP1* and *RCA* transcripts (Fig. 1A & B), whereas wild-type plants did not express transcripts for both genes (the weak signal was due to some background noise from hybridization) and the single *RCA*-overexpressing plant only expressed the *RCA* transcript (Fig. 1B). Next, we isolated genomic DNAs from wild-type and two transgenic lines, RA5 and RA9, to conduct DNA blot experiment. We found that RA5 might contain 4 transgenes and RA9 might contain 3 transgenes based on the DNA blot data (Fig. 1C). Finally, we conducted a Western blot experiment to determine the steady-state levels of RCA in lines RA5 and RA9, using anti-RCA polyclonal antibodies for Arabidopsis RCA. Compared to wild-type plants, the lines RA5 and RA9 clearly had higher amount of RCA protein than wild-type plants did (Fig. 1D).

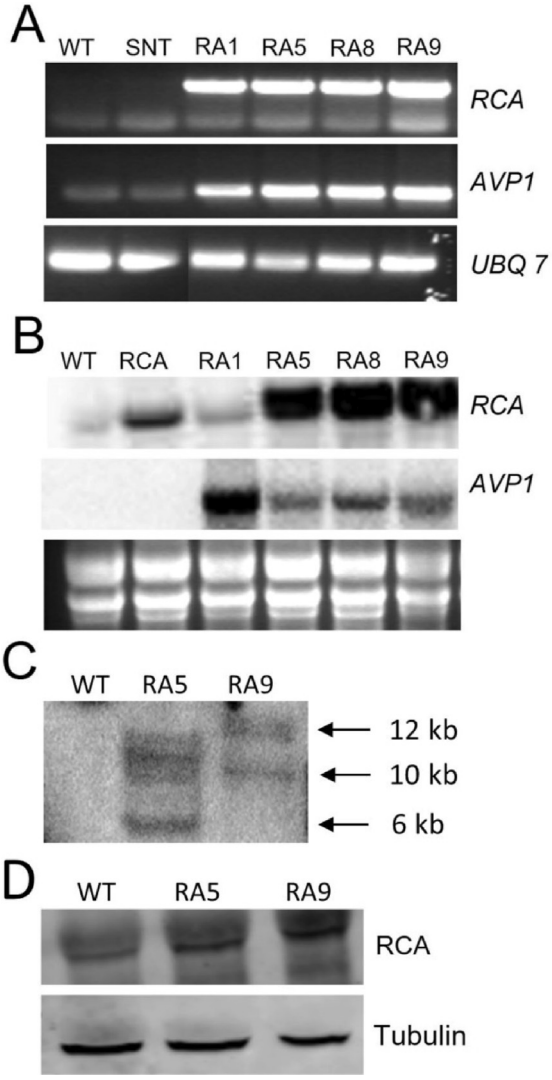

Fig. 1. Molecular analysis of *RCA/AVP1* co-overexpressing cotton plants. **A**. Analysis of *RCA/AVP1* co-overexpressing cotton plants by using the RT-PCR method. The DNA fragments amplified are labeled on the right. **B**. RNA blot analysis of *RCA/AVP1* co-overexpressing cotton plants. The names of genes used as probes are listed on the right. The ethidium bromide stained total RNAs were used as the RNA loading controls. **C.** DNA blot analysis *RCA/AVP1* co-overexpressing cotton plants. The size of the DNA fragment is marked on the right. **D**. Western blot analysis of *RCA/AVP1* co-overexpressing cotton plants. The names of the antibodies used are listed on the right. WT, wild-type plant; SNT, segregated non-transgenic plant; RCA, *RCA*-overexpressing plant; RA1, RA5, RA8 and RA9, four independent *RCA/AVP1* co-overexpressing plants.

RCA/AVP1 co-overexpressing plants are as tolerant as AVP1-overexpressing plant under combined drought and salt stresses

We previously showed that co-overexpression of *AVP1* and *RCA* in Arabidopsis made transgenic plants as tolerant as *AVP1*-overexpressing plants under drought and salt stress conditions (Wijewardene et al., 2020). To test if similar results would be duplicated in *RCA/AVP1* co-overexpressing cotton plants, we conducted physiological experiments with soil-grown cotton plants in greenhouse. We first grew cotton plants for three weeks under normal growth conditions, then divided plants into two groups: control group and treatment group. Plants in the control group were grown under normal growth conditions (i.e., no water deficit stress and no salinity stress) until the end of the experiment; whereas plants in the treatment group were irrigated with saline water and the irrigation was reduced to 25% of the amount used in the control group (i.e., combined drought and salt stresses). In addition to wild-type cotton, a segregated non-transgenic line and an *AVP1*-overexpressing line created by Pasapula et al. (2011) were used as reference lines for comparison. Under the normal growth condition, all plants grew well and there were no discernable differences in growth and appearance among all genotypes (Fig. 2A). All plants flowered around the same time, and no significant differences in net CO_2 assimilation were found among these plants except the two transgenic lines RA8 and RA9 (Fig. 2B), which showed slightly lower photosynthetic rates under normal growth condition.

When these plants were subjected to water deficit (25% water replacement) and salinity (100 mM NaCl) treatment for six weeks, all genotypes did not show much phenotypic differences in the above-ground portion (data now shown), but the biomass of the root systems of *RCA/AVP1* co-overexpressing and *AVP1*-overexpressing cotton plants were much bigger than wild-type and segregated non-transgenic plants (Fig. 2C & D). Even though all plants showed a reduction in CO_2 assimilation compared to that under normal growth conditions, *RCA/AVP1* co-overexpressing and *AVP1*-overexpressing cotton plants demonstrated higher rates of CO_2 assimilation than either wild-type or segregated non-transgenic plants, which was almost 100% higher than wild-type cotton plants in net photosynthesis (Fig. 2B). Under normal growth conditions, there were no significant differences in seed fiber yield among all genotypes (Fig. 2E), but under combined drought and salt stresses, *RCA/AVP1* co-overexpressing plants produced more bolls, resulting in 50% more seed fiber than wild-type plants (Fig. 2E).

RCA/AVP1 co-overexpressing plants produced the highest fiber yield under combined drought and heat stresses

As overexpression of *AVP1* could confer salt and drought tolerance and overexpression of *RCA* could confer heat tolerance, we tested the performance of *RCA/AVP1* co-overexpressing cotton plants under combined drought and heat stresses in a growth chamber. The moderate heat stress and water deficit established in the growth chamber were similar to the field conditions that cotton plants would normally experience in West Texas (Burke, 2017; Medlyn et al., 2002; USDA-

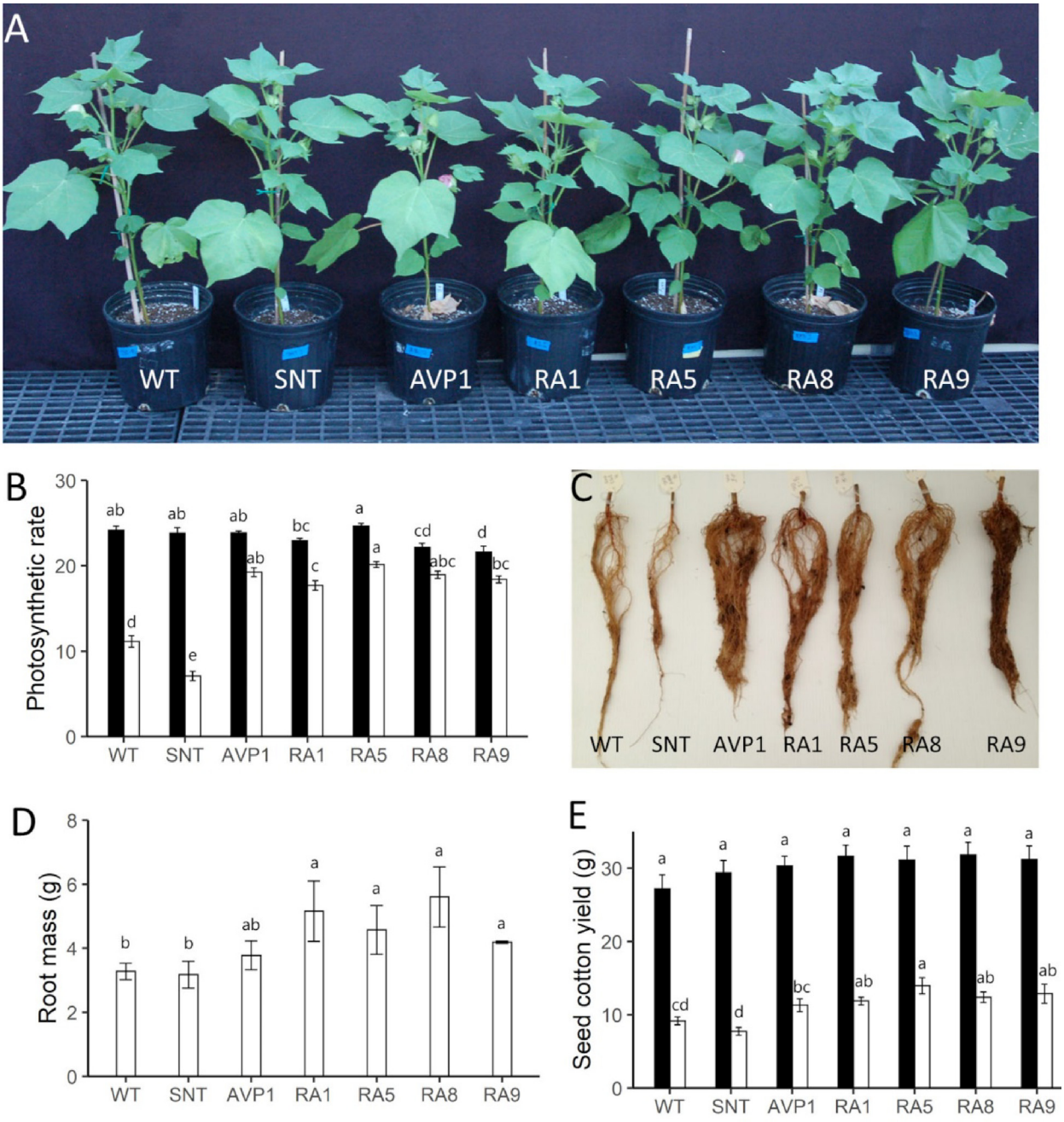

Fig. 2. Performance of *RCA/AVP1* co-overexpressing plants under combined drought and salt stresses. **A**. Phenotypes of cotton plants under normal growth condition for 6 weeks. **B**. Analyses of photosynthetic rates of cotton plants under normal growth conditions as well as under combined drought and salt stresses. Black bars, normal growth condition; white bars, under combined drought and salt stresses. **C**. Root phenotypes of cotton plants after treatment with combined drought and salt stresses. **D**. Analysis of root biomass of cotton plants after treatment with combined drought and salt stresses. **E**. Analysis of cotton seed fiber under normal growth condition as well as under combined drought and salt stresses. Black bars, normal growth condition; white bars, under combined drought and salt stresses. Results are the means ± SE (n = 10). WT, wild-type plant; SNT, segregated non-transgenic plant; RA1, RA5, RA8 and RA9, four independent *RCA/AVP1* co-overexpressing plants. Samples denoted by different letters are significantly different ($P < 0.05$, Tukey correction).

NASS, 2016). The 37 ℃ is around the afternoon temperature in the summer of West Texas, which is well above the optimal temperature of 28 ℃ for cotton. Above 30 ℃, the net CO_2 assimilation starts to decline; above 35 ℃, Rubisco activase in cotton starts to lose its activity (Crafts-Brandner and Salvucci 2000). We chose the 37 ℃ temperature for 3 h each day as the heat stress in addition to the reduced irrigation for plants grown in the growth chamber.

Following 4 weeks in the growth chamber under combined water deficit (25% water replacement) and heat stresses, gas-exchange measurements were taken before, during, and 3 h after heat stress treatment at 37 ℃ (Fig. 3). *RCA/AVP1* co-overexpressing and *AVP1*-overexpressing cotton plants had higher photosynthetic rates than wild-type plants even before the heat treatment (Fig. 3B). During heat treatment, *RCA/AVP1* co-overexpressing plants maintained the highest photosynthetic rates, followed by *AVP1*-overexpressing plants, and wild-type plants essentially stopped photosynthesizing (Fig. 2B). Interestingly, 3 h after heat stress treatment, *AVP1*-overexpressing plants reached the similar rate in photosynthesis as *RCA/AVP1* co-

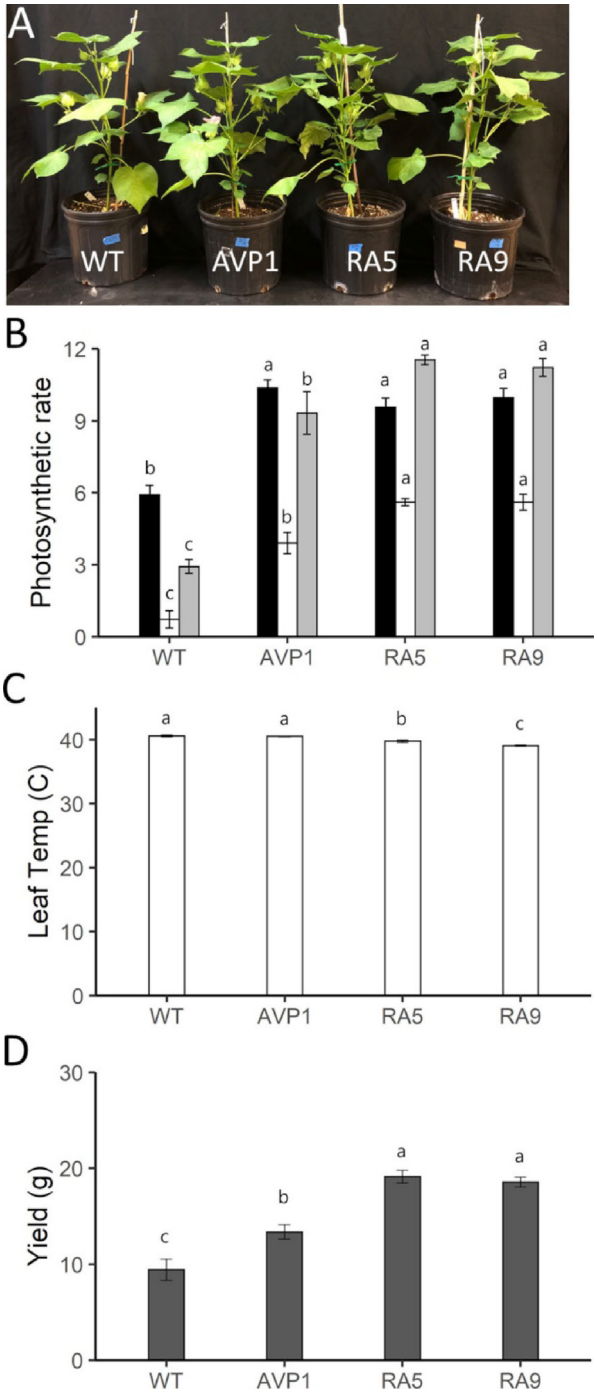

Fig 3. Performance of *RCA/AVP1* co-overexpressing plants under combined drought and heat stresses. **A**. Phenotypes of cotton plants under combined drought and heat stresses for 6 weeks. **B**. Analyses of photosynthetic rates of cotton plants under combined drought and heat stresses. Black bars, photosynthetic rates of cotton plants already under drought stress condition for 4 weeks and before the start of heat treatment each day; white bars, photosynthetic rates of cotton plants during heat treatment each day; grey bars, photosynthetic rates of cotton plants 3 h after the heat treatment. **C**. Leaf temperatures of cotton plants under combined drought and heat stresses. **D**. Analysis of seed fiber yield of cotton plants after the treatment of combined drought and heat stresses. Results are the means ± SE (n = 15). WT, wild-type plant; AVP1, *AVP1*-overexpressing plant; RA5 and RA9, two independent *RCA/AVP1* co-overexpressing plants. Samples denoted by different letters are significantly different ($P < 0.05$, Tukey correction).

overexpressing plants, to a level before the heat stress was applied, yet wild-type plants only reached a level at halfway (Fig. 3B). This result confirmed our hypothesis that overexpression of *RCA* indeed improves heat tolerance in transgenic plants when transgenic plants are under heat stress conditions.

The fact that *RCA/AVP1* co-overexpressing plants maintained higher photosynthesis compared to wild-type plants, indicating that they were in better physiological condition. We measured the leaf temperature of these plants under heat stress treatment, and we found that *RCA/AVP1* co-overexpressing plants had slightly lower leaf temperatures compared to wild-type and *AVP1*-overexpressing plants (Fig. 3C), which favored *RCA/AVP1* co-overexpressing plants in their cellular metabolism under heat stress, as they are able to maintain a lower canopy temperature. At the end of the combined drought and heat stress treatment, we found that *RCA/AVP1* co-overexpressing plants produced significantly higher seed fiber yield than wild-type plants (more than 100%, Fig. 3D). *AVP1*-overexpressing cotton plants also produced far more seed fiber than wild-type plants, but its yield was significantly lower than that of *RCA/AVP1* co-overexpressing plants (Fig. 3D). Therefore, we conclude that the *RCA* gene from creosote can indeed enhance photosynthesis in cotton under heat stress condition when overexpressed in transgenic cotton.

RCA/AVP1 co-overexpressing plants have higher carboxylation rate than wild-type and *AVP1*-overexpressing plants

To explain why *RCA/AVP1* co-overexpressing plants had higher photosynthetic rates under combined drought and salt stresses or under combined drought and heat stresses, we analyzed the Vcmax values of these plants. Vcmax values of a plant project the capacity of RuBP carboxylation (Kauwe et al. 2016; Smith et al. 2016), where an increase in Vcmax indicates higher RuBP carboxylation, leading to improved photosynthetic rates, while a lower photosynthetic rate shows reduced rate of photosynthesis due to RuBP carboxylation activation being reduced (Walker et al., 2016; Onoda et al., 2005). Under normal growth condition, there were little differences in Vcmax (Fig. 4A). However, under combined drought and salt stresses, the Vcmax dropped to half or more in wild-type and segregated non-transgenic plants, yet, the reduction was relatively less in *RCA/AVP1* co-overexpressing and *AVP1*-overexpressing plants. Similar results were obtained for plants under combined drought and heat stresses (Fig. 4B), where a 387% greater Vcmax was observed during heat treatment for *RCA/AVP1* co-overexpressing plants in comparing to wild-type plants. The calculated Vcmax values indicate that the maintenance of carboxylation rate is the primary mechanism for higher carbon fixation observed in *RCA/AVP1* co-overexpressing plants, and not due to higher electron transport.

RCA/AVP1 co-overexpressing plants produced the highest seed fiber yield in the field

To analyze the performance of the *RCA/AVP1* co-overexpressing cotton plants in field conditions, wild-type, segregated non-transgenic, *AVP1*-overexpressing, and *RCA/AVP1* co-overexpressing plants were grown in the USDA-ARS Experimental Farm in Lubbock, TX in the summers of 2016 and 2018. Cotton plants were grown under two different experimental conditions in 2016; irrigation and rain-fed conditions. The rain-fed experiments were considered close to true dryland farming conditions. In 2018, only rain-fed experiment was conducted. The summer weathers of 2016 and 2018 were dry, as from June to November in 2016, the rainfall was < 190 mm and the similar time in 2018, the rainfall was 270 mm among which the largest rainfall event was at the end of cotton growth season (Supp. Table 1).

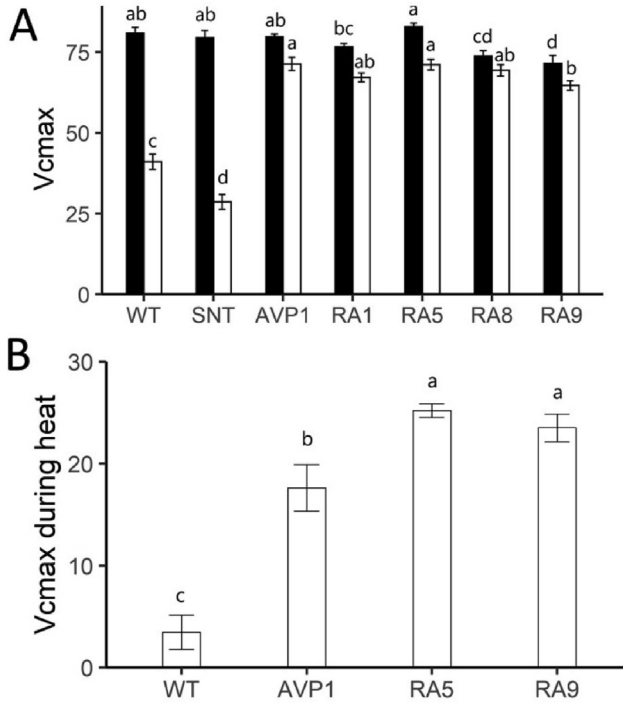

Fig 4. Photosynthesis of *RCA/AVP1* co-overexpressing plants under combined drought and salt stresses as well as under combined drought and heat stresses. **A**. Analysis of the Vcmax of cotton plants under normal growth condition as well as under combined drought and salt stresses. Black bars, normal growth condition; white bars, under combined drought and salt stresses. Results are the means ± SE (n = 10 plants). **B**. Analysis of the Vcmax of cotton plants under combined drought and heat stresses. Results are the means ± SE (n = 10). WT, wild-type plant; SNT, segregated non-transgenic plant; *AVP1*-overexpressing plant; RA1 to RA9, independent *RCA/AVP1* co-overexpressing plants. Samples denoted by different letters are significantly different ($P < 0.05$, Tukey correction).

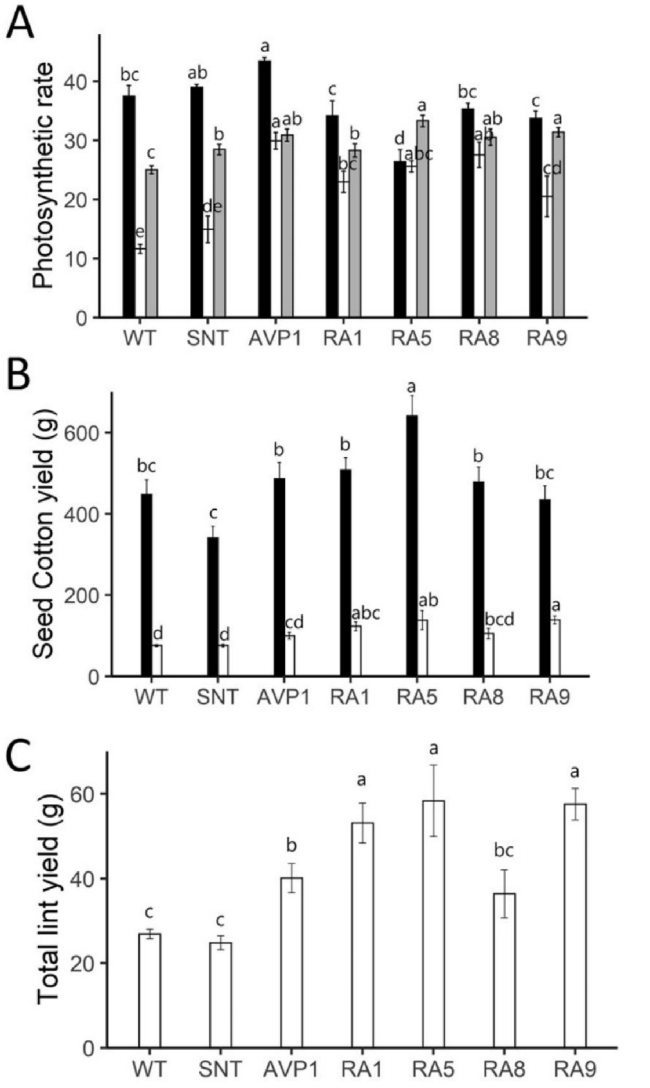

Fig 5. Performance of *RCA/AVP1* co-overexpressing plants in field condition in 2016. **A**. Analysis of photosynthetic rates of *RCA/AVP1* co-overexpressing plants in the field. Black bars, photosynthetic rates of cotton plants under irrigated condition; white bars, photosynthetic rates of cotton plants under rain-fed condition; grey bars, photosynthetic rates of cotton plants under rain-fed condition after a rainfall event. **B**. Seed fiber yield of cotton plants in the field. Black bars, seed fiber yield of cotton plants under irrigated condition; white bars, seed fiber yield of cotton plants under rain-fed condition. Results are the means ± SE (n = 432 plants). **C**. Total lint yield of cotton plants under the rain-fed condition. WT, wild-type plant; SNT, segregated non-transgenic plant; AVP1, *AVP1*-overexpressing plant; RA1, RA5, RA8, and RA9, four independent *RCA/AVP1* co-overexpressing plants. Samples denoted by different letters are significantly different ($P < 0.05$, Tukey correction).

Photosynthetic measurements were taken in the field before flowering. In 2016, there was an 80% improvement in net photosynthesis for *RCA/AVP1* co-overexpressing and *AVP1*-overexpressing plants in comparing with wild-type and segregated non-transgenic plants under rain-fed conditions (Fig. 5A). The recovery of photosynthesis after a rainfall event for transgenic plants was greater than wild-type plants. Under irrigated condition, there appeared very little differences in seed fiber yield between wild-type and transgenic plants, but the differences were bigger between segregated non-transgenic plants and transgenic plants (Fig. 5B). Under rain-fed conditions, *RCA/AVP1* co-overexpressing plants produced an average of 66% greater seed fiber yield than wild-type cotton (Fig. 5B). In comparing to wild-type plants, fiber (lint) yield was 100% higher for *RCA/AVP1* co-overexpressing plants with the exception of one line, i.e. RA8, which was about 45% higher than wild-type plants (Fig. 5C).

In 2018, a dryland experiment was conducted using irrigation only to wet the soil for seed germination. The photosynthetic measurements were taken again before flowering, and we found that *RCA/AVP1* co-overexpressing plants maintained 53% higher photosynthetic rate than wild-type and segregated non-transgenic plants (Fig. 6A). At the end of the growth season, *RCA/AVP1* co-overexpressing plants produced 76% higher seed fiber than wild-type cotton (Fig. 6B), the lint yield per plant of *RCA/AVP1* co-overexpressing plants was 91% higher than that of wild-type plants (Fig. 6C). Stomatal conductance was not affected in field grown cotton; however, a statistically higher conductance rate was found in *RCA/AVP1* co-overexpressing and *AVP1*-overexpressing cotton plants under combined drought and heat stresses in growth chamber when compared to wild-type plants (data not shown).

Defense genes are differentially regulated in the RCA/AVP1 co-overexpressing plants under combined drought and heat stresses

Due to the differences observed between wild-type and *RCA/AVP1* co-overexpressing plants under combined drought and heat stress conditions in relation to biomass and yield, we analyzed the transcript levels of a few selected marker genes that are usually upregulated

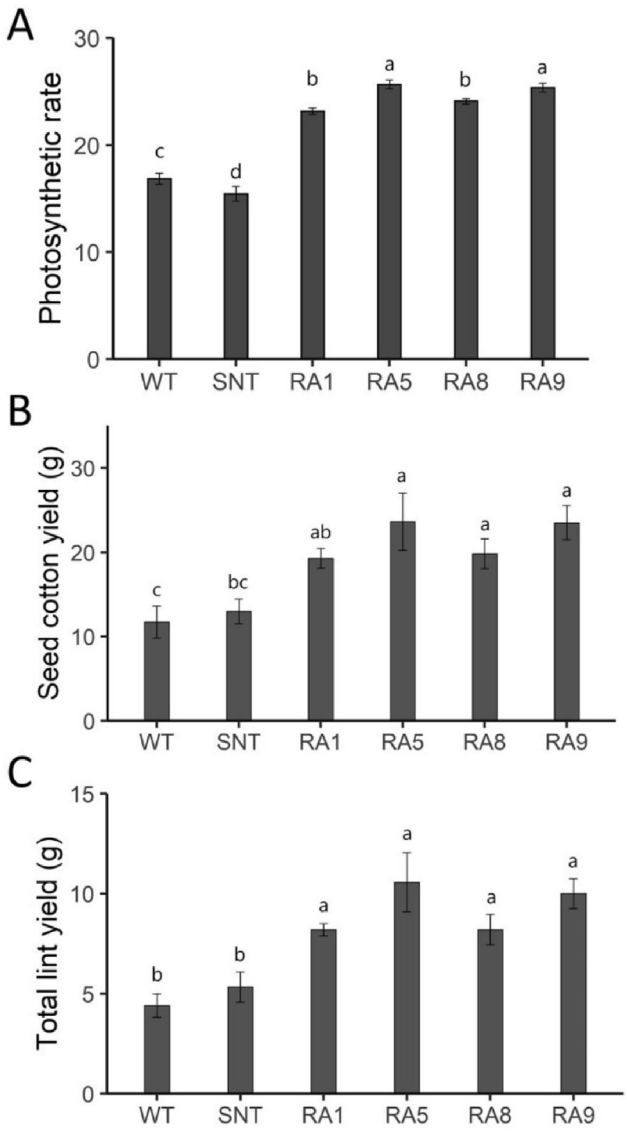

Fig 6. Performance of *RCA/AVP1* co-overexpressing plants in field condition in 2018. **A**. Analysis of photosynthetic rates of *RCA/AVP1* co-overexpressing plants under rain-fed condition. **B**. Seed fiber yield of cotton plants under rain-fed condition. Results are the means ± SE (n = 180 plants). **C**. Total lint yield of cotton plants under rain-fed condition. Results are the means ± SE (n = 180 plants). WT, wild-type plant; SNT, segregated non-transgenic plant; AVP1, *AVP1*-overexpressing plant; RA1, RA5, RA8, and RA9, four independent *RCA/AVP1* co-overexpressing plants. Samples denoted by different letters are significantly different ($P < 0.05$, Tukey correction).

under abiotic stress conditions, in particular, during drought and heat treatment. Plants from wild-type and one *RCA/AVP1* co-overexpressing line, RA9, were subjected to a period of combined drought and heat stress treatment, then total RNAs isolated from these plants were used for real-time quantitative PCR analysis. Our results showed that transcripts from six out of eight genes analyzed were up-regulated in the *RCA/AVP1* co-overexpressing line RA9 in comparing to that of wild-type cotton (Fig. 7). These marker genes include antioxidant genes *APX* (ascorbate peroxidase) and *SOD* (super-oxide dismutase), heat shock protein genes *HSP70* and *HSP90*, and transcriptional factors genes *RD29A* and *DREB26*, indicating that the *RCA/AVP1* co-overexpressing plants have higher capacities in antioxidation, protein homeostasis, and more efficient drought response than wild-type plants. Interestingly, the transcript level of an ABA biosynthetic gene *NCED3* that is normally responsive to drought stress was found to be at similar levels between wild-type and the *RCA/AVP1* co-overexpressing line RA9, and the transcript from another drought responsive gene *RD22* was found to be lower in the *RCA/AVP1* co-overexpressing line RA9, which was not expected.

Discussion

Our experiments demonstrate that co-overexpression of *RCA* and *AVP1* in cotton can significantly enhance tolerance to drought and salt stresses in greenhouse, which is consistent with the results when *AVP1* was overexpressed in tomato, rice, and cotton (Park et al. 2005; Zhao et al. 2006; Pasapula et al. 2011). Co-overexpressing *AVP1* with *SsNHX1* in rice further enhanced salt tolerance, but at a cost of decreased photosynthesis (Zhao et al., 2006). Salt-treated plants were shown to have decreased net photosynthesis, since NaCl could cause both ionic and osmotic effects that can decrease the activity of the electron transport chain, which in turn irreversibly damages PSI and PSII (Greenway and Munns 1980; Papageorgiou et al., 1998). We previously showed that overexpression of *AVP1* could protect photosynthesis to some extent when *AVP1*-overexpressing plants were subjected to NaCl treatment (Pasapula et al., 2011). Consistent with this early finding, *RCA/AVP1* co-overexpressing plants also showed enhanced net photosynthesis under salt or drought stress when compared to wild-type plants. Overexpression of *AVP1* in cotton also led to improved tolerance to drought as well as having higher stomatal conductance (Pasapula et al. 2011), which was attributed to the enhanced root development in *AVP1*-overexpressing plants (Li et al., 2005; Park et al. 2005). Therefore, it is not unexpected that *RCA/AVP1* co-overexpressing plants would be more tolerant to combined drought and salt stresses or combined drought and heat stresses, with respect to photosynthesis and having a larger root mass than wild-type plants. The higher photosynthetic rates under combined drought and salt stresses for *RCA/AVP1* co-overexpressing plants can be attributed to the overexpression of *AVP1* (Fig. 2), and the highest photosynthetic rates under combined drought and heat stresses for *RCA/AVP1* co-overexpressing plants could be attributed to the overexpression of both *AVP1* and the creosote *RCA* (Fig. 3).

Under normal growth conditions, *RCA/AVP1* co-overexpressing plants did not show a significant difference in seed fiber yield compared to wild-type cotton, but under combined drought and salt stresses, *RCA/AVP1* co-overexpressing plants consistently outperformed wild-type and *AVP1*-overexpressing cotton in three independent experiments, all conducted in the greenhouse. Our experiments clearly showed that *RCA/AVP1* co-overexpressing plants produced 50% more seed fiber than wild-type cotton, and *AVP1*-overexpressing cotton produced 30% more seed fiber than wild-type cotton (Fig. 2). The difference between *RCA/AVP1* co-overexpressing cotton and *AVP1*-overexpressing cotton was a 20% further improvement in seed fiber yield, which should be contributed by the overexpression of *RCA*. Although it is easier to understand the higher photosynthetic rates for *RCA/AVP1* co-overexpressing plants under combined drought and heat stresses, RCA's role in improving seed fiber yield for *RCA/AVP1* co-overexpressing plants under combined drought and salt stresses is not clear. Perhaps, RCA's increased carboxylation rate due to higher activation of RuBP (Vcmax) might be a reason for cotton grown under normal temperature as well as at elevated temperatures. Further, Rubisco activity and activation are negatively affected by drought stress, causing a decrease in photosynthesis. Nevertheless, since creosote is a plant adapted to hot dessert conditions, creosote RCA likely performs better than wild-type RCA under these conditions, thereby the *RCA/AVP1* co-overexpressing cotton having a better photosynthetic rate even under combined drought and salt stresses. Additionally, as creosote RCA is driven by a light inducible promoter, under optimum irradiance, the transgenic plants co-overexpressing *AVP1* and

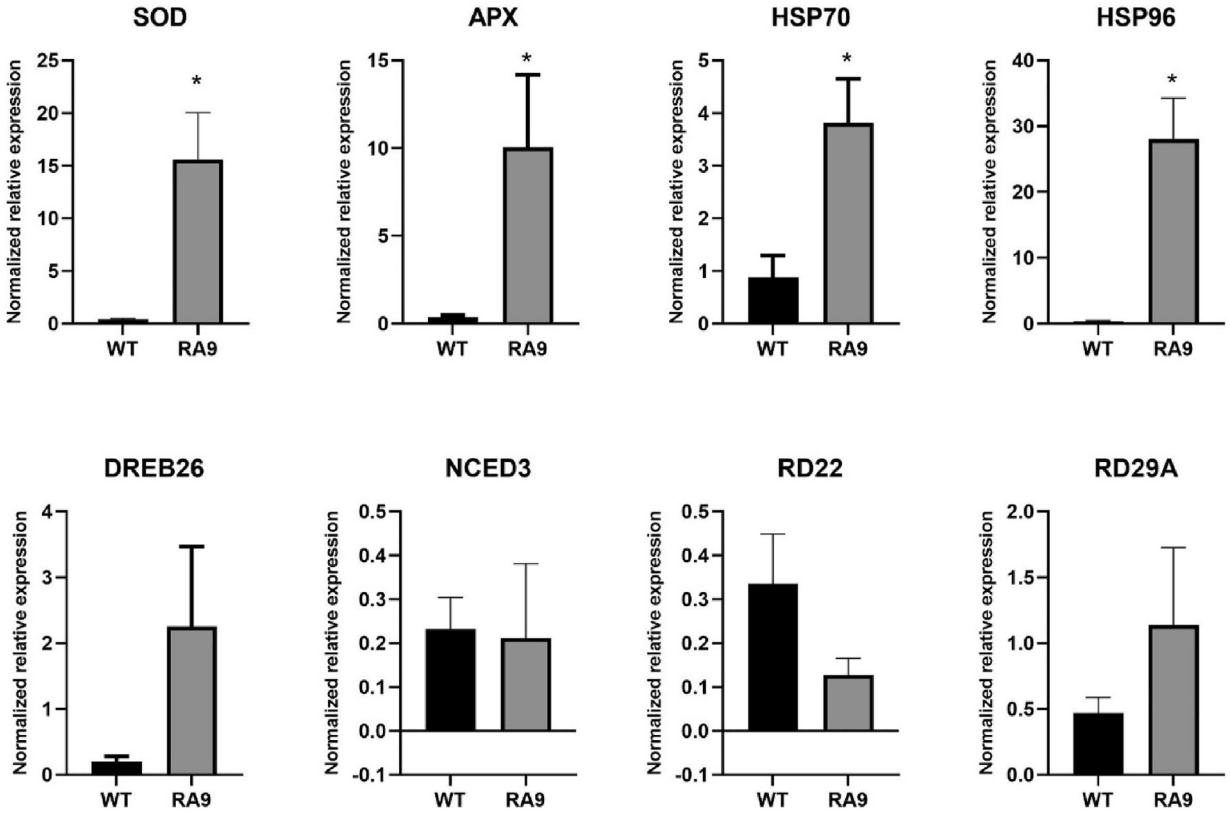

Fig. 7. Quantitative real-time PCR analysis of eight stress-related genes in wild-type and *AVP1/RCA* co-overexpressing plants under drought and heat stress conditions. Data are means ± SE (n = 3). WT, wild-type plant; RA9, *AVP1/RCA* co-overexpressing plants (line No. 9). The genes used for the quantitative real-time PCR analysis are shown above each figure.

RCA might have better adaptability to water deficit by maintaining photosynthesis, which reflects a better tolerance to drought and salt stresses.

As has been shown by others previously (Crafts-Brandner and Salvucci, 2000, 2004; Medlyn et al. 2002; Wang et al. 1992), moderate heat stress, such as the exposure to 37 ℃ used in this study, causes the inactivation of Rubisco activase, the enzyme that reduces the inactivation of Rubisco by inhibitory sugar phosphates. With the overexpression of a thermally stable form of Rubisco activase in Arabidopsis, CO_2 assimilation is enhanced through improved Rubisco activation (Kurek et al. 2007, Kumar et al. 2009). Our *RCA/AVP1* co-overexpressing cotton plants were more tolerant than wild-type plants when exposed to 37 ℃ three hours per day for 4 weeks, as indicated by their ability to maintain higher rates of CO_2 assimilation than wild-type and segregated non-transgenic plants before, during, and after heat exposure (Fig. 3). The recovery of net photosynthesis was always better for transgenic plants, especially for *RCA/AVP1* co-overexpressing cotton being closer to their value before the next heat cycle. The values of Vcmax before, during, and after heat exposure indicate that the carboxylation capacity of the plants overexpressing creosote *RCA* is always the highest in comparing to *AVP1*-overexpressing and wild-type cotton plants (Fig. 4), suggesting that the activation state of Rubisco in *RCA/AVP1* co-overexpressing plants is the reason for higher tolerance under combined drought and heat stresses. Moreover, leaf temperatures of *RCA/AVP1* co-overexpressing plants were significantly lower than *AVP1*-overexpressing cotton and wild-type cotton plants (Fig. 3B). It is possible that co-overexpression of *AVP1* and *RCA* further boosts the root development, which leads to even more effective water absorption than *AVP1*-overexpressing plants, resulting in better evaporative cooling and lower leaf temperatures. In addition to creosote RCA thermotolerance, this evaporative cooling might have also assisted to execute RCA activity more efficiently in *RCA/AVP1* co-overexpressing plants, thereby having a better tolerance to combined drought and heat stresses.

Cotton plants overexpressing *AVP1* showed enhanced photosynthesis, boll numbers, and fiber yield under field conditions (Pasapula et al. 2011). We conducted two field trials with *RCA/AVP1* co-overexpressing plants in 2016 and 2018, using one *AVP1*-overexpressing plant from Pasapula study (2011) as a reference line, in order to study the value of overexpression of the *RCA* gene from creosote. We found that under irrigated conditions, few differences in photosynthesis and seed fiber yield were seen between wild-type and transgenic cotton plants in the field (Fig. 4A). However, under rain-fed conditions in both 2016 and 2018, *RCA/AVP1* co-overexpressing cotton plants displayed higher net photosynthesis compared to wild-type and segregated non-transgenic cotton plants (Fig. 5 and Fig. 6). It is clear that data from field experiments were more varied and in wide-ranging when comparing to the data obtained from experiments conducted in greenhouse and growth chambers, as there were more variable factors in the field that can dramatically affect plant growth and development. Yet, the general conclusions drawn from both laboratory study and field study are clear: *RCA/AVP1* co-overexpressing cotton plants are more tolerant to drought, salt, and heat stresses, and *RCA/AVP1* co-overexpressing plants could produce much higher fiber yield in dryland conditions in the field. The total lint yield of *RCA/AVP1* co-overexpressing cotton was 100% higher than that of wild-type cotton in 2016 and the total lint yield of *RCA/AVP1* co-overexpressing cotton was 90% higher than that of wild-type cotton in 2018 under rain-fed conditions.

One of the key regulators in plant response to dehydration and water deficit stress is the phytohormone abscisic acid (ABA), where

a variety of ABA-induced genes are upregulated when plants are exposed to drought stress (Shinozaki and Yamaguchi-Shinozaki, 2007; Singh et al., 2017). Among these, RD22 and RD29A are some of the most common genes whose transcript levels in plants are up-regulated under drought stress conditions (Shinozaki and Yamaguchi-Shinozaki, 2007). In our qRT-PCR analysis (Fig. 7), while there was an upregulation in *RD29A* transcript levels in *RCA/AVP1* co-overexpressing line RA9, but not *RD22*. Heat shock proteins (HSPs) including HSP70 and HSP96 are a class of proteins ubiquitously present in plants, which play a pivotal role in heat stress and thermotolerance, showing an upregulation at transcript levels when conferring enhanced tolerance to heat stress (Vierling, 1991). Our data are consistent with the literature (Fig. 7). Additionally, the transcript levels of two reactive oxygen species (ROS) scavenging enzyme-encoding genes *APX* and *SOD* were highly upregulated under combined drought and heat stress conditions, suggesting that *RCA/AVP1* co-overexpressing plants might have a higher capacity in antioxidation metabolism. The transcript levels of several ABA and drought inducible genes were analyzed in this study and the results are difficult to explain. The transcript levels of two genes, *DREB26* and *RD29A*, were significantly up-regulated under combined drought and heat stresses (Fig. 7), which is in agreement with the positive roles played by these two genes under combined drought and heat stress condition. However, we cannot explain why the transcripts of the other two genes *NCED3* and *RD22* were not up-regulated in *RCA/AVP1* co-overexpressing plants. In fact, the transcript of *RD22* was reduced in the *RCA/AVP1* co-overexpressing line RA9. It appears that there is a deviation in the transcript levels of the stress related marker genes from the conventional expectation of being upregulated. The *RCA/AVP1* co-overexpressing plants showed higher transcript levels of *RCA* than *AVP1* (Fig. 1B), thus, it might be a contributing factor to higher tolerance exhibited in these plants due to a more efficient photosynthesis under the combined drought and heat stresses in comparing to wild-type plants, rather than following the canonical regulatory pathways conferring higher tolerance. Nevertheless, a sound scientific explanation for the observation is lacking at this time.

Genetic engineering for increased abiotic stress tolerance can benefit from overexpressing two or more genes that confer different beneficial traits, which can further improve crop performance under abiotic stress conditions, leading to significantly higher crop yield. The findings in our laboratory over the last 10 years provided strong evidence that this strategy might work in agriculture. For example, Shen et al. (2015) showed that co-overexpression *AVP1* and *AtNHX1* in cotton further improves drought and salt tolerance, leading to higher fiber yield in field condition. Esmaeili et al. (2021) demonstrated that co-overexpression of *AVP1* and *OsSIZ1* in cotton substantially improved drought, heat and salt tolerance, leading to the doubling of fiber yield under rain-fed conditions in the field. Our research on engineering plants like *RCA/AVP1* co-overexpressing cotton is another step towards achieving food security for the world. There are currently no crops available on the market that show high tolerance to abiotic stresses, and we hope that our research will stimulate more similar research activities, from which high yield and high stress tolerant crops will be created.

Materials and methods

Cotton transformation

The *AVP1/RCA* co-overexpression vector used by Wijewardene et al. (2020) was introduced into the *Agrobacterium tumefaciens* strain GV3101, which was then used to transform wild-type cotton Coker 312 according to the protocol by Bayley et al. (1992). In this protocol, we used a callus induction media to promote callus tissue growth, which was maintained until callus aged 6–9 months and then they were placed in a liquid culture media to induce embryo formation with a final media for root induction. Ex-plants were taken from the tissue culture media and moved into soil once true leaves and roots were formed.

Molecular confirmation of transgenic cotton plants

Putative transgenic cotton plants were first grown in 20 mm test tubes in Murashige and Skoog (MS) medium containing kanamycin (50 mg/l^{-1}), and the T_1 plants that developed lateral roots were transferred to greenhouse to obtain T_2 (second transgenic generation) plants. From the T_1 plants, genomic DNAs were extracted from young leaves using the CTAB DNA isolation method (Stefanova et al., 2013), which was used for PCR analysis of transgene in putative transgenic plants. The PCR positive lines were then used for total RNA isolation using the RNA extraction kit from Sigma (i.e., Spectrum Plant Total RNA Kit). The RNA concentration was determined by using the Nano-Drop equipment (Fisher Scientific) and 20 μg of total RNAs from each transgenic line were used for RNA blot experiment. The primer sets used for creating probes are listed in Supp. Table 2. Transgenic lines with high transcript levels for *AVP1* and *RCA* were identified and allowed to proceed to the T_3 generation for obtaining homozygous plants. Four independent transgenic lines, RA1, RA5, RA8, and RA9, were identified and used for all subsequent experiments. In the DNA blot analysis, an overnight digestion of 30 μg of genomic DNAs from WT and two *RCA/AVP1* co-overexpressing cotton plants (i.e., RA5 and RA9) was carried out with the restriction enzyme *Eco* RI, then separated by electrophoresis and blotted onto a Nylon membrane (Amerisham Hybond-N^+). A P^{32}-labelled gene-specific probe for *AVP1* was created as the hybridization probe (Supp. Table 2). DNA hybridization was conducted as described by Pasapula et al. (2011).

For Western blot analysis, two transgenic lines, RA5 and RA9, were used along with the wild-type cotton. These plants were grown under normal growth conditions for four weeks, then they were transferred to a walk-in growth chamber with a programmed heat treatment of 37 ℃ for 3.5 h and 28 ℃ for the remainder of time each day. After two weeks of heat stress treatment, the first two leaves from the top were collected and immediately put into liquid nitrogen before storing in a −80 ℃ freezer. Plant material from each genotype was ground to fine powder in a chilled mortar and pestle using liquid nitrogen, followed by total protein extraction and quantification as described in Wijewardene et al. (2020). Twenty micrograms of total proteins from wild-type, RA5 and RA9 were separated on a 10% SDS-polyacrylamide gel, after mixing the samples 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% bromophenol blue), boiling for 5 min and a brief spinning down in a microfuge before electrophoresis. Proteins from SDS-PAGE were transferred to a PVDF membrane (Sequi-blot™ PVDF from Bio-Rad, Hercules, California) overnight using Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol [v/v] pH 8.3), followed by washing the membrane three times with TBST (TBS buffer with 0.1% Tween [v/v]), after which the non-specific sites of the membrane was blocked using 5% BSA [w/v] with TBST at room temperature for one hour with continuous shaking. Then, the membrane was washed three times with TBST with continuous shaking before incubating it with the antibody raised against RCA from PhytoAB Inc. (San Jose, California) at room temperature for 1.5 h. The membrane was rinsed again with TBST three times and incubated with the alkaline phosphatase (AP) conjugated-goat anti rabbit antibodies from Bio-Rad (Hercules, California) for one hour, followed by washing and color development using the AP conjugate substrate kit of Bio-Rad (Hercules, California) according to manufacturer's instructions. Tubulin from PhytoAB Inc. (San Jose,

California) was used as the loading control to compare the band intensity of each genotype and compare with that of wild-type cotton.

Combined drought and salt stresses in greenhouse

Cotton plants were grown in 11-L pots under normal growth condition for four weeks, then drought stress was applied as 25% water replacement (i.e., 2000 ml of water for well-watered plants whereas 500 ml of water for drought stress treatment every two days). After 2 weeks under drought stress treatment, salt stress treatment began. Each salt stress treatment consisted of 2 rounds of 500 ml of 50 mM NaCl solution for irrigation plus 4 rounds of 500 ml of 100 mM NaCl solution for irrigation. After the last salt treatment, gas-exchange measurements were conducted with Li-Cor 6400 instrument (LI-COR Biosciences, Lincoln, NE, USA). Measurements were made on a full-sun day (non-irrigation day). Gas-exchange measurements were also made with well-watered plants after irrigation on the same day. Drought stress treatments were continued until harvest. Leaf area and root biomass were measured at the end of salt treatment. Total seed fiber yield was obtained for each plant. Ten replications were used in the experiment and the experiment was repeated three times.

Combined drought and heat stresses in growth chamber

Cotton plants were grown in greenhouse in the 11-L pots with Metro Mix 852 soil for 4 weeks, then they were moved into a walk-in growth chamber (i.e., Conviron BDW 120) that was set with a photon flux density of 1200 μmol m^{-2} s^{-1} for 14 h per day and darkness for 10 h. The heat treatment was applied as follows: starting from 28 ℃ at 1:00 pm and reaching to 37 ℃ at 1:30 pm, staying at 37 ℃ for 3 h, then from 37 ℃ to 28 ℃ from 4:30 pm to 5:00 pm. The temperature during the rest of the day and night periods was 28 ℃. The drought treatment was imposed immediately after moving into the growth chamber as a 25% water replacement (~250 ml water per day). At 8 weeks of time (4 weeks after the treatment of combined drought and heat stresses), gas-exchange measurements were made before the heat treatment, during the heat treatment, and 3 h after the heat treatment. Plants were left in the growth chamber under combined drought and heat stresses until harvest. Total seed fiber yield per plant was obtained at the end of the experiment. Eight replications were used in the experiment and the experiment was repeated three times.

Gas-exchange measurements

The top third nodal leaf was used for all gas exchange measurements using the Li-Cor 6400 instrument (LI-COR Biosciences, Lincoln, NE, USA). Reference CO_2 was set to 40 Pa pCO_2 (ambient CO_2 conditions of 400 ppm). For greenhouse condition, the irradiance was set to 1800 μmol m^{-2} s^{-1} and conducted on days of full sun and no irrigation. The block temperature for the leaf chamber was set at 28 ℃. In the field conditions, the irradiance was set to 2000 μmol m^{-2} s^{-1}, and the block temperature was set at 28 ℃. In the growth chamber, the irradiance was set to 1200 μmol m^{-2} s^{-1}, and the block temperature was set at 25 ℃. In the field and greenhouse, gas-exchange measurements were started at 9:00 am and ended before 12:00 pm. Vcmax was calculated from one-point measurements referenced by Kauwe et al. (2016) and Smith et al. (2016).

Field test

Field tests were conducted at the Experimental Farm of USDA-ARS Cropping Systems Research Laboratory in Lubbock, TX. In 2016, the experiment was conducted under two different conditions: well-watered and rain-fed conditions (except in the beginning the soil was wet for germination). Cotton plants were planted on June 5th with nine replications for dryland and four replications for irrigated. The number of seed sown/replicate was 45 with twin row planting and a randomized block design. Data for net photosynthesis, boll counts, node counts, and final seed fiber yield were collected for plants in one-meter plots with stand count taken within the meter quadrat. Total lint yield was obtained, and the micro gin samples were analyzed at the end of the experiments. For irrigated plants, 2.5 cm of irrigation was applied to plants on each Monday. In 2018, only dryland field test was performed, again initial irrigation was applied to get the soil wet for seed germination. Cotton plants were hand-planted on June 14th with 6 replicates in a single row and 30 seeds per replication. Photosynthesis, boll counts, node counts, stand counts, and final seed fiber yield per plant were obtained from 1 m plots.

Statistical analysis

An analysis of variance (ANOVA) and post-hoc pairwise comparisons using Tukey's honest significant difference (HSD) test were conducted in R version 4.0.2 (RStudio Team, 2016) to assess for significant differences between group means. A significance level of $\alpha = 0.05$ was used for all statistical tests.

CRediT authorship contribution statement

Jennifer Smith: was responsible for formal analysis, data curation, investigation, and writing the draft. **Inosha Wijewardene:** was responsible for investigation, validation, and editing the draft. **Yifan Cai:** was responsible for investigation and validation. **Nardana Esmaeili:** was responsible for investigation, validation, and editing the draft. **Guoxin Shen:** was responsible for conceptualization and funding acquisition. **Eric Hequet:** was responsible for funding acquisition and supervision. **Glen Ritchie:** was responsible for funding acquisition and supervision. **Paxton Payton:** was responsible for funding acquisition and supervision. **Hong Zhang:** was responsible for conceptualization, funding acquisition, supervision, and editing the draft.

Data availability

Data will be made available on request.

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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