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Expression of an Arabidopsis vacuolar H^+-pyrophosphatase gene (*AVP1*) in cotton improves drought- and salt tolerance and increases fibre yield in the field conditions

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Summary

The Arabidopsis gene *AVP1* encodes a vacuolar pyrophosphatase that functions as a proton pump on the vacuolar membrane. Overexpression of *AVP1* in Arabidopsis, tomato and rice enhances plant performance under salt and drought stress conditions, because up-regulation of the type I H^+-PPase from Arabidopsis may result in a higher proton electrochemical gradient, which facilitates enhanced sequestering of ions and sugars into the vacuole, reducing water potential and resulting in increased drought- and salt tolerance when compared to wild-type plants. Furthermore, overexpression of *AVP1* stimulates auxin transport in the root system and leads to larger root systems, which helps transgenic plants absorb water more efficiently under drought conditions. Using the same approach, *AVP1*-expressing cotton plants were created and tested for their performance under high-salt and reduced irrigation conditions. The *AVP1*-expressing cotton plants showed more vigorous growth than wild-type plants in the presence of 200 mM NaCl under hydroponic growth conditions. The soil-grown *AVP1*-expressing cotton plants also displayed significantly improved tolerance to both drought and salt stresses in greenhouse conditions. Furthermore, the fibre yield of *AVP1*-expressing cotton plants is at least 20% higher than that of wild-type plants under dry-land conditions in the field. This research indicates that *AVP1* has the potential to be used for improving crop's drought- and salt tolerance in areas where water and salinity are limiting factors for agricultural productivity.

Keywords: drought tolerance, proton pump, root development, salt stress, transgenic cotton.

Introduction

Abiotic stresses such as drought, salinity and extreme temperatures are serious threats to modern agriculture. These stresses lead to a series of morphological, physiological, biochemical and molecular changes in plants that adversely affect plant growth and productivity (Wang *et al.*, 2001). Water deficit and salinity are the major limiting factors in plant productivity, affecting more than 10 per cent of arable land on our planet and result in a yield reduction, on average, of more than 50% for most major crop plants (Bartels and Sunkar, 2005).

Cotton is a vital agricultural commodity and multibillion dollar industry that underpins US and global economies. As an important renewable resource, cotton is the world's leading natural fibre and second largest oilseed crop in

production. The production, marketing, consumption and trade of cotton-based products stimulate the economy with revenues in excess of $100 billion annually in the United States, making cotton the No. 1 value-added crop. Cotton is grown on more than 10 million acres in the United States, with the majority of production in America's semi-arid southwest. Agricultural production strategies that served the industry well in times of abundant and cheap water are no longer viable as water quality and availability has declined. Therefore, the primary challenge facing scientists is enhancing cotton's tolerance to drought and salt stresses to maintain productivity on marginal land under water-limited conditions. Improvements in this area of food and fibre production will have an enormous impact on the economies of the semi-arid southwest in the United States, as well as, marginal production regions around the world.

One way to enhance drought- and salt tolerance in plants is to increase solute concentration in the vacuoles of plant cells (osmotic adjustment), therefore increasing the vacuolar osmotic pressure with the concomitant decrease in water potential, which would favour water movement from soil into plant root cells. Two approaches have been used to increase solute contents in plant vacuoles. The first approach involves increasing the activity of a vacuolar sodium/proton (Na^+/H^+) antiporter that mediates the exchange of cytosolic Na^+ for vacuolar H^+ (Apse *et al.*, 1999). The second approach involves increasing the activity of a H^+ pump on the vacuolar membrane to move more H^+ into the vacuoles, therefore generating a higher proton electrochemical gradient ($\Delta\mu H^+$) that can be used to energize secondary transporters including vacuolar Na^+/H^+ antiporters (Gaxiola *et al.*, 2002). Both approaches enhance Na^+ accumulation in the vacuoles and reduce the potential of Na^+ toxicity in the cytoplasm, leading to higher salt tolerance in transgenic plants (Apse *et al.*, 1999; Gaxiola *et al.*, 2001). However, it appears that the second approach can simultaneously increase both salt- and drought tolerance in transgenic plants (Gaxiola *et al.*, 2002). For example, Gaxiola *et al.* (2001) showed that the drought- and salt tolerance can be significantly increased in transgenic Arabidopsis plants by overexpressing the Arabidopsis gene *AVP1* that encodes a vacuolar H^+ pyrophosphatase, an H^+ pump. In addition to Arabidopsis, overexpression of *AVP1* in tomato also displayed increased drought tolerance (Park *et al.*, 2005). The increased drought tolerance phenotype is attributed to an increased root biomass, which allows *AVP1*-overexpressing tomato plants to absorb water more efficiently and suffer less damage under severe water-deficit conditions (Park *et al.*, 2005). Furthermore, *AVP1* overexpression appears to facilitate auxin transport and regulate auxin-mediated developmental processes (Li *et al.*, 2005).

Subsequent to the pioneering work from Gaxiola's group (Gaxiola *et al.*, 2001; Li *et al.*, 2005; Park *et al.*, 2005), other groups have demonstrated that overexpression of similar genes encoding vacuolar membrane-bound pyrophosphatase (H^+ PPase or H^+ pump) can increase both salt- and drought tolerance in heterologous systems, including rice (Zhao *et al.*, 2006), tobacco (Gao *et al.*, 2006), cotton (Lv *et al.*, 2008, 2009) and maize (Li *et al.*, 2008). We report here that increased expression of the vacuolar membrane-bound H^+ pump can indeed lead to increased drought- and salt tolerance in transgenic cotton, and furthermore we show that *AVP1*-expressing cotton produces more fibre under field conditions, which represents the first step in the potential application of this gene in agriculture.

Results

Creation and molecular analysis of transgenic cotton plants that express *AVP1*

The *35S-AVP1* construct described by Gaxiola *et al.* (2001) was introduced into cotton (*Gossypium hirsutum* cv. Coker 312) via Agrobacterium-mediated transformation (Bayley *et al.*, 1992). A total of 45 independent transgenic lines were generated, and random lines were selected for genomic PCR analysis and RNA blot analysis to confirm transformation and overexpression (Figure 1a, b). Homozygous transgenic plants for two lines, 5 and 9, respectively, were selected for DNA blot analysis which indicated that both lines appeared to have a single T-DNA insertion (Figure 1c). Additionally, Western blot analysis of isolated membrane proteins showed increased levels of a 75-kDa band presumably representing the AVP1 protein in transgenic lines 5 and 9, compared to wild-type plants (Figure 1d). The antisera used were raised against the PPi binding site of the H^+-PPase (Park *et al.*, 2005). Immunocytochemical localization was used to localize H^+-PPase in wild-type and transgenic (line 5) plants (Figure 2). Strong staining was seen in leaf sections of *AVP1*-expressing cotton plants with AVP1 antibodies, particularly in the phloem parenchyma cells (pp), companion cells (cc), sieve elements (se) and early xylem (x) cells (Figure 2, D2), indicating that *AVP1* is indeed expressed in transgenic cotton.

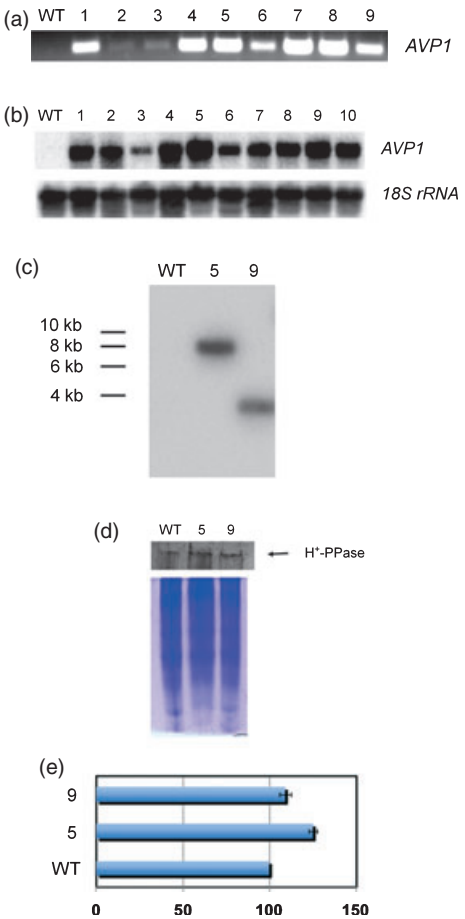

Figure 1 Molecular analysis of wild-type (WT) and *AVP1*-expressing cotton plants. a. PCR analysis using two *AVP1*-specific primers to identify nine independent transformation lines. b. RNA blot analysis using a cDNA clone for *AVP1* as the probe and the 18S rRNA as the RNA loading control. c. DNA blot analysis of wild-type and two *AVP1*-expressing cotton plants (lines 5 and 9, respectively). Cotton genomic DNA was digested with *Eco* RI that does not cut within the *AVP1* transgene. A cDNA clone for *AVP1* was used as the probe. d. Western blot analysis of wild-type and two *AVP1*-expressing cotton plants. Immunoblot: 100 μg of delipidated protein was loaded in a 7% urea gel and transferred to a PVDF membrane and subjected to Western blot analysis. The AVP1 antibody was used in the Western blot analysis. Loading control: 100 μg of microsomal fraction proteins from wild-type and transgenic plants as indicated was delipidated, separated in 7% urea gel and stained with Coomassie blue. e. Relative levels of H^+-PPase in transgenic lines 5 and 9 in comparing with wild-type. The Western blot experiments in (d) were conducted 3 times, and each time the H^+-PPase level in WT obtained by using densitometry analysis was set at 100%; therefore the bars shown in (e) are mean number ± standard deviation of per cent change in H^+-PPase content in transgenic lines 5 and 9.

AVP1 overexpression in cotton results in salt tolerance

To test whether *AVP1* overexpression enhances salt tolerance, we performed two experiments under controlled conditions in a greenhouse. In the first experiment, transgenic lines 1, 4 and 9, and wild-type Coker 312 plants were grown hydroponically in a saline medium (see Materials and methods). Prior to salt treatment, there were no phenotypic differences between wild-type and transgenic plants (Figure 3a). However, after 7 days of growth in 200 mM NaCl, *AVP1*-expressing plants were considerably larger (Figure 3b) and produced significantly more shoot and root biomass than wild-type plants (Figure 3c, d). The three *AVP1*-expressing cotton plants produced 49%, 53% and 48% more dry shoot mass, 85%, 115%, and 115% more dry root mass, respectively, compared to wild-type Coker 312. No differences were observed between wild-type Coker 312 and transgenic lines grown under control conditions. Interestingly, transgenic Arabidopsis that overexpressing *AVP1* showed increased biomass production under all conditions, including no salt control (Gaxiola *et al.*, 2001).

In the second experiment, 100 plants of each of transgenic lines 5 and 9, a segregated non-transgenic line derived from line 5 (SNT), and the parental wild-type Coker 312 were grown under greenhouse conditions in soil. Following optimal irrigation for 21 days, a slow-onset salt stress was created by irrigating with increasing concentrations of NaCl, similar to the method described by Apse *et al.* (1999) (Figure S1a). Prior to the salt treatment, wild-type and transgenic plants displayed no phenotypic differences (Figure S2). In fact, there were no differences in fresh biomass, dry biomass, boll number or fibre yield between wild-type and transgenic plants grown under control (no salt) conditions (Figure S3a–c). However, after treatment with NaCl for 48 days (from 50 mM up to 200 mM), the *AVP1* transgenic plants showed increased physical size (Figure 4a) and larger root mass (Figure 4b). *AVP1*-expressing cotton plants produced significantly more shoot mass and root mass (Figure 5a, b). Transgenic lines produced 19% and 17% more fresh shoot mass, 27% and 31% more fresh root mass (Figure 5a), 16% more dry shoot mass, and 24% and 26% more dry root mass than wild-type plants (Figure 5b). Following treatment with 200 mM NaCl for 30 days, plants were irrigated with control water (no additional NaCl) until harvest to analyse the consequence of this salt treatment to the final yield of these plants. We analysed the total boll number and fibre weight of these plants in the end and found that these two *AVP1*-expressing lines produced 21% and 25% more bolls, as well as 23% and 27% more fibre per plant when compared to wild-type plants and SNT line (Figure 5c).

In a separate experiment, we measured photosynthesis on the 28th day of a 200 mM NaCl treatment and found

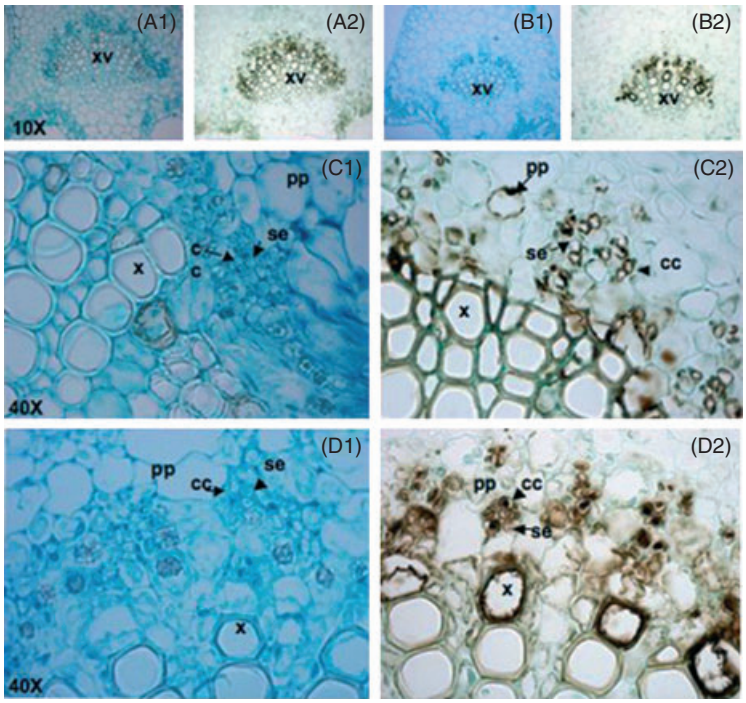

Figure 2 Immunocytochemical localization of H^+-PPase protein in leaf cross-sections of wild-type and AVP1-expressing cotton plants. A1 and C1, leaf cross-section of wild-type cotton stained with fast green. B1 and D1, leaf cross-section of transgenic cotton (line 5) stained with fast green. A2, leaf cross-section of wild-type incubated with an antisera raised against H^+-PPase. C2, higher magnification of (A2) as indicated. The signal (brown precipitated) is detected in phloem parenchyma (pp) and companion cells (cc). A weak signal is also detected in sieves elements (se) and early xylem (x). B2, leaf cross-section of transgenic cotton (line 5) incubated with an antisera raised against H^+-PPase. D2, higher magnification of (B2) as indicated. The signal is detected in phloem parenchyma (pp), companion cells (cc) and sieves elements (se). A strong signal is also detected in the early xylem cells close to the phloem.

that *AVP1*-expressing cotton plants maintained higher photosynthetic rates than wild-type control plants did under this condition (Figure 6a). The *AVP1*-expressing cotton plants also had higher stomatal conductance and transpiration rates compared to wild-type plants (Figure 6b, c). Overall, our data showed that *AVP1* overexpression in cotton considerably relieves the inhibitory effects of salinity on shoot growth and root development, and *AVP1*-overexpression increases fibre yields under saline conditions in the greenhouse.

AVP1 overexpression in cotton results in enhanced tolerance to water-deficit stress

AVP1-overexpressing plants and wild-type Coker 312 plants were grown under limited irrigation to examine the effect of *AVP1* overexpression on drought tolerance. Following optimal irrigation for 21 days, a slow-onset water-deficit stress was created by withholding water for 5 days, similar to the method described by Rivero *et al.* (2007) (Figure S1b). Starting on the 6th day, 300 mL of water was added to each plant every other day for 20 days, followed by and increased irrigation of 600 mL for 40 days. *AVP1* transgenic plants accumulated more biomass under reduced irrigation compared to wild-type plants (Figures 4d and 7). The root system of transgenic plants was also considerably larger than that of control plants (Figure 4d). The *AVP1*-expressing cotton plants produced significantly more fresh mass and dry mass, and in both cases, the difference in root biomass was significantly higher than that in shoot biomass. For example, *AVP1*-expressing cotton produced 23% and 24% more fresh shoot mass, yet they produced 36% and 41% more fresh root mass than control plants (Figure 7a). *AVP1*-transgenic cotton produced 14% and 15% more dry shoot mass, and 29% and 31% more dry root mass than control plants (Figure 7b). A group of treated plants (10 plants) were allowed to grow until the end of the experiment under this condition, and the final yield was analysed. The *AVP1*-expressing cotton plants produced 24% and 29% more bolls, and 24% and 31% more fibre, respectively, than those of wild-type and segregated non-transgenic plants in the end (Figure 7c). These experiments indicated that *AVP1* overexpression in cotton significantly improves cotton's performance under reduced irrigation conditions: the increased biomass, boll number and fibre yield. Furthermore, it is likely that the increased root system seen in *AVP1*-expressing cotton plants is a primary factor in the measured drought tolerance, similar to what was observed in transgenic tomato plants expressing *AVP1* (Park *et al.*, 2005).

AVP1-expressing cotton plants produced more boll number and higher fibre yield in the field

Field trials were conducted in cropping years 2007 and 2008 at the USDA Cropping Systems Research Laboratory in

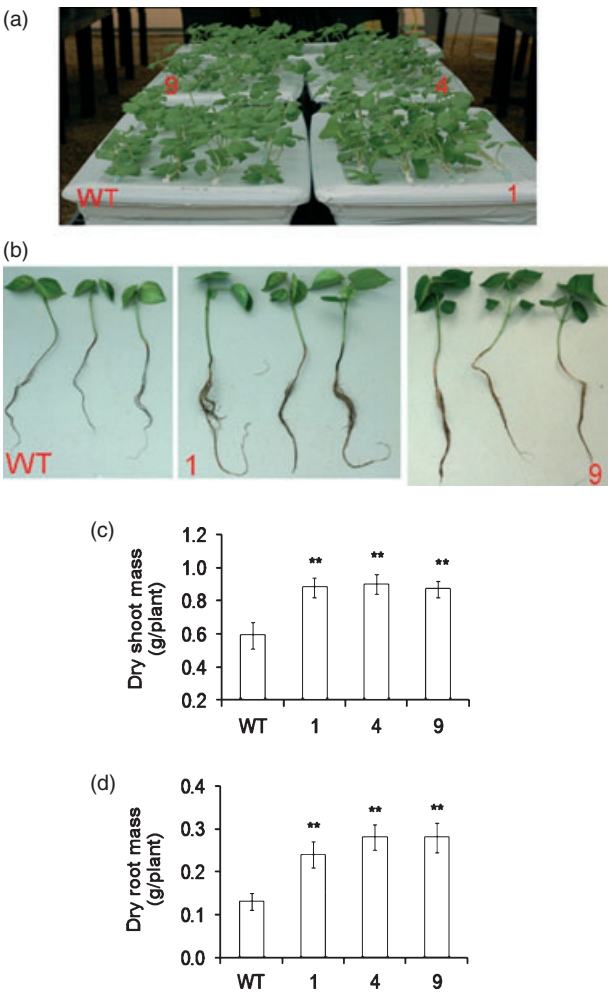

Figure 3 Salt treatment under hydroponic condition. a. Phenotype of wild-type and *AVP1*-expressing cotton plants before salt treatment. b. Phenotype of wild-type and *AVP1*-expressing cotton plants after salt treatment. WT, wild-type; 1, 4 and 9, three *AVP1*-expressing cotton plants. c. Dry shoot mass. d. Dry root mass. **statistically significant at 1% level.

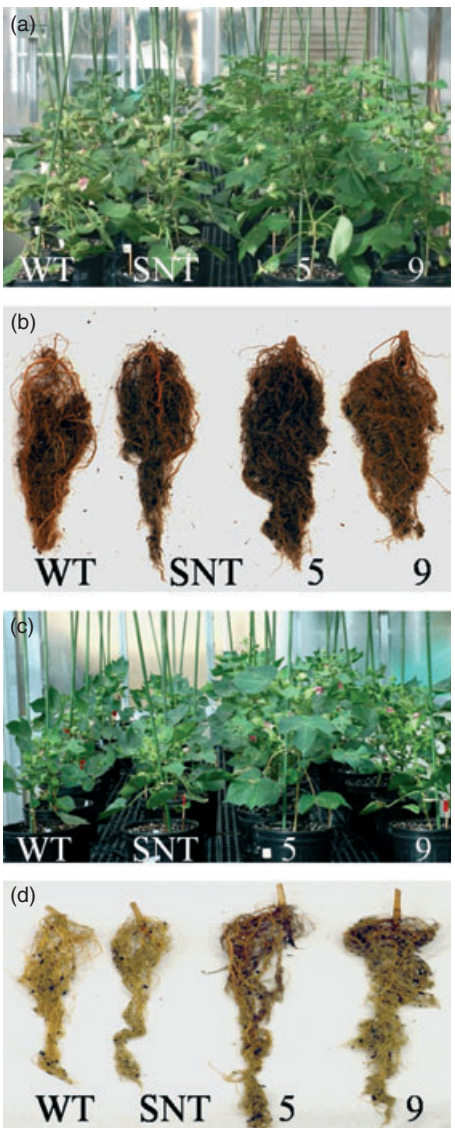

Figure 4 Phenotypes of wild-type, segregated non-transgenic and *AVP1*-expressing cotton plants during stress treatments. a. Phenotype during salt treatment. WT, wild-type plants; SNT, segregated non-transgenic plants; 5 and 9, two independent lines of *AVP1*-expressing cotton. The picture was taken 20 days into 200 mM NaCl treatment. b. Root phenotypes after salt treatment. The picture was taken 30 days into 200 mM NaCl treatment. c. Phenotype during drought treatment. The picture was taken 30 days into reduced irrigation treatment. d. Root phenotypes after drought treatment. The picture was taken 40 days into reduced irrigation treatment.

Lubbock County, Texas to evaluate the performance of *AVP1* transgenic cotton plants under a rainfed (dry land) production system. Wild-type Coker 312, three independent *AVP1*-expressing lines (i.e. 2, 5 and 9) and one *AtNHX1*-expressing line (i.e. N79, He *et al.*, 2005) were used in these experiments. Results from our field trial in crop year 2007 showed that these three *AVP1*-expressing transgenic lines demonstrated a significantly higher average boll number per plant than that of wild-type plants (Figure 8a). Consistent to this result, these same transgenic lines also showed significantly higher fibre yield per plant compared to Coker 312 (Figure 8a). The increases in boll number per plant are 31%, 34% and 30%, and the increases in fibre yield per plant are 24%, 32% and 27%, respectively, for these three lines. The *AtNHX1*-expressing cotton was shown to outperform wild-type cotton plants in the field (He *et al.*, 2005), and our data confirmed that result (*AtNHX1*-expressing cotton plants produced 23% more boll and 27% more fibre than wild-type cotton). It appears that *AVP1*-expressing cotton plants performed at least the same as *AtNHX1*-expressing cotton plants in the field. In our field trial of 2008, we obtained similar results. Again, *AVP1*-expressing cotton plants outperformed wild-type plants with respect to boll number per

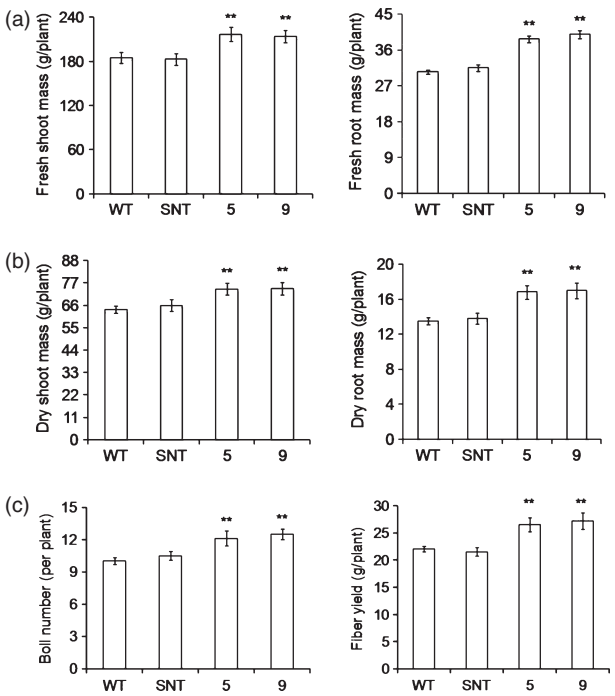

Figure 5 Biomass and yield of wild-type, segregated non-transgenic and *AVP1*-expressing cotton plants after salt treatment. a. Fresh shoot mass and fresh root mass after salt treatment. WT, wild-type plants; SNT, segregated non-transgenic plants; 5 and 9, two independent lines of *AVP1*-expressing cotton. The measurement was taken 30 days into 200 mM NaCl treatment. b. Dry shoot mass and dry root mass after salt treatment. c. Boll number and fibre yield of salt-treated plants at the end of the experiment in greenhouse. **statistically significant at 1% level.

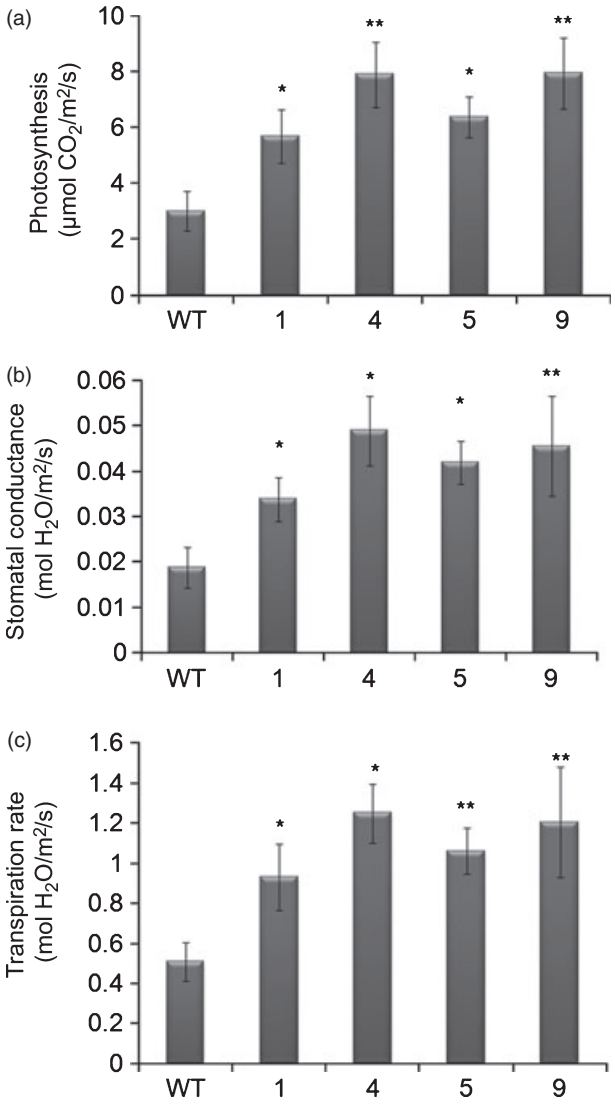

Figure 6 Photosynthetic performance of wild-type and *AVP1*-expressing cotton plants during salt treatment. a. Photosynthesis during salt treatment. WT, wild-type plants; 1, 4, 5 and 9, four independent lines of *AVP1*-expressing cotton. The measurements were taken when plants were 28 days into 200 mM salt treatment. b. Stomatal conductance under salt treatment. c. Transpiration rate under salt treatment. **statistically significant at 1% level; *statistically significant at 5% level.

plant and fibre yield per plant (Figure 8b). These three *AVP1*-expressing cotton lines produced 22%, 32% and 34% more boll per plant, and 21%, 30% and 25% more fibre per plant than wild-type cotton in the field. Again, the *AtNHX1*-expressing cotton plants produced 25% more boll and 21% more fibre than wild-type cotton. Our data clearly indicate that *AVP1* expression in cotton can increase fibre production in the field in the semi-arid land conditions of America's Southwest.

Discussion

One of the promising strategies for increasing agricultural productivity is through application of plant biotechnology, which provides tools for crop improvement (Herrera-Estrella, 2000). Applying the knowledge gained from studying model systems to improve crop productivity was the main objective of this research. Transgenic Arabidopsis plants overexpressing *AVP1* demonstrated higher tolerance to both salinity and drought compared to wild-type plants grown under similar conditions (Gaxiola *et al.*, 2001). To test whether this strategy could improve cotton's performance under salinity or drought conditions, transgenic cotton plants that express *AVP1* were generated. As expected, *AVP1*-expressing cotton plants performed better than wild-type and segregated non-transgenic cotton plants under salinity and drought conditions. For example, *AVP1*-expressing cotton plants grew much better in the presence of 200 mM NaCl in the hydroponic growth condition (Figure 3b). The increased salinity tolerance was

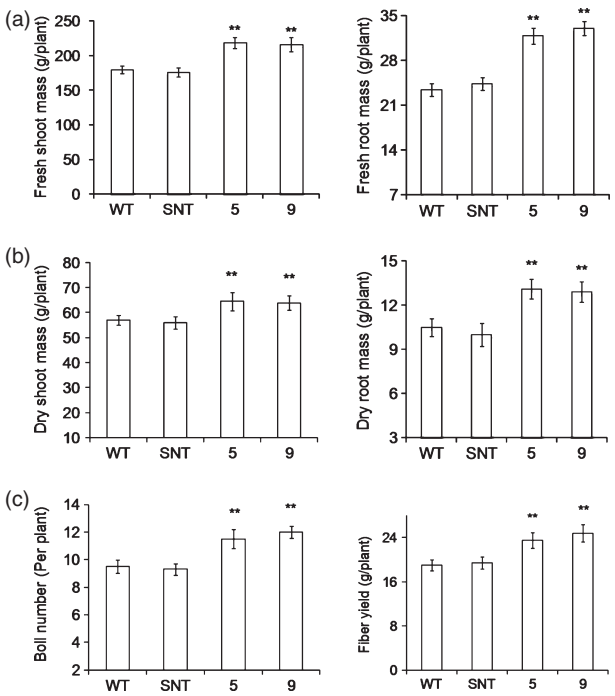

Figure 7 Biomass and yield of wild-type, segregated non-transgenic and *AVP1*-expressing cotton plants after drought treatment. a. Fresh shoot mass and fresh root mass after reduced irrigation treatment. WT, wild-type plants; SNT, segregated non-transgenic plants; 5 and 9, two independent lines of *AVP1*-expressing cotton. The measurement was taken 60 days into reduced irrigation treatment. b. Dry shoot mass and dry root mass after reduced irrigation treatment. c. Boll number and fibre yield of drought-treated plants at the end of the experiment in greenhouse. **statistically significant at 1% level.

measured by quantifying the biomass of cotton plants. The dry shoot mass and the dry root mass of all *AVP1*-expressing seedlings were significantly higher than those of wild-type plants grown under 200 mM NaCl condition for 1 week (Figure 3c, d). In particular, the dry root weights of transgenic seedlings were at least 85% or higher than those of wild-type seedlings, consistent with the finding from other systems where *AVP1*-overexpression significantly increased root development, although the enhanced root development triggered by overexpression of *AVP1* in Arabidopsis is not NaCl dependent (Gaxiola *et al.*, 2001; Li *et al.*, 2005).

The soil-grown *AVP1*-expressing cotton also displayed significantly improved salt tolerance in greenhouse conditions. After treatment with 200 mM NaCl for 30 days, they were physically much larger than the control plants (Figure 4a), and the root systems were more developed as well (Figure 4b). Fresh biomass of both shoot and root of *AVP1*-expressing plants is significantly higher than those of control plants (Figure 5a); so is the dry biomass of shoot and root of *AVP1*-expressing plants (Figure 5b). Again, the increase in root fresh weight or root dry weight is more than the increase in shoot fresh weight or shoot dry weight in *AVP1*-expressing plants (27% and 31% increase versus 17% and 19% increase in fresh weight and 24% and 26% increase vs. 16% and 16% increase in dry weight, respectively). This difference in biomass between *AVP1*-expressing plants and control plants is likely the result of the difference in photosynthesis between *AVP1*-expressing plants and control plants under salt conditions. The *AVP1*-expressing plants maintained a much higher photosynthetic rate than control plants under 200 mM NaCl condition (Figure 6a), which explains the biomass difference between *AVP1*-expressing plants and control plants. Previous studies have shown that salinity does decrease the photosynthetic activity in plants. The enhanced photosynthetic activity of *AVP1*-expressing cotton could be explained by a more efficient Na^+ vacuolar sequestration capacity in the transgenic plants. In some rice varieties, the net photosynthetic rate (measured in terms of CO_2 assimilation) of the youngest fully expanded leaf declined with increasing levels of salinity stress (Dionisio-Sese and Tobita, 2000), which was attributed to the direct effect of salt on stomatal conductance via a reduction in guard cell turgor (Dionisio-Sese and Tobita, 2000). Moradi and Ismail (2007) showed that in some salt-tolerant rice cultivars, both during seedling and reproductive stages, photosynthetic rate, stomatal conductance and transpiration rate decreased under saline conditions, and the values were even greatly decreased for the salt-sensitive cultivar. *AVP1* overexpression in cotton leads to higher yield in transgenic cotton plants after salt treatment (Figure 5c).

In addition to better performance under saline conditions, *AVP1*-expressing cotton plants are also significantly more drought resistant, which could be attributed to their larger root systems. Under reduced irrigation conditions, *AVP1*-expressing plants are physically larger than control plants (Figure 4c), and the root systems of *AVP1*-expressing plants are much larger than those of wild-type and segregated non-transgenic plants (Figure 4d). Again, the fresh shoot biomass and fresh root biomass of *AVP1*-expressing plants are significantly higher than those of control plants (Figure 7a, b). The yield of *AVP1*-expressing plants is also significantly higher than that of control plants after drought treatment (Figure 7c). Our data are consistent with the data obtained from Arabidopsis that *AVP1* overexpression leads to simultaneous increase in both salt tolerance and drought tolerance in transgenic plants (Gaxiola *et al.*, 2001). More importantly, our field

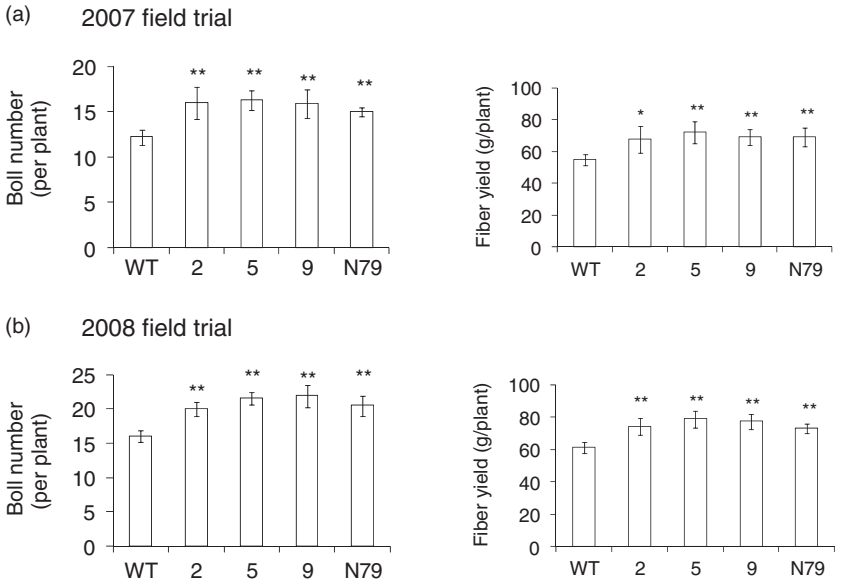

Figure 8 Boll number and fibre yield of wild-type, *AtNHX1*-expressing and *AVP1*-expressing cotton plants in the dry-land field condition. WT, wild-type plants; 2, 5 and 9, three independent lines of *AVP1*-expressing cotton; N79, *AtNHX1*-expressing line. **statistically significant at 1% level.; *statistically significant at 5% level.

study data indicated that *AVP1*-expressing plants could produce more fibre in the dry-land conditions of West Texas (Figure 8), strongly suggesting that *AVP1* could be used to engineer higher drought tolerance and salt tolerance in crops, in addition to tomato, reported by Park *et al.* (2005).

Similar results were obtained with genes that encode vacuolar H^+-pump in different species. For example, overexpression of the wheat gene *TVP1* in Arabidopsis plants improves both drought tolerance and salt tolerance in transgenic Arabidopsis (Brini *et al.*, 2007). Overexpression of *TsVP*, a gene from the salt-tolerant species *Thellungiella halophila*, improves salt tolerance in tobacco (Gao *et al.*, 2006) and in cotton (Lv *et al.*, 2008). Furthermore, this gene was shown to improve drought tolerance when overexpressed in maize (Li *et al.*, 2008) and in cotton (Lv *et al.*, 2009). Interestingly, overexpression of *TsVP* in cotton increases root biomass in transgenic cotton plants under non-stress conditions (Lv *et al.*, 2008); whereas in our study, we did not find a significant difference in biomass between *AVP1*-expressing cotton plants and control cotton plants under normal conditions (Figures S2 and S3). *Thellungiella halophila* is a close relative of Arabidopsis, and TsVP and AVP1 share 96% identity at the amino acid level (Gao *et al.*, 2006); therefore it is not likely that the difference in protein structure is responsible for the phenotypic difference between *AVP1*-expressing cotton plants and *TsVP*-expressing cotton plants under normal conditions. Nevertheless, overexpression of either *AVP1* or *TsVP* can improve both drought tolerance and salt tolerance in other plants.

The increased salt tolerance in *AVP1*-overexpressing cotton could be explained by a higher $\Delta\mu H^+$ in vacuole that activates secondary transporters including vacuolar Na^+/H^+ antiporters, whereas the increased drought tolerance could be explained by larger root systems, which enables *AVP1*-overexpressing cotton plants absorb water more efficiently. However, the molecular mechanism of how *AVP1* overexpression leads to larger root systems is not clear. Li *et al.* (2005) suggested that *AVP1* overexpression stimulates auxin polar transport in root systems, which in turn stimulates root development. There were strong evidences that H^+-PPase is also found in plasma membrane (Long *et al.*, 1995; Ratajczak *et al.*, 1999; Langhans *et al.*, 2001), which leads to a hypothesis that H^+-PPase may play a more upstream role in mediating a cascade of events that leads to the activation of the other H^+-pumps and facilitating auxin polar transport in root system (Gaxiola *et al.*, 2007).

Even though successful overexpression of a single gene in plants has been achieved many times, simultaneous overexpression of more than one gene in the same plant might be desirable. Gaxiola *et al.* (2002) suggested that simultaneous overexpression of both vacuolar Na^+/H^+ antiporter and vacuolar H^+ pump would probably be required to further increase salinity tolerance of plants beyond 200 mM NaCl. Even though overexpression of *AVP1* alone already showed significantly improved drought tolerance and salt tolerance, we are speculating that double overexpression of *AVP1* and *AtNHX1* will likely make cotton plants perform even better under drought and salt conditions, and this strategy will have a major impact on cotton

production in areas of the world where water and salinity are major limiting factors for agricultural productivity.

Materials and methods

Cotton transformation

The *S35-AVP1* construct in Agrobacterium strain GV3101 was made previously by Gaxiola *et al.* (2001) and was used to transform the wild-type Coker 312 cotton. The transformation protocol by Bayley *et al.* (1992) was followed with minor modifications (Yan *et al.*, 2004).

DNA isolation for PCR analysis and DNA blot analysis

Genomic DNA was isolated from leaves of greenhouse-grown plants by using the method of Guillemaut and Maréchal-Drouard (1992) with minor modifications. Approximately one gram of fresh leaf tissue was ground in 3 mL extraction buffer [100 mM NaOAC (pH 4.8), 50 mM EDTA (pH 8.0), 500 mM NaCl, 2% PVP (10,000 MW), 1.4% SDS and 0.25 mg/mL Ribonuclease A]. The extract was transferred to Eppendorf tubes (1.5 mL) and incubated at 65°C for 15 min; then an equal volume of 10 M ammonium acetate was added and mixed well. Tubes were incubated at 65°C for 10 min to precipitate all proteins and then centrifuged at 10 000 ***g*** for 10 min. The supernatant was collected in a separate tube, and an equal volume of cold isopropanol was added and mixed by inverting the tubes several times. Samples were left at –20°C for one hour and centrifuged at 10 000 ***g*** for 10 min to recover DNA pellets. DNA pellets were dissolved in 500 μL TE buffer (buffer solution containing 10 mM Tris and 1 mM EDTA) and extracted twice with PCI (phenol: chloroform: isoamyl alcohol = 25: 24: 1) and once with CI (chloroform: isoamyl alcohol = 24: 1). Solutions were transferred to fresh tubes, to which 1/10 volume of 3 M NaOAC and one volume of ice cold isopropanol were added and mixed well and placed at –20°C for two hours and centrifuged at 10 000 ***g*** for 12 min. The pellets were washed with 70% (v/v) ethanol and resuspended in 50 μL of DNase-free water. The genomic DNA concentration and purity was determined using Nanodrop (NanoDrop Technologies, Wilmington, DE, USA).

PCR amplification was carried out with a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) using the ExTaq polymerase (TaKaRa, Shiga, Japan). Primers were designed to amplify the partial segment of *AVP1*. The 30 μL PCR mixture contained 5 μL genomic DNA (~0.1 μg/μL), 3 μL 10× Ex*Taq* buffer, 4 μL of dNTP (2.5 mM), 1 μL of forward primer (0.1 μg/μL, 5′-GGCTCTGTTGAGGGATTCAG-3′), 1 μL of reverse primer (0.1 μg/μL, 5′-GCAATGACAGCTGGGTTTCTT-3′), 0.125 μL ExTaq polymerase enzyme (5 units/μL) and 16 μL H_2O. The PCR condition was set as follows: an initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

Genomic DNAs from wild-type and transgenic lines 5 and 9 were digested with the restriction enzyme *Eco* RI, electrophoresed (20 μg per lane) in 0.8% agarose, treated with 0.25 M NaOH, neutralized with 0.5 M Na_2HPO_4 (pH 6.5), blotted to a Biotrans Nylon membrane (ICN Biomedicals, Inc., Aurora, OH, USA) and hybridized with an *AVP1* cDNA probe. Hybridization was carried out according to the method of Church and Gilbert (1984) using probe labelled by random priming (Feinberg and Vogelstein, 1983), and the washing conditions were as follows: two times (10 min each) in 0.5% BSA, 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2) and 5.0% SDS at 63°C; then four times (5 min each) in 1 mM EDTA, 40 mM Na_2HPO4 (pH 7.2) and 1% SDS at 63°C.

Isolation of total RNAs for RNA blot analysis

About 2 grams of leaves from the cotton seedlings (5–6 leaf stage) was collected and ground into fine powder in liquid nitrogen and then transferred into tubes containing 15 mL of isolation buffer [Tris-HCl 100 mM (pH 9.0), NaCl 200 mM, 1% sarcosyl, EDTA 20 mM (pH 9.0) and 10% PVP (MW 40 000)], supplemented with 1 mL PCI. Tubes were centrifuged for 10 min at 3000 ***g*** at 4°C. Supernatant from each tube was transferred into a new tube and extracted with PCI and CI. The top layer was transferred to a new tube which consisted of an equal volume of 6 M LiCl and mixed well by inverting tubes; the tubes were placed on ice for ~1 h and then centrifuged for 10 min at 10 000 ***g*** and 4°C. The supernatant was collected in a new tube, and 2 mL of 2% potassium acetate was added and mixed well by inverting. Samples were then incubated for 5 min at 65°C and centrifuged at 10 000 ***g*** for 10 min with the supernatant being transferred to a fresh tube, and 0.1 volume of 3 M sodium acetate and three times volume of iced 100% ethanol were added. The samples were mixed by inversion and placed at –20°C for 2 h and then centrifuged at 10 000 ***g*** for 10 min at 4°C. The pellets were washed with 75% ethanol, air-dried and then resuspended in 20 μL diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration was calculated using Nanodrop (NanoDrop Technologies). The ratio of the readings at 260 nm and 280 nm (A260/A280) ranged from 1.8 to 2.1, indicating that the purity of RNAs was high.

A total of 10 μg of total RNAs per lane was loaded into each lane and separated by electrophoresis in a 1.2% formaldehyde-agarose gel and then transferred onto a Biotrans nylon membrane (ICN Biomedicals, Inc.) using capillary blot procedure. The RNAs were cross-linked to the membrane at 1200 μJ/cm^2 for 12 s, air-dried and then baked for 2 h at 80°C in a vacuum oven. The membrane was then prehybridized for 1 h at 64°C in the prehybridizing solution [1% BSA, 1 mM EDTA (pH 8.0), 0.5 mM Na_2HPO_4 (pH 7.2), 7% SDS]. Overnight hybridization at 64°C was performed with the denatured ^{32}P-labelled probe made by random priming (Feinberg and Vogelstein, 1983). The hybridization solution was the same as the prehybridization solution. The membrane was then washed with the solution I [0.5% BSA, 1 mM EDTA (pH 8.0), 40 mM Na_2HPO_4(pH 7.2), 5% SDS] at 64°C for 5 min, and two times with solution II [0.1 mM EDTA (pH 8.0), 40 mM Na_2HPO_4 (pH7.2), 1% SDS] at 64°C for 10 min each. The membrane was wrapped in saran wrap and exposed to a phosphor screen for 2–5 h. Radioactivity was detected by scanning the phosphor screen using a phosphor imager. The membrane was stripped at 74°C by washing in the stripping solution [2 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0), 0.08% SDS] 2–3 times, 10 min

each, until the radioactivity was completely lost before the membrane was used for another hybridization.

Immunolocalization

Leaves from wild-type and transgenic cotton (line 5) were fixed with FAA (3.7% formaldehyde, 50% ethanol, 5% acetic acid). Embedding and sectioning of the abaxial phloem region were performed as described by Paez-Valencia *et al.* (2008). For immunolocalization experiments, microtome sections of 10 μm were deparaffinized with xylene and rehydrated through a graded ethanol series. After deparaffination and hydration, the slides were washed in phosphate-buffered saline (PBS, pH 7.0) solution. To recover antigenicity, histological sections were immersed in antigen retrieval citraplus solution (BioGenex HK080-5K) and autoclaved for 5 min. The sections were incubated with the primary antibody raised against a keyhole limpet haemocyanin-conjugated peptide corresponding to the putative hydrophilic loop IV of the AVP1 protein (Sarafian *et al.*, 1992) at a 1:1000 dilution in PBS, as well as pre-immune sera rabbit (negative control data not shown). Three rinses with PBS were followed by incubation of the samples using the LSAB+System HRP (DAKO) following the manufacturer's instructions. After several washes, the samples were contrasted with Fast Green (Sigma).

Membrane isolation and delipidation of microsomal fractions

Leaf microsomal fractions were isolated from hydroponically grown plants as described previously (Randall and Sze, 1986). Briefly, the plant material was ground with a cold mortar and pestle, in cold homogenization buffer, containing 50 mM Hepes-BTP, pH 7.4, 250 mM sorbitol, 6 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μg/mL pepstatin and 1 μg/mL leupeptin. The homogenate was passed through four layers of cheesecloth and centrifuged at 12 000 ***g*** for 10 min at 4°C. The supernatant was subsequently centrifuged at 60 000 ***g*** for 30 min. The resulting microsomal fraction pellet was resuspended into a buffer containing 25 mM Hepes-BTP, pH 7.4, 250 mM D-Sorbitol, 1 mM DTT and 1 mM PMSF, 5 μg/mL pepstatin and 1 μg/mL leupeptin. To remove non-covalently lipids associated, 100 μg of proteins of each sample was precipitated with five volumes of cold acetone, washed three times in 1.0 mL Chloroform/methanol(2:1, vol/vol), washed once in 1.0 mL chloroform/methanol/water (10:10:3 vol/vol/vol) and once in 1.0 mL of acetone prior to air-drying at 37°C. The dried proteins were solubilized in Laemli sample buffer at 100°C (Laemli REF) without reducing agents and tracking dyes. Dithiothreitol (final concentration, 50 mM) and bromophenol blue were added to solubilized samples that were loaded on a 7% polyacrylamide/8M urea for electrophoreses. Equivalent amount of protein (100 μg) was loaded in each lane.

Western Blot Analysis

For Western blotting, equal amounts of protein (100 μg) were resolved under the same conditions as mentioned earlier and transferred to Immobilon-P membranes (Millipore, USA). After blocking with 5% defatted milk, the membranes were incubated with specific polyclonal sera raised against the pyrophosphate-binding domain of the H^+-PPase AVP1(Sarafian *et al.*, 1992) at room temperature during 12 h. After 1.5 h of incubation with a secondary antibody conjugated with alkaline phosphatase, the membranes were treated with a NBT/BCIP substrate solution (Roche, Indianapolis, IN, USA) for staining.

Cotton growth under hydroponic salt conditions

Seeds were placed on wet germination paper and placed in a germinator for 5 days at 30°C. Then, seedlings were carefully removed from paper and put into tubs containing a nutrient solution (Figure 3a) in the greenhouse for recovery. After 2 days, fertilizer (20:20:20 NPK, $CaNO_3$, $MgSO_4$ as per the manufacturers recommendation) was added. Three tubs were used for control and three tubs for salt treatment. The salt treatment was made as follows: 2 days at 50 mM, 2 days at 100 mM, 2 days at 150 mM and 7 days at 200 mM. An equal amount of water (30 L) was maintained in both control and salt-treated tubs. Plants were removed from the tubs and dried with paper towels to measure shoot length, fresh shoot weight, root length and fresh root weight measurements. After drying, the seedlings were placed in oven at 55°C for 4 weeks and then dry shoot and dry root weights were measured.

Cotton growth under salt and drought conditions in the greenhouse

Cotton seeds from wild-type plants, segregated non-transgenic plants (i.e. SNT plants) and two homozygous *AVP1*-expressing plants (lines 5 and 9, respectively) were planted into 15-gallon pots containing soil mix. These plants (>100 for each line) were allowed to grow under normal conditions for 21 days and then were divided into three groups: well-watered group as control, salt treatment group and drought treatment group. For salt treatment (Figure S1a), on day 22, plants were irrigated with 1000 mL of 50 mM NaCl solution every other day, 6 days later the concentration of NaCl was increased to 100 mM, then to 150 mM and finally to 200 mM. Plants were treated for 30 days at 200 mM NaCl; half was then harvested for biomass analysis, and the other half was irrigated with normal water again until the end of the experiment. For drought treatment (Figure S1b), on day 22, water was withheld for 5 days; irrigation was then resumed with 300 mL of water per pot every other day for 20 days; at the end of that time, the irrigation was increased to 600 mL of water every other day. After 40 days under this condition, half of the plants were harvested for biomass analysis, while the other half was allowed to continue growing under the same condition until the end of experiments. For the well-watered group, some plants were harvested on day 21 and day 69 for biomass analysis, and one group was allowed to grow under normal conditions until the end of the experiment. Once the boll was fully opened, fibre was harvested. In the biomass analysis, the shoot was cut at the soil level for weight measurements. For fresh root weight measurement, soil from the pot was washed away by rinsing the roots

with water carefully, and roots were dried on paper towel. The shoots and roots were dried in an oven of 75°C for 2 weeks before their dry weights were measured. The temperature in the greenhouse was maintained at 28 ± 2°C, and the relative humidity was maintained at 50 ± 10% throughout the growth period.

Leaf gas exchange measurements

Cotton seeds from wild-type, T_1 transgenic lines 1, 4, 5 and 9 were sterilized and germinated on sterile Stewart's germination media (Stewart and Hsu, 1977). Transgenic seeds were grown on a medium containing kanamycin (50 μg/mL) for screening transgenic plants as described earlier. Resistant plants with a complete root system, including lateral roots, were used for experiments. Each plant was transferred to a small pot containing pro-mix BX peat moss, perlite and vermiculite medium (Premier Brands, New Rochelle, NY, USA) and grown in a growth chamber until the 3–4 leaf stage. Plants were then transferred into two gallon pots containing pro-mix and placed in the greenhouse. Each pot was supplied with one Osmocote plus® (15-8-11) tablet once and were supplied with Scotts Peters excel (15-5-15 Cal-Mag) water-soluble fertilizer each week thereafter. After becoming established for 2 weeks, salt treatment was started as follows: 50 mM NaCl for 7 days, 100 mM NaCl for 7 days, 150 mM NaCl for 7 days and 200 mM of NaCl for 4 weeks. Leaf gas exchange measurements were taken during this stage. To characterize photosynthetic performance, gas exchange measurements were taken with a portable photosynthesis system (Li-6400, LI-COR Inc., Lincoln, NE, USA). Measurements were taken on young, fully expanded, intact leaves of greenhouse-grown, salt-treated and control plants. Net CO_2 assimilation rate, stomatal conductance and transpiration were assessed at a CO_2 concentration of 400 μmol/mol, ~50% relative humidity, 28°C chamber temperature, 500 μmol/s air flow and a photon flux density of 1500 μmol/m^2/s. The instrument was stabilized according to manufacturer guidelines. Steady-state levels of reference CO_2 and reference H_2O were observed before taking measurements. The IRGAs (infra-red gas analysers) were matched manually before taking measurements. Five measurements were taken for each sample.

Field experiment

Wild-type, one *AtNHX1*-expressing transgenic line (N79) and three *AVP1*-expressing transgenic lines (2, 5, 9) were field-tested at the USDA-ARS experimental farm in Lubbock, TX. The land was slightly moist during the time of sowing, and no further irrigation was provided to the dry land. Standard agronomical practices were performed for weed control and pesticide application. Boll number per plant and fibre yield per plant were analysed at the end of the growth season.

Statistical analysis

Statistic analyses were performed using Microsoft Office Excel 2007. All *P* values are from comparison between transgenic and wild type. $^{*}P < 0.05$, $^{**}P < 0.01$ are the two significance levels shown in the data.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 The designs for salt and drought stress treatments.

Figure S2 Phenotype (A) and biomass (B) of wild-type, segregated non-transgenic, and *AVP1*-expressing cotton plants before stress treatment.

Figure S3 Biomass and yield of well-watered control plants in greenhouse.

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