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REGULAR ARTICLE

EXPRESSION OF A FULL LENGTH *ARABIDOPSIS* VACUOLAR H^+-PYROPHOSPHATASE (*AVP1*) GENE IN TOBACCO (*NICOTIANA TABBACUM*) TO INCREASE TOLERANCE TO DROUGHT AND SALT STRESSES

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SUMMARY

Among various abiotic stresses salinity and drought are the two major factors limiting the crop productivity. Genetically engineered salt and drought tolerant plants could provide an avenue to the reclamation of farmlands lost to agriculture because of salinity and a lack of rainfall. The Arabidopsis gene *AVP1* encodes a vacuolar pyrophosphatase that functions as a proton pump and generates an electrochemical gradient in vacuole, thereby activating vacuolar membrane-antiporters including Na^+/H^+ antiporter, which helps in sequestration of Na^+ into vacuole. In addition, over-expression of *AVP1* gene increase vegetative growth by auxin transport and enhances auxin mediated root development, consequently achieving higher water absorption and retention capacities. The goal of present work is amplification of full length *AVP1* (3.2kb) gene, from *Arabidopsis thaliana* genomic DNA through PCR, its cloning into a suitable plant expression vector and transformation in tobacco through *Agrobacterium* mediated transformation method for its characterization. PCR analysis showed the successful transformation of this gene in *Nicotiana tabaccum*. Screening of these putative transgenic plants against different salinity levels (50-250mM NaCl) showed that transgenic plants were tolerant to 250mM NaCl whereas the control plants showed wilting within 36-48 hours of salt treatment. Under periodic drought stress treatment transgenic (*AVP1*) plants were significantly more tolerant than wild type plants. Similarly the results of salinity and drought tolerance experiment in sand under saline and water regime conditions confirmed that introns play a key role in gene expression and regulations and improve the growth of plants. These resistant phenotypes are associated with increased internal stores of solutes.

Keywords: *AVP1*, Proton pump, introns, Na^+/H^+ antiporter, salt and drought tolerance.

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1. Introduction

Environmental stresses such as salt, drought and chilling adversely affect the growth of plants and have endured as the most serious factors limiting yield of agricultural crops, which are particularly sensitive to these factors. These,

environmental stresses cause severe changes in the growth, physiology, and metabolism of plants thus threatening the cultivation of plants around the globe (Lunde *et al.,* 2007). According to an estimate, at present about 20 per cent of irrigated land in the world is affected by salinity. Similarly drought and high temperatures can significantly reduce crop yields. Due to these environmental stresses, the loss of cultivable land is likely to increase over the next 20 years and invade the world food supply. Crops yield losses in Pakistan due to drought and salinity are feared to reach $ 28.5 million lost per year, while total annual economic damage is estimated at $300 million. A yield loss of crops in moderately saline areas of the country average about 65 per cent. Grain productivity through green revolution has reached the ceiling, whereas the world population continues to grow (Akhtar *et al.,* 2008). Therefore, improving food crops yield in normal and less productive soils, including saline soils is highly desirable to feed the ever-increasing population.

These environmental factors, such as salt and drought affects the plants through three primary mechanisms. First, salt decreases the osmotic potential of the soil solution effectively creating water stress on the plant. Halophytes cope with this situation by actively taking up Na^+, which act as an osmoticum to maintain the water potential gradients necessary for continuous water uptake. Glycophytes, however, generally exclude Na^+ because Na^+ is not sequestered as readily into vacuoles as in halophytes (Ehret and Plant, 1999). It can result in severe ion toxicity, which is the second effect of these factors on plants. These plants must also generate higher level of osmotically active compounds in the cells in order to sustain adequate gradients for water uptake. Lastly, interaction of salts with mineral nutrition may result in nutrient imbalances and deficiencies. To induce tolerance against toxic Na^+ sensed by plants, regulation of K^+ uptake and/or prevention of Na^+ entry, efflux of Na^+ from the cell, and utilization of Na^+ for osmotic adjustment are strategies commonly used by plants to maintain desirable K^+/Na^+ ratios in the cytosol. Osmotic adjustment is recognized as an effective component of drought and salt resistance in several crop plants (Morgan, 1984; Ludlow and Muchow, 1990; Hasegawa *et al*., 2000; Munns, 2002). Osmotic adjustment involves net accumulation of solutes in cell in response to a fall in water potential of the cell's environment. This osmotic adjustment can be attained by transferring salt tolerance gene having the ability to express compatible osmolytes or gene acting as a Na^+/H^+ antiporter (Zhu, 2003).

Vacuolar sequestration of Na^+ is an important and cost-effective strategy for osmotic adjustment that reduces the Na^+ concentration in the cytosol. The expression and activity of Na^+/H^+ antiporters as well as V-type H^+-ATPase and H^+-PPase are responsible for Na^+ sequestration into the vacuole (Gaxiola *et al.,* 2001). These phosphatases generate the necessary proton gradient required for activity of Na^+/H^+ antiporters and the activity of membrane associated PPase which was first reported for the crude membrane fraction prepared from plants in mid-1970s and then it was also described the location of H^+-PPase in vacuolar membranes in first half of the 1980 (Rea *et al*., 1993).

The vacuolar transporters are an integrated part of a complex cellular network, enabling the plant to react properly to changing environmental conditions, store nutrients and energy in time of plentiful supply, and maintain optimal metabolic conditions in the cytosol (Martinoia *et al*., 2007). The H^+-pyrophosphatase (V-PPase) of plant vacuolar membrane catalyzes the electrogenic translocation of H^+ from the cytosol to vacuole lumen, in parallel with the vacuolar H^+-ATPase located in the same membrane, establishes the inside-acid, inside-positive H^+-electrochemical potential difference responsible for energizing the H^+-coupled transport of solutes into the vacuole. In the view of above reports, Gaxiola *et al.* (2001) demonstrated

that transgenic plants over-expressing the vacuolar H^+-pyrophosphatase are much more resistant to high concentrations of NaCl and to water scarcity than isogenic wild-type strains and characterization of *AVP1* over-expressing plants revealed a dramatic enhancement of their root development with obvious implications for their ability to withstand drought (Li *et al*., 2005). It is also appealing to hypothesize that the increased root proliferation and apoplast/ rhizosphere acidification capacities evident in *Arabidopsis*, tomato and rice plants engineered to over-express the H^+-PPase *AVP1* (Li *et al.,* 2005; Zhao *et al.,* 2006) could be instrumental in producing plants that exhibit increased resilience to mineral deficiencies.

Almost all previous report about the function and characterization of *AVP1* are with cDNA but there is no report describing the characterizations of full length AVP1 gene with reference to salinity and drought tolerance Introns, derived from the term "intragenic regions", are non-coding sections of DNA and play a significant role in gene regulation and expression. Introns may affect gene expression by increasing the time required to transcribe the gene. One way for extended transcription times to affect the behavior of a gene expression program is through a negative feedback loop (Ian *et al*., 2008). As many reports previously revealed about inducing salinity tolerance by over-expression of *AVP1* were based on cDNA, but no reports studied full length genes without removing introns. Approximately 80% of *Arabidopsis* genes contain introns. Introns facilitate the evolution of new proteins by exon shuffling (Long *et al*., 1995) and allow multiple proteins to be produced from a single gene through alternative splicing and they often have important functions in gene regulation. Positive and negative regulatory sequences have been identified within specific introns (Bruhat *et al*., 1990; Deyholos and Sieburth, 2000) and introns are generally required for abundant expression of many genes in plants (Callis *et al*., 1987), nematodes (Okkema *et al*., 1993) and insects (Meredith and Storti, 1993). Thus full length gene *AVP1* in tobacco plants having a total size of 3.2 kb and 16 introns was cloned under strong plant expressible promoter CaMV35S. The 35S promoter is a constitutive promoter, causing high levels of gene expression in plants (Tang *et al*., 2003).

2. Materials and Methods

This research work was carried out at the National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad to explore the potential of full length *AVP1* gene of *Arabidopsis thaliana* for conferring salt and drought tolerance in *Nicotiana tabaccum*.

PCR amplification, cloning and transformation of *AVP 1* gene

The full length *AVP1* gene was amplified using pair of specific primers (5′-TCGCGCGAAGCGGTTCTCT-3′and 5′-CCTC GGATTGAGTTTAGAAG-3′) designed from the sequence of *AVP1* gene available at The *Arabidopsis* Information Resource (TAIR), Accession # 2036134. The amplified gene (3.2kb) was cloned in the T/A cloning vector (pTZ57R/T, Fermentas) at the *Eco*R1 site and named as p57ZSI. Expression cassette bearing double 35S promoter, polylinker site and CaMV terminator was lifted from pJIT60 with *Sac*I-*Xho*I and cloned into a binary vector pGreen0029 to form pGreenZSA. The cloned *AVPI* gene from p57ZS1 was lifted with *Eco*RI-*Sal*I and cloned in pGreenZSA at *Eco*RI/*Sal*I sites. The resultant plant expression/binary vector was named as pZS1. pZS1 was introduced into *Agrobacterium tumefaciens* strain LBA4404 (already having a helper plasmid pSoup) via electroporation using Electroporator (BTX Model ECM600). The transformants were selected on LB plates containing tetracycline (10µg/ml), kanamycin (50 µg/ml) and rifampicin (50 µg/ml).

Generation of Transgenic *Arabidopsis* Plants

AVP1 gene was transformed into *Nicotiana tabacum* cv. Samsun through *Agrobacterium*

tumefaciens leaf disc method. *Agrobacterium* culture was grown at 25°C on shaker at 250 rpm in liquid LB medium containing tetracycline (10 µg/ml), kanamycin (50 µg/ml) and rifampicin (50 µg/ml) to an OD_{600} = 1.0. NAA 0.1 mg/l, BAP 1.0 mg/l kanamycin 50 mg/l. Leaf discs were dipped in bacterial suspension for 15-20 minutes., blotted dry on sterile filter paper and co-cultivated on co-cultivation (CC) medium (MS salts and vitamins containing NAA 0.1 mg/l, BAP 1.0 mg/l, sucrose 3% and phytagar 1.5%.) for 2-3 days. After co-cultivation, the leaf discs were washed with washing medium (CC medium without phytagar but having cefotaxime 250 mg/l). Leaf discs were transferred to selection cum regeneration medium (CC medium having cefotaxime 250 mg/l and kanamycin 50 mg/l). Regenerated plants were removed from culture vessels and hardened in sterilized sand before transplanting to soil in pots. Transgenic plants were screened by PCR using kanamycin resistant gene specific primers.

Screening for salt and drought tolerance
NaCl Stress Treatment

Plants were grown and maintained in a containment glass-house at 25°C in a 16/8 h light/dark cycle till flowering and seed setting. Transgenic plants were germinated in small plastic pots containing soil. Five weeks old plants were subjected to saline stress by providing nutrient solution (1/8 strength Murashige and Skoog salt mixture) supplemented with NaCl, incrementally increasing with each successive watering from 50mM through 100, 150, and 200 to a final concentration of 250mM NaCl.

Water Stress Treatment

For drought stress, transgenic plants were deprived of water for 3, 6, 9 and 12 days. After 12 days of water deprivation, plants were grown for another 12 days under a fully watered regime at 25°C.

3. Results

Arabidopsis vacuolar pyrophosphatase (*AVP1*) full length gene was amplified, cloned and expressed in *Nicotiana tabacum* to explore the potential of full length gene for conferring salt tolerance in crops. In the previously reported studies, cDNA clones of *AVP1* have been used and evaluated.

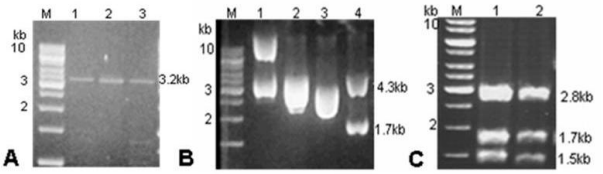

Fig. 1. (A) Lane M represents 1kb DNA ladder, lane 1, 2, 3, represent the amplification of AVP1 gene from *Arabidopsis thaliana* total genomic DNA through PCR. (B, C) Restriction analysis of plasmid p57ZSI developed by the cloning of PCR amplified product in pTZ57R/T with *Hind* III, *Hind* III and *EcoR* I.

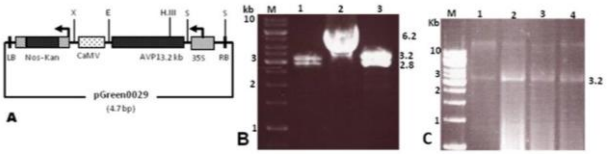

Fig. 2. Different steps in cloning of AVP1 gene from p57ZSI into pGREEN-ZSA. A) Theoretical skectch of AVP1 and pGREEN0029 (named pZSI. B) Digestion of p57ZSI and pGREEN-ZSA with *Eco*RI-*Sal*1, lane 1, 1kb DNA ladder; lane 2, restriction of p57ZSI with *Eco*RI-*Sal*1 lane 3, restriction of pGREEN-ZSA with *EcoR*I-*Sal*1 C) Transformation of pZSI in *Agrobacterium tumefaciens* LBA4404 was also confirmed with PCR fig 2-C

Amplification and cloning of *AVP1* gene in pTZ57R/T, pGREEN expression vector.

The full length *AVP1* gene of approximately 3.2kb was amplified (figure1-A), then cloned in pTZ57R/T vector and was named as p57ZSI. The cloning of *AVP1* in pTZ57R/T plasmid was confirmed with *Hind*III digestion giving rise to DNA fragments of 1.7kb and 4.3kb (figure1-B) and finally clone was also confirmed by digestion of p57ZS1 with *Eco*R1 and *Hind*III resulting three bands of 2.8kb, 1.7kb and 1.5kb as shown in figure1-C. The cloned *AVP1* gene from p57ZS1 and pGREEN expression cassette from pGREENZSA clone (already available in lab) were lifted with *Eco*R1-*Sal*I fig 2-B and ligated and transformed in *Agrobacterium tumefaciens* LBA4404 via electroporation using BTX ECM 630 Electroporation System and

named as pZSI (Zahid, Sher Afzal, Ibrahim). Transformation of pZSI in *Agrobacterium tumefaciens* LBA4404 was also confirmed with PCR fig 2-C.

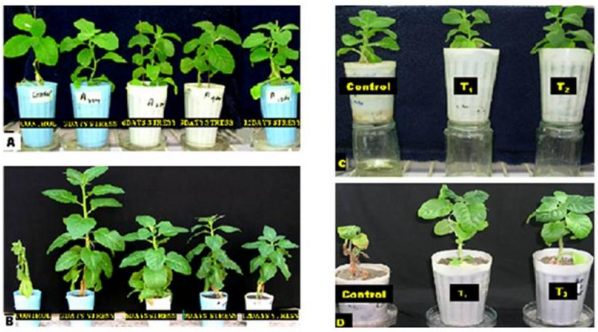

Fig. 3. AVP1 transformed tobacco plants exposed to drought stress as well as 250mM NaCl salt stress (A) Plants before stress. (B) Effect of periodic drought stress on putative transgenic plants after 24 days along with non transformed control. (C) Transgenic *AVP1* plants before exposure to salt stress. (D) Effect of 250 mM salt stress on putative transgenic plants after 15 days along with non transformed control plants.

The rapid production of large numbers of genetically identical plantlets

About 70 explants (leaf discs) of *Nicotiana tabaccum* L. cv. Samsun were placed in solidified MS0 medium, transformed, incubated and co-cultivated with recombinant A. *tumefaciens* strain having *AVP1* gene with expression vector. On selection media only 38 explants could survive with an average transformation efficiency of 54.28 %. The rest of bleached explants indicated that these were not transformed or desired expression level was absent. The first signs of plantlet regeneration were evident after 10-15 days from the date of infection with *Agrobacterium tumefaciens* strain LBA4404. These transgenic plants were confirmed via PCR analysis using kanamycin resistant gene as well as *AVP1* specific primers as shown in fig (4-A, B).

Transgenic plants with AVP1 gene are salt-tolerant

The transgenic plants expressing *AVP1* are more salt tolerant than wild-type plants. Plants from *AVP1* transgenic lines grow well in up to 250 mM NaCl concentration, whereas wild-type plants grow poorly and exhibited chlorosis at same concentration. After 10 days in these conditions wild-type plants died, whereas the transgenic plants continued to grow well as shown in figure 3-D.

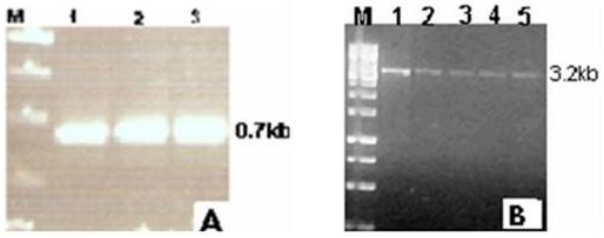

Fig. 4: PCR Analysis of transgenic plants (A) Lane M represents 100bp DNA ladder, Lane 1,2 and 3 represents 0.7kb fragment of kanamycine gene (B) Lane M represents 1kb DNA ladder, lane 1 is control plasmid DNA while lane 2, 3, 4, 5 are the amplification of 3.2kb AVP1 gene.

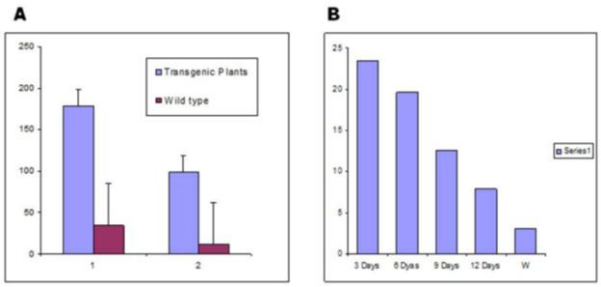

Fig. 5: A.) Mean values for fresh and dry weight of shoot and root (g/plant) of Tobbaco plants (*Nicotiana tobbacum* L.) When 20 day-old plants were subjected to non-saline and saline conditions in sand culture (Means ±S.E; n=4) B) Mean values for fresh and dry weight of shoot and root (g/plant) of Tobbaco plants (*Nicotiana tobbacum* L.) when 20 day-old plants were subjected to water regime conditions for 24 days conditions in sand culture (Means ±S.E; n=4)

Transgenic plants with *AVP1* gene are drought-tolerant

The wild types and *AVP1* transgenic plants were exposed to drought stress by growing plants under conditions of water deprivation. Transgenic lines were subjected for 3days to 6, 9 and 12 days of drought stress. Transgenic plants deprived of water for 3 days had no noticeable effect on plant growth while the control plants showed signs of wilting. However, transgenic plants exposed to 6, 9 and 12 days of drought stress showed signs of wilting. It was observed that wilting was more pronounced in transgenic lines exposed to 12 days of drought compared to transgenic lines exposed to 6 or 9 days of drought stress. When these transgenic plants were watered after 12 days of water deprivation, all the transgenic lines recovered from drought stress while the

control plants did not show any recovery and exhibited permanent wilting. The transgenic plants exposed to 12 days of drought stress took 2-3 days longer to recover from wilting compared transgenic lines exposed to 6 and 9 days of drought stress. Plant growth was least affected in transgenic plants exposed to three days drought stress and growth retardation became more pronounced as the exposure time to drought stress was increased from six to nine or twelve days as shown in figure 3-D.

4. Discussion

Drought and salinity are major constraints in the world and considerable factors affecting the crop production and food security, and adversely impact the socio-economic fabric of many developing countries. Water scarcity, declining water quality for irrigation and soil salinity are problems, becoming more acute (Flowers, 2004). In the present work, it has been shown that over-expression of the *Arabidopsis* vacuolar H^+-pyrophosphatase (*AVP1*) gene in transgenic *Nicotiana tabaccum* plants results in salt and drought tolerance. The enhanced tolerance to salinity and drought in transgenic plants with increased levels of *AVP1* is explained most easily by an enhanced uptake of ions into the vacuole. Presumably, the greater *AVP1* activity provides increased H^+ to drive the secondary active uptake of cations into the lumen of the vacuole. If so, there must be a compensatory transport of anions to maintain electroneutrality (Gaxiola, 1999). The resulting elevated vacuolar solute content would confer greater water retention, permitting plants to survive under conditions of low soil water potentials. Furthermore, at high Na^+ concentrations, the increased H^+ gradient could also enhance the driving force for *AtNHX1*-mediated Na^+/H^+ exchange, thereby contributing to the Na^+ sequestration into the vacuole of *AVP1* transgenic plants. Presumably, any toxic effects intrinsic to Na^+ are mitigated by this sequestration in the vacuole.

The advancement of science in the field of genomics and proteomics has led to much vital information which is now being used by scientists throughout the world. The *Arabidopsis* genome-sequencing project (Adam, 2000) has led to the identification of a plant proton pump *Arabidopsis* vacuolar pyrophosphatase *AVP1*, Na^+/H^+ antiporter gene, *AtNHX1*, and *SOS1* (Apse *et al.*, 1999; Gaxiola *et al.*, 1999; Yokoi *et al.*, 2002; Leigh 1994) which plays a key role in sequestration of Na+ ion against their electrochemical potential gradient (Blumwald *et al.*, 2000). It is the fact that all higher organisms possess introns and the more complex organisms possess a higher proportion of introns indicates that they serve at least minor and possibly major, functions. The research has found a high level of conservation in some introns, indicating that they have some selective advantage. How many introns display this conservation, is not yet known (Sun et al., 2000). According to evolutionist Patrusky (1992) "Nature, for reasons as yet unknown, created the intron, and evolution has chosen to keep it and ultimately has found new ways to use it. Some DNA behaves as exons when expressed by one pathway, but as introns when expressed by another pathway. Both pathways can operate simultaneously, resulting in greater protein product variety.

In view of emerging importance of introns, we transfer full length *AVP1* gene which contains introns and exons. The idea was that it would improve abiotic stress in transgenic plants. It is well established that introns results in increased stability and expression of genes (Buchman *et al.*, 1988; Chung *et al.*, 1989; Okkema *et al.*, 1993). During growth of tobacco plant which is most sensitive plant against salinity, we observed the high growth rate, which shows consistency with Li *et al.*, 2005 that *Arabidopsis* H^+-PPase *AVP1* regulates auxin-mediated organ development of the plant, which means transport of auxin controls developmental events in plants in addition to maintaining vacuolar pH.

When transgenic plants were exposed to salinity and periodic drought stress, they showed better efficiency, which directly co-relates with the over-expression of vacuolar H^+-pyrophosphatase (Gaxioal et *al.*, 2001). Wild-type plants displayed progressive chlorosis and

a general growth inhibition when treated with high salt concentrations or when deprived of water. Almost in many transgenic lines, *AVP1* gene expressed by inducing salinity tolerance to plants. But as it mentioned that few transgenic plants could not survive. The reason could be the integration of gene at inappropriate position due to which that gene could not express itself. Obviously from the result it is now clear that overexpression of full length *AVP1* gene helps to manage nutrition imbalance as well as osmotic adjustment and make plant tolerant against salinity. We describe these properties to the increased accumulation of solutes. The net increase in the concentration of solutes in the cell must lead to an increase in the uptake of water so that these transgenic plants can maintain turgor.

Similarly, results of drought stress analysis, during which plants were exposed to different level of drought stress, also show a positive response in inducing drought stress tolerance. The growth rate of different level of drought stress was different in transgenic lines as compared to wild type. It means those plants which were on three days water stress were more tall than 6 days and similar conditions were observed in 9 days water stress and 12 days water stress plants. From here we can conclude that *AVP1* gene expressed and shows positive results towards inducing drought stress tolerance. This study was, therefore, undertaken to see behavior and role of *AVP1* gene with introns in enhancing salt tolerance characteristics in the targeted plant. Moreover, our results suggest that the genetic manipulation of vacuolar proton-pumping pyrophosphatases in economically important crops could provide an important avenue for crop improvement.

Conclusion

Introns play a very important role in gene regulation and expression in plants. So transformation of salt, drought and cold tolerant gene (*AVP1*) with introns is an important aspects for improving the crops against abiotic stresses.

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