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Research Article

ECTOPIC OVEREXPRESSION OF BARLEY *PIP2;4* CONFERS SALT TOLERANCE IN ARABIDOPSIS

Jayeeta Mitra, Jay Prakash Awasthi and Sanjib Kumar Panda*

Plant Molecular Biotechnology Laboratory, Department of Life Science and Bioinformatics, Assam University, Silchar-788011, Assam, India

*Corresponding author, email address: drskpanda@gmail.com

Abstract

In the present study *HvPIP2;4* was overexpressed in *Arabidopsis thaliana* to engineer enhanced salt tolerance. Barley Aquaporin was selected since barley shows fairly good tolerance to drought, salt stress and low temperature compared to many other crops including rice, and it was thought that analysis of barley aquaporin will provide a good insight into the molecular mechanisms involved in transport of water & their efficacy during abiotic stress condition. Arabidopsis line expressing *HvPIP2;4* from annual crop plant *Hordeum vulgare* (Barley) under the control of constitutive promoter was used to analyze the expression of *HvPIP2*; 4 and its efficacy during salt stress when NaCl concentration gradually increased. The pattern of expression of *Hv*PIP2; 4 were found to be NaCl dose dependent during salt stress. The constitutive expression of *HvPIP2;4* enhanced salt stress tolerance in *Arabidopsis*. *HvPIP2;4* played a dominant role in improving plant salt tolerance. It may be very well presumed that overexpression of *HvPIP2;4* in crop plant might benefit them by enhancing their salt tolerance capacity.

Keywords: Aquaporin; *PIP2;4*; Overexpression; Barley; Arabidopsis

Introduction

Water plays a very important role in plant growth and development, a fundamental requirement of the proper growth of plants is adequate uptake of water and its flow across the membrane (Agre and Homer, 2000). Vital processes such as cellular respiration and photosynthesis requires the presence of water (Taiz and Zeiger, 2006). Water transport within and between plants tissues uses both the apoplastic and the symplastic routes; therefore, a fairly large number of cellular membranes are to be crossed by the water molecules (Quigley *et al.*, 2002). Aquaporins, a family of water channel proteins controls and maintains the radial component of symplastic route (Amodeo *et al.*, 1999).Water gets transported across the membrane either through diffusion via the lipid bilayer and/or through the water channel proteins aquaporins (Preston *et al.*, 1992). Peter Agre and his team first discovered the phenomenon of water transport facility via aquaporins in John Hopkins University in early 1990s. The aquaporins facilitate in the rapid and regulated movement of water across the membrane. The presence of aquaporins has been found in bacteria, fungi, animals and plants. Due to their universal presence they are supposed to play an important part in life cycle (Heymann and Engel, 1999). Plant aquaporin was first discovered by Maurel *et al.*, 1993 thereafter a large number of plant aquaporin has also been reported from other plant species such as Arabidopsis 35, Tomato 47, Maize 36, Rice 33 and 71 in Cotton (Johanson *et al*., 2001; Chaumont *et al.*, 2011; Sakurai *et al*., 2005; Park *et a*l., 2010). Some selective aquaporins increases 10-20 times the hydraulic conductivity of the membrane. In various studies it was found that a rise in concentrations of salt has shown to inhibit root water uptake due to reduction in root hydraulic conductance and thereby increasing the transmembrane diffusion efficiency of water (Obroucheva *et al.*, 2010). Several measures such as heterologous co expression in oocytes, interaction of proteins and their detection in mutant yeast, overexpression of certain genes in plants and inhibitors have been utilized to analyze the functionality of aquaporins. Among abiotic stressors salt stress plays a major role for the reduction of crop yield worldwide (Kronzucker and Britto, 2011). Salinity had affected about half of the world's irrigated land and more than 20% of the cultivated land (Mahajan and Tuteja, 2005).To cope with the increasing food requirement minimization of losses incurred due to abiotic stress is a major concern for all

nations. To mitigate stress ion homeostasis and water needs to be regulated so that water deficit and/or ion toxicity can be avoided. With the discovery of plant aquaporin it was thought that they play an integral role in adaptation of salinity and drought stress by metabolically controlling the flow of water (Maurel *et al.*, 2002, Baiges *et al.*, 2002). Barley *(Hordeum vulgare* L.) is diploid, contains 14 chromosomes, is self-pollinating and it belongs to the grass family. Barley is fairly good salt and drought tolerant plant, many barley varieties have been evaluated from the stand point of salt tolerance. Several PIP genes have been identified in Barley *Hv*PIP1; 3, *Hv*PIP1; 6 and *Hv*PIP2; 1 have already been characterized and their functionality as water channels have been verified in *Xenopus laevis* oocytes (Hollenbach and Dietz, 1995; Katsuhara *et al.*, 2002; Katsuhara and Shibasaka 2007; Wei *et al.*, 2007). Expressed Sequence tag (EST) were analyzed for barley and it was found that a large number of *PIP1* and *PIP2* gene exist in barley similarly as found in other plant species (Katsuhara *et al.*, 2008). These findings lead us in studying and characterizing new *HvPIP*s so that we can have a clear idea about the water transport mechanism controlled by *HvPIP* aquaporin in barley plants. It is believed that functional characterization will give new insights regarding the role of *HvPIP2;4* gene. In the current study an independent transgenic *Arabidopsis* line carrying *HvPIP2;4* under the control of constitutive promoter was obtained, and the expression patterns of *HvPIP2;4* in transgenic *Arabidopsis* exposed to salinity was analyzed.

Materials and Methods

Plant Materials and Treatment Conditions

Barley (*Hordeum vulgare* cv. NP21) seeds were procured from Indian Agricultural Research Institute, Pusa, New Delhi and germinated in dark for 3 days on two layers of moist filter paper disk placed on a Petri dish at 30±1°C. After 3 days, the germinated seeds were transferred to Hoagland's nutrient medium (pH 6.2) and grown hydroponically. The seedlings were grown in a growth chamber under continuous white light provided with cool, fluorescent white tubes, for 7 days. *Arabidopsis thaliana*, ecotype Columbia (Col-0) was kept at 22°C in a soil-peat mixture for 10h under light and watered twice a week with tap water.

Isolation of HvPIP2; 4 Target Gene

Total RNA isolation was done as described by (Bilgin *et al.*, 2009). One microgram of total RNA was reverse transcribed to cDNA by using the cDNA Synthesis Kit (Thermo Fischer Scientific, Waltham, MA, USA) followed by cloning in pTZR/T(Thermo Fisher Scientific, Waltham, MA, USA) and sequenced. It was found to show 100% homology with *HvPIP2;4* gene reported by Panda *et al* (2005).

Binary Vector Preparation and Transformation in to the Model Plant

HvPIP2;4 was sub cloned into a constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter/CaMV strain Cabb B-D polyadenylation signal cassette, within the intermediate vector pRT101 (Töpfer *et al.*, 1987). Using the restriction site HindIII, the CaMV35S promoter-*Hv*PIP2;4-35S terminator cassette was subsequently transferred to T-DNA portion of plant binary vector pCAMBIA 2301 (11.6 kb). Neomycin phospho-transferase gene (*nptII*) selectable marker for plant transformation and b-glucuronidase gene (gus) from *E. coli* beta-glucuronidase gene, it is a gene fusion marker and used for gene expression analysis are present in the T-DNA region of pCAMBIA2301 and are regulated by cauliflower mosaic virus (CaMV) 35S promoter (Fig.1). The resulting recombinant plant binary vector was labeled as pCAMBIA2301-35S::*HvPIP2;4* (13.1kb), and electroporation at 1250V with capacitance of 25mF and resistance of 400 ohm was used to transfer the recombinant plant binary vector pCAMBIA 2301-35S::*HvPIP2;4* into *A.tumifaciens* GV3101 strain (Sambrook and Green 2001, Nagel *et al.*, 1990). *Arabidopsis thaliana* (ecotype Columbia) was transformed inplanta via floral dipping method with the construct (Clough and Bent, 1998). The T_1 transgenic lines were screened on 1/2 MS Medium (from Duchefa, Haarlem, Netherlands) supplemented with 50mg/l Kanamycin (Duchefa, Haarlem, Netherlands). The selection of transgenic was continued until T_3 generations to bearing transgenic homozygote lines that have a single T-DNA locus (35S::*Hv*PIP2; 4).

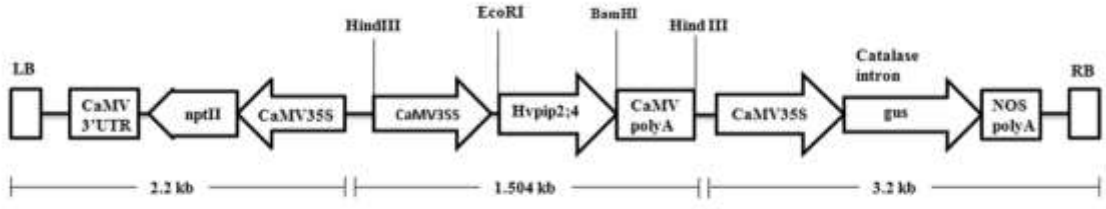

Fig. 1: T DNA Region of pCAMBIA 2301 *HvPIP2;4* (1.504bp).The 873 bp fragment containing *Hv*PIP 2;4 under control of CaMV35S promoter and NOS terminator.LB and RB: left border and right border of T-DNA region, NOS T :nos terminator, 35P:CaMV35S promoter, nptII: neomycinphosphotransferase-II.

Screening of the Putative Transformed Plants

For screening of the putative transformed plants Genomic DNA was isolated from T_3 transformed putative *Hv*PIP2; 4 overexpressing lines and non- transformed plants using CTAB method (Rogers and Bendich, 1988). The presence of *nptII* was detected by polymerase chain reaction (PCR) in T_3 transformed putative plants. Primers (forward primer 5'-CCACCATGATATTCGGCAAC-3' and reverse primer 5'-GTGGAGAGGCTATTCGGCTA-3') amplified 0.54-kb fragment of *nptII*. PCR condition was 94ºC for 5 min; 1cycle, 94ºC, 1min, 58ºC, 1min, 72ºC, 1min; 35cycles, 72ºC, 7min; 1cycle. PCR fragments were analyzed on 1% agarose gel and stained with 10mg/ml ethidium bromide.

Phenotypic Changes under Salt Stress

The 4 days old germinating seedlings were transferred to 1/2 MS media liquid and plants were grown at 23°C. The photoperiod lasted for 16 hour with 8 hours in dark, under fluorescent illumination supplemented by incandescent light yielding an intensity of 100-150mE/m2×s. Salt stress of (0mM, 100mM and 200mM) was imposed hydroponically for 48hrs and phenotypic changes were recorded.

Measurement of Growth Parameters under Salt Stress

Determination of Plant root growth. The 4 days old germinated seedlings were transferred to 1/2 MS Medium liquid supplemented with (0mM, 100mM and 200mM) NaCl for 1 week and the difference in root length of the wild type WT and T_3 independent transgenic line of *Arabidopsis* seedlings expressing *HvPIP2;4* was measured by using ImageJ software (Schneider *et al.*, 2012). Mean data was collected from at least three independent experiments for wild type and T_3 Kanamycin selected transgenic *Arabidopsis* line.

Determination of Chlorophyll Content

The 10 day old germinated seedlings were transferred to 1/2 MS liquid supplemented with (0mM, 100mM, and 200mM) NaCl for 5 days**.** In order to measure the chlorophyll content, 150mg of shoot tissue was grounded with liquid nitrogen and the powder was transferred into centrifuge tube. 2.5 ml of 80% acetone was added into the tube and mixed well and placed in dark for 15- 30 min. After centrifugation at 4^0C for 15 min (3,000 rpm) the supernatant was transferred to a new centrifuge tube and the absorbance was read spectrophotometrically (Bacman coulter DU 730 UV- Vis Spectrophotometer) at 663 nm and 645 nm (Arnon, 1949).

Determination of Dry and Fresh Biomass

To determine fresh biomass the wild type and transgenic samples were weighed after sampling, for the dry biomass, samples were dried in oven at 65ºC for 48 h and then weighed to determine the dry biomass.

Relative Water Content (RWC) Analysis

Leaf segment were excised from fully expanded leaves and fresh weight (FW) was recorded. The segments were then allowed to float on deionized water for about 4hours and turgid weight (TW) was recorded. The leaf segments were dried at 80ºC for 24 hours dry weight (DW) recorded and RWC calculated.

Results

Molecular Analyses of Transgenic Plants

The presence of the expected 540bp amplified product corresponding to *nptII* in kanamycin-resistant T_3 transgenic plant was detected by PCR analysis. Amplification was not found in the control untransformed plant (Fig 2).

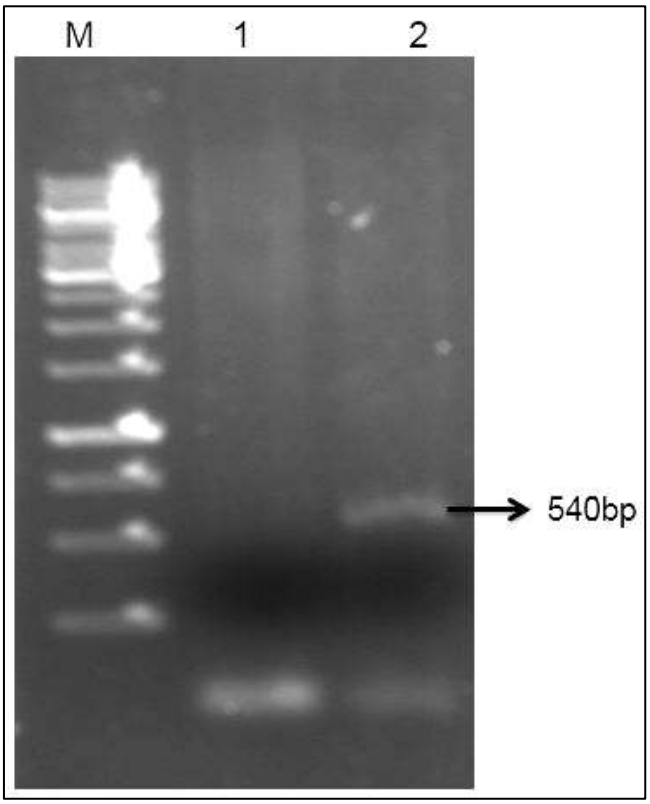

Fig. 2: Molecular analysis of T3 transformed plants. PCR amplification of the 540bp fragment of the *nptII*gene.Lane M 1Kb Marker, lane 1 DNA from untransformed plants (negative control), lane 2 DNA from independently transformed plants.

Growth of Transgenic Arabidopsis Overexpressing HvPIP2;4 under Salt Stress

To validate the function of *HvPIP2;4* on model plant *Arabidopsis* during salt stress, independent T_3 homozygous *Arabidopsis* line expressing *HvPIP 2;4* through constitutive CaMV35S promoter were subjected to salt stress. The differences in their growth and survival were monitored and scored (Fig. 3).

Phenotypic Observation

Phenotypic observation clearly showed the transgenic ecotypes were more tolerant than wild type. Growth of the

plant stunted with time and increasing NaCl concentration (0mM, 100mM, and 200mM) in both wild type and transgenic plants. After 48hrs with the increase in the concentration of NaCl both wild type and transgenic ecotypes were affected but the transgenic ecotypes showed more resilience (Fig. 4).

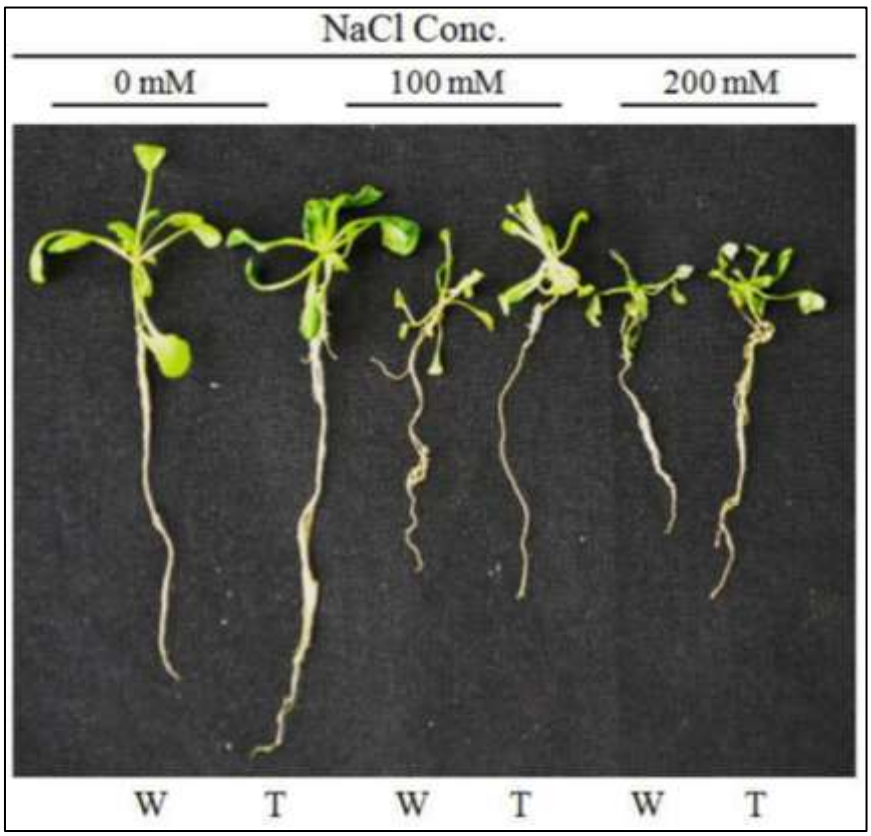

Fig. 3: Studying the physiological changes in transgenic *Arabidopsis* line under salt stress. Effect of Salt stress on wild type (WT Col-0) and transgenic *Arabidopsis* line expressing *HvPIP2;4* constitutively. NaCl induced morphological changes was visible in 10 days old WT and transgenic lines after exposure to (0mM, 100mM, 200mM) NaCl for 48h.

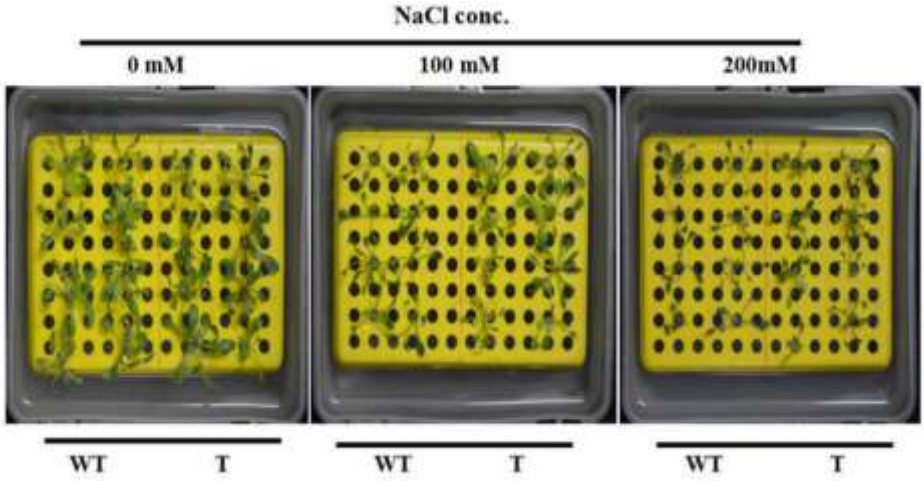

Fig. 4: Studying the physiological changes in transgenic *Arabidopsis* line under salt stress. Effect of Salt stress on wild type (WT Col-0) and transgenic *Arabidopsis* lines expressing *HvPIP2;4* constitutively. NaCl induced morphological changes was visible in 15 days old WT and transgenic line after exposure to (0mM, 100mM, 200mM) NaCl for 48h.

Estimation of Fresh Weight and Dry Weight

The transformed ecotypes showed an increase in dry weight and fresh weight compared to wild types. With the onset of stress (100mM & 200mM) a decrease in the fresh weight and dry weight was observed in both the wild type and transgenic ecotypes. However the decrease in both fresh weight and dry weight was found to be more in wild types than in transgenic ones (Fig 5 & Fig. 6).

Physiological Growth Parameters

The Physiological growth parameter (root length was scored in WT and independent T_3 homozygous transgenic *Arabidopsis* line expressing *HvPIP2;4* constitutively (35S::*HvPIP2;4*). It was subjected to (0mM, 100mM, 200mM) NaCl conc. After 7 days of salt stress the difference in root length was measured. Under control unstressed condition also a significant difference in the root length was found between WT and transgenic *Arabidopsis* line expressing *HvPIP2;4* constitutively. Inhibition of root growth were clearly observed with the increase in NaCl concentration (0mM, 100mM & 200mM NaCl) in WT and T_3 transgenic line, there was inhibition in the root growth rate of both WT and transgenic line under salt stress (100mM and 200mM) NaCl With increase in NaCl concentration the root growth of both wild type and transgenic plants were affected but in comparison to wild type transgenic ecotypes had higher root length than the wild type hence they were able to survive (Fig. 7).

Relative Water Content

The relative water content is an indicator for water status in leaves. Under unstressed condition no significant difference was observed in the relative water content of wild type and transgenic ecotype with transgenic ecotype showing a slight increase in water content than the wild types. With the onset of stress (100mM and 200mM) NaCl there was a decrease in the plant water content of both wild type and transgenic ecotypes. The transgenic ecotype showed a less decrease in the water content than in wild type (Fig. 8).

Physiological Studies

Estimation of chlorophyll content. All the chlorophyll pigments showed higher presence in transgenic ecotypes right from the unstressed condition to gradual increase in stress (100mMNaCl and 200mMNaCl). With the onset of stressed conditions there was a decrease in amount of pigments but the transgenic ecotypes still retained higher amount of chlorophyll pigments than the wild ones (Fig. 9, 10, 11 & 12).

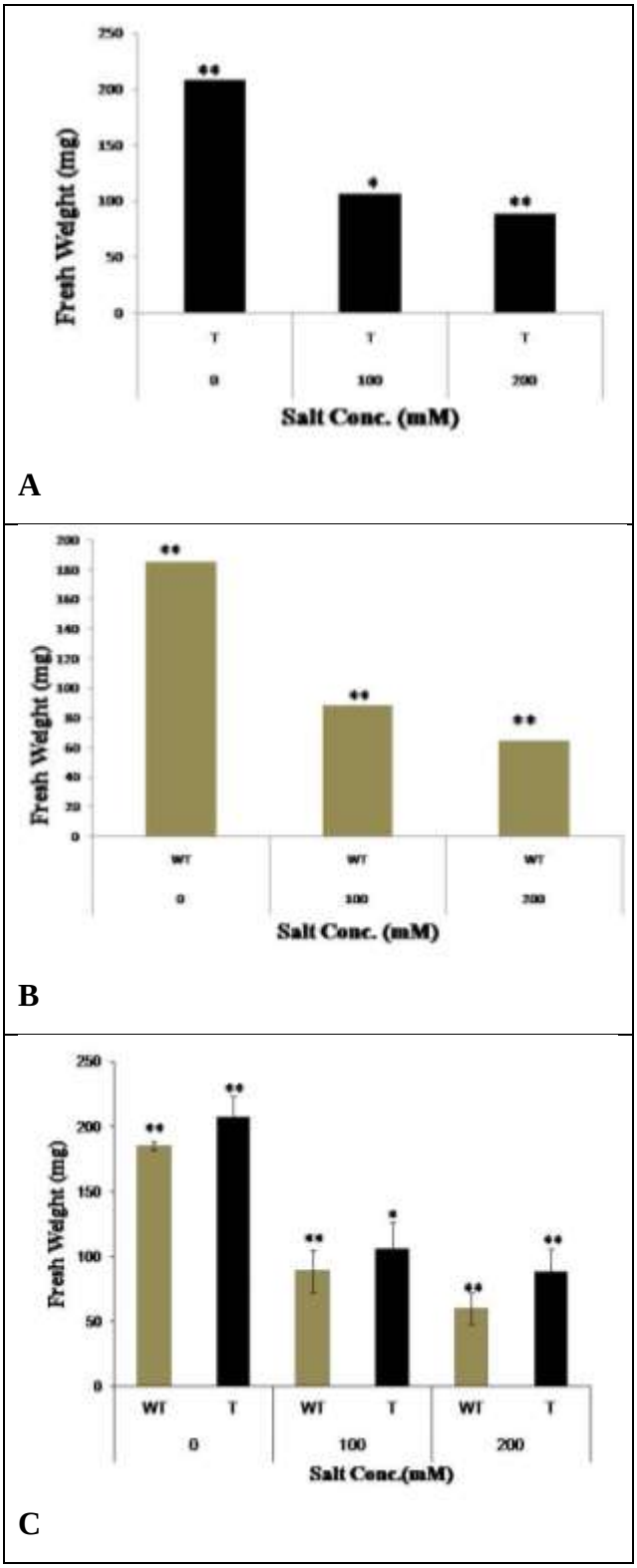

Fig.5: Changes in Fresh weight of the transgenic Arabidopsis line (A.), wild type (B.) and changes between Wild type line and Transgenic (C.) under different concentrations of Salt stress in hydroponic medium.

[The values are represented as the means ± SE (n = 3) of at least three independent experiments, where n is the no of times experiment repeated and SE denotes Standard Error. Statistically significant values at P≤ 0.05 and P≤0.01 using Tukey analysis are indicated by '*' and '**' respectively.]

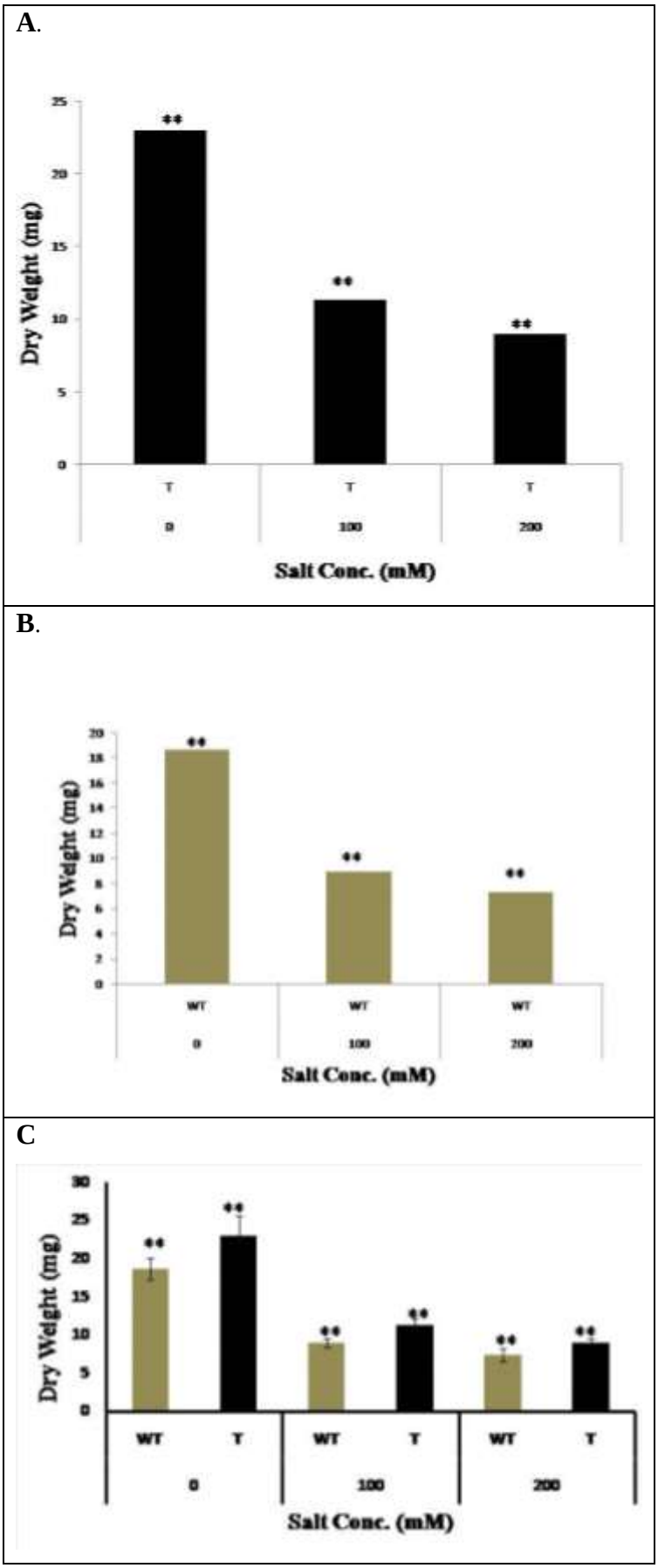

Fig. 6: Changes in dry weight of the transgenic Arabidopsis line (A.), the wild type (B.) and the changes between Wild Type and Transgenic Arabidopsis line (C.) under different concentrations of Salt stress in hydroponic medium.

[The values are represented as the means ± SE (n = 3) of at least three independent experiments, where n is the no of times experiment repeated and SE denotes Standard Error. Statistically significant values at P≤ 0.05 and P≤0.01 using Tukey analysis are indicated by '*' and '**' respectively.]

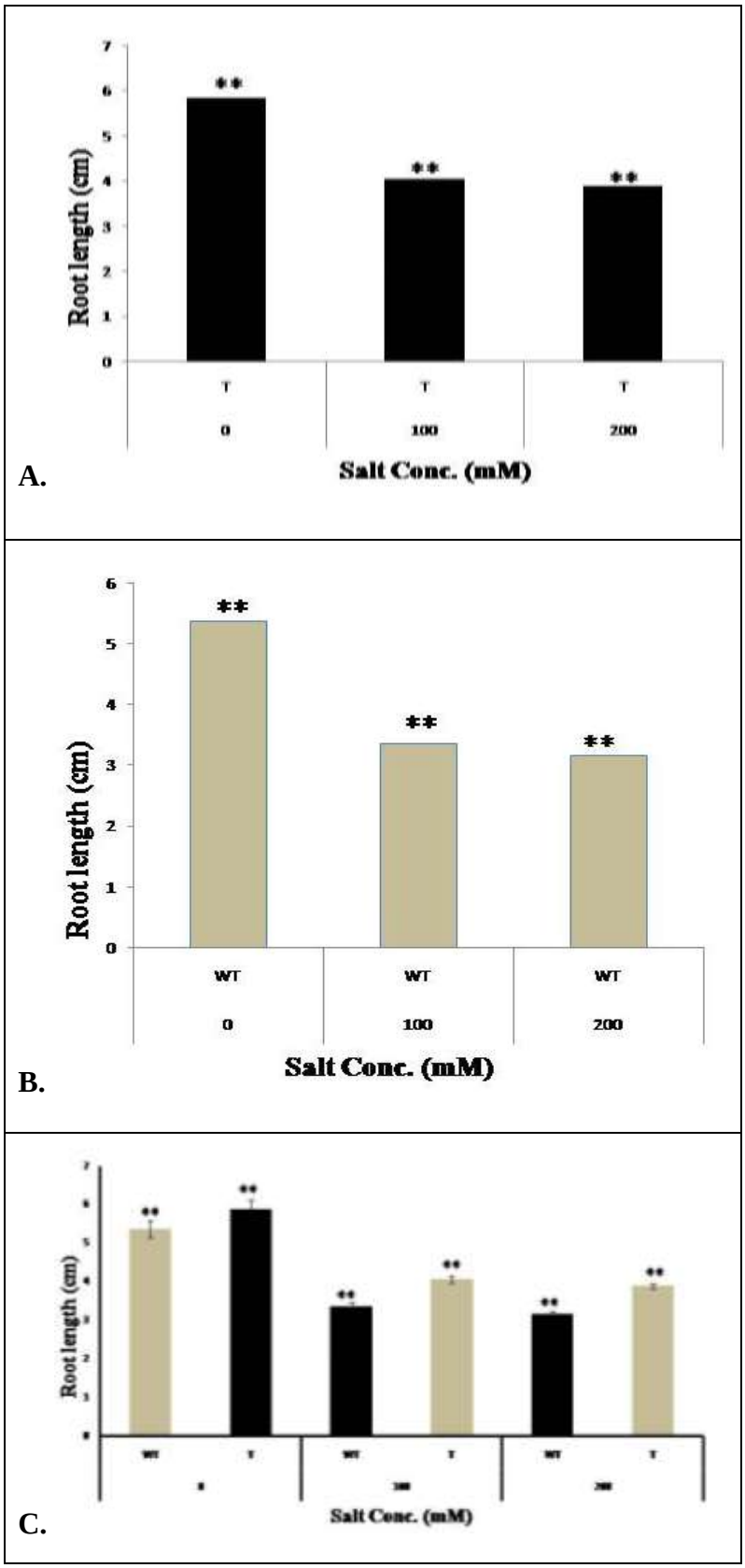

Fig. 7: Effect of salt stress on root growth of transgenic Arabidopsis line. Root growth inhibition in transgenic Arabidopsis plant (35S:: *HvPIP2;4*) (A.) and wild type (WT Col-0) (B.) upon salt stress (0mM.100mM,200mM) was studied. The 4 day old germinating seedlings was transferred to (0mM, 100mM and 200mM) NaCl stress and root length measured was plotted in a graph. (C.) Changes between Wild Type and Transgenic line

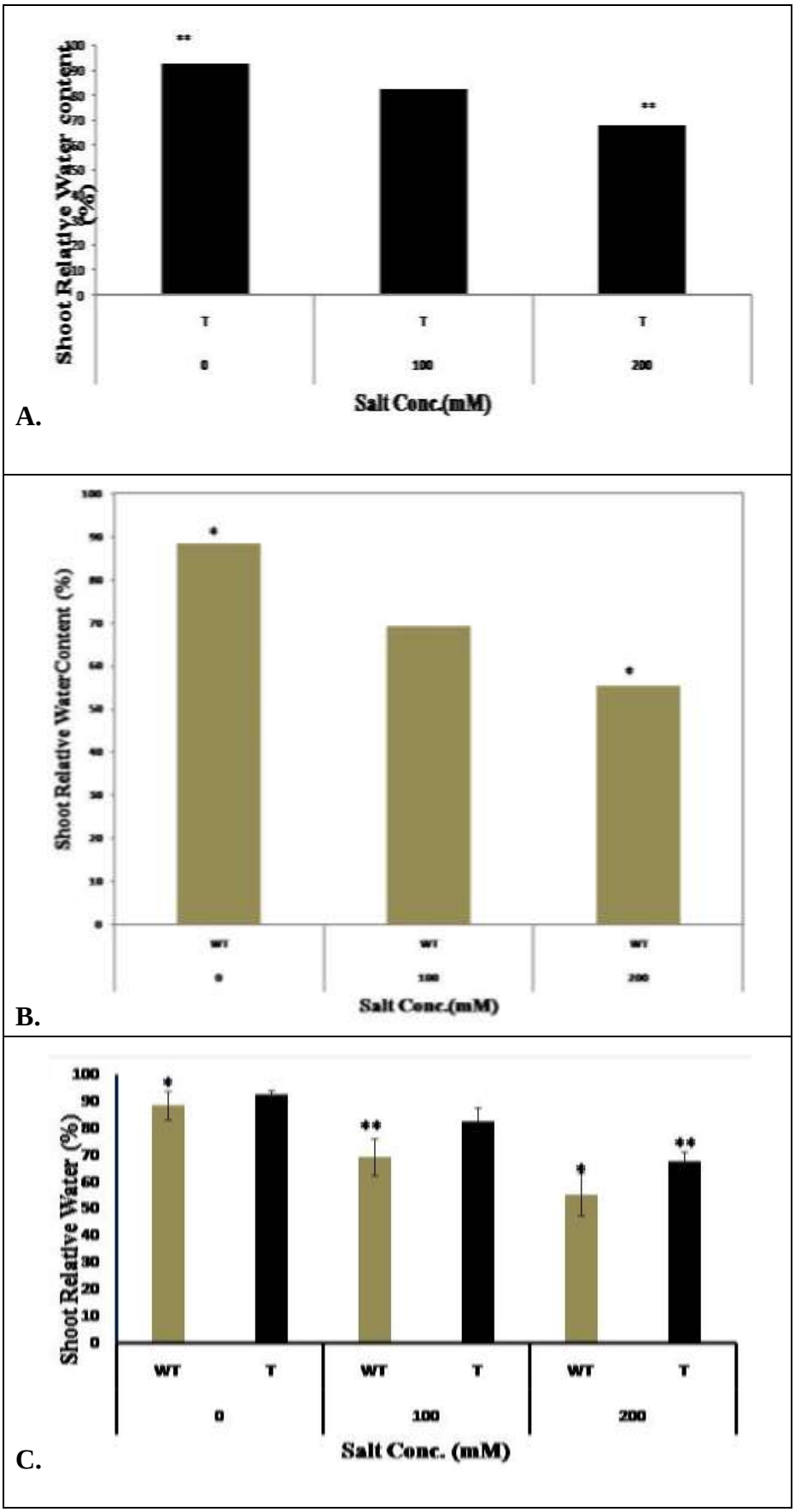

Fig. 8: Changes in Relative water content (%) of the transgenic Arabidopsis line (A.), wild type (B.) and between Transgenic and Wild type (C.) under different concentrations of Salt stress in hydroponic medium.

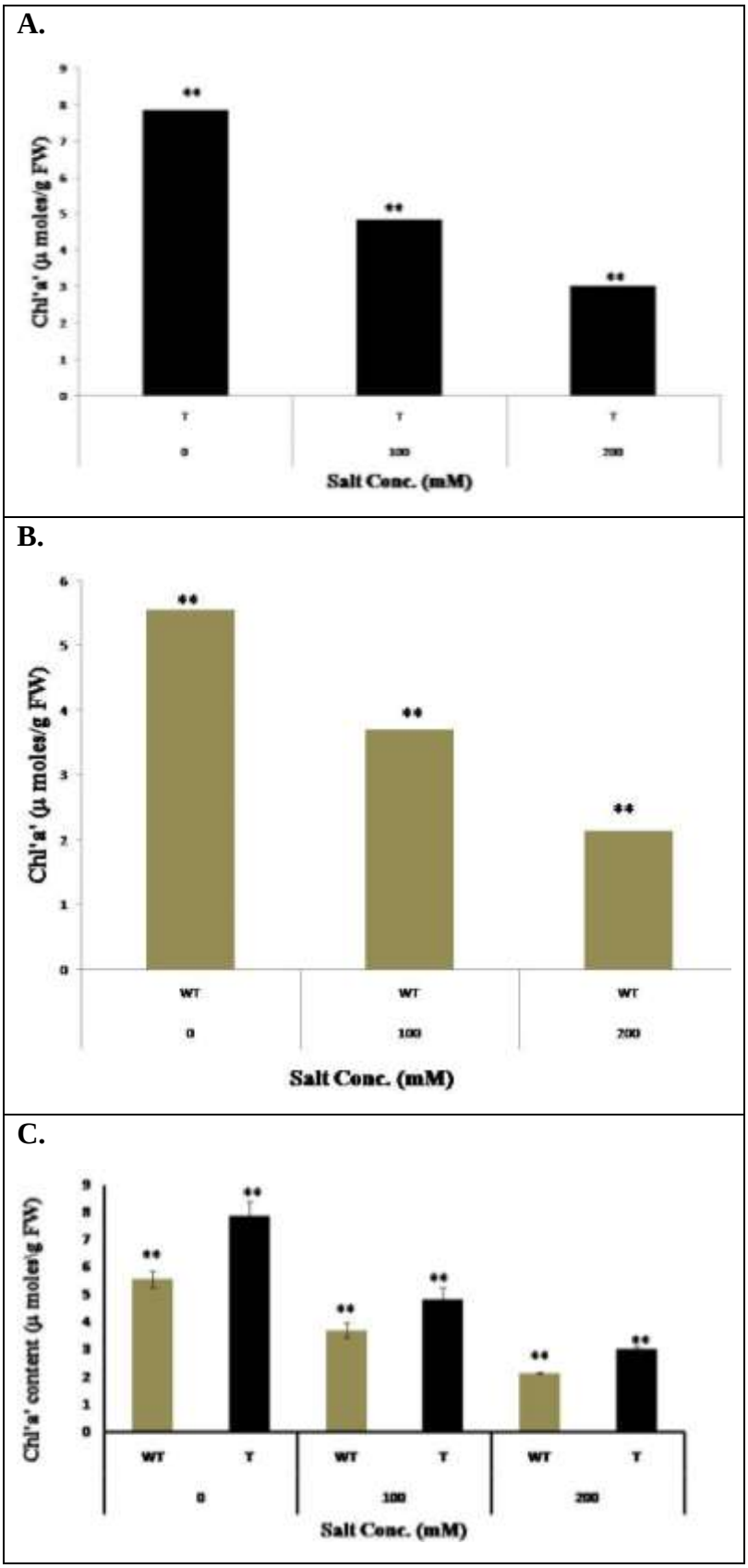

Fig. 9: Effect of Salt stress on Chlorophyll-a of transgenic Arabidopsis (A.), Wild type (B.) and between Wild type and transgenic Arabidopsis (C.)

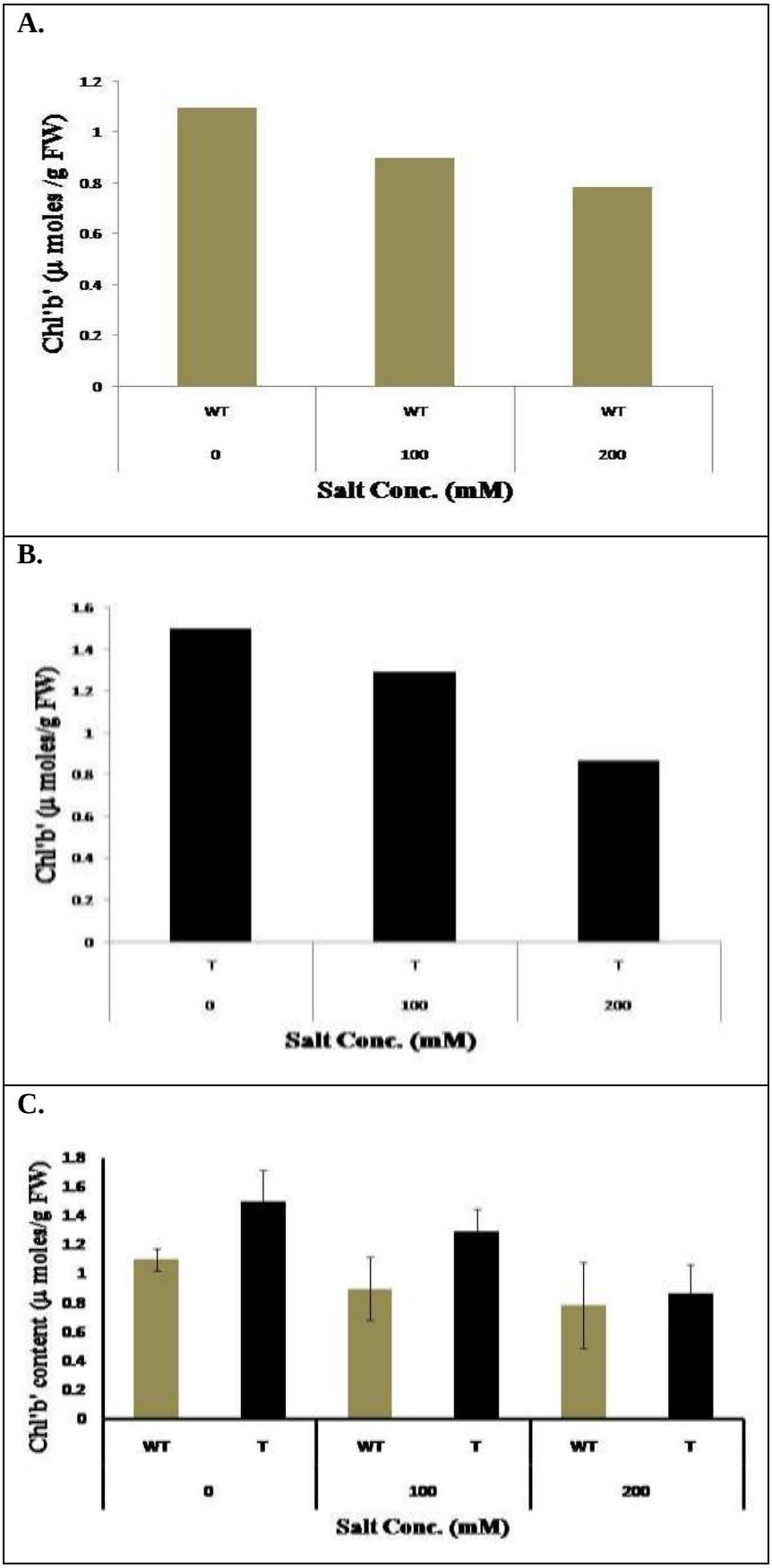

Fig. 10: Effect of Salt stress on chlorophyll-b, Wild type (A.), transgenic Arabidopsis (B.) and in between Wild type and Transgenic plants (C.).

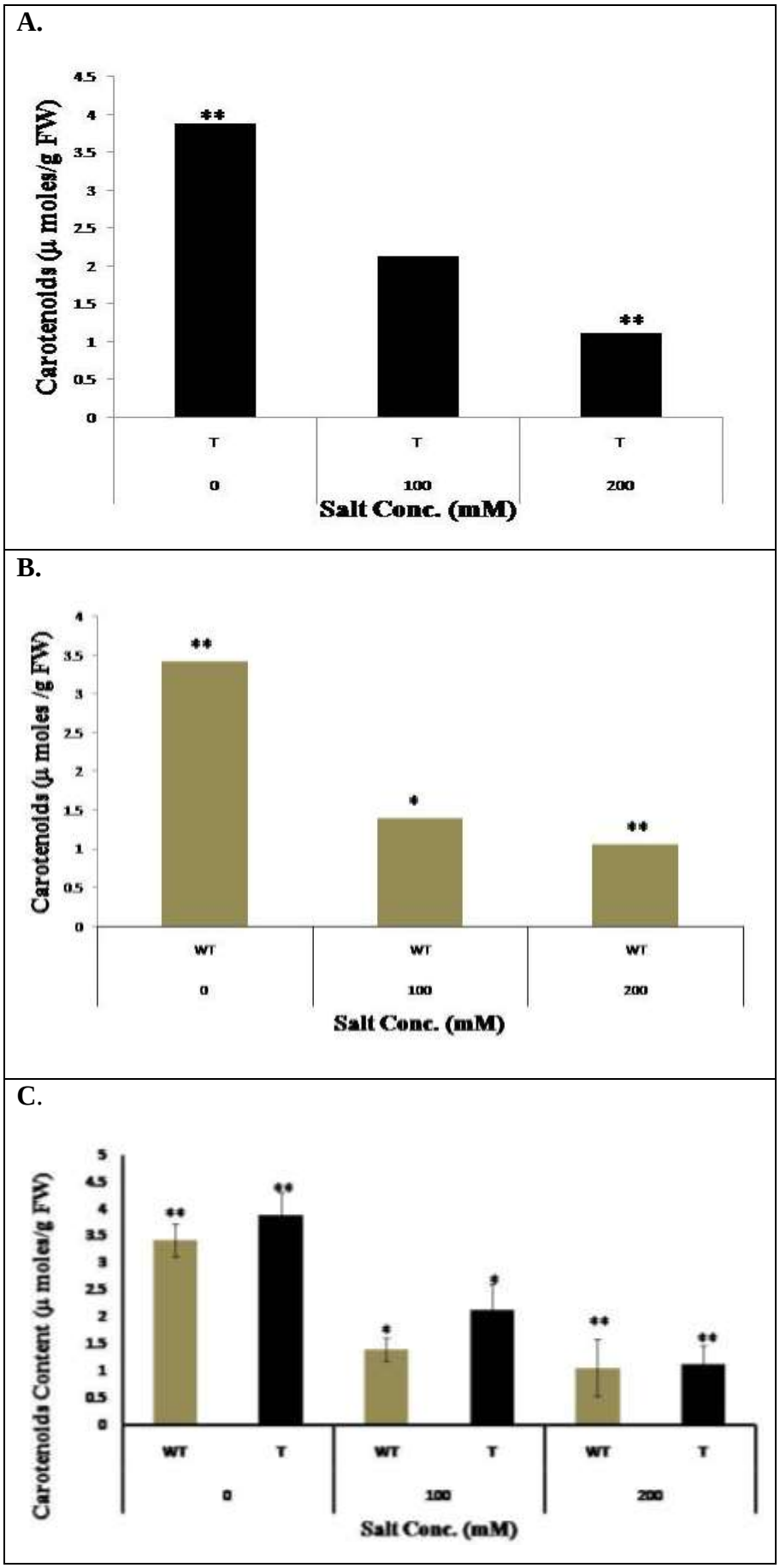

Fig. 11: Effect of Salt stress on Carotenoid content of transgenic plant (A.), Wild type (B.) and between Wild type and transgenic Arabidopsis line (C.).

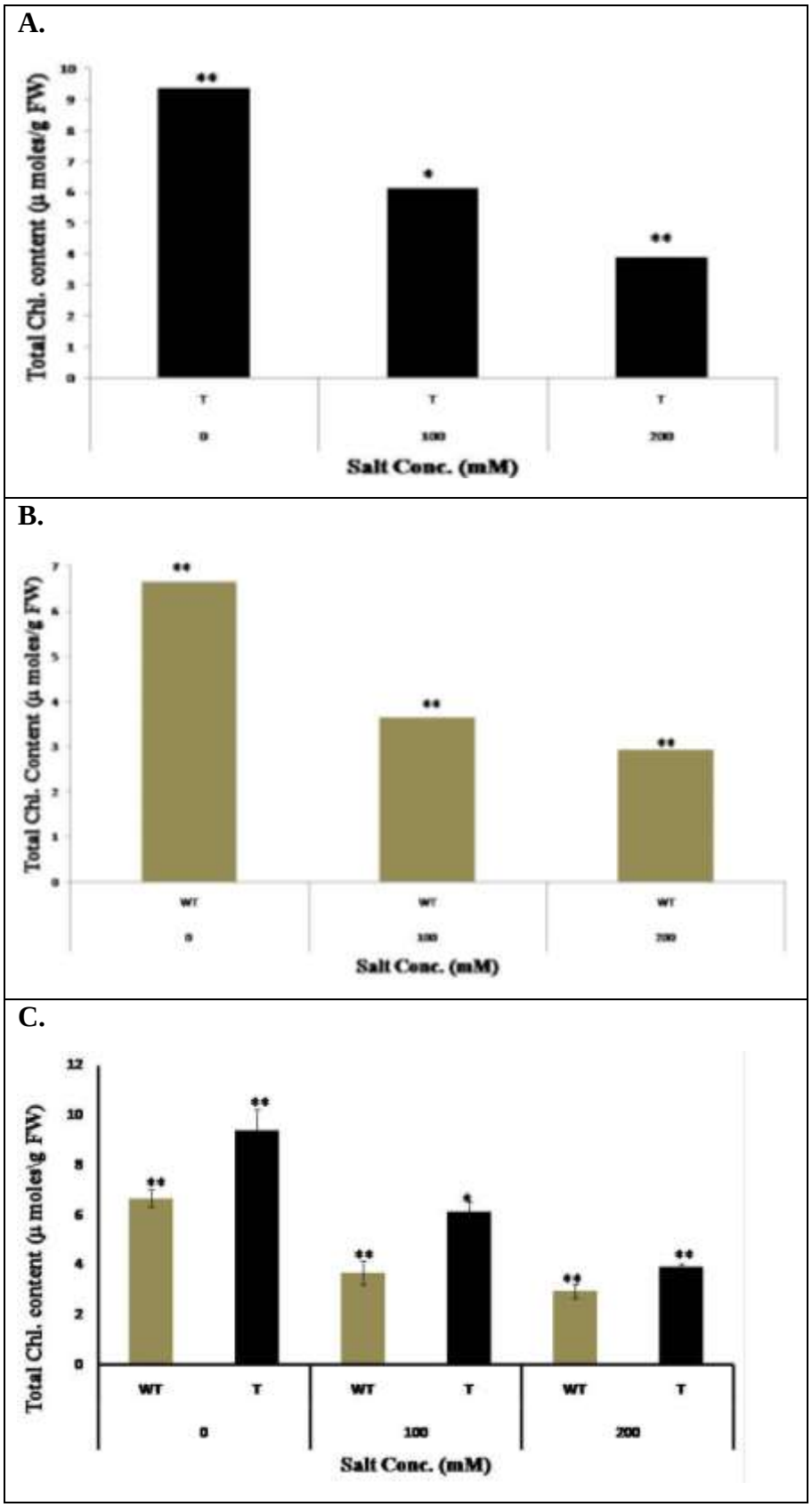

Fig. 12: Effect of Total Chlorophyll Content of transgenic Arabidopsis line (A.), Wild type (B.) and between Wild type and transgenic Arabidopsis (C.).

Discussion

Plants need to maintain their water homeostasis for maintaining better growth and development and also for mitigating osmotic stress caused by abiotic sources such as salinity and drought. In order to maintain proper water homeostasis it is very essential for the plants to take up water via roots. Genetic evidences of the importance of aquaporin especially PIP in the water uptake by roots were known from the works of several workers, like (Martre *et al.*, 2002; Siefritz *et al.*, 2002). In these studies antisense constructs were used to knockdown PIP expression in tobacco and *Arabidopsis* and *PIP2;2* genes were knocked out from *Arabidopsis* using T-DNA inserts. The knock down of PIP expression in Tobacco and *Arabidopsis* and knock out of PIP 2;2 genes in *Arabidopsis* resulted in a significant reduction in root hydraulic conductance. In various studies it was found that a rise in concentrations of salt has shown to inhibit root water uptake due to reduction in root hydraulic conductance in several plant species including Arabidopsis and maize (Azaizeh and Steudle, 1991; Peyrano *et al*., 1997; Carvajal, 1999; Martinez-Ballesta, 2003; Martinez-Ballesta, 2000; Boursiac *et al*., 2005) but a contradictory report also existed and it suggested that salinity stress does not hamper the increase or decrease of hydraulic resistance and in spite of stressed condition the plants seems to grown in a similar manner to that of unstressed plant (Boursiac *et al*., 2005). These studies motivated us in our quest of understanding the components of water transport via *Hv*PIP2;4 aquaporin gene. The present study was ventured to figure out the potential of *HvPIP2;4* in conferring abiotic stress tolerance in *Arabidopsis*. Here we have overexpressed Barley aquaporin *HvPIP2;4* in *Arabidopsis* and this was authenticated for salt tolerance. Inplanta transformation system was used to introduce *Hv*PIP 2;4 gene with a binary vector pCAMBIA 2301::CAMV35S:*Hv*PIP2;4:Poly A.

For confirmation of the transgenic event the putative transgenic plants were analysed through molecular biology techniques. Genomic DNA analysis was done using *nptII* gene as marker. The PCR analysis detected the presence of the expected 540bp amplified product corresponding to *nptII* (Fig. 2) in transformed plants. So, for further physiological and biochemical analysis putative transformants were taken under consideration.

Transgenic *Arabidopsis* plant expressing *HvPIP2;4* showed improved tolerance to salinity.

Physiological analysis was performed to determine the salt tolerance of 35S:*HvPIP2;4* transgenic *Arabidopsis* line. Under unstressed conditions no significant difference of growth was found between wild type and transgenic *Arabidopsis* line. Overexpression of *HvPIP 2;4* conferred salt tolerance to transgenic *Arabidopsis* line. The transgenic grew better than the wild type in 100 and 200mM NaCl stressed condition. There was previous report that rapid water transportability was shown by PIP 2 protein (Boursaic *et al.*, 2005, Munns and Passioura, 1984) and this phenomenon was also reported in Rice Plants *Os*PIP2-2 (Martre *et al.*, 2002). The above data indicates that transgenic plants over expressing *HvPIP2;4* displayed high tolerance to mild salt stress. This shows that *HvPIP2;4* could possibly have intrinsic water trans- portability inplanta and play a important role in regulating water homeostasis.

The root length of the transgenic 35S: *HvPIP2;4* Arabidopsis varieties was more than that of the wild type even in unstressed conditions.

Because Aqp gene mediate and regulate rapid transmembrane water flow during growth and development, *HvPIP2;4* overexpressing plants may be more efficient in regulating water transport across membranes under stressed conditions. It is speculated that the physiological effects are beneficial for plants in maintaining the protein machinery that regulates nutrient uptake and distribution.

With the gradual increase in stress (100 and 200mM) there was a reduction in the root length both in the transgenic and wild varieties.

Salinity stress affects the vital photosynthetic system components including the chlorophyll content. In Transgenic *HvPIP2;4* varieties Chlorophyll pigments were found to be more than that in wild type. With the gradual increase in stress (100mM & 200mM NaCl) there was a decrease in the amount of Chlorophyll content. Due to the presence of more chlorophyll pigment in *HvPIP2;4* transgenic lines the transgenic plants showed better growth and development than wild types and they could mitigate stress easily. By measuring the biomass of Arabidopsis seedlings the increase in salinity tolerance was quantified. After harvest the salt stressed 35S:*HvPIP2;4* expressing *Arabidopsis* line yielded significantly more fresh weight than the stressed wild type control. The relative water content is an indicator for water status in leaves, Our results indicated that *Hv*PIP 2;4 transformed *Arabidopsis* lines had higher relative water content than the wild type. These results suggested that *Hv*PIP2;4 expression enhanced the osmotic regulation capability of transgenes and had thus enhanced the salt stress tolerance in transgenic *Arabidopsis* this phenomenon has also been reported from OsPIP1-1 and OsPIP2-2 (Guo *et al*., 2006). A similar result was obtained when *Ta*NIP gene was introduced in Arabidopsis and transgenic Arabidopsis showed enhanced salt tolerance.

In this study aquaporin is found to play a very important role in mitigating salt stress tolerance similar results were obtained from the works of (Gao *et al.*, 2010, Guo et al 2006, Jhang et al 2007, Sade et al 2010, Ayadi et al 2011)

This result suggested that the *HvPIP2;4* gene play important role in regulating water homeostasis and that *HvPIP2;4* gene could possibly have intrinsic water transportability *in planta*. The expression pattern showed NaCl dose dependency during stressed condition. Salt tolerance was enhanced costitutively in transgenic *Arabidopsis* and *HvPIP2;4* played a constructive role in increasing plant salt tolerance. It may be very well presumed that overexpression of *HvPIP2;4* in crop plant might benefit them by enhancing their salt tolerance capacity.

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