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ORIGINAL ARTICLE

Expression of the *Arabidopsis* vacuolar H^+-pyrophosphatase gene *AVP1* in peanut to improve drought and salt tolerance

Hua Qin · Qiang Gu · Sundaram Kuppu · Li Sun · Xunlu Zhu · Neelam Mishra · Rongbin Hu · Guoxin Shen · Junling Zhang · Yizheng Zhang · Longfu Zhu · Xianlong Zhang · Mark Burow · Paxton Payton · Hong Zhang

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Abstract The *Arabidopsis* gene *AVP1* encodes an H^+-pyrophosphatase that functions as a proton pump at the vacuolar membranes, generating a proton gradient across vacuolar membranes, which serves as the driving force for many secondary transporters on vacuolar membranes such as Na^+/H^+-antiporters. Overexpression of *AVP1* could improve drought tolerance and salt tolerance in transgenic plants, suggesting a possible way in improving drought and salt tolerance in crops. The *AVP1* was therefore introduced into peanut by Agrobacterium-mediated transformation. Analysis of *AVP1*-expressing peanut indicated that *AVP1*-overexpression in peanut could improve both drought and salt tolerance in greenhouse and growth chamber conditions, as *AVP1*-overexpressing peanuts produced more biomass and maintained higher photosynthetic rates under both drought and salt conditions. In the field, *AVP1*-overexpressing peanuts also outperformed wild-type plants by having higher photosynthetic rates and producing higher yields under low irrigation conditions.

Keywords Drought tolerance · Peanut transformation · Salt resistance · Transgenic plants · Yield improvement

Abbreviations

AVP1	*Arabidopsis* vacuolar pyrophosphatase 1
IPT	Isopentenyltransferase

H. Qin · Y. Zhang
College of Life Sciences, Sichuan University, Chengdu, Sichuan, China

Q. Gu · S. Kuppu · L. Sun · X. Zhu · N. Mishra · R. Hu · H. Zhang (✉)
Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA
e-mail: hong.zhang@ttu.edu

G. Shen
Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang, China

J. Zhang · M. Burow
Texas AgriLife Research Center, Texas A&M University, Lubbock, TX 79403, USA

L. Zhu · X. Zhang
College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, China

P. Payton
USDA-ARS Cropping Systems Research Laboratory, Lubbock, TX 79415, USA

Introduction

Drought and salinity are the two major environmental factors that cause huge crop losses worldwide annually (Boyer 1982; Boyer and Westgate 2004). The climate change is increasing the earth's surface temperature, and it affects rainfall patterns and increases the chance of having extreme weather conditions in many places on earth, which negatively affects agricultural production in the world (Long and Ort 2010; Battisti and Naylor 2009). Yet, the pressure from world population growth demands more food production from our decreasing crop lands. In fact, we must increase food production by at least 50 % within the next 20–30 years, as the world population is expected to reach 9 billion by 2050 (FAOUN 2010). The real challenge that we face today is to develop technologies that will increase food production under various stressful conditions such as drought and salinity, and with limited crop land to meet human demand.

Traditional breeding approach has been used to select drought and salt tolerant crop varieties for a long time. It has been largely successful in modern agriculture and will remain as a tool. However, due to the lengthy time required for introgressing the genes found into cultivated varieties and limitation in the same species, other approaches must be sought. With the advent of molecular biology, genetic engineering has emerged as a powerful approach that may revolutionize agriculture in terms of identifying functional genes and deploying these genes into crops in much shorter times. Indeed, the development of herbicide-resistant crops and Bt-containing crops are the testament of powerful impact of genetic engineering on today's agriculture. Over the last 20 years, many genes that confer drought tolerance and salt tolerance have been found and tested in the field, and a few are in the final stages of the approval process for commercial release (Lemaux 2008, 2009; Mittler and Blumwald 2010; Castiglioni et al. 2008; Yang et al. 2010).

Among the first group of genes identified for improving crops are transcription factor genes that control gene expression under stress conditions (Century et al. 2008). For example, the DREB/CBF class of transcription factor genes activate gene expression in response to drought and temperature stresses (Mittler and Blumwald 2010; Yang et al. 2010). Overexpression of some members of this class of genes could increase both drought and heat tolerance in transgenic plants (Qin et al. 2007). Therefore, this class of genes may be useful in creating heat- and drought-tolerant crops in the future. Besides the transcription factor genes, other types of genes have also been found to play critical roles in conferring stress tolerance. Some of these genes encode enzymes in stress signal transduction pathways, hormone biosynthesis pathways, or functional proteins that play protective roles under stress conditions (Lemaux 2008, 2009; Mittler and Blumwald 2010). One example is that the production of cytokinin under water-deficit conditions could confer increased drought tolerance in transgenic plants (Rivero et al. 2007; Peleg et al. 2011). We also demonstrated that transgenic peanut plants that contain the P_{SARK}*::IPT* construct are significantly more drought-tolerant than wild-type control plants under laboratory and field conditions (Qin et al. 2011). The *IPT* gene encodes an isopentenyltransferase that plays a critical role in cytokinin biosynthesis, and when the *IPT* gene is under the control of a water-deficit inducible promoter, i.e. P_{SARK}, transgenic plants demonstrate a significantly increased drought tolerance phenotype (Rivero et al. 2007; Peleg et al. 2011; Qin et al. 2011).

Peanut ranks No. 5 among the major oilseed crops in the world and is very nutritious in various vitamins and ions (http://www.peanutusa.com). The top peanut producing countries are China, India, and USA, and yet the production of peanut in these countries is under threat due to decreasing availability of water and increasing salinity in soils. The yield and quality (i.e. taste) are adversely affected by drought and salinity (Stansell and Pallas 1985; Hashim et al. 1993; Lamb et al. 1997; Craufurd et al. 1999). Making peanut drought- and salt-tolerant will ensure that the peanut production can be maintained in these and other peanut producing countries in a sustainable way. One method to make peanut drought- and salt-tolerant is to increase the proton pump activity on the vacuolar membranes, which transports proton into vacuoles (Gaxiola et al. 2001). Increased proton chemical gradient across vacuolar membranes will energize the secondary transporters such as sodium/proton antiporters, leading to decreased water potential inside the vacuoles and increased salt tolerance (Park et al. 2005). Interestingly, overexpression of the *Arabidopsis* proton pump gene *AVP1* not only led to increased salt tolerance but also increased auxin polar transport in roots, leading to robust root development, and therefore significantly increased drought tolerance in transgenic plants (Li et al. 2005). In fact, overexpression of *AVP1* in heterologous systems could lead to increased drought tolerance and salt tolerance (Zhao et al. 2006; Pasapula et al. 2011). We have therefore introduced *AVP1* into peanut in an effort to improve drought and salt tolerance in peanut. Our data indicate that, indeed, *AVP1* is a promising gene to improve drought and salt tolerance in peanut.

Materials and methods

Peanut transformation

The peanut (New Mexico Valencia A variety) was transformed with the Agrobacterium strain LBA4404 harboring the *35S-AVP1* construct (Gaxiola et al. 2001). Agrobacterium cultures were grown overnight on a rotary shaker at 28 °C in the dark in a LB medium (pH 7.2) containing the antibiotics rifampicin (50 mg l^{-1}), spectinomycin (100 mg l^{-1}), and streptomycin (25 mg l^{-1}). The cultures were centrifuged and resuspended in fresh MS medium (Murashige and Skoog 1962) plus 100 μM acetosyringone to a final concentration of 10^8 cells per ml of culture medium ($A_{620} = 0.6$) before used for peanut transformation. The transformation protocol was described by Qin et al. (2011).

Molecular analysis of transgenic peanut plants

DNA isolation and PCR analysis

Genomic DNAs were isolated from fresh peanut leaves of greenhouse-grown plants by using the PowerPlant™ DNA

Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). The DNA concentration was determined using the Nanodrop instrument (NanoDrop Technologies, Wilmington, DE, USA). The polymerase chain reaction (PCR) was used to confirm transformants by using the 35S promoter specific primer p35S-F1 and the *AVP1* specific primer AVP1-R1. PCR amplification was carried out in a total volume of 25 μl containing 100 ng of genomic DNA, 1 U of GoTaq DNA polymerase (Promega, Madison, WI, USA) and 0.4 μM of each forward and reverse primer. Cycling conditions comprised an initial denaturation at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 50 s and a final extension of 10 min at 72 °C. The amplified products were electrophoresed on 1.2 % agarose gel and visualized in the presence of ethidium bromide with a gel documentation system.

The sequences of the primers used in PCR are: p35S-F1, CAAGACCCTTCCTCTATATAAGG, and AVP1-R1, CG TATGTAGCAATCTTCATCCCAA.

RNA blot analysis

Total RNAs were isolated from fresh leaves of peanut seedlings using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, MO, USA). A total of 12 μg of total RNAs per sample was separated by electrophoresis in a 1 % (w/v) formaldehyde-agarose gel, and transferred onto a Biotrans™ nylon membrane (MP Biomedicals, Solon, OH, USA). The hybridization condition was described in the protocol of Church and Gilbert (1984).

Plant growth under salt condition in the greenhouse

Seeds of wild-type and four *AVP1*-expressing plants (lines 1, 2, 4, and 6) were planted into 16-l pots filled with promix BX peat moss, perlite, and vermiculite. These plants were allowed to grow under normal conditions for 14 days, then salt stress was imposed. The salt treatment was conducted in an incremental manner, starting with 400 ml of 30 mM NaCl for 6 days followed by 6 days of 60 mM NaCl and 10 days of 90 mM NaCl. At that point, 500 ml of 120 mM NaCl was applied for 10 days until the end of the experiment. During salt treatment, one leaf from each plant was taken for PCR analysis of the presence of *AVP1*, and at least six PCR positive lines from transgenic plants were chosen for data analysis. After the salt treatment, biomass was determined by measuring fresh weight and dry weight. The fresh weight of each individual plant was measured immediately after harvest. Dry weight was measured after 48 h of drying at 60 °C in an air oven. The temperature in the greenhouse was maintained at 25 ± 2 °C, and the relative humidity was maintained at 50 ± 10 % throughout the growth period. A duplicate set of plants that were irrigated with normal water were used as controls.

Plant growth under reduced irrigation condition in growth chamber

Seeds of wild-type and four *AVP1*-expressing plants were planted into 11-l pots filled with soil mix, germinated in a growth chamber under controlled conditions (25 °C, 500 μmol photons m^{-2} s^{-1}, 16 h photoperiod) for 15 days, and grown for another 15 days without irrigation. After that, half of the wild-type and transgenic plants were selected to receive 300 ml of water every 3 days (designated as optimal watering condition), whereas the other half of the plants received 150 ml of water every 6 days (reduced irrigation condition). Plants were grown for another 45 days. No water drained out of the pots in these treatments. During this time, one leaf from each plant was taken for PCR analysis for the presence of *AVP1*, and at least six PCR positive lines from transgenic plants were chosen for data analysis. After the reduced irrigation treatment, biomass was determined by measuring fresh weight and dry weight. The fresh weight of each individual plant was measured immediately after harvest. Dry weight was measured after 48 h of drying at 60 °C in an air oven.

Gas exchange measurements

Gas exchange measurements were conducted on the third nodal leaf using a LI-COR 6400 infrared gas exchange analyzer (Licor, Lincoln, NE, USA). Leaf-to-air vapor pressure deficit was set to ambient conditions, block temperature was 25 °C, the CO_2 concentration was set at 400 μmol mol^{-1} and maintained for all measurements across pots. Irradiance was set to 1,500 μmol m^{-2} s^{-1} using a light-emitting diode LI-6400-002 (Licor). Leaves were first equilibrated in the chamber for at least 5 min prior to logging measurements of gas exchange. Field measurements of photosynthesis were taken under an irradiance of 2,000 μmol photons m^{-2} s^{-1} and 370 μmol mol^{-1} CO_2 in the leaf chamber of a LI-COR 6400. Leaf temperature was maintained at 25 °C. Gas exchange measurements were carried out in the middle of August, starting at 0900 hours and continuing until 1200 hours.

Leaf chlorophyll measurement

The relative content of chlorophyll was measured by using a portable chlorophyll meter SPAD-502PLUS (Konica Minolta, Japan). The mean of three readings was taken on one side of the midrib of each primary leaf blade, midway between the leaf base and tip from individual leaf arrangement.

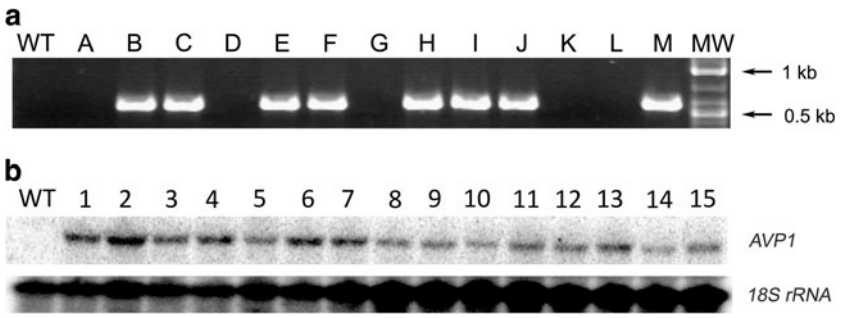

Fig. 1 Molecular analysis of transgenic peanut plants. **a** PCR analysis of transgenic peanut plants. *WT* wild-type plant, *lanes A–M* 13 independent putative transgenic lines, *MW* DNA molecular weight markers. The 35S promoter specific primer and *AVP1*-specific primers were used in the PCR analysis. **b** RNA blot analysis of transgenic peanut plants. *Lanes 1–15*, 15 independent transgenic peanut lines that were tested positive in the PCR experiment. A cDNA clone for *AVP1* was used as the probe, and the 18S rRNA was used as the RNA loading control

Peanut growth in the field

Peanut plants were field-grown at the Experimental Farm of the USDA-ARS Cropping Systems Research Laboratory in Lubbock, TX, in the middle of May in both 2009 and 2010. Wild-type and four transgenic lines 1, 2, 4, and 6 were used in 2009, and wild-type, segregated non-transgenic plants (NT), and the four transgenic lines 1, 2, 4, and 6 were used in 2010. In 2009, one treatment was used: low irrigation. In 2010, two treatments were used: low irrigation (19 mm per week) and high irrigation (38 mm per week). Seeds were sown into 2-m rows at a density of 20 seeds m^{-1} and a 100-cm row spacing in a 16-row block in 2009 and a 24-row block in 2010. Individual plants were randomly selected each time from *AVP1*-expressing peanut lines for gas exchange analysis in 2009. However, in 2010, all transgenic plants were tested for the presence of transgene *AVP1* by using the PCR method. The weight of peanut seeds and the dry weight of above-ground biomass were collected at the end of experiments.

Oil and fatty acid analysis in peanut seeds

Oil content analysis

The time domain nuclear magnetic resonance (TD-NMR) experiments were carried out on a Bruker Minispec MQ10 NMR Analyzer. The instrument was calibrated by eight pure peanut oil calibration standards at the concentrations of 0, 10, 20, 30, 40, 50, 60, and 70 %. Data were acquired using the Minispec software (Bruker) and the data oil program (Bruker). The parameters were set up based on the description of Qin et al. (2011).

Fatty acid composition analysis

The fatty acids of peanut seeds were converted to their methyl esters, which were extracted into hexane and

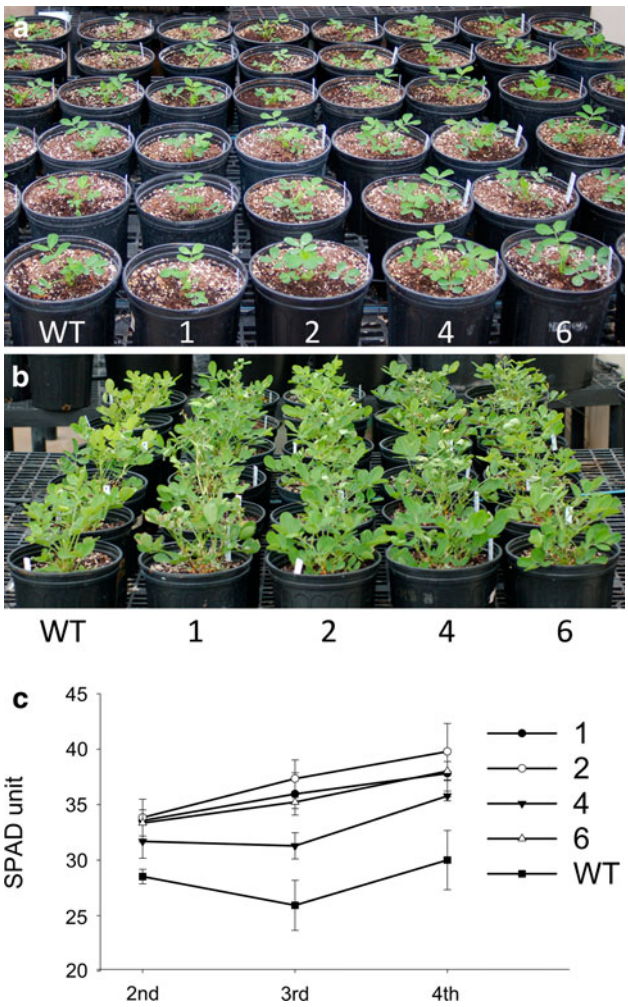

Fig. 2 Phenotypes of wild-type and *AVP1*-expressing peanut plants before and after salt treatment in greenhouse. **a** Fifteen-day-old peanut plants under normal irrigation conditions. **b** Phenotypes of wild-type and transgenic peanut plants under salt treatment conditions for 32 days. **c** SPAD values of wild-type and transgenic peanut plants at 3 different leaf positions after salt treatment for 31 days. *WT* wild-type plant, *1, 2, 4, 6* four independent transgenic peanut lines. *Bar* standard error; $n = 6$ for each line

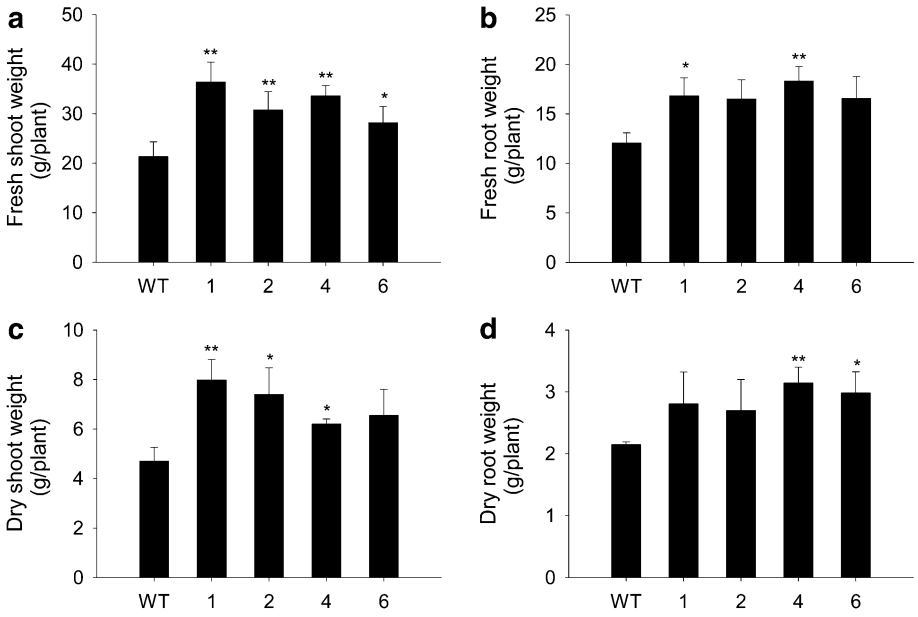

Fig. 3 Biomass of wild-type and *AVP1*-expressing peanut plants after salt treatment for 32 days. **a** Fresh shoot weight of peanut plants. **b** Fresh root weight of peanut plants. **c** Dry shoot weight of peanut plants. **d** Dry root weight of peanut plants. *WT* wild-type plant, *1, 2, 4, 6* four independent transgenic peanut lines. *Bar* standard error; *significant at 5 %; **significant at 1 %; $n = 6$ for each line

analyzed by HP 5890 gas chromatography (GC) with a flame ionization detector (FID) and a capillary column (DB-Wax, 30 m length, 0.53 mm i.d., 0.50 μm film thickness) (Qin et al. 2011). Fatty acids were identified by comparison with fatty acid methyl ester standards (68A) purchased from Nu-Chek Prep, and were quantified by using the peak area percentage as a ratio to the total area of all methyl esters present.

Statistical analysis

Salt treatments and reduced irrigation experiments in greenhouse and growth chamber were performed two times with 4 independent lines and 6–8 plants for each line each time. Means of one representative experiment are presented. In field-grown plants, 40 plants were used for each line, among which 12 plants were used for photosynthesis analysis. The statistical significance of differences in the mean values of the examined parameters between the transgenic and wild-type plants was determined using the Student's *t* test ($*P < 0.05$, $**P < 0.01$).

Results

Creation and molecular analysis of *AVP1*-transgenic peanut plants

We used the *35S-AVP1* construct of Gaxiola et al. (2001) to transform peanut by using the Agrobacterium-mediated transformation method (Sharma and Anjaiah 2000). A total of 40 independent transgenic lines were obtained, and then we isolated genomic DNAs from these 40 plants and conducted PCR experiments using the *AVP1*-specific primers. It appeared that only about 60 % of these 40 plants were transgenic plants, as we could not detect an expected 0.6-kb DNA fragment from 40 % of them in the PCR analysis (an example of the PCR results is shown in Fig. 1a). However, all PCR positive lines contained *AVP1* transcript based on RNA blot analysis (Fig. 1b), suggesting that *AVP1* is expressed in all transgenic lines. Four lines 1, 2, 4, and 6 that were shown to express *AVP1* at relatively high levels were chosen for detailed physiological studies.

Expression of *AVP1* in peanut enhances salt tolerance

Under normal growth conditions, wild-type and transgenic peanut plants grew similarly in greenhouse, showed no phenotypic differences and produced the same amount of biomass in the greenhouse (data not shown). There were no differences in photosynthetic rate, transpiration and stomatal conductance between wild-type and transgenic plants after these plants were grown in the greenhouse for one and half months (data not shown). To test whether *AVP1*-expressing plants were more salt tolerant, we performed a salt tolerance test in the greenhouse. Following optimal irrigation for 14 days, a slow-onset salt stress was created by irrigation with increasing concentrations of NaCl. Prior to the salt treatment, wild-type and transgenic plants did not display any phenotypic differences (Fig. 2a); however, after treatment with NaCl for 32 days (from 30 up to 120 mM), the *AVP1*-expressing peanut plants were physically larger in size and were greener (Fig. 2b). The color of the transgenic plants suggested more chlorophyll contents in the leaves of *AVP1*-expressing peanut plants. We analyzed this by measuring the SPAD values of these plants, as

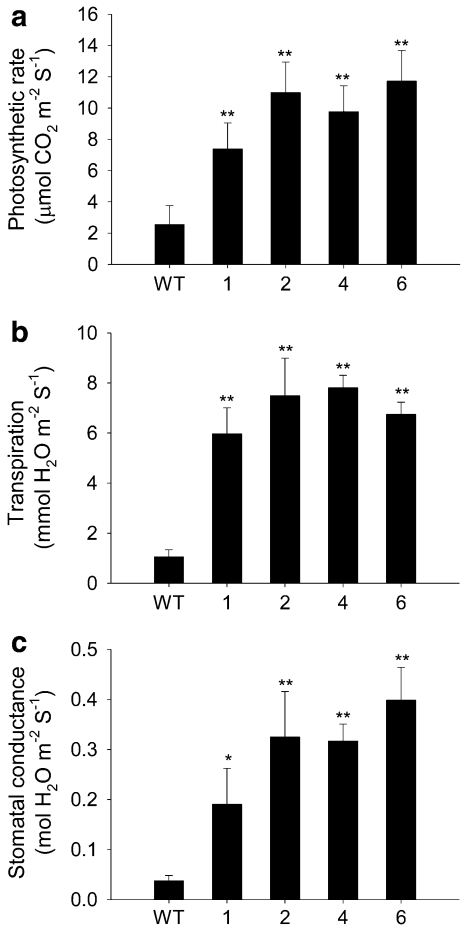

Fig. 4 Photosynthetic performance of peanut plants grown in greenhouse under salt treatment for 31 days. **a** Photosynthetic rates of peanut plants. **b** Transpiration rates of peanut plants. **c** Stomatal conductance of peanut plants. *WT* wild-type plant, *1, 2, 4, 6* four independent transgenic peanut lines. *Bar* standard error; *significant at 5 %; **significant at 1 %; $n = 6$ for each line

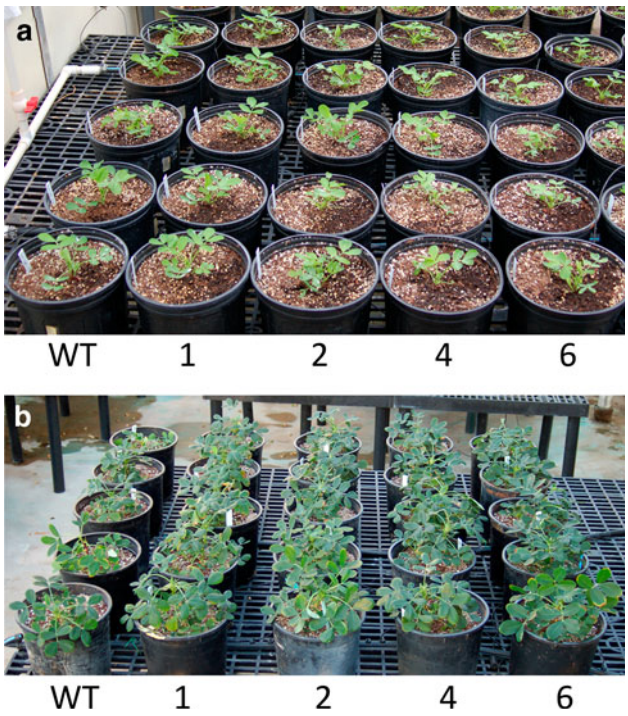

Fig. 5 Phenotypes of wild-type and *AVP1*-expressing peanut plants before and after drought treatment in a growth chamber. **a** Fifteen-day-old peanut plants under normal irrigation condition. **b** Peanut plants under reduced irrigation conditions for 60 days. *WT* wild-type plant, *1, 2, 4, 6* four independent transgenic peanut lines

SPAD values (or the leaf greenness) are positively correlated with the content of chlorophyll, and they provide a reliable estimate to the level of chlorophyll (Tobias et al. 1994; Chang and Robison 2003). We measured the SPAD values of three leaves, from the 2nd to the 4th leaf of each transgenic plant, and our data showed that indeed every leaf from transgenic plants displayed higher SPAD values (Fig. 2c).

After salt treatment, transgenic plants produced 30 % more fresh shoot weight and fresh root weight than wild-type plants (Fig. 3a, b). Similar results were also obtained for dry shoot weight and dry root weight (Fig. 3c, d). The photosynthetic performance of wild-type and *AVP1*-expressing peanut plants under salt treatment was measured, and these data were collected after these plants were grown under salt condition for 32 days. The *AVP1*-expressing plants displayed photosynthetic rates that were about two-fold higher than those of wild-type plants (Fig. 4a). The transpiration rates and stomatal conductance of *AVP1*-expressing plants were also significantly higher than those of wild-type plants (four-fold higher, Fig. 4b, c).

Expression of *AVP1* in peanuts enhances drought tolerance

To test if *AVP1*-expressing peanut plants were also drought tolerant, we first grew these plants under normal irrigation condition, i.e. 300 ml of water every 3 days, for 15 days in a growth chamber, and no phenotypic difference was noticed between *AVP1*-expressing plants and wild-type plants at this time (Fig. 5a). Then, we stopped irrigation for 15 days before a reduced irrigation scheme was applied to these plants: 150 ml of water every 6 days. After 45 days of continued growth under reduced irrigation, *AVP1*-expressing plants looked bigger and bushier than wild-type plants (Fig. 5b). The fresh shoot weight and the fresh root weight of *AVP1*-expressing plants were significantly higher than those of wild-type plants (22 and 26 %, respectively) (Fig. 6a, b). The dry shoot weight and the dry root weight of *AVP1*-expressing plants were also higher than those of wild-type plants (9 and 16 %, respectively) (Fig. 6c, d).

Before these plants were harvested for biomass analysis, we measured the photosynthetic performance of these

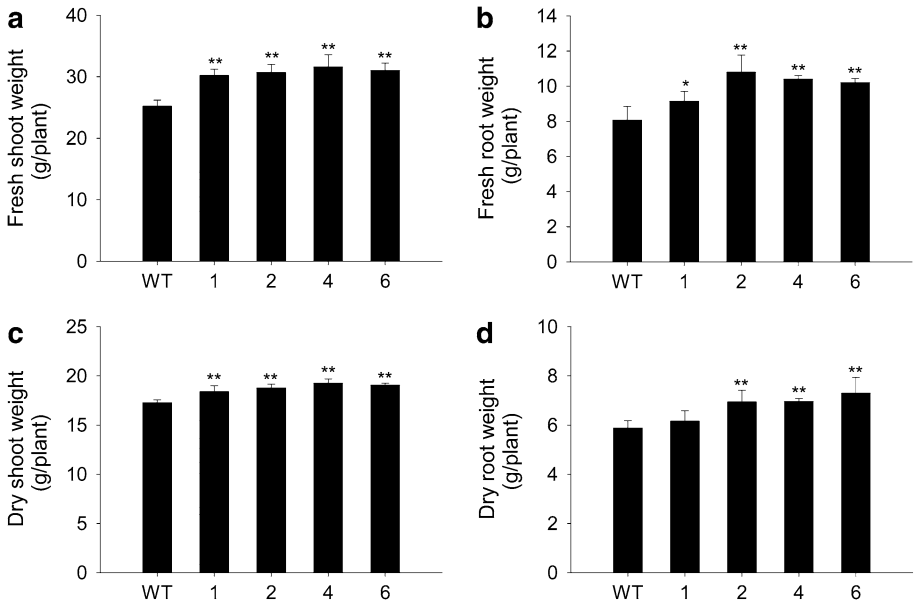

Fig. 6 Biomass analysis of wild-type and *AVP1*-expressing peanut plants in a growth chamber under reduced irrigation conditions for 60 days. **a** Fresh shoot weight of peanut plants. **b** Fresh root weight of peanut plants. **c** Dry shoot weight of peanut plants. **d** Dry root weight of peanut plants. *WT* wild-type plant, *1, 2, 4, 6* four independent transgenic peanut lines. *Bar* standard error; *significant at 5 %; **significant at 1 %; $n = 6$ for each line

plants. The *AVP1*-expressing plants displayed photosynthetic rates that were at least two-fold higher than those of the wild-type plants (Fig. 7a). The transpiration rates of *AVP1*-expressing plants were also significantly higher than those of control plants (Fig. 7b). Similar results were obtained for stomatal conductance (Fig. 7c).

AVP1-expressing peanut plants produce more seeds under low irrigation conditions in the field

Because peanuts did not set seeds well in our greenhouse, we had to grow them in the field. The T_0 transgenic peanuts were obtained in 2008 and the T_1 seeds were grown in field again in 2009 at the Experimental Farm of USDA-Cropping System Laboratory in Lubbock, TX. We treated these field-grown plants as doing a drought tolerance test because we purposely reduced irrigation during the growing season (19 mm per week). In the end, we compared the yields of *AVP1*-expressing plants and wild-type plants and we found that *AVP1*-expressing plants produced 37 % higher yield on average (Fig. 8a).

In 2010, we grew the T_2 seeds at a different site in the same farm. This time we added a well irrigation control (38 mm per week), and the irrigation for this control was 2 times more than the drought-treated plants received. Moreover, we conducted PCR experiments with DNAs isolated from each plant and made sure that only PCR-positive lines would be considered as transgenic plants. The plants, which derived from transgenic lines but did not contain transgene *AVP1* based on PCR analysis, were treated as another group, the segregated non-transgenic plants (NT). The year 2010 was very dry and hot in September in Lubbock, TX (Table 1), which might be the reason that the overall yields for all

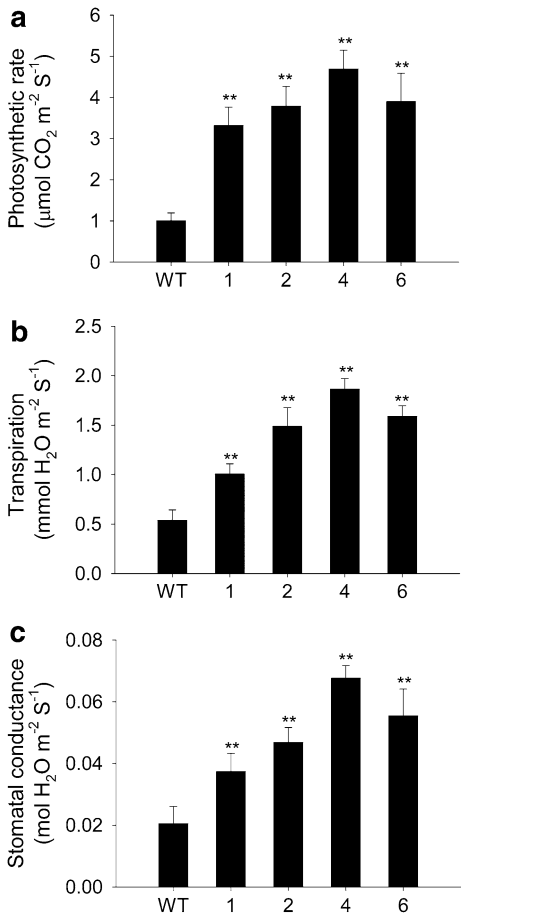

Fig. 7 Photosynthetic performance of peanut plants grown in a growth chamber under reduced irrigation for 59 days. **a** Photosynthetic rates of peanut plants. **b** Transpiration rates of peanut plants. **c** Stomatal conductance of peanut plants. *WT* wild-type plant, *1, 2, 4, 6* four independent transgenic peanut lines. *Bar* standard error; **significant at 1 %; $n = 6$ for each line

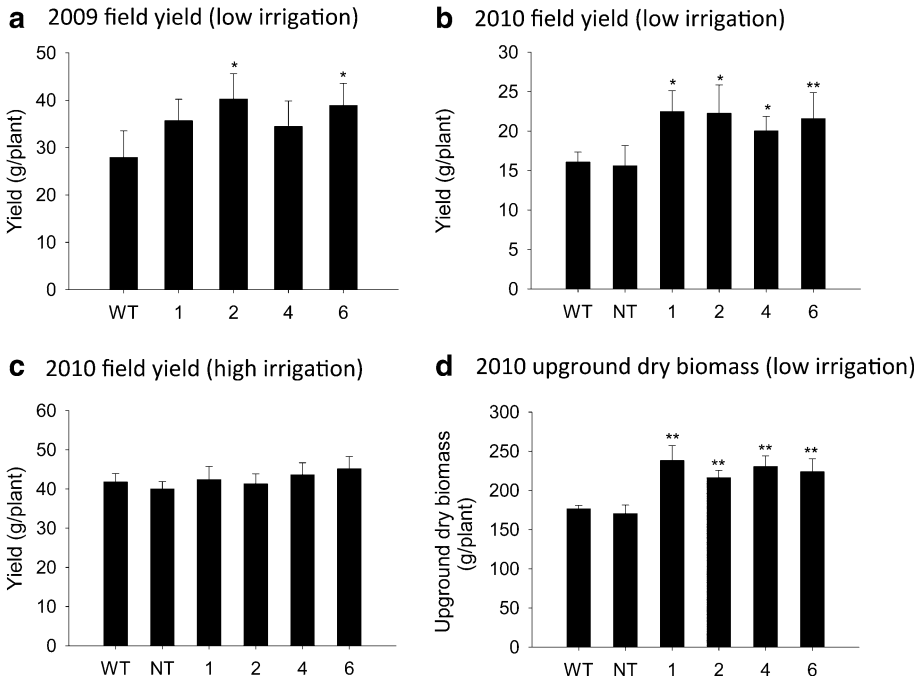

Fig. 8 Peanut yields from wild-type, non-transgenic and transgenic peanut plants in field conditions. **a** Yield in 2009. **b** Yield from the low irrigation group in the 2010 field. **c** Yield from the high irrigation group in the 2010 field. **d** Upground dry biomass from the low irrigation group in 2010. *WT* wild-type plant, *NT* segregated non-transgenic plants, *1, 2, 4, 6* four independent transgenic peanut lines. *Bar* standard error; *significant at 5 %; **significant at 1 %; $n = 40$ for each line

peanut plants were lower than the previous year (comparing Fig. 8a, b). However, the four independent *AVP1*-expressing lines still produced yields that were significantly higher than those of the control plants (Fig. 8b). In the high irrigation group, we did not see yield differences between *AVP1*-expressing plants and control plants (Fig. 8c). At the end of the growing season, we measured the biomass of the drought treated plants, and we found that *AVP1*-expressing plants produced much larger biomass than control plants (Fig. 8d).

The photosynthetic rates of *AVP1*-expressing plants were generally higher than those of control plants (Fig. 9a), and similar results were obtained for transpiration rates and stomatal conductance (Fig. 9b, c). These differences were smaller than what were observed for these plants under reduced irrigation conditions in greenhouse and growth chamber (compared to Figs. 4 and 7). The low irrigation treatment caused considerable yield reductions among all plants, but the penalty was more severe in wild-type plants than that in *AVP1*-expressing peanut plants (62 % reduction in wild-type vs. 50 % reduction in *AVP1*-expressing plants, Fig. 8b, c).

No major changes in the oil content of *AVP1*-expressing plants

There were no major differences in the oil content between wild-type and transgenic plants (Table 2). The contents of major fatty acids in peanut, such as palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$), were also similar between wild-type and transgenic plants (Table 2). The minor fatty acid such as stearic acid, gadoleic acid, behenic acid and lignoceric acid varied a little between wild-type and transgenic peanut plants; however, this difference in minor amino acid composition was also detected between the wild-type and the segregated non-transgenic lines (NT) (Table 2). It is obvious that the introduction of *AVP1* into peanut plants does not affect the oil content and major fatty acid compositions.

Discussion

In this report, we show that overexpression of *AVP1* in peanut leads to improved drought and salt tolerance. Our results are consistent with our work on cotton (Pasapula et al. 2011), supporting the claim that *AVP1*-overexpression could lead to increased drought and salt tolerance in transgenic plants (Gaxiola et al. 2002). Many studies in the literature also support this notion. The *AVP1*-expressing plants generated larger biomass than wild-type plants after salt treatment (Figs. 2, 3). These differences are clearly due to the differences in photosynthetic rates, transpiration, and stomatal conductance between *AVP1*-expressing and wild-type plants (Fig. 4). Wild-type plants looked pale after salt treatment (Fig. 2b), and they contained much less chlorophyll than *AVP1*-expressing plants (Fig. 2c), which explains why *AVP1*-expressing plants maintained higher photosynthetic rates after and during salt treatment. Similar data were obtained after drought treatment for 2 months: *AVP1*-expressing plants outperformed wild-type plants during drought treatment (Figs. 5, 6, 7).

Table 1 Average monthly high and low temperature and total precipitation for Lubbock, TX, USA, in 2009 and 2010

Lubbock	Average high temperature (°C)	Average low temperature (°C)	Precipitation (cm)
2009			
January	15.39	−4.17	0.33
February	18.78	0.56	1.85
March	22.22	4.17	0.94
April	24.83	7.56	3.84
May	28.17	13.17	1.73
June	33.83	19.00	6.20
July	34.28	20.28	4.29
August	34.78	19.56	1.19
September	28.50	14.17	6.25
October	22.22	6.83	1.98
November	20.67	2.33	0.33
December	9.11	−4.67	3.76
2010			
January	11.78	−3.37	3.58
February	9.89	−1.83	4.52
March	18.83	2.61	7.24
April	23.11	9.06	11.81
May	28.06	13.61	2.90
June	34.39	20.00	6.48
July	30.28	20.11	18.14
August	34.00	19.61	3.38
September	31.06	16.56	2.36
October	26.33	8.83	6.63
November	19.39	1.50	0.18
December	15.89	−1.00	0.00

Source: National Weather Service Weather Forecast Office, Lubbock, TX (http://www.srh.noaa.gov/lub)

We performed salt and drought tolerance tests twice in a greenhouse and a growth chamber, respectively, and our results were highly reproducible. When *AVP1*-expressing peanut plants were grown in field with reduced irrigation, they also outperformed wild-type plants by displaying higher photosynthetic rates, transpiration, and stomatal conductance than control plants (Fig. 9). Due to limited seeds from T_0 plants in 2008, we did not have a high irrigation control for peanuts grown in 2009. We also did not conduct PCR analysis for each transgenic peanut in the field to confirm the presence of the transgene *AVP1*, which explains why the error bars were large and why 2 out of 4 transgenic lines did not show increased yield at significant level (Fig. 8a). To resolve these problems, we added a high irrigation group for the 2010 experiment, and we also conducted a PCR experiment to verify each transgenic peanut grown in the field. All PCR-negative plants were treated as the NT group, the segregated non-transgenic plants. This time all

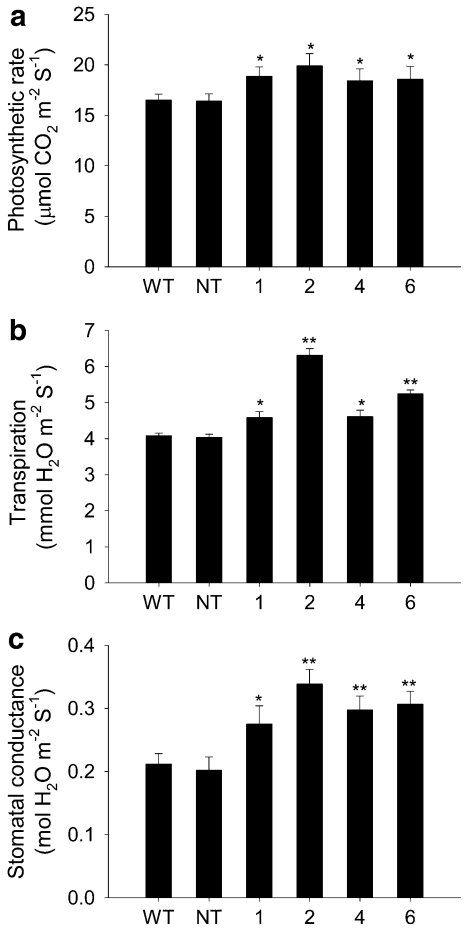

Fig. 9 Photosynthetic performance of field-grown peanut plants under low irrigation conditions in 2010. **a** Photosynthetic rates of peanut plants. **b** Transpiration rates of peanut plants. **c** Stomatal conductance of peanut plants. *WT* wild-type plant, *NT* segregated non-transgenic plants, *1, 2, 4, 6* four independent transgenic peanut lines. *Bar* standard error; *significant at 5 %; **significant at 1 %; $n = 12$ for each line

transgenic plants demonstrated higher yield than control plants (WT and NT) at significant levels, three lines at 5 % and one line at 1 % (Fig. 8b). The upground dry biomass from *AVP1*-expressing peanut plants significantly outweighed those from control plants, all at the 1 % level (Fig. 8d). These results were consistent with those obtained from the greenhouse-grown and growth chamber-grown plants. The differences in yield and biomass between *AVP1*-expressing plants and control plants were likely due to differences in photosynthetic performance of these plants.

The vacuolar proton pump gene *AVP1* and its homologs from other species have been introduced into several important crops, and, in all cases, increased expression of the vacuolar proton pump genes could increase drought and salt tolerance in transgenic plants. For example, Park et al. (2005) introduced *AVP1* into tomato and significantly improved drought tolerance in transgenic tomato plants.

Table 2 Oil content and fatty acid composition of wild-type and *AVP1*-expressing plants grown under well irrigated condition in 2010

Sample	Oil content	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$	$C_{24:0}$
WT	47.77 ± 0.90	10.70 ± 0.26	2.82 ± 0.35	38.90 ± 1.46	39.46 ± 1.60	1.51 ± 0.15	1.29 ± 0.07	3.51 ± 0.36	1.52 ± 0.18
NT	47.59 ± 1.14	10.39 ± 0.36	2.19 ± 0.18*	39.29 ± 1.53	39.86 ± 1.74	1.41 ± 0.05	1.56 ± 0.15**	3.79 ± 0.48	1.98 ± 0.40*
1	48.61 ± 0.63	10.66 ± 0.55	2.66 ± 0.29	39.87 ± 2.22	40.17 ± 1.78	1.30 ± 0.083*	1.35 ± 0.18	2.75 ± 0.36**	1.69 ± 0.20
2	4854 ± 0.23	11.29 ± 0.44*	2.36 ± 0.12*	39.96 ± 1.57	39.43 ± 0.39	1.42 ± 0.10	1.33 ± 0.12	3.50 ± 0.57	1.85 ± 0.29*
4	47.13 ± 0.36	10.77 ± 0.52	2.46 ± 0.24	38.63 ± 1.18	39.38 ± 1.03	1.36 ± 0.075	1.49 ± 0.26	3.33 ± 0.49	2.03 ± 0.24**
6	48.85 ± 0.45	10.55 ± 0.22	3.10 ± 0.35	42.69 ± 0.68**	35.70 ± 0.50**	160 ± 0.088	1.31 ± 0.086	3.33 ± 0.16	1.74 ± 0.14*

Oil content and fatty acid composition are in percentage (%). $C_{16:0}$ palmitic acid; $C_{18:0}$ stearic acid; $C_{18:1}$ oleic acid; $C_{18:2}$ linoleic acid; $C_{20:0}$ arachidic acid; $C_{20:1}$ gadoleic acid; $C_{22:0}$ behenic acid; $C_{24:0}$ lignoceric acid. *WT* wild-type, *NT* segregated non-transgenic, *1, 2, 4, 6* four independent *AVP1*-expressing peanut lines. The standard errors are given

* Significant at 5 %; ** significant at 1 %

Zhao et al. (2006) expressed *AVP1* in rice and increased salt tolerance in rice. Li et al. (2008) introduced *TsVP*, an *AVP1* homolog from *Thellungiella halophila*, into maize and showed that overexpression of *TsVP* in maize could lead to increased drought tolerance. Lv et al. (2008, 2009) and Pasapula et al. (2011) introduced *TsVP* and *AVP1*, respectively, into cotton, and demonstrated that overexpression of *TsVP* or *AVP1* could confer higher tolerance to salt and drought stresses and produce higher yields in field conditions. These experiments strongly suggest that *AVP1* and its homologs are excellent target genes that could improve agricultural productivities in areas where water is limited.

We recently reported that, by expressing a cytokinin production gene *IPT* in peanut, we could significantly improve drought tolerance in peanut (Qin et al. 2011), which was only the second report in literature to genetically engineer peanut for improved drought tolerance. The first report by Bhatnagar-Mathur et al. (2007) was to express an *Arabidopsis* transcription factor gene *AtDREB1A* in peanut, but unfortunately, there was no evidence that *AtDREB1A*-expressing peanut plants were more drought tolerant under water-deficit or reduced irrigation conditions. In this report, we demonstrated that by overexpressing *AVP1*, we could simultaneously increase both drought and salt tolerance in peanut, which represents a major progress made in engineering peanut for better agronomic traits. Whether *AVP1*-expressing peanut plants can increase peanut yield under reduced irrigation condition in large-scale field trials is yet to be tested, and how these plants would perform in comparing with *IPT*-expressing peanut in the field will be very informative for genetic engineering peanut in the future.

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