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AN EFFICIENT *IN-VITRO AGROBACTERIUM* -MEDIATED TRANSFORMATION PROTOCOL FOR RAISING SALINITY TOLERANT TRANSGENIC PLANTS IN FINGERMILLET [*ELEUSINE CORACANA* (L.) GAERTN.]

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Abstract

An efficient reproducible protocol has been established for raising salinity tolerant fingermillet through *in vitro Agrobacterium* mediated transformation. To enhance the salinity tolerance in fingermillet a double gene construct of *PgNHX1* (from *Pennisetum glaucum*) and *AVP1* (from *Arabidopsis thaliana*) was developed using the plant binary expression vector pCAMBIA 1301. This was then mobilized into electro competent *Agrobacterium tumefaciens* strain EHA105 and used for transforming fingermillet var. GPU28. GUS histochemical assay was monitored for confirmation of callus transformation. The putative transformants were acclimatized in incubation chamber for two weeks and then transplanted in greenhouse for further acclimatization. Putative transgenics were confirmed by physiological analysis and PCR amplification of genomic DNA using primers. The putative transgenic plants showed higher salt tolerance of 300mM compared to treated wild type plants. This is the first study in fingermillet reporting the expression of double gene construct of *PgNHX1* and *AVP1* for salinity tolerance.

Key words : Transgenics, fingermillet, transformation, salt tolerance.

Introduction

Finger millet [*Eleusine coracana* (L.) Gaertn] is one of the important food source for millions of people, widely grown as cereal crop in the arid areas of Africa and Asia. It has nutritional qualities superior to that of rice and is on par with wheat (Latha *et al*., 2005). But salinity is one of the major environmental constraints severely affecting the crop production. Even though the plants have developed various physiological and biochemical strategies to tolerate stress condition, it is necessary to increase the stress tolerance level of crops by overexpressing the gene of interest so as to survive under severe stress condition. Nearly 20% of the world's cultivated area and nearly half of the world's irrigated lands are affected by salinity (Zhu 2001). High salt concentration decreases the osmotic potential of soil solution creating water stress in plants and finally causes ion toxicity. The primary cause of ion toxicity in crop plants is Na^+ and hence maintenance of cellular Na^+ concentration is critical for optimal metabolic activity under salt stress (Tester and Davenport, 2003). Sequestration of excess cytosolic Na^+ into vacuole is one of the most important successful strategy towards the maintenance of ion homeostasis in most of the plants that averts the deleterious effects of Na^+ in the cytosol by maintaining a higher ratio of K^+/Na^+. Transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter (*NHX1*) driven by the electrochemical gradient of protons generated by the vacuolar H^+- translocating enzymes, H^+- ATPase and H^+-PPase (*AVP1*) (Yamaguchi and Blumwald 2005). In recent years many experiments in different plant species have been carried out demonstrating the importance of vacuolar antiporters *NHX1* and *AVP1* in plant salt tolerance (Gaxiola *et al*., 1999, 2001; Apse *et al*., 1999; Zhang and Blumwald, 2001; Soliman *et al*., 2009; Xue *et al*., 2004; Yin *et al*.., 2004; Ohta *et al*., 2002; Verma *et al*., 2007; Zhao *et al*., 2006 a,b; Guo *et al*., 2006; Gao *et al*., 2006; Park *et al*., 2005; Bao *et al*.,

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2009). Thus, recent report on transporters emphasizes the importance of *NHX1* and *AVP1* in Na^+ sequestration into the vacuole.

Monocots are usually recalcitrant to *Agrobacterium* mediated transformation due to the absence of phenolic compounds, other genetic and environmental factors. However successful transformations have been carried out in cereals by including various factors during co-cultivation process. In our study, we have optimized efficient transformation protocol for raising transgenic fingermillet that are resistant to salinity stress by co-expressing vacuolar antiporter genes*PgNHX1* and *AVP1*. The fingermillet transgenics co-expressing *PgNHX1* and *AVP1* exhibited higher level of salinity tolerance compared to treated wild type plants. The reason for this could be the increased activity and higher efficiency of the Na^+/H^+ antiporter driven by the proton gradient generated by *AVP1* H^+ pyrophosphatase.

Materials and Methods

Plant transformation vector construct

A high efficiency monocot plant transformation binary vector pCAMBIA 1301, the widely used vector was used for making the construct in the present study. The T-DNA region contains the right border, expression cassette for β –glucuronidase (GUS) a reporter gene, LacZα, a α subunit of bacterial lactase, MCS, a multiple cloning site, expression cassette for Hygromycin Phospho Transferase II (HPTII) a selectable marker and the left border. The *PgNHX1* gene was obtained from Dr. M.K. Reddy, ICGEB, New Delhi and the second gene *AVP1* was obtained from Dr. R A Gaxiola, University of Connecticut, Farmington, CT. For developing double gene construct of *PgNHX1* and *AVP1*, the *AVP1* gene cassette was partial digested with *HindIII* from PCB302::*AVP1* vector, the full length 3.3 Kb fragment was inserted into pCAMBIA 1301 at *HindIII* site. *NHX1* gene cassette was then incorporated at *EcoRI* and *SalI*, the binary plant expression vector pCAMBIA 1301 containing *NHX1*+ *AVP1* was obtained. This was confirmed and was mobilized into electro-competent *Agrobacterium tumefaciens* strain EHA105 (Competant cells were prepared according to Shen and Forde, 1989) by applying an electric pulse using a Gene PulserÔ with pulse controller unit (Bio-Rad). Good transformation efficiency was obtained at a field strength of 12.5 kV/cm, a capacitance of 25 µF and resistors of 400 W in parallel with the sample. These confirmed transformed cultures were used for finger millet transformation.

Plant material, transformation and growth conditions

For *in vitro Agrobacterium* mediated gene transfer, mature seeds of finger millet var. GPU-28 were used for callus induction and the embryogenic calli obtained were used as explants for the transformation. The finger millet seeds were soaked overnight and surface sterilized with carbendazim for five minutes, washed with distilled water and then finally treated with 0.1% mercuric chloride for 30 seconds followed by 4-5 times wash in autoclaved distilled water. Healthy seeds of uniform size were selected and placed on MS (Murashige and Skoog, 1962) nutrient medium with 2.5mg/L 2,4-D + 0.5mg/L BAP fortified with 3 percent sucrose and were solidified with 0.8% agar agar. The pH of the media was adjusted to 5.8 before autoclaving (121°C and 15 lbs per sq. inch). The callus initiation was observed after one week of culture, which was allowed to grow on the same medium for four weeks. Later, the calli were transferred to MS medium supplemented with 2.0 mg/L 2,4 – D and allowed to proliferate for another three weeks under dark condition (to avoid regeneration from callus) that were suitable as explants for transformation. The *Agrobacterium tumefaciens* strain EHA 105 harboring the binary vector pCAMBIA 1301 with genes of interest was grown in liquid LB medium until the OD reached to 0.6 to 0.8 at 600nm. The culture was centrifuged at 2500rpm for 5 minutes and pellet obtained was dissolved in half strength MS and made to a final volume until the OD reached to 0.6. To this culture 1% of the filter sterilized tobacco leaf extract and 150µM acetosyringone was added to improve the transformation efficiency. The embryogenic calli obtained from mature seeds were soaked in this medium for 5 minutes, blotted dry to remove excess bacteria, inoculated on co-cultivation medium (MS medium+2mg 2,4-D+150µM acetosyringone+1% filter sterilized tobacco leaf extract) and kept under dark at 25°Cfor two days, so that *Agrobacterium* infects the explant and thus transfers the DNA to plant tissue. The explants were then washed with medium containing bacteriostatic agent (400mg cefotaxime) to kill the *Agrobacterium* and sub cultured on callus proliferation medium (MS medium + 2mg 2,4-D+400mg Cefotaxime) for 2 days at 25°C so that the transformed tissue proliferates. The proliferated calli were transferred to the selection medium containing hygromycin (25mg), on which they were grown for eight weeks, being sub cultured onto fresh selection medium every two weeks. The surviving resistant calli were then cultured on regeneration medium (MS medium+80mM NH_4NO_3+ 25mg Hygromycin) until the plantlets reached about 1-2 cm in length. Later they were transferred to

fresh regeneration medium containing 20mg hygromycin. The regeneration was carried out at 24±2°C under fluorescent light (150 – 200 μ E $m^{-2}s^{-1}$). The putative transformants were then cultured in half strength MS media for 2 weeks for stronger root formation. The rooted putative transformants were transplanted into small pots containing soil rite and covered with plastic bags to prevent dehydration. The plastic bags were gradually opened to decrease humidity, allowing hardening off, of the plants. After one week, the plastic bags were removed and the plants transplanted to pots containing soil and allowed to grow to maturity in the greenhouse (fig. 1).

GUS histochemical assay

GUS assay (Jefferson *et al*., 1987) was carried out after six days of co-cultivation with *Agrobacterium* for initial confirmation of transformation. The calli were incubated overnight at 37°C in 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) substrate solution containing 50mM sodium phosphate buffer, pH 7.0, 0.1% Triton X 100, 5mM each of potassium ferrocyanide and potassium ferricyanide. Development of blue color was noticed in transformed calli (fig. 2).

Analysis of putative transformants

Physiological analysis of T_0 transformants

Leaf senescence assay

Healthy leaf bits from the putative T_0 transformants and wild type were placed on MS medium supplemented with different salt concentration ranging from 0mM to 450mM for three days. The effect of salt stress on leaf bits were visualized by phenotypic observation.

Chlorophyll stability index

The leaf bits (50mg) of the wildtype and putative T_0 double transgenics of *NHX1+AVP1*were imposed salt stress by floating the leaf bits in 200mM and 400mM NaCl solution for 72hrs. The leaf bits were then incubated in acetone: DMSO (1:1) solution (5 ml). The extract was taken and absorbance was recorded for total chlorophyll, expressed as mg g^{-1} fresh weight. Then percent reduction in total chlorophyll content and Chlorophyll Stability Index (CSI) was calculated as given below.

Total chlorophyll content = (A. 652/34.5) x (volume/ Fresh weight) Percent Reduction over Control = [(Abs. control - Stressed sample)/Abs. control] x100 Chlorophyll stability index =100-R

Membrane stability

Percent leakage, which reflects loss of membrane integrity, was quantified by Sullivan's method. The leaf bits of the wild type and double gene putative transformants, which had undergone 400mM of salt stress for three days were incubated in 10 ml of distilled water for 10 minutes. Initial electric conductivity (ECa) was taken using EC-TDS analyzer (ELICO-CM183). Then the leaf bits were kept for 10 minutes at 65 ^{0}C and the second ECb was taken, finally it was boiled for 15 minutes which was considered as final ECc. The percent leakage was calculated using the formula,

Percent leakage = [ECb-ECa/ECc] x 100

Table 1 : Survival of explants in different medium supplemented with 25mg/L hygromycin as selection pressure.

Medium	Number of surviving explants		
	Event 1	Event 2	Event 3
Callus induction medium	90	81	118
Selection medium	4	2	4
Regeneration medium	1	1	1
Number of shoots regenerated	6	5	6

Molecular analysis of putative transformants by PCR

The double transgenic plants were used for PCR analysis. 50ng of genomic DNA was used as template DNA from wild type and double transgenic lines. Genomic DNA was extracted by CTAB (Cetyl Trimethyl Ammonium Bromide) method as described by Doyle and Doyle (1998). 35s forward and *NHX1* reverse primer, 2x35s forward primer and *AVP1* specific reverse primer and hpt gene specific forward and reverse primers were used. The PCR reaction was performed at 95°C for 5 minutes followed by 30cycles of denaturation at 95°C (1 minute), annealing at 58°C (45 seconds), extension at 72°C (1minute) and then final extension at 72°C for 10 minutes.

Results and Discussion

Raising of fingermillet transformants

Fingermillet variety GPU28 was used in the present experimental studies. For *In vitro Agrobacterium* mediated transformation, seeds were used as explant for callus induction on MS medium fortified with 2mg 2, 4-D and 0.5mg BAP. Almost all the seeds induced callus within one week of inoculation, after four weeks the callus were transferred to medium containing 2mg 2, 4-D and allowed to proliferate for another three weeks. These calli were then used for *Agrobacterium* infection. The *Agrobacterium tumefaciens* strain EHA 105 harboring the binary vector pCAMBIA 1301 with hygromycin resistance gene (hpt) as plant selection marker, a gene

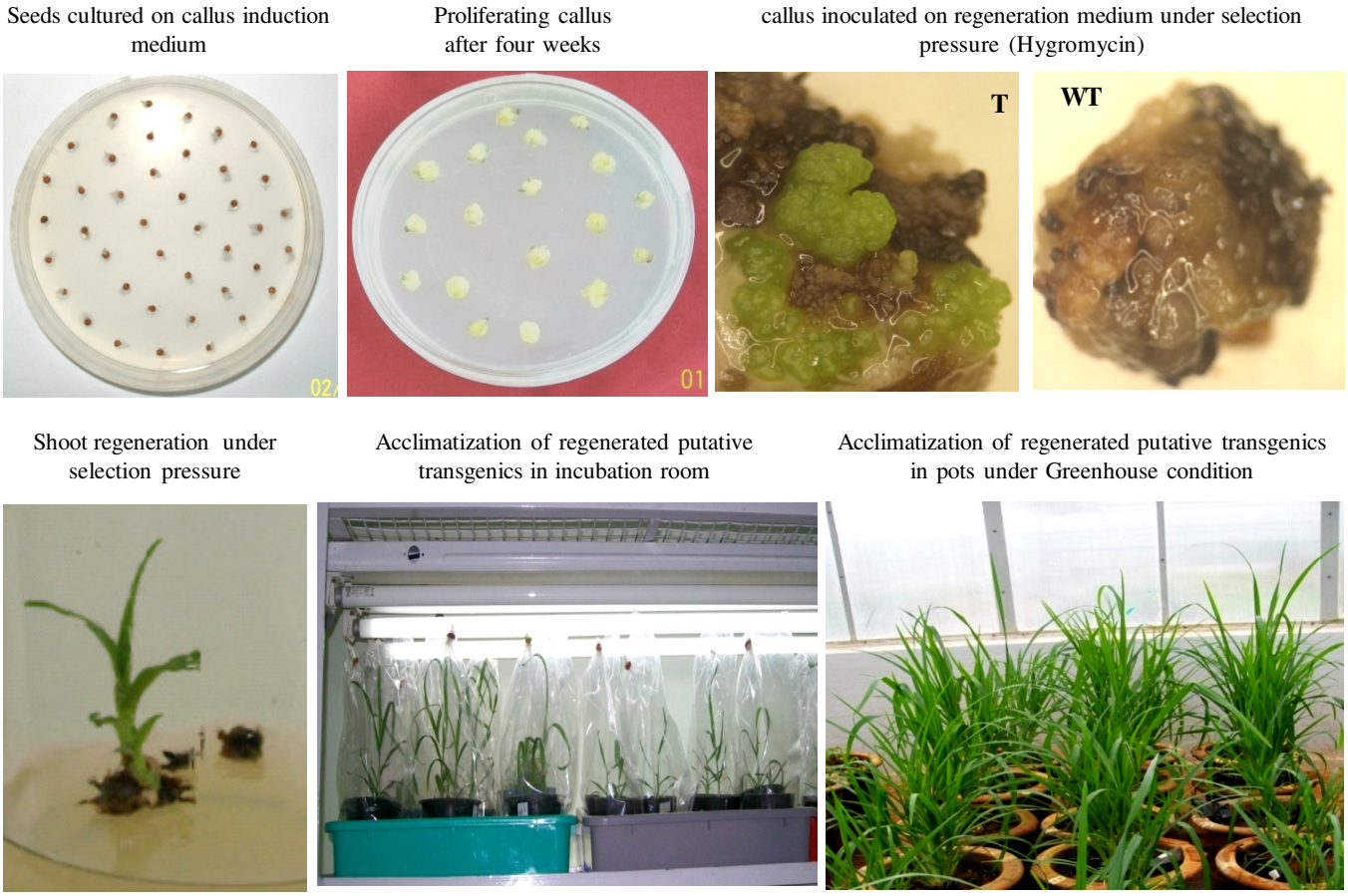

Fig. 1 : *In vitro Agrobacterium* mediated transformation in fingermillet starting from callus to regeneration of putative transformants in green house.

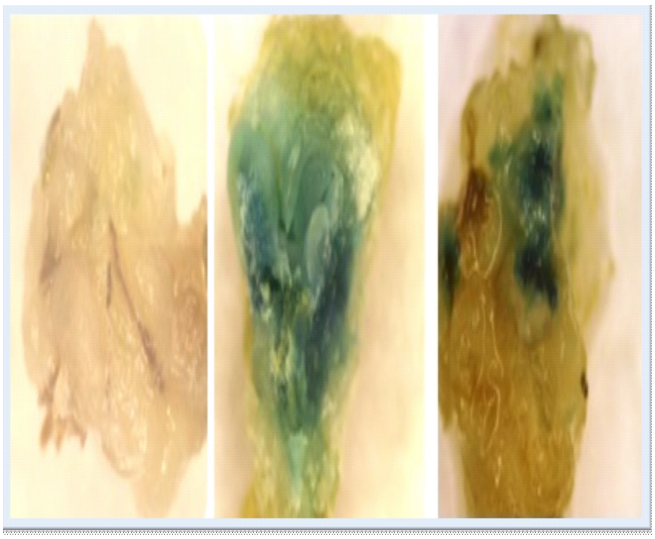

Fig. 2 : GUS expression in callus explants of finger millet transformed with double gene construct of *PgNHX1* and *AVP1* in figure (b) and (c) and (a) represents non transformed wild type callus.

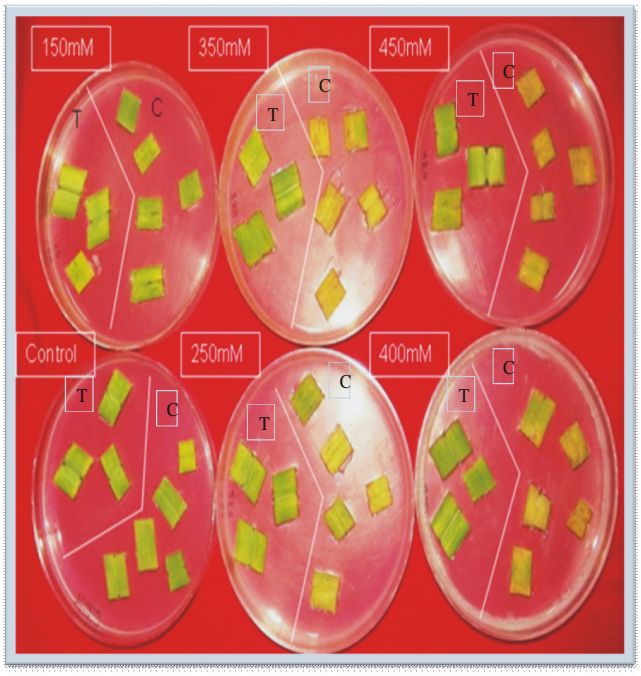

Fig. 3 : Leaf senescence bioassay in wild type treated (C) and T_0 transgenics (T) using different NaCl concentration. (photographs taken 4 days after treatment).

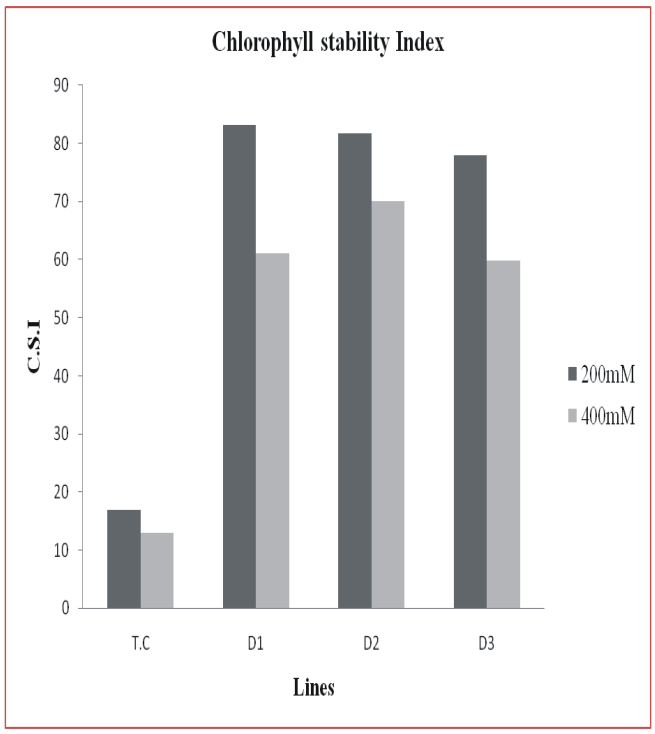

Fig. 4 : Chlorophyll stability index of wild type and T_0 transgenic plants under salt stress. TC- treated wild type and D1-D3 are treated transgenics.

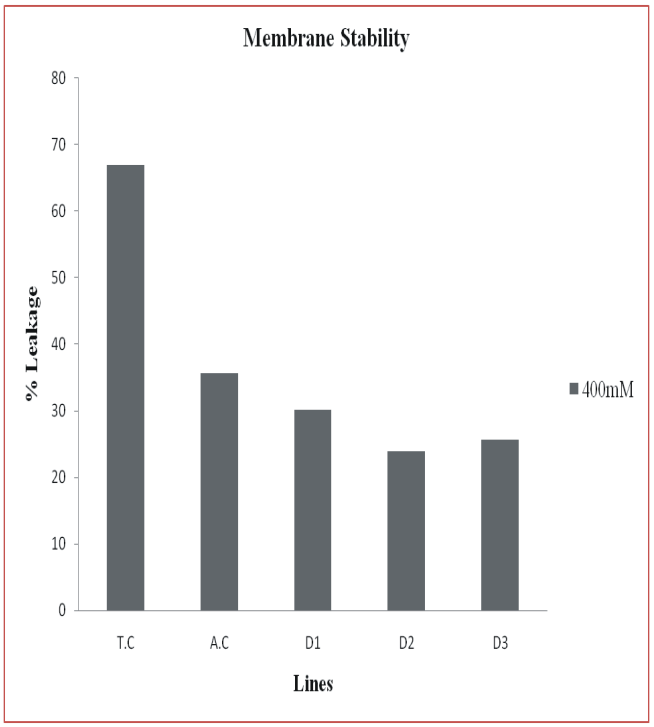

Fig. 5 : Membrane Stability of wild type and T_0 transgenic plants under salt stress.TC- treated wild type, AC- Absolute control and D1-D3 are treated transgenics.

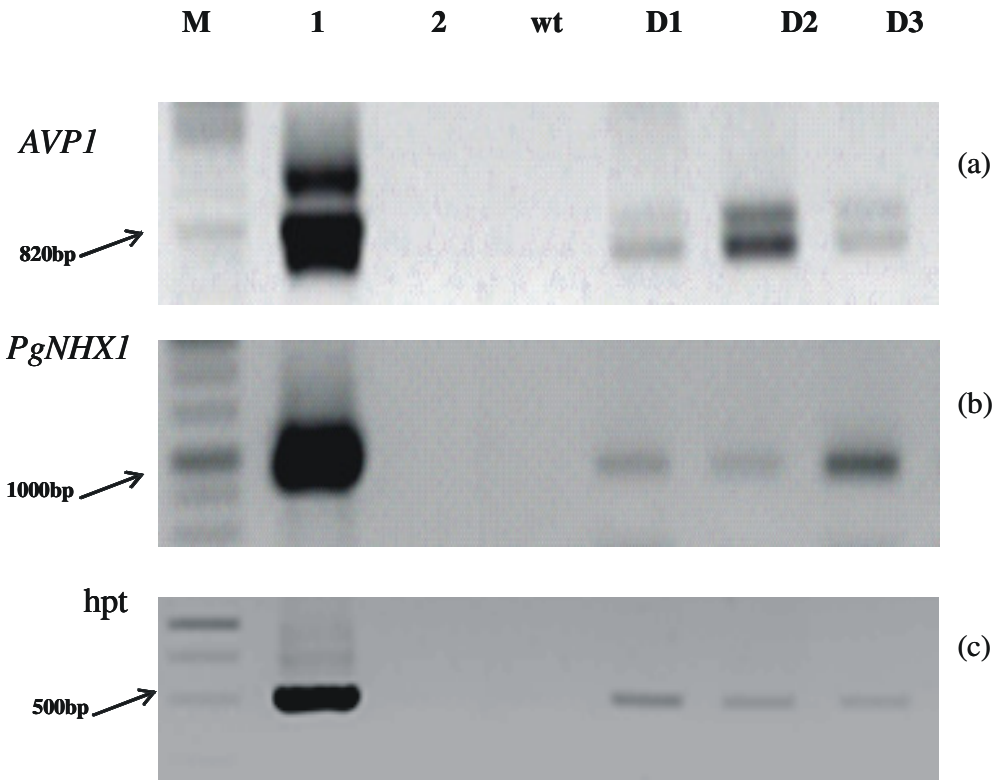

Fig. 6 : PCR analysis of T_0 transgenics of *PgNHX1* + *AVP1*, (a) fragment of length 820bp was detected with 2x35s promoter forward and gene specific reverse primer of *AVP1* (lanes D1 to D3), (b) fragment length of 1000bp of *PgNHX1* using 35s promoter forward and gene specific reverse primer (lanes D1 to D3) and (c) fragment length of 500bp was obtained for *hptII* gene specific forward and reverse primers (lanes D1 to D3). Lane M:1kb ladder, Lane 1: positive control, Lane 2:negative control, wt: DNA from wild type.

for b-glucuronidase (gus A) as scorable marker and with genes of interest *viz.*, *PgNHX1+AVP1* was used for plant transformation as explained in materials and methods. In each event of plant transformation nearly six plants regenerated under 25mg/l hygromycin selection pressure as shown in table 1. Three independent double transgenic lines were selected for physiological and molecular analysis.

Leaf senescence assay

Leaf samples of wild type and T_0 double transgenic plants were placed on MS medium supplemented with different salt concentrations ranging from 0mM to 450mM NaCl to assess the salinity tolerant level of double transgenics under *in vitro* salt stress condition. Figure 3 clearly depicts the tolerance level of transgenic leaf samples even under a high concentration of 400mM NaCl whereas the wild type treated plants showed severe chlorosis. This bioassay was carried out to assess the phenotypic changes in leaf samples subjected to salt stress.

Chlorophyll stability index

CSI of putative T_0 double transgenics of *PgNHX1* + *AVP1* and the wild type plants was assessed by imposing salt stress of 200mM and 400mM to leaf samples for 72hrs. The CSI was higher in double transgenic lines than wild type treated plants with CSI of around 80 in transgenics compared to 18 in treated control plants subjected to 200mM salt. Similarly in 300mM salt treated plants, the chlorophyll stability index was around 63 in double transgenics compared to CSI of 13 in treated wild type plants (fig. 4). Thus, transgenics lines co-expressing *PgNHX1* and *AVP1* showed higher CSI than wild type treated plants.

Membrane stability

Electrolyte leakage indicates the extent of damage caused to the cell membrane under salt stress. Hence, membrane stability was studied to assess the primary injury faced by the membrane under salt stress of 400mM in transgenics and wild type leaf samples. The transgenic lines co-expressing *PgNHX1* and *AVP1* exhibited 24 % to 30 % leakage compared to 67% leakage in treated wild type plants. Thus significant decrease in electrolyte leakage in transgenic plants was observed compared to treated wild type (fig. 5).

PCR analysis

PCR was performed with hpt II primers and 35s promoter forward and *PgNHX1*, *AVP1* gene specific reverse primers. Amplification of the expected fragments (fig. 6) confirmed the successful integration of *PgNHX1* and *AVP1*.

Agrobacterium mediated plant transformation is one of the most important aspect for introducing specific DNA fragments into plant genomes. Naturally monocots are recalcitrant to *Agrobacterium* infection but successful attempts have been made in the past years in developing transgenic crops by incorporating phenolics and modifying genetic and environmental aspects during transformation process. Low efficiency of transformation is one of the major problem in developing transgenics in monocots. In this regard we have developed an efficient reproducible protocol for regenerating salinity resistance fingermillet by integrating double gene *viz.*, *PgNHX1* and *AVP1* in the plant genome. Similar work using *Suaeda salsa NHX1* and *Arabidopsis AVP1* has been carried out in rice (Zhao *et al.*, 2006). This is the first report in fingermillet where we have co-expressed vacuolar genes involved in sequestration of excess sodium into the vacuole. In the present work, the *Agrobacterium* infected calli were cultured on hygromycin containing MS medium supplemented with four times the normal level of ammonium nitrate without any growth regulator. The plantlet regeneration on medium fortified with ammonium nitrate has been already reported (Poddar *et al.*, 1997), which resulted in increase in number of plantlet regeneration from the callus. The putative transgenics thus obtained were all PCR positive and exhibited higher level of salt tolerance than treated wild type plants.

Abbreviations used

NHX1: Sodium hydrogen exchanger

AVP1: Arabidopsis vacuolar pyrophosphatase

2,4-D: 2,4-Dichlorophenoxy acetic acid

BAP: Benzyl amino purine

DMSO: Dimethylsulfoxide

PCR: Polymerase chain reaction

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