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Improvement of Salt Tolerance in Putatively Transgenic Rice Plants Overexpressing *AVP*1, a Vacuolar H^+-Pyrophosphatase

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ABSTRACT

Alleviating salt stress in plants is an important aspect of crop improvement. Salt stress affects plant growth and development in many different ways. To maintain growth and productivity plants must adapt to stress conditions and exercise specific tolerance mechanisms. In the present study, *AVP1*, a vacuolar H^+-PPase gene from *Arabidopsis thaliana* was overexpressed in rice (var-Vikas) by *Agrobacterium* mediated *In Planta* transformation technique. To screen putative T_1 plants for salt tolerance, stringent salt screening test was followed and root and shoot growth of T_1 putative transformants was used as a selection criterion. Some of the transgenics showed significantly higher root and shoot growth compared to wild type. PCR analysis confirmed the integration of the transgene in the rice genomic DNA. Physiological studies such as chlorophyll (Chl) estimation, membrane integrity, cell viability tests were also conducted to assess their levels of tolerance at T_1 generation. Some of the T_1 transformants showed lower percent reduction in Chl content, higher cell viability after NaCl treatment compared to wild type (WT). These results clearly demonstrate that transgenic rice plants overexpressing *AVP1*, a vacuolar H^+ proton pump have better salt-tolerance.

Keywords: *Agrobacterium*, *in planta*, leaf bioassay, screening
Abbreviations: ***AVP1***, *Arabidopsis* vacuolar pyrophosphatase; **Chl**, chlorophyll; **PCR**, polymerase chain reaction; **TTC**, 2,3,5-trichloro triphenyl tetrazolium chloride; **WT**, wildtype

INTRODUCTION

Salinity is a soil condition characterized by a high concentration of soluble salts. Soils are classified as saline when the EC is 4 dS/m or more (USDA-ARS), which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa, which significantly reduces the yield of most crops (Munns and Tester 2008). Reducing the spread of salinization and increasing the salt tolerance of high yielding crops are important global issues. Salinity always results in high concentration of Na^+ in external medium compared to other nutrients such as K^+ and other cations, altering the ionic balance of soil solution (Tester and Davenport 2003). The important mechanisms that contribute Na^+ tolerance of plants, are the processes involved in establishing ionic and osmotic homeostasis. Vacuoles occupy 90% volume of plant cells. Plant vacuoles play an important role in the maintenance of turgor pressure and storage of ions and metabolites. Vacuolar membrane of plant cell contains two types of H^+-pumps, a vacuolar H^+-ATPase (EC 3.6.1.3) and a vacuolar H^+-pyrophosphatase (PPase; EC 3.6.1.1). Vacuolar H^+-PPase consists of a single polypeptide in *Arabidopsis* encoded by *AVP1*. *AVP1* generates a proton gradient across the vacuolar membrane thus increasing the sequestration of ions inside the vacuole. Manipulating the vacuolar H^+-pyrophosphatsase (H^+-PPase) to improve Na^+ homeostasis is recognized as an attractive strategy in plants. Recently, H^+-PPase of *Arabidopsis thaliana* (Gaxiola *et al.* 2001) *AVP1*, *Suaeda salsa* (Guo *et al.* 2001) *SsVP1*, wheat *TVP1* (Brini *et al.* 2007) has been overexpressed in *Arabidopsis thaliana* and the H^+-PPase of *Rhodospirillum rubrum* (D'yakova *et al.* 2006) *RPP* and *Thellungiella halophila TsVP1* in tobacco have led to enhanced salt tolerance in transgenic plants. Further, the efficacy of the gene has been more recently evaluated for salt and drought tolerance in cotton (Pasapula *et al*. 2011) and bentgrass (Li *et al*. 2010).

Rice is India's predominant crop and is a staple food of the people of the country. However its productivity is limited by salinity. Rice is the most salt sensitive crop among cereals. Therefore if salt tolerance were to be enhanced in Rice leading to improvement in yield, it would be highly beneficial for Rice productivity in saline areas. To achieve this, *AVP1* was introduced into rice using a tissue culture-independent *in planta* transformation protocol developed by our group in several crop species (Manoj Kumar *et al.* 2009). The main objective of the study was to improve the salt tolerance of a rice genotype grown in the coastal areas. We report the possibility of tolerance to higher levels of salt in rice overexpressing the *AVP1* gene.

MATERIALS AND METHODS

Plant material

A rice genotype from the coastal area viz., 'Vikas' was used for transformation studies. Seeds were soaked overnight in distilled water and were surface sterilized first with 1% Bavistin for 10 min and later with 0.1% $HgCl_2$ for few seconds. After treatment with each sterilant, the seeds were washed thoroughly with distilled water. They were later put for germination in Petri dishes at 30°C. Two-day old seedlings were taken as *ex plants* for *Agrobacterium* infection.

Vector for transformation

The *AVP1* gene construct was obtained from Dr. Roberto A Gaxiola, University of Connecticut. This gene was cloned into *Sma*I site of the pRT-103 vector, released with *Hind*III and cloned into plant transformation vector pCB-302 (**Fig. 1**).

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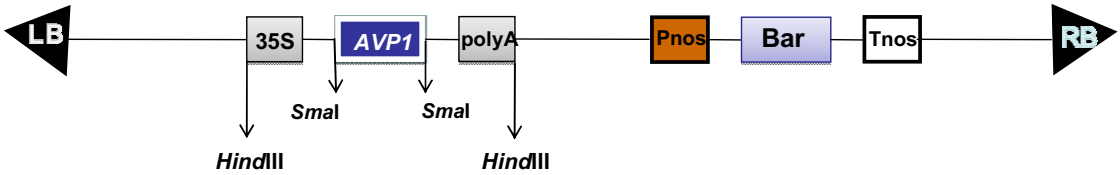

Fig. 1 T-DNA map of the binary vector pCB302 harboring *AVP1* and *bar* as the gene of interest and marker gene respectively.

Transformation and development of transformants

Transformation and generation of the primary transformants was accomplished using the tissue-culture independent *in planta* transformation procedure (Rohini and Rao 2001) as standardized in rice (data not shown; unpublished). The seedlings with just emerging plumule were infected by wounding at the meristem with a sterile needle and subsequent immersion in the culture of *Agrobacterium* for 1 h. Following infection, the seedlings were washed briefly with sterile water and later transferred to autoclaved soilrite (vermiculite equivalent) moistened with water for germination under aseptic conditions in the growth room in wide mouth capped glass jars of 300 mL capacity, 5 seedlings/jar. After 5 to 6 d, the seedlings were transferred to soilrite in pots and were allowed to grow under growth room conditions for 10 d before they were transferred to the greenhouse.

Analysis of the T_1 generation for the selection of putative transformants

1. Seed germination assay for salinity tolerance

Both WT as well as T_1 generation seeds were surface sterilized with 0.1% $HgCl_2$ for 10 min and with 0.5% Bavistin for 30 min, washed thoroughly with sterile water and soaked overnight. After 24 h, just germinated seedlings of uniform size were selected and soaked in 250 mM NaCl for 3 h as pre-treatment and transferred to Petri dishes with 0.6% Agar media containing 450 mM NaCl. Salt treatment was continued for 9 d. Then seedlings were transferred to Petri dishes with filter paper rinsed with water for recovery for 4 d. Seedlings which recovered well were selected and raised.

2. Leaf senescence assay for salt tolerance

Six Leaf bits of known weight (50 mg) from individual putative transformants and WT were transferred to MS salt solidified with 0.6% agar and 450 mM NaCl for 72 h under dark conditions. After 72 h, based on scoring the extent of chlorosis symptoms (Eker *et al.* 2006), lines which remained green were selected for further molecular and physiological analysis.

3. Molecular analysis

Tissues from the progeny plants were analyzed for the presence of the introduced genes. Genomic DNA was isolated following the procedure of Dellaporta *et al.* (1983) from fresh leaf tissue of the greenhouse-grown plants.

DNA amplification by PCR (Polymerase chain reaction) was carried out using 2 sets of primers; marker specific (*bar*) forward (5′-TGCAACATCGTCAACCACTA-3′) and reverse (5′-ACAGCA ACCACGCTGTTGAA-3′) primers and 35S (promoter specific) forward (5′-TCCTTCGCAAAGACCCTTC-3′) and gene specific reverse (5′-GAGCAGCGCATGATGCTTCAG-3′) primers (Gaxiola *et al*. 2001) were also used in order to avoid the interference of endogenous *AVP1* gene.

Physiological analysis of T_1 transformants

1. Chlorophyll estimation

The leaf bits (50 mg) of the WT and *AVP1* putative transformants were incubated in acetone: DMSO (1:1) solution (8 mL) over night. The extract was taken and absorbance was recorded at 553, 645 and 663 nm, using UV-Vis spectrophotometer (UV 2450, Shimadzu). Chl content was estimated by substituting the absorbance values in the formulae given below. Total Chl was expressed as mg g^{-1} fresh weight (Arnon 1949).

Chl *a*: $12.7(A_{663}) - 2.69(A_{645})$ V/weight * 1000

Chl *b*: $22.9(A_{645}) - 4.68(A_{663})$ V/weight * 1000

Total Chl (mg g^{-1} FW) = (Chl *a* + Chl *b*)

Each sample was taken in triplicates, total chlorophyll calculated and the mean data presented.

2. Membrane permeability based on leakage of solutes from leaf sample

Cell membrane integrity was measured in the wild type and 17 putative transformants through recording the optical density of cell contents in to the medium in which leaf discs were incubated (Towill and Mazur 1975). A known weight (50 mg) of leaf samples were taken and incubated in water for 3 h. Leakage was recorded by reading the absorbance at 273 nm (initial absorbance). The leaf bits were later kept in hot water bath (65°C) for 15 min and absorbance was recorded at 273 nm (final absorbance) using UV-2450 visible Spectrophotometer (Shimadzu). The leakage was calculated using the following formula:

$$\text{Leakage \%} = \frac{\text{Initial absorbance}}{\text{Final absorbance}} \times 100$$

Each putative plant was analysed in triplicates and the average calculated and presented.

3. Screening for cell viability by TTC reduction test

Cell viability of the untransformed (control) and 17 putative transformants were assessed using the property of 2, 3, 5-trichloro triphenyl tetrazolium chloride (TTC) reduction by respiratory enzymes, which converts colourless TTC to red colour formazone. The red formazone formed in the tissue (reflection of mitochondrial activity and cell viability) was extracted with methoxy ethanol and its absorbance was recorded at 485 nm (Kalina and Parmer 1986). Each sample was taken in triplicates and the average presented.

RESULTS

Development of transgenic rice overexpressing *AVP1*

In planta transformation protocol was followed to develop primary transformants. A total of 100 rice seeds were surface sterilized and allowed to germinate for 24 h, pricked randomly on embryo axis with a needle. Wounded seeds were infected with *Agrobacterium*, cultured in the AB minimal medium. After 45 min of infection, seeds were placed on sterile soilrite and allowed to grow for 4 d in the growth chamber. After establishment in the growth chamber, seedlings were hardened in green house before shifting them to pots. Around 19 T_0 plants got established in greenhouse, with an average of 16 panicles in each plant. The seeds produced by these plants were selected for analysis in the T_1 generation.

Standardization of a stringent salt screening test for salt tolerance

In planta transformation protocol gives rise to a large number of T_1 seeds and a high throughput screening protocol to select high expressing lines is required to screen such a huge number of plants. In this direction, a stringent salt

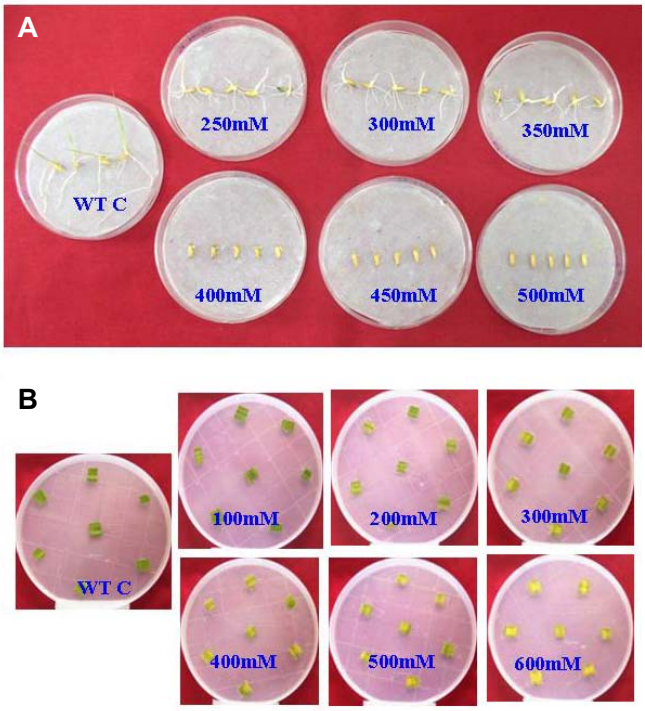

Fig. 2 (**A**) Standardization of seed germination assay for salt tolerance on different concentrations of NaCl. (**B**) Standardization of leaf senescence assay for salt tolerance on different concentrations of NaCl.

screening was standardized for T_1 transformants using WT seeds of rice (var. 'Vikas'). To examine whether the over expression of the vacuolar H^+-pyrophosphatase conferred salt tolerance to the plants, the putative T_1 seeds were screened at two levels, *viz*., at seed level (primary screening) and at plant level (secondary screening).

Standardization of screening strategies for the selection of putative transformants

1. Seed level

Germinating WT seedlings of uniform size were selected and subjected to a pretreatment of 250 mM NaCl for 3h. The seedlings were later transferred to Petri plates containing 0.6% agar and different concentrations of NaCl for 9 d and shifted to water for recovery. There was significant reduction of root and shoot lengths of seedlings at 400 mM NaCl concentration which were treated for 9 d (**Fig. 2A**). As a stringent screening, 450 mM was used as preliminary screening concentration.

2. Plant level

A stringent salt screening test at plant level was standardized in the WT plants following the primary seed level screening. The WT leaf samples were used for standardization. A known weight (50 mg) of leaf samples from WT were treated with different concentrations of NaCl (in mM) (200, 250, 300, 350, 400, 450 and 500) on 0.6% agar + ½ strength MS salts for 72 h and looked for the extent of chlorosis. There was severe chlorosis in WT leaf bits at 450 mM (**Fig. 2B**). Hence 450 mM was used as a stringent screening concentration.

Stringent screening test for salt tolerance for analysis of putative transformants in the T_1 generation

1. Seed level screening

Around 1640 putative T_1 seeds were screened. The growth of both WT as well as transgenics was inhibited in agar media. However the inhibition of growth was more in WT seedlings compared to T_1 seedlings (**Fig. 3A**). Four days

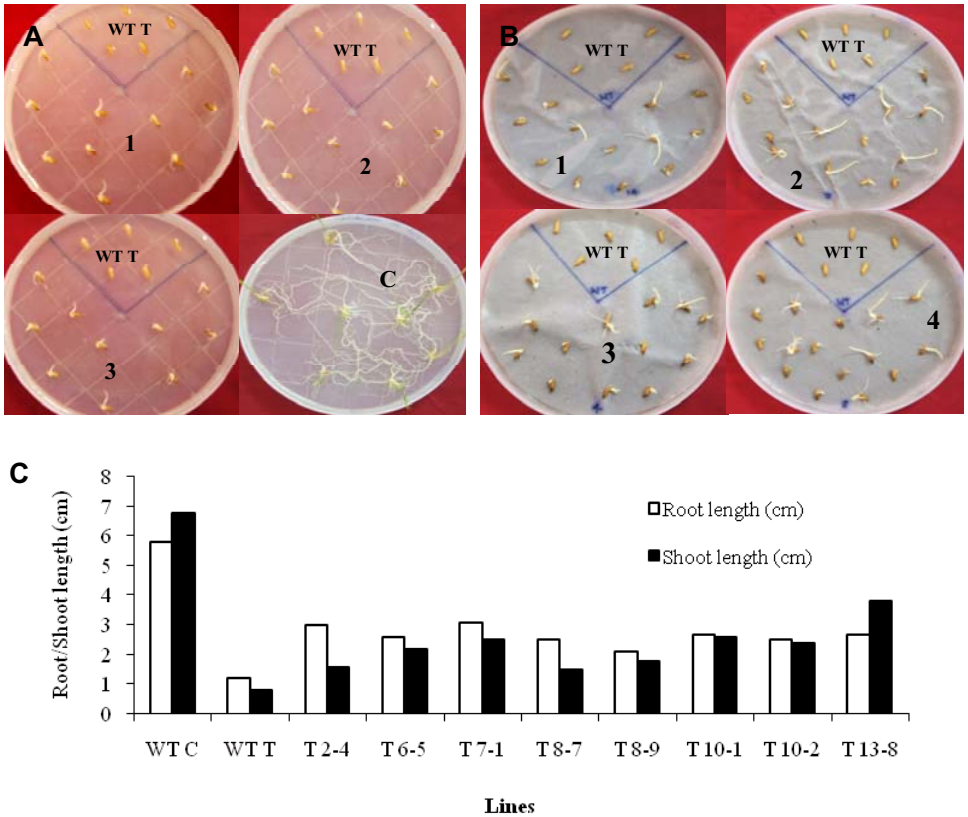

Fig. 3 Screening of T1 generation seeds by germination assay on salt for the selection of putative transformants. (**A**) T1 generation and WT seeds were screened for tolerance on 0.6% agar containing 450 mM NaCl following pretreatment for 3 h in 250 mM NaCl. (**B**) Recovery of the seeds following 9 days of salt stress. WTT: WT treated; 1-4: seeds from different T_0 plants. (**C**) Graphical representation of the root and shoot lengths of the T_1 generation and WT seedlings following salt stress when compared to the treated wild type.

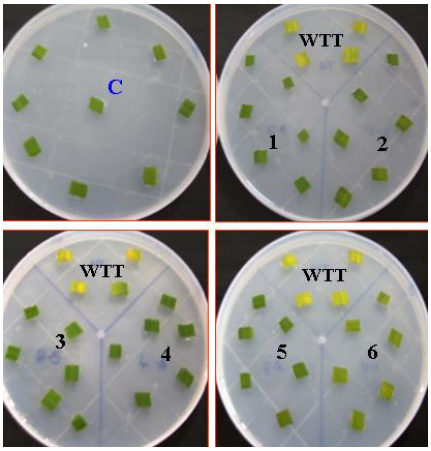

Fig. 4 Leaf bioassay for stress-induced senescence in T_1 generation plants. Resistance to salt-induced senescence in detached leaves of transgenic rice plants compared to the wild type. C: absolute control; WTT: wild type treated; 1-6: putative transformants.

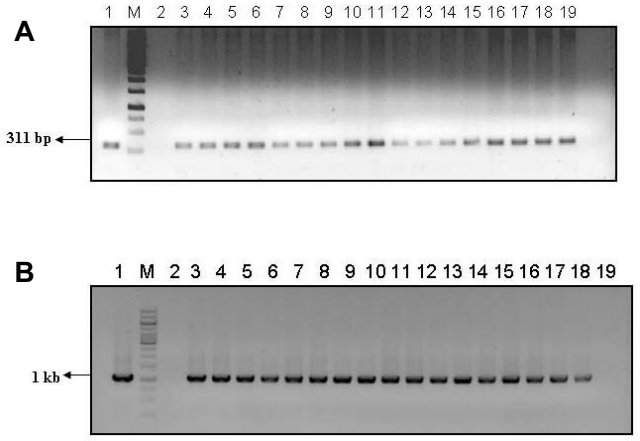

Fig. 5 Analysis of the T_1 generation plants using (**A**) *bar* gene-specific primers (**B**) 35S promoter-*AVP1* gene specific primers. Lane M: 1-kb ladder; Lane 1: positive control; Lane 2: DNA of the WT plant; Lanes 3-19: DNA of putative transformants.

after recovery, around 214 seedlings of the putative T_1 transformants had recovered (**Fig. 3B**). Some of the putative transgenics showed significantly higher root and shoot growth compared to WT (**Fig. 3C**). Out of 1640 T_1 seedlings 84 seedlings were selected on salt media (seed level/ primary screening) and transferred to pots in greenhouse for further analysis.

2. Secondary screening (plant level screening)

Plant level screening was carried out by leaf senescence assay on all the 84 putative transformants following the seed level screening. Selection of plants was based on the extent of chlorosis. Accordingly, 17 lines were found to be tolerant i.e., remained greener compared to the treated WT which showed chlorosis (**Fig. 4**).

Molecular characterization of putative T_1 transformants

The selected plants following seed and plant level screening were analyzed further at molecular level. To reconfirm the transformed nature of plants, PCR was performed with *bar* (marker-specific) primers and 35s promoter forward and *AVP1* gene specific reverse primers. When *bar*-specific primers were used, all the 17 tested plants showed amplification of the gene (**Fig. 5A**). To further confirm the transgenic nature of these plants, 35s promoter forward and gene specific reverse primers were used. Amplification of the expected fragment confirmed the transgenic nature (**Fig. 5B**). However the amplification was not observed in WT.

Physiological analysis of T_1 transformants

1. Chlorophyll estimation

Based on the PCR analysis, 17 transgenic lines were selected to study tolerance of plants to salinity at physiological level. Leaf samples of putative transformants were exposed to 450 mM NaCl for 72 h and tolerance was assessed in terms of percent reduction in total chl (**Fig. 6A**) over non stressed control after NaCl treatment.

There was a significant difference in percent reduction of Chl among putative transgenics and WT treated with respect to their non-stressed control. Compared to WT, transgenics had less reduction in Chl with 27-50%, whereas WT had 68% reduction. Among the putative transgenics, Line T 8-9 had less reduction with 27% compared to WT treated. On an average, putative transgenic lines had 41.3 ± 5.7% reduction in Chl compared to WT treated with 68.0 ± 7.9% reduction in chl upon salt treatment.

2. Membrane permeability measurements

Change in the membrane permeability is considered as a primary injury under salt stress. This is caused by both ionic as well as osmotic effects of salt injury, leading to electrolyte leakage and is determined based on this parameter. The percent leakage was significantly less in putative transgenic lines T 2-4, T 6-5, T 7-1 and T 13-8 compared to WT treated (**Fig. 6B**). Putative transgenics had an average percent leakage of 8.55 ± 2.29% where as WT treated had 23.48 ± 1.49% leakage.

2. Cell viability test

Cell viability of the untransformed (control) and the putative transgenics was assessed using the property of 2,3,5-trichloro triphenyl tetrazolium chloride (TTC) reduction by respiratory enzymes, which converts colourless TTC to red colour formazone and its absorbance is recorded at 485 nm. The absorbance is a direct reflection of cell viability and mitochondrial activity. The cell viability was almost same in putative transgenics as well as WT under control/unstressed condition. The average absorbance of unstressed putative transgenics was 0.63 and wild type was 0.68 at A_{485}. After imposition of salt stress treatment, the cell viability was reduced to a lesser extent in putative transgenic lines in comparison to non-transgenic line (WT). Putative transgenic lines showed an average absorbance of 0.38 ± 0.07 at A_{485} whereas WT stressed showed an average absorbance of 0.13 ± 0.04. All the selected putative transgenic lines showed higher viability after imposing salt stress compared to non-transgenic plants (**Fig. 6C**).

DISCUSSION

Global warming, population growth, environmental stresses, diminishing land resource associated with increasing demand are considered as serious challenges in future (Valliyodan and Nguyan 2006). Amongst various environmental stresses, salinity is considered as major one especially in tropical countries. Salt stress affects plant growth and development in many different ways. To maintain growth and productivity, plants must adapt to stress conditions and exercise specific tolerance mechanisms. One mechanism involves removal of Na^+ from the cytoplasm by transporting it into the vacuole via Na^+/H^+ exchangers driven by the electrochemical gradient of protons (H^+) generated by the tonoplast H^+-ATPase (V-ATPase) and H^+ pyrophosphatase (V-PPase) (Niu *et al.* 1995; Qiu *et al.* 2004). In plants Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across membrane and have a variety of functions, including

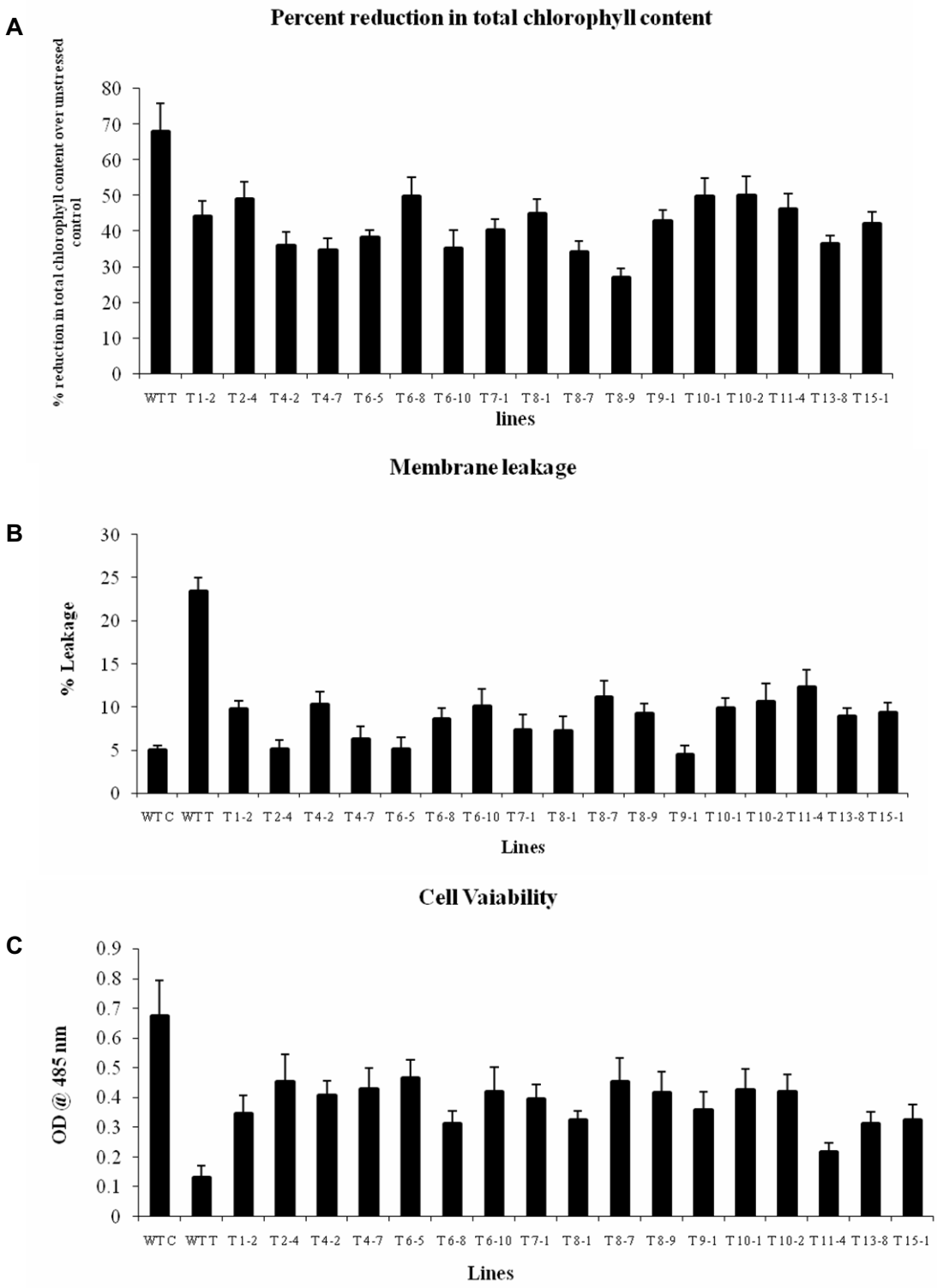

Fig. 6 Physiological analysis of the transformants. (**A**) Chlorophyll stability in the NaCl treated leaf bits compared to the treated and untreated WT. (**B**) Membrane integrity of the salt-treated leaves in transgenics when compared to the WT. (**C**) Graph showing cell viability in terms of absorbance at 485 nm in transgenics and WT under salt stress when compared to the WT. TTC test for cell viability was performed in transgenics and WT after salt treatment for 96 h.

maintenance of cellular ion homeostasis and regulation of cytoplasmic pH and cell turgour (Horie and Schroede 2004). Increasing evidence has demonstrated that vacuolar H^+-pyrophosphatase proton pump plays a crucial role in plant salt tolerance. Improvement in salt tolerance evoked by overexpression of *AVP1* was observed in tomato and *Medicago* (Yang *et al.* 2007; Bao *et al.* 2009). Increased salt tolerance was also observed in transgenic rice carrying the *TVP1* (H^+-PPase from wheat) and *SsVP1* (H^+-PPase *from Suaeda salsa*) (Guo *et al.* 2001; Brini *et al.* 2007), cotton (Pasapula *et al*. 2011) and bentgrass (Li *et al*. 2010). These results indicate that expression of a single H^+-pyrophosphatase gene in plants can be effective in reducing Na^+ toxicity. Gaxiola *et al*. (2001) showed that transgenic *Arabidopsis* plants expressing higher levels of the vacuolar proton-pumping pyrophosphatase, *AVP1*, are more resistant to salt and drought than are wild-type plants. These resistant phenotypes are associated with increased internal stores of solutes. In the present study, it was envisaged to transfer *AVP1* into rice with an aim to improve the salt tolerance of a coastal rice var. 'Vikas'.

Though transgenics in rice have been developed globally using the *in vitro* regeneration, response of rice seems to be genotype dependent. This problem can be overcome

by using tissue culture independent transformation strategies that avoid tissue culture steps. This '*in planta*' transformation strategy was first standardized in *Arabidopsis* (Feldmann and Marks 1987). The methodology has also been extended to other crops that are not amenable to tissue culture like rice (Supartana *et al.* 2005), soybean (Chee *et al.* 1989), etc. In our laboratory, we have developed one such *in planta* strategy where *Agrobacterium* is targeted to the apical meristem and the transformants are allowed to grow in many recalcitrant species like cotton, groundnut (Rohini and Rao 2000), etc.

In the present study, this transformation strategy was extended to rice and primary transformants developed harboring *AVP1* gene. These T_0 transformants were allowed to set seed and T_1 seeds were collected. Since the *in planta* transformation produces chimeras in the T_0 generation, the analysis of the transformants has to be carried out in the T_1 generation. Therefore, it requires standardization of stringent screening techniques to identify the putative transformants.

Yamaguchi and Blumwald (2005) have emphasized the need for routine stress tolerance screens to obtain potential salt tolerant lines from among the putative T_1 transformants. This procedure has been generally followed in many studies including those dealing with overexpression of genes for ion homeostasis (Zhao *et al.* 2006; Chen *et al.* 2007). Techniques for salt tolerance screening either adopt laboratory-based methods like root and shoot growth; salt induced leaf damage and germination test or use physiological tests in intact seedlings in the green house (Ohta *et al.* 2002; Chen *et al.* 2007; Bao *et al.* 2009). Up to early 2000, in most studies related to overexpression of *AVP1* gene, the salt tolerance selection screens have been introduced, only after molecular confirmation of the integration of the gene. However, it was observed that not all the plants selected based on molecular analysis showed salt tolerance. A radically different approach was followed by Zhao *et al.* (2006) with transgenic rice carrying a vacuolar antiporter, *NHX1*. The plants were initially selected based on stress tolerance up to three generations and later confirmed the transgenic nature at molecular level.

In this study, stringent salt screening techniques similar to Zhao's approach was followed and root and shoot growth of putative T_1 primary transformants was used as a selection criterion. Only those seedlings which had not only recovered upon transfer to normal media, but had a root and shoot length of more than 10% compared to WT treated seedlings were selected and transferred to green house. These plants were subjected to the plant level screening once they were established in the greenhouse. Leaf senescence bioassay further allowed the identification of tolerant, moderately tolerant and susceptible plants. This variation between the two screening strategies shows the importance of high stringency screening techniques in the analysis of putative transformants. Subsequently, 17 T_1 generation plants which were tolerant at both the levels of screening i.e., at the seed and plant level screening test were used for molecular confirmation by PCR. The advantage of imposing such a stringent technique at two levels prior molecular analysis of the putative T_1 transformants can been seen from the fact that 100% of the finally selected seedlings tested positive in the PCR test. The selected plants also exhibited tolerance at physiological level. Under salt stress, the selected transgenic plants showed greater membrane integrity with less leakage. The plants also showed higher chlorophyll stability when compared to the WT. Molecular analysis using PCR further confirmed the transgenic nature of all the selected plants. However, copy number of the T-DNA needs to be checked for the inheritance and stability of the transgene.

The present study not only demonstrates that overexpression of *AVP*1 results in salt tolerance but also provides immense scope for further detailed analysis of the plants for the identification of promising lines. Further, relevance of the *AVP*1 gene in these plants can also be analysed for drought tolerance.

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