

Co-overexpression of *AVP1* and *AtNHX1* in Cotton Further Improves Drought and Salt Tolerance in Transgenic Cotton Plants

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Abstract Salinity and drought are two major environmental stresses that limit the growth and productivity of cotton. To improve cotton's drought and salt tolerance, transgenic cotton plants expressing the *Arabidopsis* vacuolar Na^+/H^+ antiporter gene *AtNHX1* and H^+ -pyrophosphatase gene *AVP1* were produced by cross-pollination of two single-gene-overexpressing plants. The salt tolerance and drought tolerance were further enhanced by simultaneously overexpressing *AVP1* and *AtNHX1* in comparison to *AVP1* or *AtNHX1* single-gene-overexpressing plants and to wild-type plants. Plant height, boll number, and fiber yield of *AVP1/AtNHX1*-co-overexpressing plants were higher than those of *AVP1*-overexpressing, *AtNHX1*-overexpressing, segregated non-transgenic line,

and wild-type plants under saline and drought conditions. The photosynthetic rate of *AVP1/AtNHX1*-co-overexpressing plants was significantly higher than that of single-gene-overexpressing and wild-type plants under 200 mM NaCl treatment. In addition, the root systems of *AVP1/AtNHX1*-co-overexpressing plants were larger than those of single-gene-overexpressing and wild-type plants, which was likely due to increased auxin polar transport in the root systems of the *AVP1/AtNHX1*-co-overexpressing plants. Moreover, these *AVP1/AtNHX1*-co-overexpressing cotton plants produced 24 % higher fiber yield under low-irrigation conditions and 35 % higher fiber yield under dryland conditions as compared to wild-type cotton in the field.

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Introduction

Drought and salinity are two major environmental factors that negatively affect plant growth and productivity (Bartels and Sunkar 2005). Plants, on the other hand, have evolved several strategies to deal with these stresses (Bray 1997). One strategy is to activate stress-related transcriptional factors that initiate expression of a set of genes, which allows plants to adapt to the stressful conditions and repair the damages caused by these stresses (Mittler and Blumwald 2010). Other strategies include quick changes in cellular metabolisms such as changes in enzyme activities and changes in ion transporter activities, which allow cellular homeostasis to be reestablished under stressful conditions (Bartels and Sunkar 2005). In the salt stress response, for example, plants may take two actions:

actively exporting Na^+ out of plant cell through the action of the plasma membrane-bound Na^+/H^+ antiporter or actively importing Na^+ into plant vacuoles through the action of the vacuolar membrane-bound Na^+/H^+ antiporter (Agarwal et al. 2013; Munns and Tester 2008). Transporting Na^+ out of the cytoplasm or into the vacuoles is one of the most efficient mechanisms in salinity tolerance, which could reduce Na^+ toxicity in the cytoplasm and at the same time reduce water potential in vacuoles, thus increasing salt tolerance by enhancing water uptake under saline conditions (Agarwal et al. 2013). Consequently, overexpressing genes that encode the plasma membrane-bound Na^+/H^+ antiporter or the vacuolar membrane-bound Na^+/H^+ antiporter could make plants more salt tolerant. Indeed, over the last 15 years, numerous studies have confirmed this mechanism. For examples, through overexpression of the *Arabidopsis* vacuolar membrane-bound Na^+/H^+ antiporter gene *AtNHX1*, salt-tolerant *Arabidopsis*, rapeseed, and tomato were obtained (Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001) and through overexpression of the *Arabidopsis* plasma membrane-bound Na^+/H^+ antiporter gene *SOS1*, salt-tolerant *Arabidopsis* plants were obtained (Shi et al. 2002).

The activities of *SOS1* and *AtNHX1* are energized by the proton electrochemical gradient (PEG) across membranes that are generated by the plasma membrane-bound proton pumps (e.g., H^+ -adenosine triphosphatase) and the vacuolar membrane-bound proton pumps, respectively (Gaxiola et al. 2007). Theoretically, increased expression of genes that encode the plasma membrane-bound proton pump or the vacuolar membrane-bound pump would also increase salt tolerance. Indeed, overexpression of the *Arabidopsis* gene *AVP1* that encodes a vacuolar membrane-bound proton pump, H^+ -pyrophosphatase, leads to increased salt tolerance in *Arabidopsis* and tomato (Gaxiola et al. 2001; Park et al. 2005). Interestingly, overexpression of *AVP1* also leads to increased drought tolerance (Gaxiola et al. 2001; Park et al. 2005), and this phenotype was attributed to stronger auxin polar transport in the root systems of *AVP1*-overexpressing plants, which leads to robust root development that helps water absorption under water-deficit conditions (Li et al. 2005). Overexpression of *AVP1* results in significantly improved salt and drought tolerance in transgenic plants, indicating a great potential for using *AVP1* to improve stress tolerance in crops. As expected, drought- and salt-tolerant crops such as rice, cotton, and peanut were obtained when *AVP1* was introduced into these crops (Pasapula et al. 2011; Qin et al. 2013; Zhao et al. 2006). However, the salt tolerance conferred by overexpressing *AtNHX1* or *AVP1* appears limited to around 200 mM NaCl, suggesting that the PEG generated by native proton pumps on vacuolar membranes might become limiting in the *AtNHX1*-overexpressing plants or the

Na^+/H^+ antiporters like *AtNHX1* might become limiting in *AVP1*-overexpressing plants under high salt concentrations. Consequently, Gaxiola et al. (2002) proposed that coupled overexpression of *AVP1* and *AtNHX1* might further increase drought and salt tolerance in transgenic plants.

Cotton is a vital economic crop, accounting for most natural fiber supply in the world. Cotton is regarded as one of the relatively more salt- and drought-tolerant crops; therefore, it is a choice for the reclamation of saline, arid, and semiarid land. However, cotton's growth and development, especially its yield and fiber quality, are negatively affected by salt and drought stresses (Dong 2012). Thus, improving cotton's tolerance to salt and drought will have a huge positive impact on cotton's quality and production. Although traditional breeding has been successful in bringing out today's cotton varieties that are widely grown over the world, it is becoming more difficult in finding drought-tolerant alleles or salt-tolerant alleles that would allow introgression of these tolerant alleles into cultivated cotton varieties. Finding genes that confer both drought and salt tolerance at the same time is even more difficult. The genetic engineering approach, however, offers an efficient way to improve drought and salt tolerance in cotton. As described above, *AVP1* and *AtNHX1* are key enzymes involved in salt tolerance in *Arabidopsis*, which have been successfully demonstrated in previous studies. For instance, *AtNHX1* overexpression confers increased salt tolerance in cotton (He et al. 2005) and *AVP1* overexpression leads to significantly improved drought and salt tolerance in cotton (Pasapula et al. 2011). However, to further improve drought and salt tolerance in cotton, co-overexpression of *AVP1* and *AtNHX1* might be needed.

With the intention to test if an enhanced PEG coupled with an increased Na^+/H^+ antiporter activity in the vacuolar membranes would further improve salt and drought tolerance, *AVP1/AtNHX1*-co-overexpressing cotton plants were generated by crossing *AVP1*-overexpressing cotton with *AtNHX1*-overexpressing cotton plants. Our data indicate that *AVP1/AtNHX1*-co-overexpressing cotton plants are indeed more drought and salt tolerant than *AVP1* or *AtNHX1* single-gene-overexpressing plants. The *AVP1/AtNHX1*-co-overexpressing plants maintained the highest photosynthetic rate under saline conditions, and they produced the highest biomass under water-deficit conditions. More importantly, they produced the highest yields under dryland and reduced-irrigation conditions in the field. This work confirmed the hypothesis of Gaxiola et al. (2002) that coupled overexpression of a vacuolar proton pump gene and a vacuolar Na^+/H^+ antiporter gene could further improve drought and salt tolerance in transgenic plants.

Materials and Methods

Plant Materials

The *AtNHX1* and *AVP1* single-gene transgenic cotton plants, N79 and 5, were made by He et al. (2005) and Pasapula et al. (2011), respectively, with Coker 312 as their background genotype. The *AVP1/AtNHX1*-co-overexpressing cotton plants were generated by crossing the *AVP1*-overexpressing line 5 to the *AtNHX1*-overexpressing line N79. The F₁ plants from the cross between N79 (♀) and 5 (♂) were named CoO1, and the cross between 5 (♀) and N79 (♂) were named CoO2. A segregated non-transgenic (SNT) line derived from line 5 was used as a negative control, and the wild-type (WT) cotton used in this study was Coker 312. After confirmation by DNA and RNA blot analysis, the CoO1 plants that overexpress both *AVP1* and *AtNHX1* were used for subsequent physiological analysis. All CoO1 plants used in the physiological experiments were also verified by PCR to insure the presence of the transgenes *AVP1* and *AtNHX1*.

Growth Conditions and Stress Treatments

WT, SNT, 5, N79, and CoO1 plants were used in all stress treatment experiments. Cotton seeds were first germinated in 50×80-cm trays and grown in a greenhouse at 28±2 °C. For salt treatment, 2-week-old cotton plants were irrigated with salt solutions in an incremental manner: 50 mM NaCl for 7 days (200 ml daily), then 100 mM for 7 days, 150 mM for 7 days, and finally 200 mM for 21 days. The experiment was repeated four times. Plant biomass was analyzed 3 weeks after the addition of 200 mM NaCl. Fiber yield (with seeds) and boll number were determined after the cotton matured completely at the end of experiment. The well-watered control plants were placed under the same growth conditions except being irrigated with normal water. For drought experiment, 21-day-old cotton plants were withheld irrigation for 5 days, and then plants were rewatered with 500 ml of water per pot every other day for 34 days. Plant height was measured on day 20, day 30, and day 40. Plant biomass was analyzed 39 days after the start of reduced irrigation. The irrigation was increased to 800 ml of water every other day after day 60. Fiber yield and boll number per plant were determined after all bolls were fully opened.

DNA and RNA Blot Analysis

Genomic DNAs and total RNAs were isolated from the leaves of WT, SNT, *AVP1*-overexpressing line 5, *AtNHX1*-overexpressing line N79, and *AVP1/AtNHX1*-co-overexpressing lines CoO1 and CoO2 using protocols described by He et al. (2005) and Pasapula et al. (2011). The experimental conditions for DNA blot and RNA blot analyses were also

the same as those described by He et al. (2005) and Pasapula et al. (2011).

Leaf Gas Exchange Measurements

Leaf gas exchange measurements were taken during salt treatment (10 days into 200 M NaCl treatment) with a portable photosynthesis system (Li-6400, LI-COR Inc., Lincoln, NE, USA). Young, fully expanded, intact leaves of salt-treated and control plants were used for gas exchange measurements. Net CO₂ assimilation rate, stomatal conductance, and transpiration were assessed with the Li-6400 setting at a CO₂ concentration of 400 μmol/mol, relative humidity 50 %, chamber temperature 28 °C, air flow 500 μmol/s, and photon flux density 1,500 μmol/m²/s. Five replicates were taken for each sample.

Naphthylphthalamic Acid Treatment

Cotton seeds of WT, SNT, *AVP1*-overexpressing line 5, *AtNHX1*-overexpressing line N79, and *AVP1/AtNHX1*-co-overexpressing lines CoO1 were surface sterilized with 5 % bleach and rinsed with sterile water. The sterilized seeds were then grown in 2×22-cm tubes containing MS medium with or without 100 μM of naphthylphthalamic acid (NPA). The root development was examined 7 days after planting.

Field Experiment

WT, SNT, *AVP1*-overexpressing line 5, *AtNHX1*-overexpressing line N79, and *AVP1/AtNHX1*-co-overexpressing plants were field-tested at the USDA-ARS Experimental Farm in Lubbock, TX, USA in 2010. Cotton plants were tested under dryland and low-irrigation conditions, and three independent replicates, each with 30 plants, in randomized plots for each condition. For the dryland condition, plants were grown in soil without further irrigation after sowing. For the low-irrigation condition, plants were irrigated using drip irrigation; the irrigation level was 25 % replacement of estimated evapotranspiration minus rainfall in the Lubbock area during the experimental year. The frequency of irrigation in the absence of rain was about every 7 days. Standard agronomical practices were performed for weed control and pesticide application. Boll number per plant and fiber yield per plant were analyzed at the end of the growth season after all bolls had opened.

Statistical Analysis

Statistical analysis was performed using the SPSS package program version 11.5 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way ANOVA, followed by the least significant difference (LSD) test at a 95 % confidence level ($p < 0.05$). The values are reported as means with their standard error for all results.

Results

Generation and Molecular Characterization of *AVP1/AtNHX1*-Co-overexpressing Cotton Plants

To investigate the effect of co-overexpression of the vacuolar H^+ -pyrophosphatase gene *AVP1* and the vacuolar Na^+/H^+ antiporter gene *AtNHX1* in cotton, *AVP1/AtNHX1*-co-overexpressing plants were created by crossing *AVP1*-overexpressing plants (line 5, Pasapula et al. 2011) to *AtNHX1*-overexpressing plants (line N79, He et al. 2005). The F_1 plants created were analyzed by using RNA blot and DNA blot analysis. Two independent F_1 populations, CoO1 and CoO2, contained both *AVP1* and *AtNHX1* transcript (Fig. 1a), whereas the parental line 5 contained *AVP1* transcript only and the parental line N79 contained *AtNHX1* transcript only. WT and SNT plants contained neither *AVP1* nor *AtNHX1* transcript. Furthermore, when genomic DNAs were isolated from these plants, the *AVP1* transgene was found only in the *AVP1*-transgenic line 5 and CoO1 plants and the *AtNHX1* transgene was found only in the *AtNHX1*-transgenic line N79 and CoO1 plants (Fig. 1b), confirming that the F_1 progenies of the crossing between *AVP1*-transgenic cotton and *AtNHX1*-transgenic cotton indeed contained both transgenes and both transgenes were expressed in the F_1 progenies.

Co-overexpression of *AVP1* and *AtNHX1* in Cotton Leads to Greater Salt Tolerance than Transgenic Cotton Overexpressing only *AVP1* or *AtNHX1*

The F_1 progenies from the crossing between *AVP1*-transgenic cotton and *AtNHX1*-transgenic cotton contained both transgenes, suggesting that these F_1 plants could be used as *AVP1/AtNHX1*-co-overexpressing plants for physiological analyses. We therefore used these F_1 plants for a salt tolerance test. In this experiment, we used WT and SNT as negative controls and *AVP1*-transgenic line 5 and *AtNHX1*-transgenic line N79 as reference lines. Cotton plants were first grown in a greenhouse with normal irrigation for 14 days, and then salt stress was applied by irrigating plants with increasing concentrations of NaCl. Before salt treatment or irrigation with water, there were no phenotypic differences among these plants (Fig. 2). However, 42 days after the start of NaCl treatment (50 mM for 7 days, 100 mM for 7 days, 150 mM for 7 days, and 200 mM for 21 days), the *AVP1/AtNHX1*-co-overexpressing plants demonstrated the best growth among all plants (Fig. 3a). The biomass analysis indicated that *AVP1/AtNHX1*-co-overexpressing plants produced significantly more fresh and dry shoot biomass during this time (Fig. 3b). After the 200 mM NaCl treatment for 21 days, normal water was used again for irrigation until the end of the experiment. Although all cotton plants survived the salt treatment, WT, SNT, and *AVP1*-transgenic line 5 developed earlier

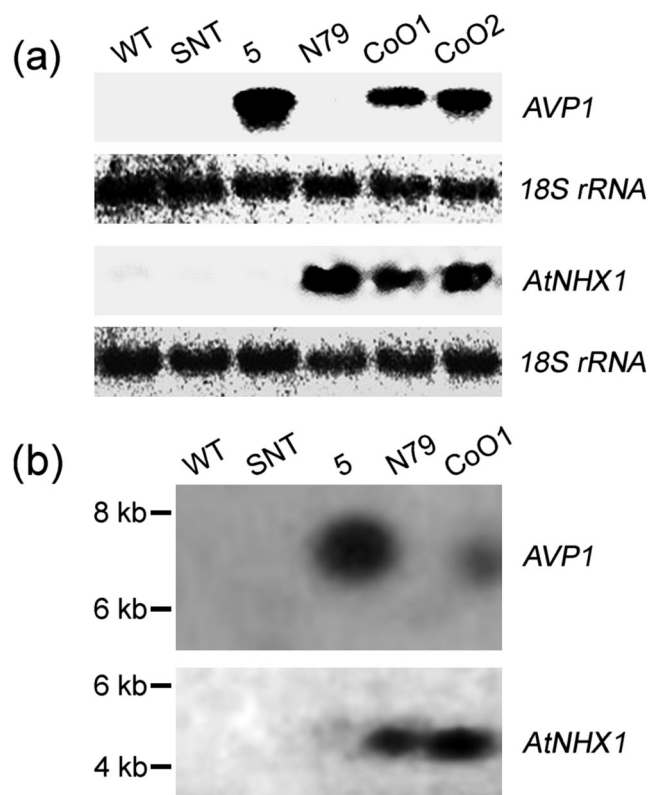


Fig. 1 Molecular analysis of cotton plants. **a** RNA blot analysis and **b** DNA blot analysis. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVP1*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* and *CoO2* two *AVP1/AtNHX1*-co-overexpressing populations. A cDNA fragment of *AtNHX1* and a cDNA fragment of *AVP1* were used as the hybridization probes. The 18S rRNA was used as the RNA loading control

senescence phenotypes in bracts and main stem leaves in comparison with the *AtNHX1*-transgenic line N79 and *AVP1/AtNHX1*-co-overexpressing plants (Fig. 4a). Our data confirmed that *AtNHX1* overexpression could indeed increase salt tolerance, but in terms of final boll numbers and fiber yields, *AVP1/AtNHX1*-co-overexpressing plants produced significantly more bolls and higher yield than single-gene-overexpressing plants, which in turn produced more bolls and higher yields than WT and SNT plants (Fig. 4b).

The Photosynthetic Capacity of *AVP1/AtNHX1*-Co-overexpressing Plants Is Stronger than That of *AVP1* or *AtNHX1* Single-Gene Overexpressing Plants

The better growth of *AVP1/AtNHX1*-co-overexpressing plants under saline conditions and higher fiber yield after salt treatment (Fig. 3 and 4) indicated that *AVP1/AtNHX1*-co-overexpressing plants must have better photosynthesis under salt stress conditions. We therefore measured photosynthetic rates of *AVP1/AtNHX1*-co-overexpressing plants during salt treatment (i.e., on the 10th day into

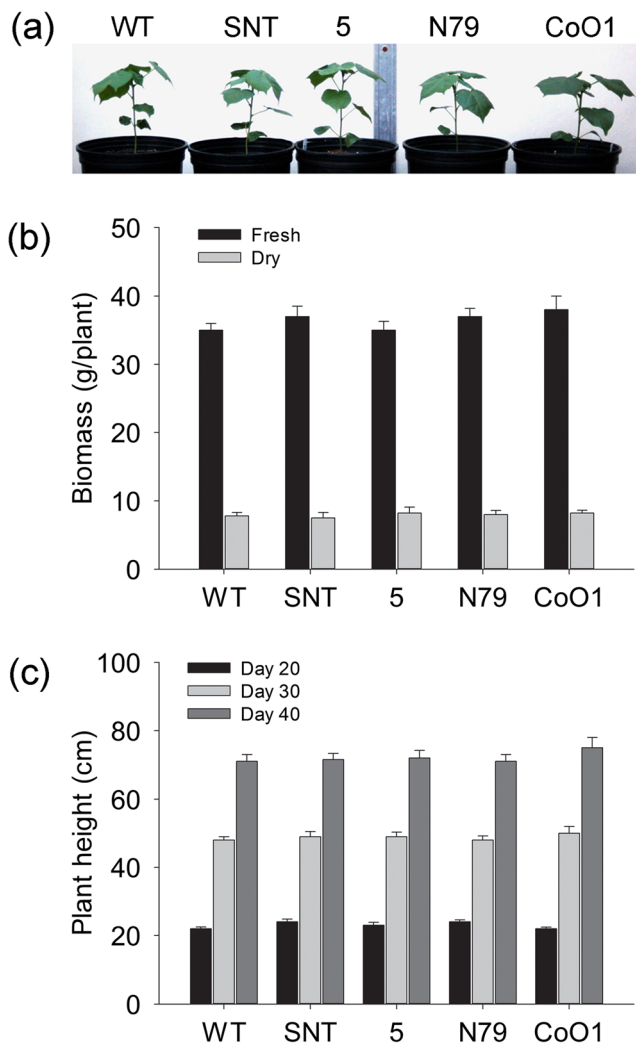


Fig. 2 Phenotype, biomass, and plant height of cotton plants. **a** Phenotypes of 20-day-old cotton plants. **b** Fresh and dry shoot biomass of 20-day-old cotton plants. **c** Plant height under normal growth condition. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVPI*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVPI/AtNHX1*-co-overexpressing plants

the 200 mM NaCl treatment). Our data indicated that *AVPI/AtNHX1*-co-overexpressing plants had the highest photosynthetic rate among all plants being analyzed (Fig. 5a). Furthermore, *AVPI/AtNHX1*-co-overexpressing plants also had the highest stomatal conductance and the highest transpiration among these plants (Fig. 5b, c), although *AVPI*-overexpressing and *AtNHX1*-overexpressing plants also had significantly higher photosynthetic rates, stomatal conductance, and transpiration than WT and SNT plants. Overall, our data clearly demonstrated that co-overexpression of *AVPI* and *AtNHX1* in cotton considerably relieved the inhibition of salinity on plant growth and development and increased boll number and fiber yield under saline condition in greenhouse.

Co-overexpression of *AVPI* and *AtNHX1* in Cotton Confers Higher Drought Tolerance than Overexpression of *AVPI* or *AtNHX1* Alone

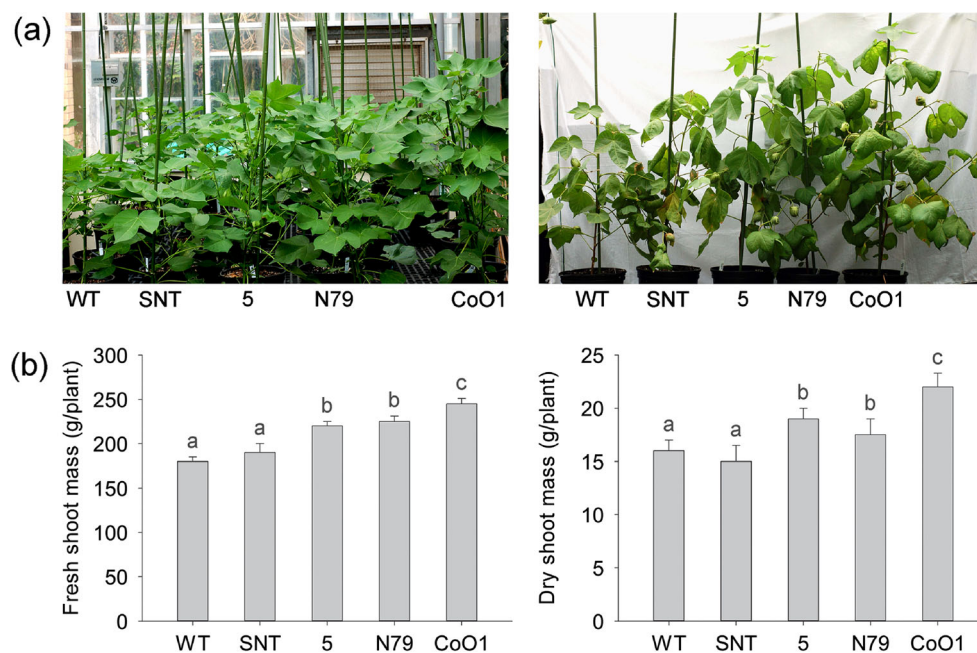
To test how *AVPI/AtNHX1*-co-overexpressing cotton plants would perform under reduced-irrigation conditions, especially in comparing their growth to *AVPI*-overexpressing and *AtNHX1*-overexpressing cotton plants under water deficit conditions, we conducted drought tolerance test in greenhouse. A slow-onset water deficit stress was created on 21-day-old cotton plants following a method described by Rivero et al. (2007). After stopping irrigation for 5 days, 500 ml of water was added to each pot every other day for 34 days, and then irrigation was increased to 800 ml of water for the next 50 days. Plant heights of *AVPI*-overexpressing and *AtNHX1*-overexpressing cotton plants were notably higher than those of WT and SNT plants just 10 days into reduced irrigation (Fig. 6a), and there were no distinct differences between these two single-gene overexpression plants during drought treatment. The plant height of *AVPI/AtNHX1*-co-overexpressing plants, however, was considerably higher than that of *AVPI*-overexpressing and *AtNHX1*-overexpression plants after drought treatment (Fig. 6b). The biomass analysis data also indicated that *AVPI/AtNHX1*-co-overexpressing plants produced significantly more fresh shoot mass and dry shoot mass than *AVPI*-overexpressing and *AtNHX1*-overexpression plants (Fig. 7a, b). Furthermore, the boll number and fiber yield of *AVPI/AtNHX1*-co-overexpressing plants were also statistically higher than those of WT, SNT, and *AVPI*- or *AtNHX1*-overexpressing plants (Fig. 7c, d).

The Larger Root Systems of *AVPI/AtNHX1*-Co-overexpressing Plants under Salt or Drought Conditions Might Be Due to More Active Auxin Transport in Roots

The *AVPI*-overexpressing cotton developed larger root systems under both drought and salt conditions (Pasapula et al. 2011), which likely contributed to their increased tolerance under these conditions. We examined if similar larger root systems would develop in *AVPI/AtNHX1*-co-overexpressing cotton plants under salt and drought conditions. Indeed, *AVPI/AtNHX1*-co-overexpressing cotton plants developed the largest root systems under salt and drought conditions, in comparison with *AVPI*-overexpressing, *AtNHX1*-overexpressing, and control plants (Fig. 8a, c). The fresh biomass and dry biomass of *AVPI/AtNHX1*-co-overexpressing cotton plants were considerably higher than those of *AVPI*-overexpressing, *AtNHX1*-overexpressing, and control plants (Fig. 8b, d).

It was reported that the larger root systems of *AVPI*-overexpressing *Arabidopsis* plants were due to increased auxin polar transport in roots, which promoted root development in

Fig. 3 Phenotypes of cotton plants under salt treatment. **a** Phenotypes of cotton plants under salt treatment for 42 days. **b** Biomass of cotton plants after salt treatment for 42 days. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVPI*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVPI/AtNHX1*-co-overexpressing plants



transgenic plants (Li et al. 2005). To test if *AVPI/AtNHX1*-co-overexpressing cotton plants also have such a capacity in having higher auxin transport in root systems, we studied how *AVPI/AtNHX1*-co-overexpressing cotton plants would respond to the presence of the auxin transport inhibitor NPA in the media. All cotton plants developed robust lateral root

systems in the absence of NPA (Fig. 9a); however, in the presence of 100 μ M of NPA, the taproot of WT and *AtNHX1*-overexpressing plants lost the ability to grow downward, whereas the tap root of *AVPI*-overexpressing and *AVPI/AtNHX1*-co-overexpressing plants could still grow downward (Fig. 9b), although the development of lateral root

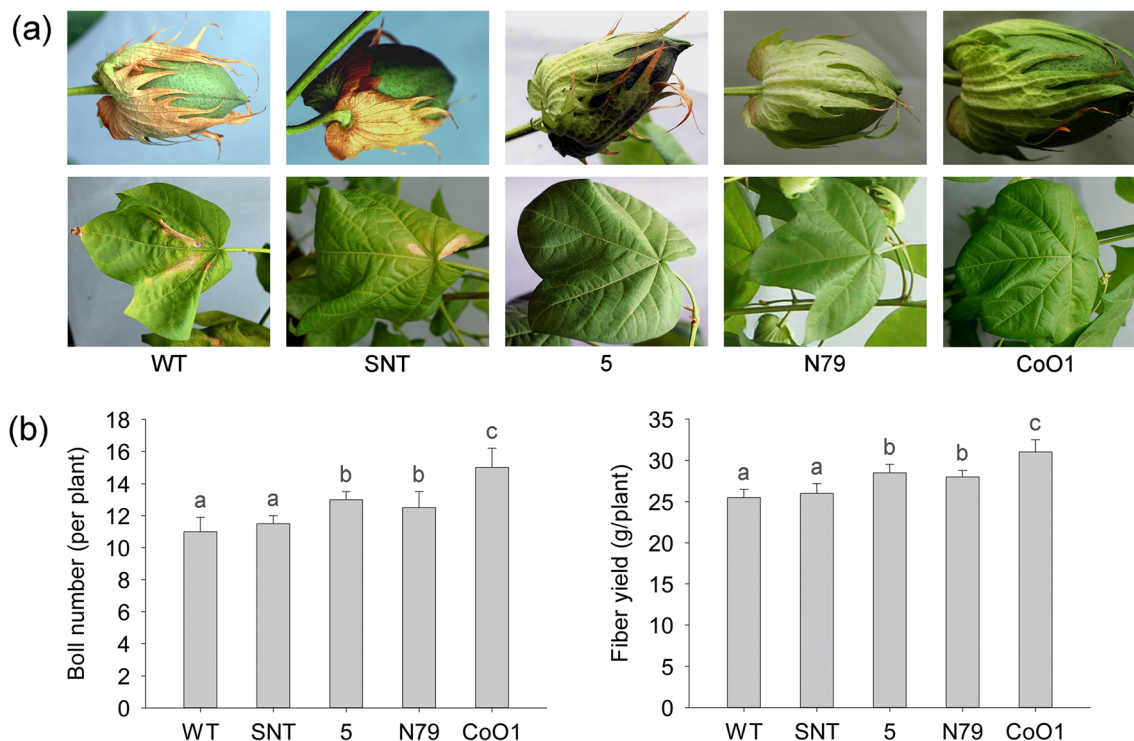


Fig. 4 Phenotypes and yields of cotton plants under salt treatment. **a** Phenotypes of cotton plants after salt treatment. **b** Boll numbers and fiber yields of cotton plants after salt treatment. *WT* wild type, *SNT* segregated

non-transgenic line, *5* *AVPI*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVPI/AtNHX1*-co-overexpressing plants

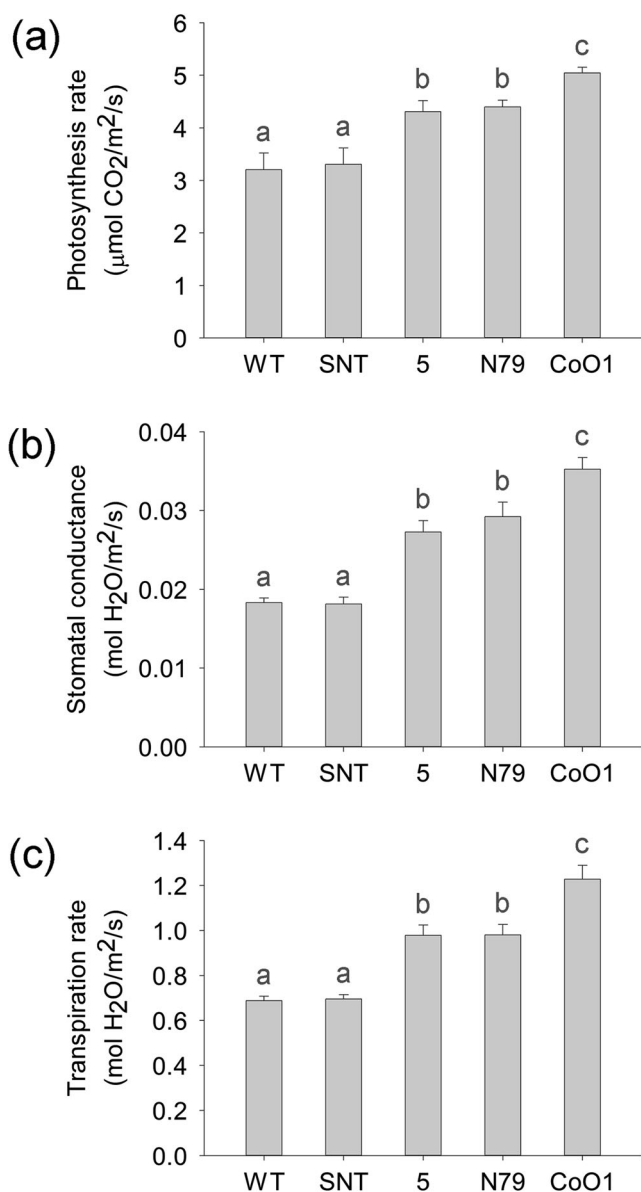


Fig. 5 Photosynthetic performance of cotton plants during salt treatment. **a** Photosynthetic rates of cotton plants during salt treatment. **b** Stomatal conductance of cotton plants during salt treatment. **c** Transpiration rates of cotton plants during salt treatment. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVP1*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVP1/AtNHX1*-co-overexpressing plants. The measurements were taken when plants were 28 days into salt treatment (7 days into 200 mM NaCl treatment). Each data point represents the mean of three independent biological replicates per treatment (mean \pm standard error). Values not sharing a common letter are significantly different at $p < 0.05$

systems was completely inhibited by this high concentration of NPA. Our data indicated that *AVP1* overexpression could partially overcome the inhibitory effect of NPA on root development in transgenic cotton plants, which suggested that either increased auxin transport or higher auxin concentration in the root systems of these transgenic cotton plants might be responsible for the observed larger root systems in *AVP1*-

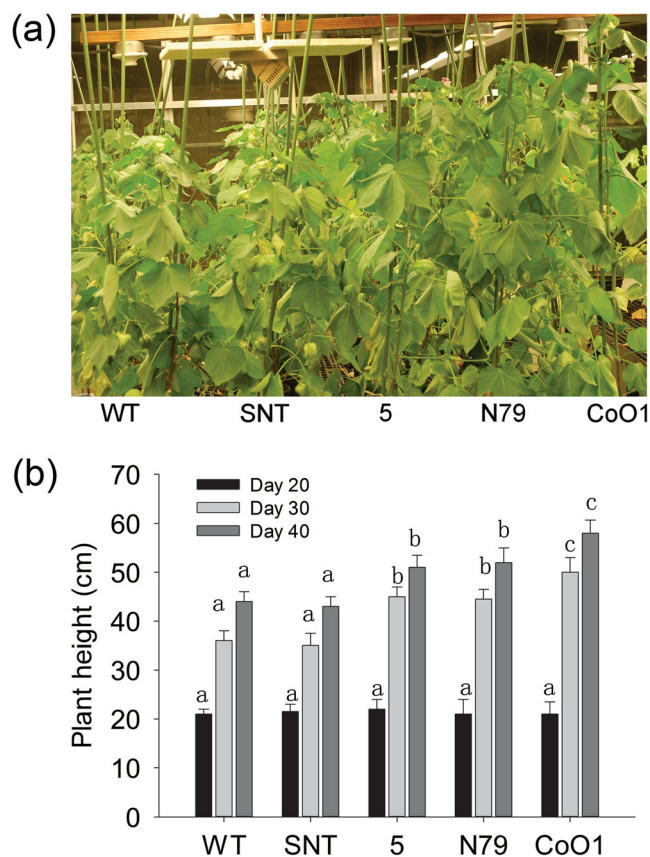


Fig. 6 Phenotypes and plant heights of cotton plants under drought treatment. **a** Phenotypes of cotton plants under drought treatment for 39 days. **b** Cotton heights at different stages of drought treatment. Day 20, no drought stress applied yet; day 30, cotton plants had been drought treated for 10 days; day 40, cotton plants had been drought treated for 20 days. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVP1*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVP1/AtNHX1*-co-overexpressing plants. Each data point represents the mean of three independent biological replicates per treatment (mean \pm standard error). Values not sharing a common letter are significantly different at $p < 0.05$

overexpressing and *AVP1/AtNHX1*-co-overexpressing cotton plants under saline or drought conditions.

AVP1/AtNHX1-Co-overexpressing Cotton Plants Produce More Bolls and Higher Fiber Yield under Low-Irrigation and Dryland Conditions in the Field

Previously, we showed that *AVP1* overexpression led to increased fiber yield under low-irrigation and dryland field conditions in West Texas (Pasapula et al. 2011). To test how *AVP1/AtNHX1*-co-overexpressing cotton would perform in field conditions, especially as compared to *AVP1*-overexpressing and *AtNHX1*-overexpressing plants, we conducted field experiments with these transgenic cotton plants at the USDA-ARS Experimental Farm in Lubbock, TX, USA in 2010 (Fig. 10). As expected, the *AVP1* and *AtNHX1* single-gene-overexpressing plants, 5 and N79, produced significantly higher average boll number and fiber yield per plant than

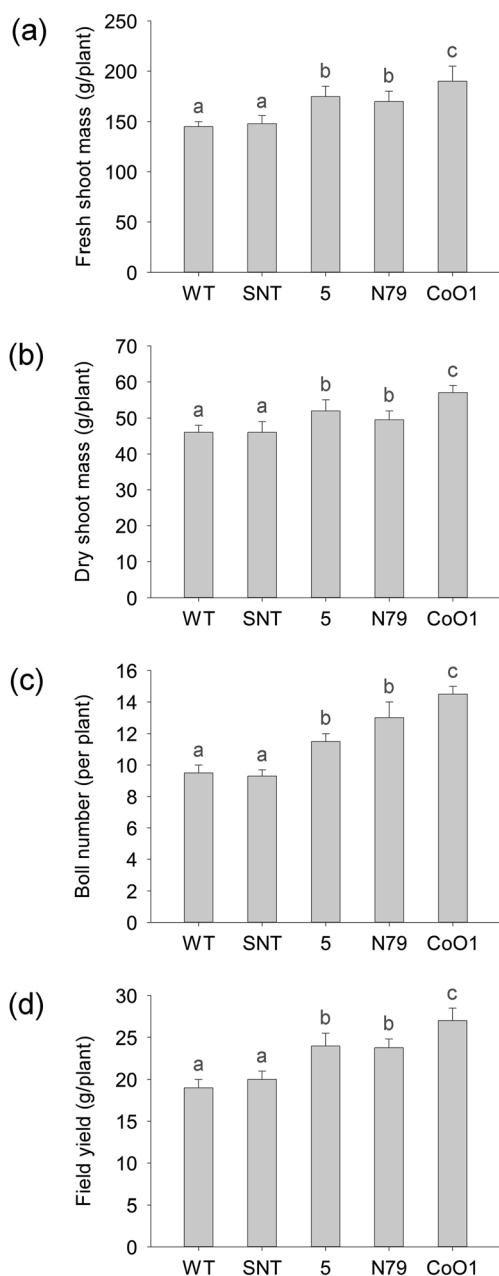


Fig. 7 Biomass analysis and cotton yields after drought treatment. **a** Fresh shoot mass of cotton plants after drought treatment. **b** Dry shoot mass of cotton plants after drought treatment. **c** Boll numbers of cotton plants after drought treatment. **d** Fiber yields of cotton plants after drought treatment. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVP1*-transgenic line *5*, *N79* *AtNHX1*-transgenic line *79*, *CoO1* *AVP1/AtNHX1*-co-overexpressing plants. Each data point represents the mean of three independent biological replicates per treatment (mean±standard error). Values not sharing a common letter are significantly different at $p < 0.05$

those of wild-type and *SNT* plants (Fig. 10), while no statistical difference was found between these two single-gene-overexpressing lines. The *AVP1/AtNHX1*-co-overexpressing cotton plants, however, performed even better than *AVP1* and *AtNHX1* single-gene-overexpressing plants. The boll number

and fiber yield of *AVP1/AtNHX1*-co-overexpressing cotton plants were statistically higher than those of *AVP1* and *AtNHX1* single-gene-overexpressing plants under both dry-land (Fig. 10b) and low-irrigation (Fig. 10d) conditions. The *AVP1/AtNHX1*-co-overexpressing cotton plants produced 24.3 % more bolls and 24.2 % more fiber under low-irrigation condition and 41.6 % more bolls and 36.3 % more fiber under dryland conditions than *WT* and *SNT* plants. These data clearly indicated that simultaneously overexpressing *AVP1* and *AtNHX1* in cotton could significantly increase fiber production in the field under low-irrigation or dryland conditions.

Discussion

Since drought and salinity are two major abiotic stresses affecting plant productivity, improving drought and salt tolerance in crops became one of the most important objectives in agriculture. By using the model system, a number of key genes involved in plant salt or drought tolerance have been cloned and introduced into crops through transgenic technology, which led to limited success in improving salt or drought tolerance, or tolerance to both salt and drought in several crops (Deikman et al. 2012; Mittler and Blumwald 2010). Among them, the *Arabidopsis* vacuolar Na^+/H^+ antiporter gene *AtNHX1* and H^+ -pyrophosphatase gene *AVP1* are the two best studied ones. Overexpression of *AtNHX1* leads to significantly improved salt tolerance in transgenic plants (Apse et al. 1999; He et al. 2005; Li et al. 2010; Zhang and Blumwald 2001; Zhang et al. 2001), and overexpression of *AVP1* leads to improved salt and drought tolerance (Banjara et al. 2011; Gaxiola et al. 2001; Li et al. 2005, 2010; Park et al. 2005; Pasapula et al. 2011; Zhao et al. 2006). However, overexpression of *AtNHX1* alone does not lead to increased drought tolerance, and the increased salt tolerance by overexpressing *AVP1* appears to be limited to around 200 mM NaCl (Pasapula et al. 2011). Because the vacuolar Na^+/H^+ antiporter activity depends on the vacuolar proton pump activity, Gaxiola et al. (2002) proposed that by co-overexpressing the vacuolar Na^+/H^+ antiporter gene *AtNHX1* and the vacuolar H^+ -pyrophosphatase gene *AVP1*, it is possible to further increase salt and drought tolerance in transgenic plants.

To confirm this hypothesis, Zhao et al. (2006) co-overexpressed the vacuolar Na^+/H^+ antiporter gene *SsNHX1* (from *Suaeda salsa*) and *AVP1* in rice and provided evidence that co-overexpression of *SsNHX1* and *AVP1* could confer greater performance to transgenic plants than *SsNHX1* single-gene-overexpressing plants under saline conditions. However, there were no field studies with the transgenic rice expressing *SsNHX1* and *AVP1*. Furthermore, no *AVP1* single-gene-overexpressing rice was used as a reference line, so it is

Fig. 8 Root phenotypes and biomass of cotton plants under salt or drought treatments. **a** Root phenotypes of cotton plants after salt treatment for 51 days. **b** Fresh root mass and dry root mass of cotton plants after salt treatment. **c** Root phenotypes of cotton plants after drought treatment for 45 days. **d** Fresh root mass and dry root mass of cotton plants after drought treatment. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVP1*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVP1/AtNHX1*-co-overexpressing plants. Each data point represents the mean of three independent biological replicates per treatment (mean±standard error). Values not sharing a common letter are significantly different at $p < 0.05$

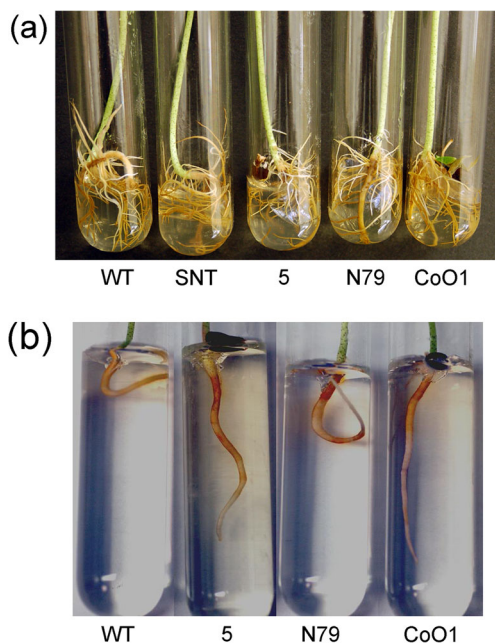
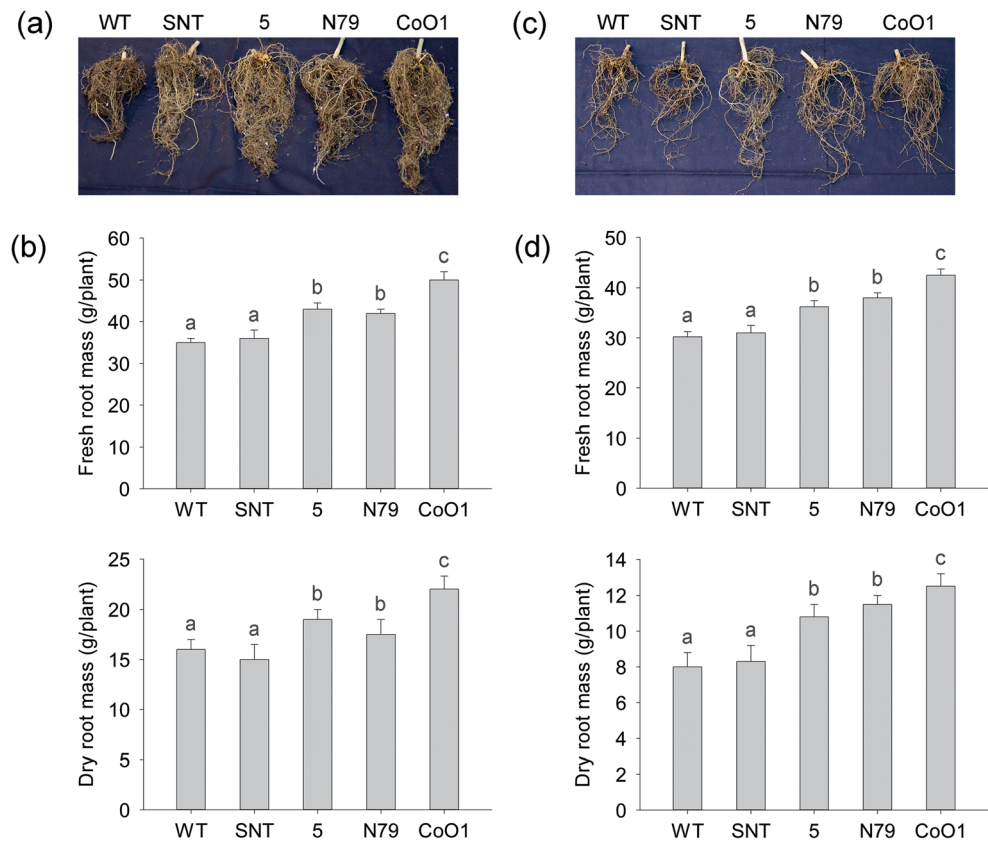
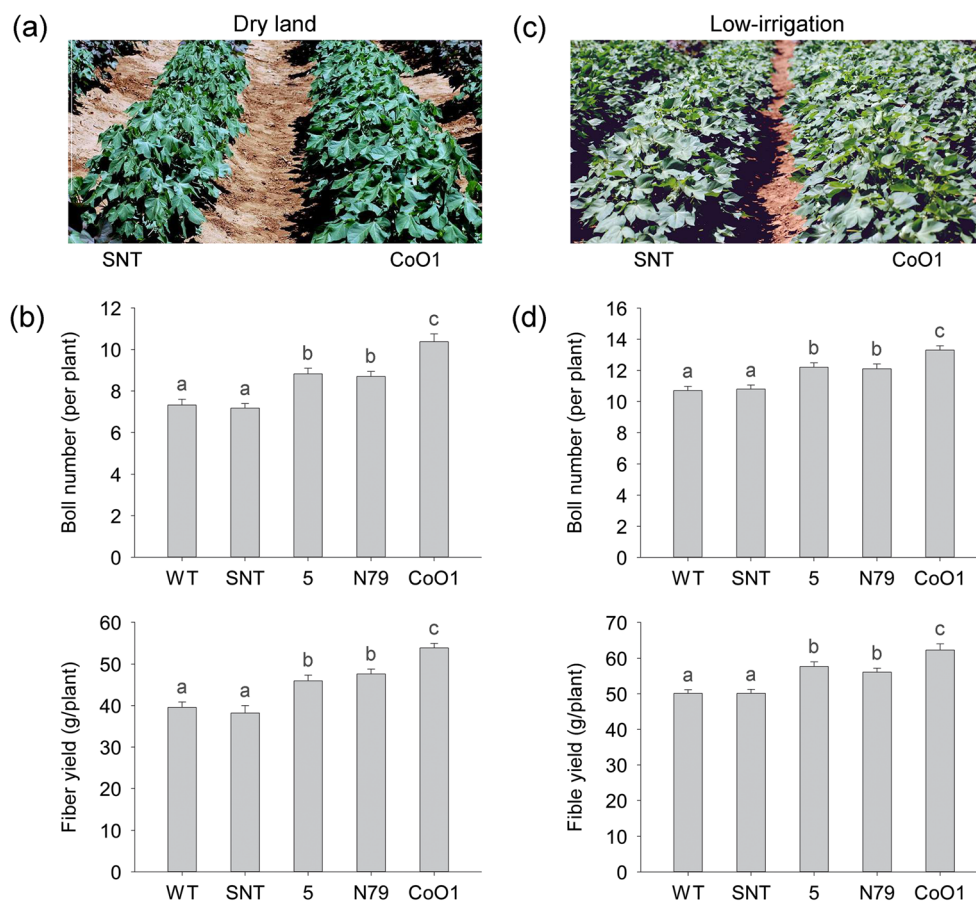


Fig. 9 Root phenotypes of cotton plants in the presence or absence of NPA. **a** Root phenotypes of cotton plants in the absence of NPA. **b** Root phenotypes of cotton plants in the presence of 100 μM of NPA. *WT* wild type, *SNT* segregated non-transgenic line, *5* *AVP1*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVP1/AtNHX1*-co-overexpressing plants

difficult to estimate if there were any improvements or how much improvements there were by overexpressing both genes instead of just overexpressing *AVP1* alone. Also, there were no drought tolerance tests conducted with the *SsNHX1/AVP1*-co-overexpressing plants; it is difficult to tell if there was a significant improvement in drought tolerance with overexpressing *SsNHX1* and *AVP1*. Eight years has passed since the publication of co-overexpression of *SsNHX1* and *AVP1* in rice (Zhao et al. 2006), but no follow-up experiments were reported regarding the performance of these *SsNHX1/AVP1*-co-overexpressing rice plants in field or under water-deficit conditions.

Other people have also tried to co-overexpress a Na^+/H^+ antiporter gene and a pyrophosphatase gene in various plants. For examples, Liu et al. (2010) co-overexpressed the rice *OsNHX1* and the rice *OsVP1* in transgenic rice and they also found significantly improved salt and drought tolerance in transgenic rice plants. Bhaskaran and Savithamma (2011) co-overexpressed the *Pennisetum glaucum* vacuolar Na^+/H^+ antiporter gene *PgNHX1* and *AVP1* in tomato and improved tomato's salt tolerance. Gouiaa et al. (2012) co-overexpressed the wheat vacuolar Na^+/H^+ antiporter gene *TNHXS1* and the wheat pyrophosphatase gene *TVP1* in tobacco and improved tobacco's salt tolerance. Finally, Liu et al. (2012) co-overexpressed the *Suaeda corniculata* Na^+/H^+ antiporter gene *ScNHX1* and the vacuolar pyrophosphatase gene *ScVP* in

Fig. 10 Field performances of cotton plants. **a** Phenotypes of SNT and *AVP1/AtNHX1*-co-overexpressing plants in dryland conditions. **b** Boll numbers and fiber yields of cotton plants in dryland conditions. **c** Phenotypes of SNT and *AVP1/AtNHX1*-co-overexpressing plants in low-irrigation conditions. **d** Boll numbers and fiber yields of cotton plants in low-irrigation conditions. *WT* wild type, *SNT* segregated non-transgenic line, 5 *AVP1*-transgenic line 5, *N79* *AtNHX1*-transgenic line 79, *CoO1* *AVP1/AtNHX1*-co-overexpressing plants. Each data point represents the mean of 30 independent biological replicates per treatment (mean \pm standard error). Values not sharing a common letter are significantly different at $p < 0.05$



alfalfa and dramatically improved alfalfa's tolerance to salt and saline-alkali stress. However, except in one case, none of these studies provided information on how their transgenic plants would respond to water-deficit stress. More importantly, none of these studies addressed the critical issue that how these transgenic plants would behave in the field.

In this report, we provided clear evidence that co-overexpression of *AVP1* and *AtNHX1* can indeed further improve drought and salt tolerance in transgenic cotton. In our research, we compared the performance of *AVP1/AtNHX1*-co-overexpressing cotton with *AVP1* single-gene-overexpressing cotton and *AtNHX1* single-gene-overexpressing cotton, in addition to WT and SNT plants. Our data indicate that *AVP1/AtNHX1*-co-overexpressing cotton produced significantly more biomass than all other plants under salt stress conditions (Fig. 3) and produced the highest yields among all other plants as well (Fig. 4). *AVP1/AtNHX1*-co-overexpressing cotton maintained the highest photosynthetic rate than any other plants during salt treatment (Fig. 5). *AVP1/AtNHX1*-co-overexpressing cotton grew taller (Fig. 6) and produced more biomass (Fig. 7a, b) and higher yields (Fig. 7c and d) than any other cotton plants under reduced-irrigation conditions. The *AVP1/AtNHX1*-co-overexpressing cotton plants produced the biggest root biomass than

any other cotton plants under salt and drought conditions (Fig. 8), which is likely caused by higher auxin polar transport in *AVP1/AtNHX1*-co-overexpressing cotton that stimulates root development, as the root systems from *AVP1/AtNHX1*-co-overexpressing cotton are the least inhibited by auxin transport inhibitor NPA (Fig. 9). Finally, *AVP1/AtNHX1*-co-overexpressing cotton produced the highest yields among all cotton plants under both reduced-irrigation conditions and dryland conditions (Fig. 10). In summary, co-overexpression of *AVP1* and *AtNHX1* leads to higher drought and salt tolerance than *AVP1* or *AtNHX1* single-gene overexpression in transgenic plants.

Although there are many other genes that confer increased drought tolerance or salt tolerance when overexpressed in laboratory conditions, very few have been proven to be effective in field conditions. Based on our own experience working with transgenic cotton plants, *IPT*-expressing cotton appears to work better than *AVP1*-expressing cotton under reduced-irrigation conditions in greenhouse as well as in field conditions (Kuppu et al. 2013; Pasapula et al. 2011). However, with the stacked overexpression of *AVP1* and *AtNHX1* in cotton, we obtained similarly increased drought tolerance from *AVP1/AtNHX1*-co-overexpressing cotton plants as

those from *IPT*-expressing cotton plants. Even better, the *AVP1/AtNHX1*-co-overexpressing cotton plants are also salt tolerant, whereas the *IPT*-expressing cotton plants are not. In fact, pyramiding transgenes appears an excellent approach to increase stress tolerance or to increase tolerance to multiple stresses. Wei et al. (2011) showed that by simultaneous overexpression of the vacuolar pyrophosphatase gene *TsVP* and the glycinebetaine biosynthetic gene *BetA*, it was possible to further improve drought tolerance in transgenic maize. Our research confirmed Gaxiola's hypothesis that pyramiding *AVP1* and *AtNHX1* would be a better choice than overexpressing *AVP1* or *AtNHX1* alone (Gaxiola et al. 2002). But our approach in testing this hypothesis is not the best, as it will be very difficult to introgress both *AVP1* and *AtNHX1* into cultivated cotton varieties. What we need to do in the future is to create a single DNA construct containing both *AVP1* and *AtNHX1*, retransform Coker 312 cotton, and then breed transgenic Coker 312 containing the *AVP1/AtNHX1* construct into commercial cotton varieties. With the proof-of-concept proven, it is just a matter of time that we will be able to see commercial varieties that are more drought and salt tolerant because they contain *AVP1* and *AtNHX1*.

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