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RESEARCH

Improved Growth, Drought Tolerance, and Ultrastructural Evidence of Increased Turgidity in Tobacco Plants Overexpressing *Arabidopsis* Vacuolar Pyrophosphatase (AVP1)

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Abstract An increasing volume of evidence indicating the mechanisms of drought tolerance of AVP1-overexpressing transgenic plants has been reported. In the present study, we are reporting the experiments conducted for the drought tolerance of AVP1 overexpressing plants and WT tobacco plants in three water regimes named as "fully watered," "less-watered," and "desiccated". Results suggest that AVP1 plants exhibited greater vigor and drought tolerance in quantitative terms i.e., increase in size and weight of shoots and capsules. AVP1 plants produced more seeds than WT across all three water regimes. The less-watered regime was found to produce the greatest contrast. AVP1 overexpression enhanced solute accumulation in vacuoles resulting in an increase in water retention and turgor of the cell. The ultrastructure study of AVP1 overexpressing cells and WT leaf cells revealed that AVP1 plants displayed more turgid and hyperosmotic cells than WT. Moreover, guard cells in the AVP1 plants exhibited thick cell walls, few vacuoles, and deep and close stomata, whereas WT plants showed larger vacuoles and relatively open stomata aperture with no significant difference in size and number of the cells per unit area.

Keywords AVP1 overexpression · Less-watered drought · Ultrastructure · Tobacco

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Introduction

Plants exhibit several strategies to deal with life in extremely dry environments, namely avoidance, resistance, or tolerance to desiccation [1]. Desiccation tolerance has been defined as the ability of an organism to equilibrate its internal water potential with that of moderately dry air, and then resume normal function after rehydration [2]. Desiccation tolerant flowering plants require a slow drying time for mechanisms to be established that protect membranes and organelles during desiccation.

The first plant stress symptom induced by drought is often a rapid inhibition of shoot growth and to a lesser extent root growth. Partial or complete stomatal closure follows close behind with associated reductions in transpiration and CO_2 uptake for photosynthesis. If not relieved, drought then leads to interrupted reproductive development, premature leaf senescence, wilting, desiccation, and death [3, 4].

The reduction of growth under water deficit conditions is accomplished by a reduction in cell division rate and an increase in cell wall stiffening which inhibits cell wall expansion [5]. The stiffening of the cell wall poses a problem for drying cells. In order to tolerate desiccation, any cell with a large water-filled vacuole must overcome or limit the mechanical stress caused by its shrinking during drying. In resurrection plants, cell walls exhibited a significant increase in xyloglucans and un-esterified pectin.

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These changes enhance the tensile strength of the cell wall [6].

The development of genetically engineered plants by the modified expression of selected genes seems to be a viable option to hasten the breeding of improved plants. The role of the *A. thaliana* vPPase (AVP1) with a deduced protein having isoelectric point of 4.95 and MW of 81 kDa has been studied [7]. It was found that the AVP1 is responsible for improved drought and salt tolerance of transgenic plants when overexpressed through genetic engineering. Salt and drought tolerance of AVP1 plants is accomplished by increased solute accumulation in the vacuoles resulting in an increase in water retention. Hence, an increase in the electrochemical gradient across the vacuolar membrane should enhance the tolerance of plants to salt and drought stresses. Several other vacuolar H^+-pyrophosphatase genes have also been isolated from other plant species including halophytes and characterized for their role in increasing drought and salt tolerance of plants. Some selective reports describing plant phenotypes with the overexpression of vacuolar H^+-pyrophosphatase are reviewed here (Table 1).

Among drought stress induced by water deficit, desiccation is the most extreme condition that a plant can face during its life. In most of natural field conditions specifically rainfed areas, plants face limited water drought but many research papers (Table 1) report the comparison of transgenic and wild type plants at full and no water conditions. As a result, wild type in most cases does not recover after prolonged drought, and a comparison cannot be made. Thus, transgenic plants with modified expression of a particular gene known to induce drought tolerance in plants have to be compared with non-transgenic wild-type plants, which can survive on a limited water supply and die at complete desiccation. This paper demonstrates a quantitative account of drought tolerance exhibited by transgenic plants with overexpression of AVP1 gene in tobacco at fully watered, less-watered, and desiccation regimes in comparison with wild type. We also hypothesize that AVP1 overexpressing plants with increased turgor pressure of the cell (as a result of more solute and water retention in vacuoles) must have an adapted morphology that can be revealed through ultrastructure studies.

Table 1 Selective reports on transgenic plants overexpressing the vacuolar $H^{(+)}$-pyrophosphatase gene

Gene	Source	Phenotypes	Host plant	Reference
AVP1	*Arabidopsis thaliana*	Increased growth, leaf size, and plant fresh weight Independent to cytokinin dependent growth	*Arabidopsis*	[8]
MdVHP1	Apple	Enhanced tolerance to salt, PEG-mimic drought, cold and heat	Apple calluses and tomato	[9]
MdVHP1	Apple	$Na^{(+)}$ and malate accumulation, slightly increased soluble sugar accumulation	Apple callus and tomato fruit	[10]
Vacuolar $H^{(+)}$-pyrophosphatase	*Suaeda corniculata*	Enhanced salt, saline-alkali and drought tolerance	*Arabidopsis thaliana*	[11]
OVP1	*Oryza sativa*	Improved cold tolerance	Rice	[12]
AVP1	*Arabidopsis thaliana*	Improved salt, drought tolerance in field conditions; increased fibre yield	Cotton	[13]
AVP1	*Arabidopsis thaliana*	Increased salt tolerance and improved biomass production	Creeping bent grass	[14]
TsVP	*Thellungiella halophila*	Enhanced drought stress resistance	Cotton	[15]
TsVP	*Thellungiella halophila*	Enhanced salt tolerance and improves growth and photosynthetic performance	Cotton	[16]
TVP1	Wheat	Improved salt and drought tolerance	*Arabidopsis thaliana*	[17]
H^+-PPase	*Thellungiella halophila*	Improved salt tolerance	Tobacco	[18]
SsVP	*Suaeda salsa*	Increased salt and drought tolerance	*Arabidopsis thaliana*	[19]
AVP1	*Arabidopsis thaliana*	Increased root biomass and enhanced recovery from soil water deficit stress	Tomato	[20]
AVP1	*Arabidopsis thaliana*	Drought and salt tolerance	*Arabidopsis thaliana*	[21]

Materials and Methods

Plant Transformation and Growth Conditions

The gene construct having AVP1 open reading frame (2.3 kb) in the pPZP212 plant transformation vector [21] with the 35S tandem promoter and poly-adenylation signal at the HindIII site was used. *Nicotiana tabacum* (cv. Samsun) seeds were surface sterilized and germinated on ½ strength MS medium with 3 % sucrose and 1 % agar. Fully expanded leaves were cut into pieces and the transformation was carried out by *Agrobacterium tumefaciens* method using strain LBA 4404 [22]. Transgenic plants were selected on medium containing 25 mg/L of kanamycin. Transgenic and WT plants were grown in 16 h of light and 8 h of dark at 25/21 °C with relative humidity of 60–70 % in a phytotron.

Selection of Transgenic Plants with Single Gene Copy Number

The T0 seeds of 32 transgenic plants were germinated to produce T1 plants on kanamycin selection medium. Twenty five individual plants of T1 generation were selected randomly and 200 seeds from each of 25 individual T1 plants were grown on kanamycin-containing medium for the selection of plants with single copy number gene. Six plants which were showing the Mendelian's inheritance with a ratio of 1:3 for death and survival on kanamycin medium were selected. Consequently, one representative line with best phenotype and 100 % survival on selection medium was selected in the T2 generation for further study. Selected transgenic plants in T1 and T2 generations also confirmed the gene transformation through amplification of kanamycin [neomycin phosphotransferase (npt II)] gene with specific primers custom synthesized with the sequence as reported earlier [23].

Northern Blotting

Total RNA was isolated from leaves of transgenic and WT tobacco plants [24] and separated on 1.5 % formaldehyde agarose gel. RNA gel was blotted onto nitrocellulose membrane and hybridized with a P^{32} radiolabeled AVP1 gene probe which was amplified by PCR from AVP1 gene (Gene Bank accession number M81892) with specific primers (5′-CTACATACGCTAATGCT, 657–674 bp and 5′-ATTGTGTCATGGGTTGGCTTACC, 1,299–1,321 bp) from coding sequence. Membranes were exposed to X-ray film for 48 h at −70 °C and developed.

Protein Extraction from Leaves and Membranes

Total plant proteins were extracted using 0.5 g leaf powder with 0.2 mL extraction buffer (30 % sucrose, 0.5 M KCl, 25 mM EDTA, 5 mM DDT, 0.25 M Tris–HCl pH 8) and 0.5 mL solubilization buffer (20 % glycerol, 1 mM EDTA, 1 mM DDT, 10 mM Tris–HCl pH 7.6). The mixture was centrifuged at 2,000 rpm for 5 min to remove debris and the supernatant was again centrifuged at 14,000 rpm in a bench top centrifuge (Eppendorf 5415R) for 40 min. Soluble proteins were collected as supernatant and the pellet containing membranes was re-suspended in the solubilization buffer with agitation overnight. All processes were conducted at 4 °C.

Protein Determination, SDS-PAGE, and Western Blotting

Protein concentration was measured according to the Bradford procedure [25] using BSA as standard. Proteins (5 µg per lane) were separated on 8 % acrylamide gels and then electro-blotted onto nitrocellulose membranes for immuno-detection using the ECL Plus Western Detection system (RPN 2132 Amersham Biosciences) according to the manufacturer's instructions. An antiserum used at a 1:1,000 dilution was raised against a keyhole limpet hemocyanin-conjugated peptide (CTKAADVGADLVGKIE) corresponding to the PPi-binding site of the *Arabidopsis* H^+-PPase which is conserved among all the reported plant H^+-PPase [26].

Induction of Drought Stress and Recovery

Transgenic and WT tobacco plants were grown in pots with ten liter capacity containing soil Metro-Mix 200 (Sun Gro, USA). At the age of six weeks, five plants each of transgenic and WT lines were maintained at three different water regimes, named as fully watered, less-watered and dessicated. Fully watered plants received a supply of 1000 mL of water every third day. Gradual drought (less-watered) was induced with the supply of a restricted quantity of water i.e. 300 mL per pot. Sudden drought (dessicated) was induced by complete withholding of water. Water stress was considered to start from day 8 of last watering when soil in the pots was dry. Plants were re-watered up to full saturation on 18th day of stress when plants exhibited severe wilting of leaves and continued until harvest after 5 weeks of re-watering. This period was considered as recovery phase from drought.

Quantitative Measurements of Growth and Productivity

Shoots of five plants, along with capsules, from each water regime were cut from the base at the time of harvest and their fresh weight was recorded. Dry weight of shoots and capsules were recorded after drying them in an air oven at 80 °C for 7 days. Dry weight of shoots and capsules was

recorded. Seeds were dehusked and their dry weight was also recorded separately. Means of two genotypes were compared within each treatment separately by Least Significant Difference (LSD) method (α, 0.05).

Ultrastructure Studies

Electron microscopy was performed by a modified method as described [27–29].

Results

Molecular Characterization of AVP1 Overexpressing Transgenic Plants

Transgenic lines were confirmed for the presence of kanamycin resistance gene (nptII) at all stages of selection. The amplification of the desired size product (730 bp) representing the nptII gene was found to be completely linked with the selection of transgenic plants on kanamycin-containing medium. No amplification was detected in the untransformed and negative controls.

Overexpression of AVP1 gene in transgenic plants was confirmed by northern and western blot analysis (Fig. 1). Both the blots exhibited single band corresponding to AVP1 transcript and protein in northern and western blots, respectively. The bands on northern and western blots were quantified using Quantity One software from BIO RAD and in both cases the relative % adjusted volume (intensity mm^2) was almost three times higher in AVP1 transgenic than that of WT.

Drought Tolerance of AVP1 Plants

The vigor and flowering of AVP1 overexpressing plants and WT plants at different water regimes were compared. Three water regimes described their level of tolerance to drought at different developmental stages. In the fully watered regime, the phenotype of transgenic plants was found to be more vigorous for leaf size and number of capsules as shown in Fig. 2. The quantitative data (Fig. 8) of the fully watered regime confirmed the better phenotype of transgenic plants for fresh and dry weight of shoots and capsule, capsules alone, and of seeds as compared to WT.

Figures 3 and 4 represent the whole cascade of stress events for both the WT and AVP1 overexpressing tobacco plants, respectively. Stress was perceived to start on day eight of withdrawal of water upon drying of soil in the pots. The WT plants maintained on the desiccated regime seemed wilted with droopy stems as the stress progressed. The conditions turned even worse and plants were completely desiccated by the 17th day of stress. However, the growth of WT plants in the less-watered regime was comparable to WT plants in the fully watered regime. Although the WT plants in the less-watered regime seemed adapted with less quantity of available water and flowered, there was a negative effect on number and size of capsules

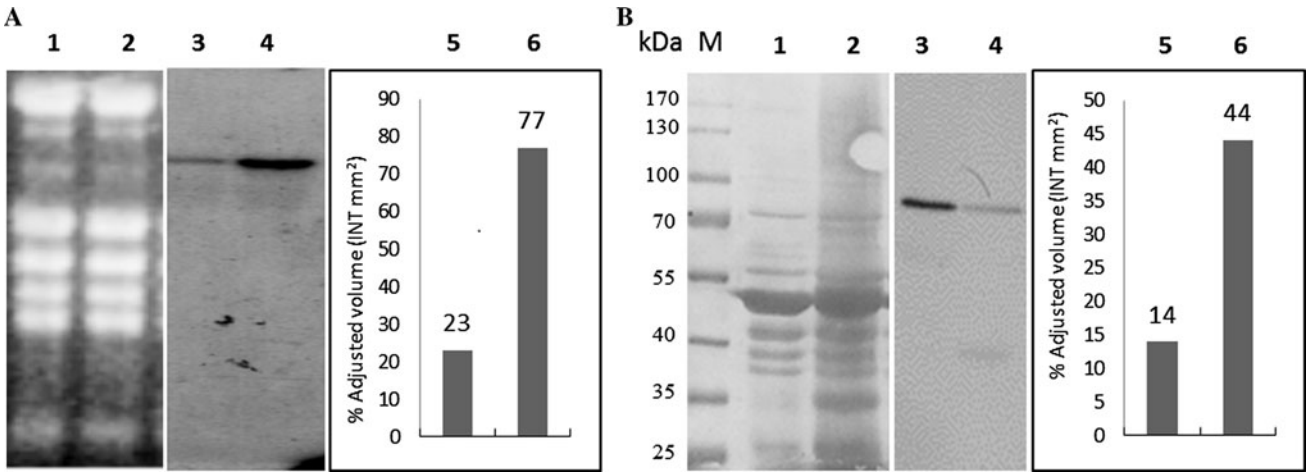

Fig. 1 Molecular characterization of AVP1 transgenic tobacco plants. **A** Northern blot: total RNA was extracted from young leaves of WT and transgenic plants and probed with 664 bp PCR amplified fragment from AVP1 cDNA. *Lanes 1*, *3*, and *5* correspond to AVP1 transgenic and *2*, *4*, and *6* correspond to WT lines. *Lane 1–2*, ethidium bromide stained total RNA bands from WT and control plants shown as loading control; *3–4* northern blot; *5–6*, the pixel density of band per square millimeter was calculated and background pixel density was subtracted (Global method) with Quantity One software from BIORAD of WT and transgenic plants. **B** Western blot: total proteins were extracted from young leaves of WT and transgenic plants, hybridized with anti-AVP1 as described in materials and methods. *M*, protein molecular weight marker; *lane 1–2*, ponceau stained nitrocellulose membrane; *3–4* western blot; *5–6* the pixel density of band per square millimeter (calculated as described earlier) of WT and transgenic plants

Fig. 2 AVP1 overexpressing transgenic plants shows improved growth of whole plant, larger leaves, and increased number of capsules than WT under normal growth conditions

formed. Figure 3 is the representative of this observation and has been confirmed through quantitative analysis as shown in Figs. 7 and 8.

The AVP1 overexpressing tobacco plants had larger leaves than WT and exhibited gradual wilting as the stress progressed in both less-watered and desiccated regimes (Figs. 3, 4). An important observation was the phenotype of AVP1 plants in the desiccated regime which flowered on the first day of stress i.e., day 8 of withholding of water. These plants continued to grow and showed a remarkable level of tolerance to drought stress as depicted by their phenotypes shown in Fig. 4. Older leaves senesced and young growing leaves became dehydrated but remained green and preserved. These plants did survive, however the WT that were treated in the same manner did not (Fig. 3). Similarly, adaptations and negative effects on growth were seen in the AVP1 plants under the less-watered regime.

Recovery from Drought

Plants were re-watered from the 18th day of stress and continued until harvest at full saturation. The WT plants exhibited irreversible damage caused by drought and could not recover after re-watering. In those plants, the emergence of a few new shoots was observed from the nodes on the stem but the leaves could not be rehydrated. The WT plants maintained under the less-watered regime recovered and completed their normal growth and development up to harvest. The fully watered controls of WT were maintained throughout and compared for phenotypes as shown in Fig. 5.

AVP1 plants showed drought tolerance under the less and desiccated water regimes. They recovered after wilting immediately within hours of being watered and escalated their growth at a faster rate than the normal controls of

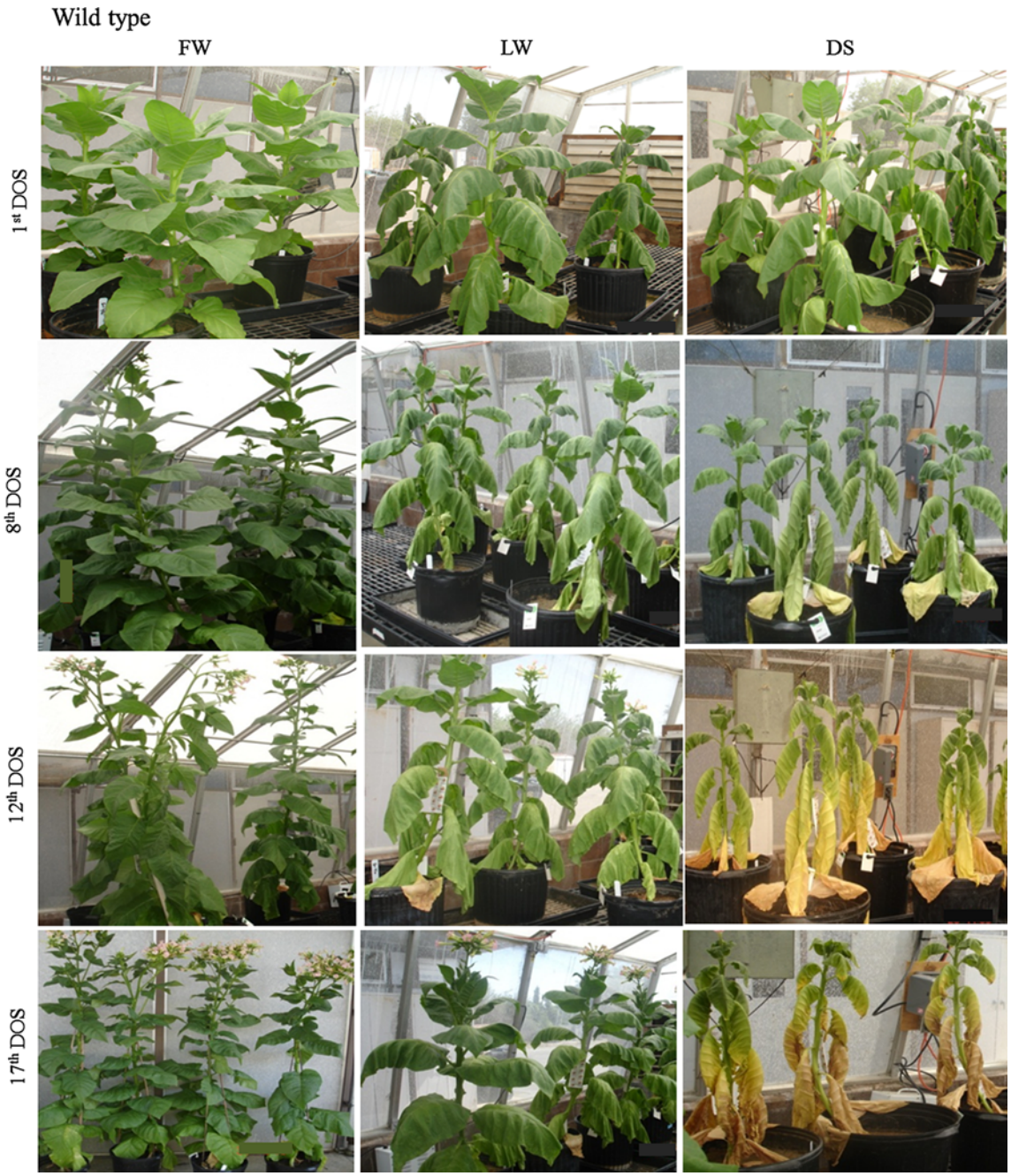

Fig. 3 Phenotypes of WT plants under fully watered, less-watered, and desiccated water regimes on 1st, 8th, 12th, and 17th day of stress. WT plants showed an increase in severity of drought symptoms under desiccation, whereas these plants showed moderate symptoms in the less-watered regime. The fully watered regime was maintained for comparison. *DOS* day of stress, *FW* fully watered regime, *LW* less-watered regime, *DS* desiccated water regime

their counterparts (Fig. 6). It was observed that the size of the capsules which emerged after re-watering in the less-watered regime was larger than the capsules from plants at fully watered and desiccation regimes at the same time. On the whole, the number and size of capsules in AVP1 plants were greater than the capsules produced by WT plants under all regimes (Fig. 7). However, WT plants in desiccation regime could not survive for comparison. Altogether, AVP1 plants not only recovered but it is suggested that increasing the number and size of capsules resulted in less damage than normally expected by drought. Furthermore, upon recovery, plants of both genotypes (WT and AVP1) showed emergence of new shoots from the middle of the stem kept in less-watered regime (Figs. 4, 5).

Fig. 4 Phenotypes of AVP1 overexpressing plants under fully watered, less-watered, and desiccated water regimes on 1st, 8th, 12th, and 17th day of stress. AVP1 plants showed drought tolerance under desiccation and less-watered regimes. The fully watered regime was maintained for comparison. *DOS* day of stress, *FW* fully watered regime, *LW* less-watered regime, *DS* desiccated water regime

Biomass and Seed Productivity of AVP1 Plants as a Measure of Drought Tolerance

Quantitative data was recorded for the fresh and dry weight of shoots and capsules, dry weight of capsules alone and weight of seeds. The data is presented in Fig. 8.

No significant difference was found in the fresh and dry weight of the shoots from AVP1 and WT plants kept at the fully watered regime. A substantial difference did result in AVP1 plants recovered from desiccation to their counterparts in less-watered regime. These showed increased shoot and capsules dry weight. This disparity was even more marked when the dry weight of seeds were compared. AVP1 plants produced more capsules resulting in a larger number of seeds than the WT tobacco plants without any stress conditions. However, the less-watered AVP1 plants

Fig. 5 Phenotypes of WT plants on the day of rewatering and then one, two, and five weeks after rewatering. WT plants under desiccation could not recover and died, however they showed complete recovery from stress under the less-watered regime. The fully watered regime was maintained for comparison *WAR* week after rewatering, *FW* fully watered regime, *LW* less-watered regime, *DS* desiccated water regime

Fig. 6 Phenotypes of AVP1 overexpressing plants on the day of rewatering and then one, two, and five weeks after rewatering. AVP1 plants showed drought tolerance and complete recovery under desiccated and less-watered regimes. The fully watered regime was maintained for comparison. *WAR* week after rewatering, *FW* fully watered regime, *LW* less-watered regime, *DS* desiccated water regime

showed low yield compared to their fully watered control group and to the WT control group. At harvest, the AVP1 plants which recovered from desiccation had a yield reduction of up to 40–50 % from their fully watered controls and 20–30 % to the WT fully watered controls (data not shown). This is based on the weight of the seeds harvested after maturity at the end of their normal life cycle (Fig. 8). Statistically, a significant difference was found among the means of WT and AVP1 plants at all water regimes as well as re-watered plants from less-watered and

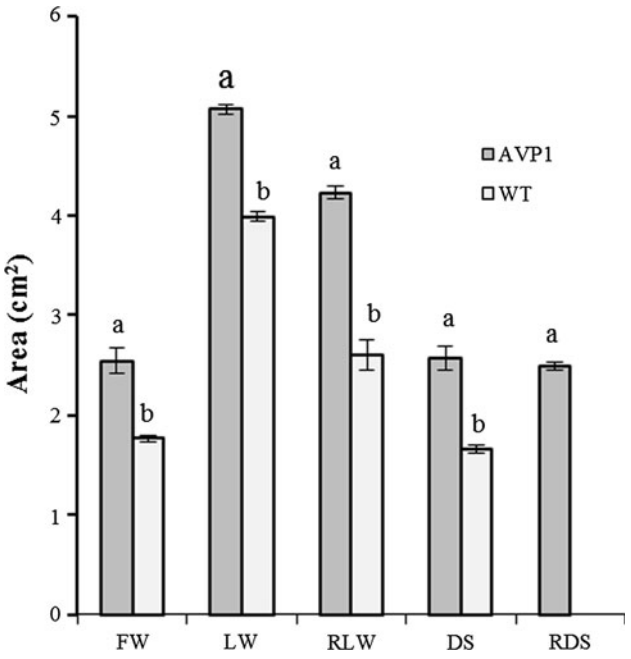

Fig. 7 Columns represent area (cm^2) of capsules produced by WT and AVP1 plants under different water regimes and after rewatering. Area was calculated by ImageJ 1.45 s software available online by Wayne Rasband, National Institute of Health, USA. Means of two genotypes i.e., AVP1 and WT were compared within each treatment separately. All 2 means are significantly different from one another by LSD ($\alpha = 0.05$) and are shown as different letters on each column in the figure. *Error bars* represent standard error of means ($n = 5$). Data was collected five week after rewatering at the time of harvest. *FW* fully watered regime, *LW* less-watered regime, *RLW* emerged after rewatering in plants under less-watered regime, *DS* desiccated water regime, *RDS* emerged after rewatering in plants under desiccated water regime

desiccated regimes. There was one exception at fully watered regime where no significant difference was found among fresh weight of shoots and capsules among WT and AVP1 plants.

Ultrastructure Studies of AVP1 Overexpressing Plant Cells

Ultrastructure studies were undertaken for leaf sections of the AVP1 overexpressing and WT plants by transmission electron microscope. Photosynthesizing leaves were used for this purpose. Images collected from the electron micrographs revealed no significant discrepancy amid the size and number of cells per unit area in both genotypes. However, in the AVP1 plants, micrographs gave the impression of shaped cells with smooth edges and seem to be more rigid and filled with a large vacuole (Fig. 9A) than those of the WT plants, which look as if they were not as stiff. The WT tobacco leaf cells were relatively asymmetrical with lopsided appearances (Fig. 9B).

AVP1 guard cells possessed thick and dense cell walls and few vacuoles inside (Fig. 9C, D); in contrast, the WT plants displayed relatively thin cell walls and larger vacuoles. Moreover, the stomatal aperture was deep and guard cells of AVP1 were closed whereas comparatively it was shallow and opened in WT.

Discussion

The AVP1 protein with primary accession number P31414 is almost similar in size and has homology with at least

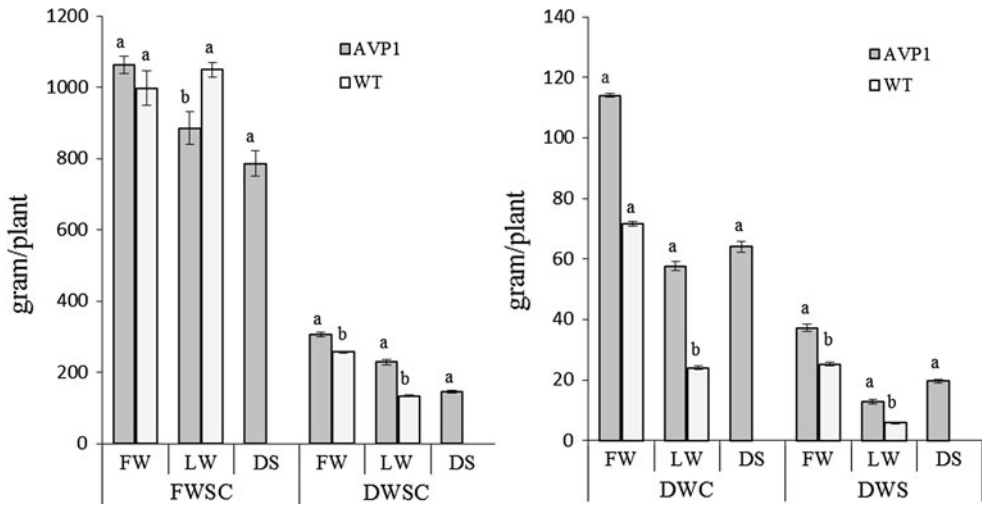

Fig. 8 Biomass produced by WT and AVP1 overexpressing plants at fully watered (FW), less-watered (LW), and desiccated (DS) water regimes as described in materials and methods. Data was collected five week after rewatering at the time of harvest. *Error bars* represent standard error of means ($n = 5$). Means of two genotypes i.e., AVP1 and WT were compared within each treatment separately. All 2 means are significantly different from one another by LSD ($\alpha = 0.05$) and are shown as different *letters* on each column in the figure. *FWSC* fresh weight of seeds and capsules, *DWSC* dry weight of seeds and capsules, *DWC* dry weight of capsules, *DWS* dry weight of seeds

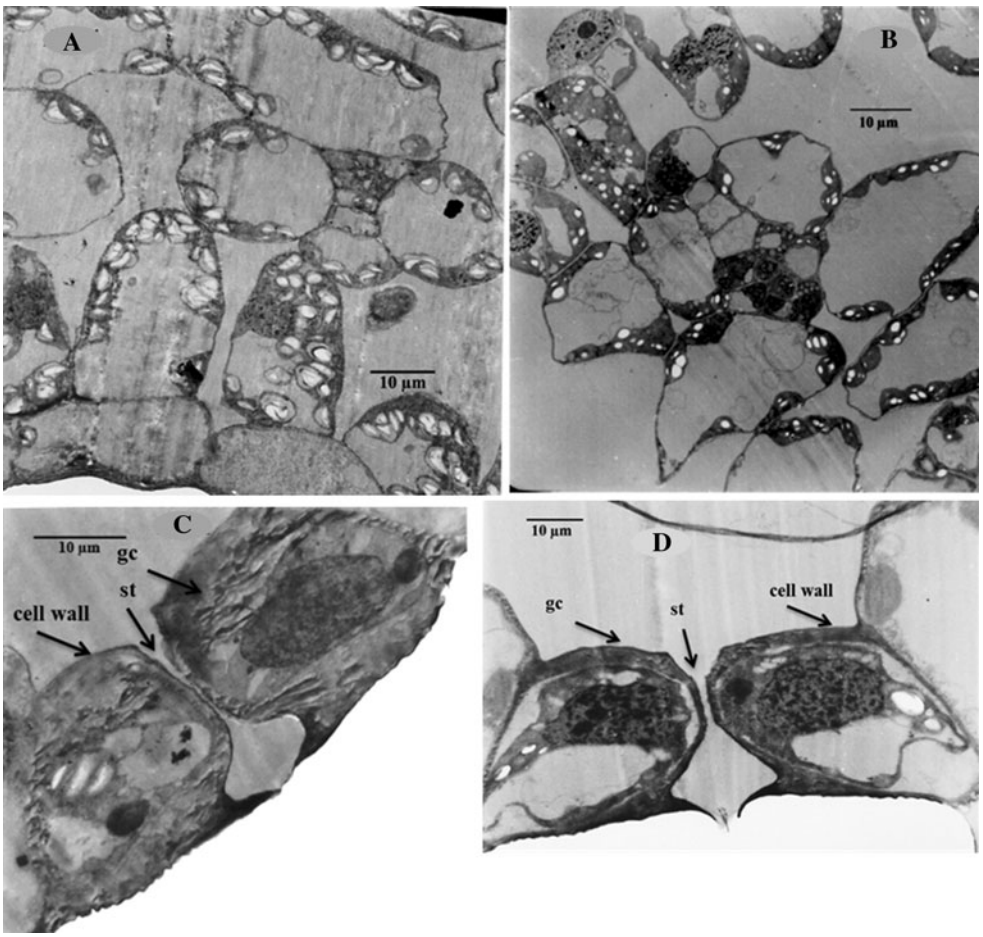

Fig. 9 Electron micrographs showed ultrastructure of leaf cells of AVP1 transgenic (**A**) and WT (**B**) plants under control conditions; displayed shape, number, and size of cells per unit area. Morphological appearance of guard cells in AVP1 (**C**) and WT (**D**) cells indicated difference in cell wall thickness, size, and shape. *s* starch, *v* vacuole, *gs* guard cell, *st* stomata

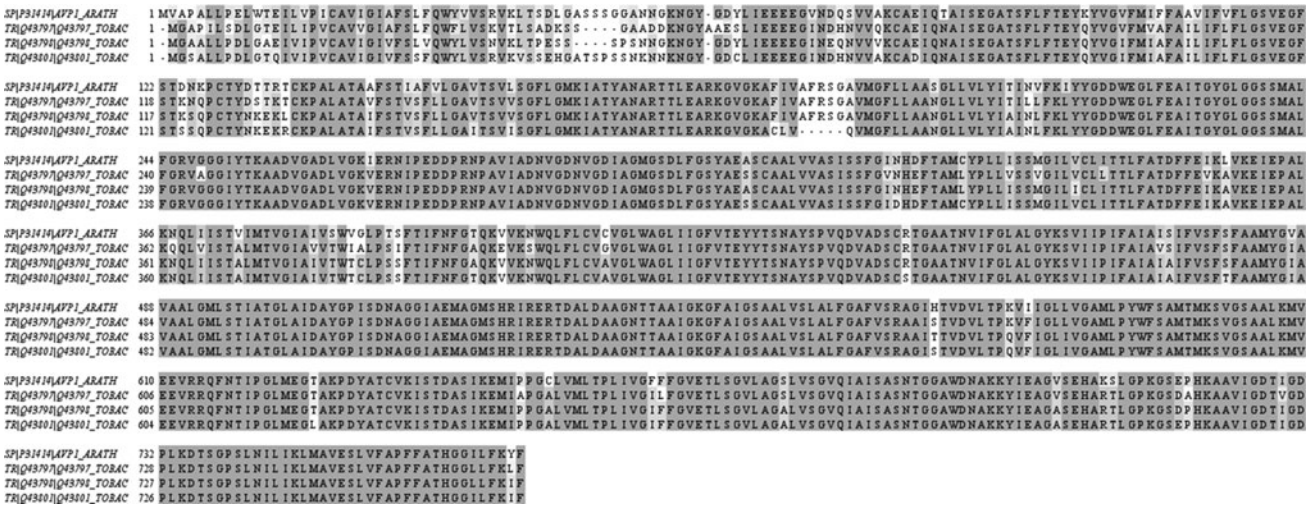

Fig. 10 Alignment of *Arabidopsis* vacuolar pyrophosphatase (AVP1) with three vacuolar pyrophosphatase gene(s) in *Nicotiana tabacum*

three of its homologs in *Nicotiana tabacum* Uniprot/Trembl accession numbers Q43797 (87 %), Q43798 (90 %), and Q43801 (89 %), respectively. It was presumed that tobacco VP1 co-migrated with the overexpressed AVP1. The homology between tobacco VP1 and AVP1 was assessed using multiple sequence alignment tool

Clustal Omega on EMBL database as shown in Fig. 10 [30]. The antibody used was raised against the conserved domain of vacuolar type membrane pyrophosphatase [26], which exhibited high specificity and no probability of cross reaction with any other protein [20, 21].

So far, studies conducted for the assessment of drought tolerance of genetically engineered plants [31, 32] (Table 1) are mostly based on withholding of water for certain periods of time rather than limiting the supply of water with few exceptions [33]. In our study, we assumed that with limited water supply, plants of both genotypes will provide the opportunity to study slow progression of drought stress and WT plants will not die. If so, this will lead to establish a comparison of transgenic and non-transgenic plants, which is not possible in the case of complete withholding of water as WT plants die at extreme condition of water desiccation. As expected, our results demonstrated that under the less-watered regime the drought symptoms like wilting of leaves and cessation of growth progressed slowly, consequently leading to the late emergence of few flowers. Our study suggested that plants of both genotypes adapted themselves by slowing down their growth and upon rewatering, recovered quickly as compared to the desiccated plants. We speculated that growing with a faster rate could be an adaptation by plants, to compensate the growth impediment during a drought period and to produce as much seeds and/or biomass as possible before the end of their normal life cycle. Also, an increase in the size and/or surface area of leaves in the AVP1 overexpressing plants consequently increased absorption of sunlight and photosynthetic efficiency thus resulting in improved biomass [34]. This effect was more evident in AVP1 plants which showed increased leaf size and high number of productive capsules. Whereas WT plants exhibited a greater number of leaves, they produced fewer numbers of productive capsules. Our results are in agreement with studies [8, 13, 14, 20] affirming that AVP1 overexpression improves overall plant growth, and it can be advocated that vigor can be a contributing factor of drought tolerance.

AVP1 overexpressing plants and WT plants under the less-watered regime exhibited the appearance of new shoots upon re-watering (Fig. 8), thus contributing to decrease in the yield penalty. More clearly, plants showed slow growth rate when facing less-watered stress but upon rewatering at full saturation, plants exhibited a greater increase in growth rate than the control plants maintained at fully watered status. Our study suggested that this growth pattern has been adapted by plants as a survival mechanism. These changes were visible and followed during whole period of stress, upon rewatering and recovery (Figs. 2–6).

Our results also suggested that the stress tolerant phenotypes of the AVP1 overexpressing plants owing to many features (Table 1) also attributed to the strong structural stability evolved due to increased turgidity in the AVP1 overexpressing cells.

In order to support this theory, we provided ultrastructural evidence showing that AVP1 transgenic tobacco plants exhibited well-shaped cells, which were notably turgid; these features indicate the AVP1 driven enhanced turgor pressure of the cell. This is the first report of direct visualization of turgidity in AVP1 overexpressing cells.

The rigid cell wall also played a central role in determining the characteristic shapes of plant cells. [35]. Anatomy of AVP1 transgenic plants revealed guard cells with thick walls, smaller vacuole, and closed stomata than WT plants having thin walls, large vacuole, and relatively open stomata. This indicates that turgor pressure of the guard cells decreased due to smaller vacuole size in transgenic plants. These findings are consistent with the previous report which showed a great number of small vacuoles in guard cells of the closed stomata and only a few big ones in the guard cells of the fully opened stomata [36]. Moreover, stomatal aperture is positively related to the turgor pressure of the guard cells but negatively related to the pressure of adjacent subsidiary or epidermal cells [37–40].

Vacuolar pyrophosphatases are involved in regulation of turgor pressure of the guard cells and in the stomatal movement [41]. The accumulation of H^+ in the vacuoles is dependent on vacuolar membrane proteins, V-ATPase and H^+-PPase [42]. Studies revealed that cytosolic pH could act as a second messenger in ABA signaling in guard cells [43]. The H^+ electrochemical gradient across the vacuolar membrane mediates the movement of other ions, such as K^+, Cl^-, sucrose, or malate between vacuoles and the cytoplasm, resulting in the changes of the water potential in guard cells [44], which drives water influx and efflux across the vacuoles. Such a process results in the changes of turgor pressure in the guard cells driving stomatal movement. This could be one of the mechanisms of stress tolerance in AVP1 overexpressing plants.

In conclusion, our results showed that plants managed on the less-watered regime experienced a slow progression of moderate drought stress and adjusted their rate of growth and productivity accordingly. This proves to be the best strategy to assess plant drought tolerance. Additionally, electron micrographs showing leaf ultrastructure presents the physical evidence of increased turgidity and endorses the previous reports describing AVP1 gene function (Table 1). Thus, the presence of the AVP1 gene was found to be effective to improve plant species with drought tolerance and better yields.

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