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GENETIC ENGINEERING OF SUGARCANE WITH THE RICE TONOPLAST H^+-PPASE (*OVP2*) GENE TO IMPROVE SUCROSE CONTENT AND SALT TOLERANCE

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Abstract

The present study describes successful transformation of sugarcane (*Saccharum officinarum*), CP-77/400 variety with the rice vacuolar H^+-PPase (*OVP2*) gene. Transgenic and control plants were then tested for biochemical and salt tolerance evaluation. First, sugarcane calli of the selected variety were induced on callus induction media as optimized previously. Various concentrations of acetosyringone and cefatoxime were used along with different co-cultivation periods to obtain maximum transformation efficiency. Maximum transformation efficiency (25 %) was achieved with 60 µM. Different concentrations of cefatoxime were used for washing the calli after co-cultivation to remove excess bacteria. Out of several concentrations, 400 mg/l resulted in higher transformation frequency (26%). Highest transformation frequency of 21 % was achieved when co-cultivation period was extended to four days. Selection of transformants was achieved with various levels of hygromycine as selective antibiotic. The hygromycine concentration of 50 mg/l resulted in higher transformation frequency of 20%. The transgenic shoots were then confirmed through PCR and RT-PCR analysis. It was anticipated that constitutive expression of the *OVP2* gene might accelerate the function of the vacuole localized sucrose antiporters resulting higher sucrose accumulation in the vacuole. No significant differences were found for soluble sugar contents between the non-transgenic control and the transgenic lines except T5 line that showed high sucrose content than that of the control. Transgenic and control plants were subjected to salt stress (NaCl) at concentrations of 0 mM and 100 mM. Some of the transgenic lines exhibited high chlorophyll and proline contents than that of the control plants at 100 mM stress. These results showed that expression of the *OVP2* gene improved total soluble sugar content and salt tolerance in transgenic lines.

Key words: *Saccharum officinarum*, *OVP2* gene, PCR, RT-PCR analysis, Salt tolerance

Introduction

Sugarcane is one of the important crops throughout the world. It is used as a major source of edible sugars in a large number of consumer products. In recent years, to meet the needs of the growing population, there has been a rapid increase in the use of sugar that resulted huge rise in prices of sugar and their products (Schmidhuber, 2006). This has also affected the growth and development of sugar and related industries with serious implications on the poor farmers and consumers. Being an agricultural country and the fifth largest sugarcane growing nation in the world, Pakistan lacks behind many countries and per hectare yield is the lowest all over the world (Ali *et al.*, 2008). Pakistan produces sugarcane with an average yield of 47.33 tons per hectare which is far below the actual potential of best sugarcane varieties in the world. It has been reported that yield potential of our existing sugarcane varieties decreased due to several reasons including improper crop rotation, low soil fertility, segregation, and susceptibility to diseases, insect attack and abiotic stresses (Malik, 1990).

Sugarcane production and the net sugar accumulation are impacted by exposure to biotic and abiotic stresses and the variable enzyme activities involved in sugar metabolism. Among abiotic stresses, salt stress is a major factor limiting sugarcane yield and sugar accumulation. It is estimated that salinity or sodicity negatively affects about one million hectares of land under sugarcane production globally. Sugarcane, the prime source of sugar production in Pakistan, suffers significant growth and yield losses above a threshold ECe of 5 dS m^{-1} (Rozeff, 1998). It has been reported that under saline conditions, sugarcane exhibits stunted or no growth and the yield falls to 50% or even more to its true potential (Subbarao & Shaw, 1985). Salt stress exerts negative effects on sugar production in the form of reduced cane growth and yield and lower sucrose accumulation in the stalk (Wahid *et al.*, 1997).

Research on sucrose metabolism and accumulation in sink tissues is of prime importance that needs full exploration so that the sugar accumulation potential of our existing varieties could be improved. In sugarcane, sucrose is the sugar translocated in the phloem (Hatch & Glasziou, 1964) to sinks, where it is used for metabolism, cell growth, respiration, and storage (Hawker, 1985). Inside the sink cells, vacuole stores most of the sucrose, and then mobilizes it to other parts. The vacuolar storage of sucrose is mediated by sucrose transporters localized to the vacuolar membranes. However, these sucrose transporters need a transmembrane gradient of proton (H^+) between the vacuole and the cytosol to function. This proton (H^+) gradient is generated by two vacuolar membrane transporters, namely the H^+-ATPase and H^+-PPase (Swart, 2005; Martinoia *et al.*, 2007; Krebs *et al.*, 2010). Swart (2005) compared different sugarcane varieties and reported a strong association between sucrose accumulation and the H^+-PPase activity. This work revealed an important role of H^+-PPase in sucrose storage. Moreover, Wu & Birch (2007) reported that the sucrose storing potential of sugarcane might be more than double the levels accumulated by the current commercial cultivars. The research findings suggest that targeting

more sucrose towards the vacuole may be one of the best strategies to achieve the goal of producing better sugarcane varieties (Rohwer & Botha, 2001). Therefore, genetic manipulation of the proton gradient or transport activity may result in increased sucrose transport and accumulation in the vacuole.

Conventional breeding programs have been extensively used for developing disease resistance and increasing sucrose content in sugarcane (Zhou, 2013). These breeding programs have led to improved yields, but not to the extent as in other major cereals, such as rice, maize and wheat. According to Grof & Campbell (2001), this low yield increases were probably due to the low gene pool used, as most current cultivars have been derived from *Saccharum officinarum* and *Saccharum spontanium*, with limited inter-crossing and selection cycles (Grivet & Arruda, 2001). Grof & Campbell (2001) argued that the existing cultivars have limited natural genetic potential of increasing sugar content through conventional breeding and selection. With the advent of recombinant DNA technology and molecular tools, transgenic approaches to increase sucrose yield in sugarcane are of tremendous importance, as they overcome the intrinsic difficulties associated with sugarcane breeding (Grof & Campbell, 2001).

The vacuolar H^+-PPase has also been reported in a number of studies for its role in excess Na^+ ion sequestration via Na^+/H^+ antiporter channel in the vacuole resulting increased salt tolerance in plants (Brini *et al.,* 2007; Pasapula *et al.,* 2011; Ahmed *et al.,* 2016). In view of the potential importance of H^+-PPase in both sugar accumulation and salinity tolerance, genetic manipulation of this should increase sucrose accumulation in the vacuole. Genetic engineering of vacuolar membrane H^+-PPase activity may enhance an increased proton gradient. In this study, the rice tonoplast H^+-PPase (*OVP2*) gene was expressed in sugarcane. We further evaluated the transgenic and non-transgenic control plants for molecular, biochemical and salt stress assays.

Experimental procedures

Plant material: The sugarcane variety (CP 77/400) has previously been selected out of several varieties as the best tissue culture responsive and was used in the subsequent transformation experiments. This variety was kindly provided by SCRI (Sugar Crop Research Institute), Mardan.

Callus induction: For callus induction, the inner immature leaf whorls from 6 month old sugarcane plants were collected and used as explants. Explants were sterilized and inoculated on MS basal salt medium containing 10% coconut water and 5 mg/l 2,4-D (Ullah *et al.,* 2016). The calli were grown for three subcultures and were then used for transformation experiment.

Cloning of the *OVP2* gene fragment in pGreen expression vector: The purified released gene fragment was subcloned in an expression vector (pGreenII0129) which was kindly provided by JIRCAS, Japan under the Material Transfer Agreement (MTA). The pGreen1110129 vector was selected as an expression vector because of its unique characteristics which make it more efficient as compared to other expression vectors. It is a small sized vector and easy to manipulate for transformation experiments as compared to other large sized vectors such as PBI. Furthermore, it is a high copy number plasmid. It was digested accordingly with *Hin*dIII and *Bam*HI to facilitate sticky end cloning.

Transformation of sugarcane calli with Agrobacterium and co-cultivation: Calli were prepared by inoculating inner immature leaf whorls on MS medium containing 10% coconut water and 2 mg/l 2,4.D (Ullah *et al.,* 2016). Calli were grown for three subcultures and then were used for transformation. The transformed colonies were picked and were further cultured in 5 ml LB medium containing proper antibiotic concentrations. The cultures were kept for 6-8 hours in a shaking incubator at a temperature of 28ºC and 160 rpm shaking. After incubation, 25 ml MS medium containing 50 µM, 60 µM, 70 µM and 80 µM acetosyringone concentrations were added to 200 µl of the overnight culture of each sample. The calli were then inoculated in the transformation cultures for 80 seconds. The calli were then dried on sterile tissue paper. Sterilized whatman No.1 filter papers of the plate size were kept above the media in the co-cultivation plates. The calli were then spread on the filter paper with approximately 8-10 calli per each plate. The plates were covered with aluminium foil and were kept for 2-4 days at 28ºC. After co-cultivation, the calli were washed with appropriate cefatoxime concentrations to remove excess agrobacterium and were then transferred to selection medium.

Regeneration and selection of transformations: For regeneration of sugarcane calli, the previously optimized protocol was used (Ullah *et al.,* 2016) with addition of the required antibiotics. The calli, after co-cultvation, were placed on selection media (SIM: 1 mg/l BAP + 2.0 mg/l NAA) containing 50 mg/l hygromycine and 400 mg/l cefatoxime) for selection of transformation events. The plates were observed on daily basis and were sub-cultured after every two weeks. After two subcultures, the calli were inoculated on regeneration media containing proper concentrations of BAP (1 mg/l) and NAA (2 mg/l) as previously reported (Ullah *et al.,* 2016). The cultures were kept for 16/8 h light/dark photoperiods at 25 ± 1ºC for 4 weeks. The regeneration media was also supplemented with hygromycine for selection of the transformed shoots. The primary shoots, emerged from the base were excised and all the dead shoots were discarded. The trimmed shoots were further sub-cultured on the same optimized shoot induction media containing appropriate concentrations of hygromycine.

Molecular analysis of the transgenic plants: The *OVP2* gene was confirmed through standard PCR. Genomic DNA was extracted from randomly selected transformants and the non-transgenic control plants by the CTAB method (Rogers & Bendich, 1988). The *OVP2* gene-specific primers were used for PCR amplification. Sequences of forward and reverse primers were 50-ATGGTGGCGCCTGCTTTGTTACCG-30 and 50-

GAACAGAGGTAACAGCACCA-30, respectively. PCR reaction contained 25 µl reaction volume. The PCR conditions were used as initial denaturation at 95ºC for 5 min followed by 40 cycles of denaturation. at 95ºC for 1 min, annealing at. 57ºC for 30 s, extension at 72ºC for 1 min, and final extension. at 72ºC for 10 min. The PCR product of the *OVP2* gene was then sequenced (Macrogen, South Korea).

RT-PCR Analysis: For *OVP2* gene expression analysis, total RNA was extracted, using the CTAB method. For removal of contaminating genomic DNA, the RNA was treated with DNase I. A total of 2 µg RNA was used for reverse transcription (Reverse Transcription System, Promega). Gene specific primers were used to amplify the *OVP2* from the cDNA. The actin gene was used as internal control and was amplified from the total cDNA using specific primers (5´-CCTCCAATCCAAACACTGTA-3´, 5´-TGGACTCTGGTGATGGTGTC-3´).

Total soluble sugars analysis: Total soluble sugars (glucose, fructose and sucrose) were analysed spectrophotometrically as reported (Bergmeyer & Bernt, 1974). Fresh leaf samples of about 50 mg from all transgenic and control plants were frozen in liquid nitrogen. The frozen leaf samples were then mixed with 500 µl buffer (HM buffer [100 mM HEPES, 20 mM $MgCl_2$, and 80% absolute ethanol). Samples were incubated overnight at 80ºC. After incubation, samples were centrifuged at 10,000 rpm for 15 min. Supernatants were transferred to fresh micro centrifuge tubes. The glucose, fructose and sucrose concentrations were then determined against D-glucose (blank) spectrophotometrically at their corresponding wavelengths.

Experimental design for salt stress evaluation: Transgenic and non-transgenic lines were subjected to salt stress at the growth room level. As the sugarcane plants, acclimatized to soil were small, therefore, two salt concentrations (0 mM and 100 mM NaCl) were used for this experiment. After acclimatization to soil condition in the growth room, all control and transgenic plants in soil pots were kept in a completely randomized design (CRD). The experiment consisted of three replications with five pots of control and each transgenic line. The pots were irrigated with normal tap water for one month. After vegetative growth for one month, salt stress evaluation was started. All plants were irrigated with either normal tap water or water with 100 mM NaCl for two weeks. After two weeks, chlorophyll and proline contents were determined on fresh leaves of transgenic and non-transgenic plants.

Analysis of chlorophyll content: Leaf chlorophyll contents of all plant were measured with a chlorophyll meter (SPAD-502 Konica Minolta, Japan). Three SPAD readings were taken from three fully expanded leaves at three different positions. Thus the chlorophyll data of each plant is represented by an average of nine SPAD values.

Analysis of proline content: Proline was calculated according to the procedure of (Bates *et al.*, 1973). A sample of 0.5 ml was mixed with 0.5 ml methanol: ddH_2O and 1ml acetic acid was also added. Sample was mixed with 1ml ninhydrin solution and then heated at a temperature of 100ºC for 45 min. Upon cooling of the sample, 5 ml toluene was added. Two layers appeared in the sample and the upper layer was evaluated for proline determination. Absorbance was calculated at 520 nm with Spectrophotometer using toluene as blank.

Statistical analysis: Data of biochemical and salt stress evaluation tests were subjected to Analysis of Variance (ANOVA) and student t-test at a significance level of $\alpha = 0.05$.

Results

Construct preparation: The *OVP2* gene and cDNA sizes were about 5.9 kb and 2.4 kb, respectively. The purified cDNA fragment was then cloned in the pBluescript SK vector. The confirmed amplified plasmid was digested with two different restriction enzymes i.e., *Hin*dIII and *Bam*HI. The purified released gene fragment was sub-cloned in an expression vector (pGreenII0129) which was also accordingly digested with *Hin*dIII and *Bam*HI to facilitate sticky end cloning. The T-DNA region of the pGreen vector harbouring the *OVP2* is shown (Fig. 1).

Selection of transformed agrobacterium: Transformed colonies were picked from agrobacterium plates and were cultured in the liquid LB containing 25 mg/l hygromycine (Fig. 2A). The pGreen plasmid was extracted from the liquid agrobacterium cultures. Restriction digestion of the plasmid with *Hin*dIII and *Bam*HI released fragment of the *OVP2* gene (Fig. 2B).

Callus induction: Already optimized callus induction media (MS + 10 % coconut water + 5 mg/l 2,4D) previously reported (Ullah *et al.*, 2016) resulted in efficient callus induction. Callus induction frequency was as high as reported previously (Fig. 3).

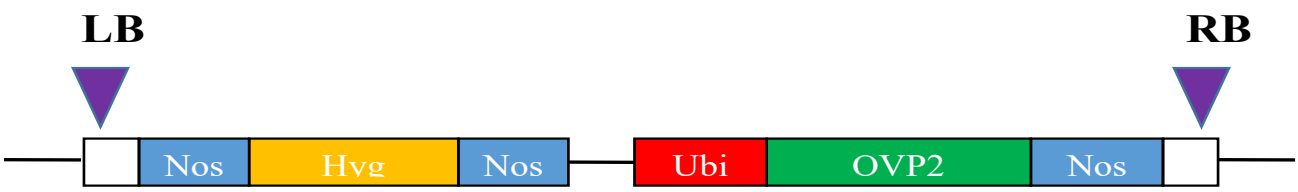

Fig. 1. T-DNA region of pGreen vector harboring *OVP2* and *Hyg* genes. The *OVP2* gene is driven by *Ubi* promoter, and the gene for hygromycine (*Hyg*) is driven by nopaline synthase promoter (*NosP*). LB and RB, Right and Left border sequences of the T-DNA region, respectively.

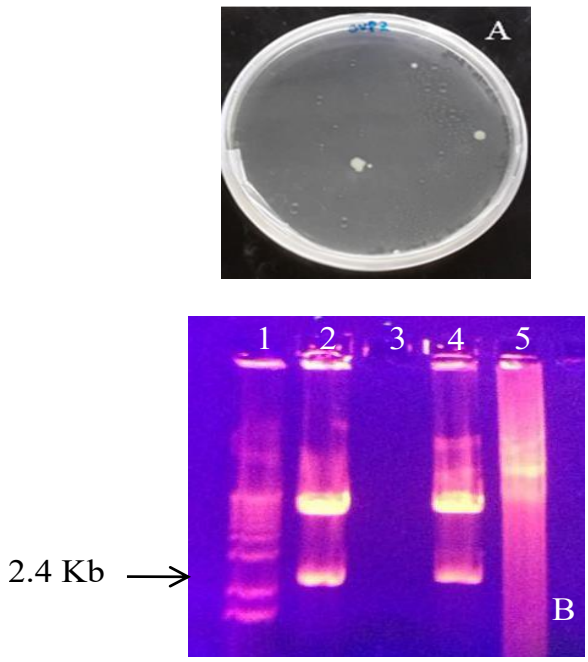

Fig. 2. (A) Transformed colonies of Agrobacterium. (B) Restriction digestion of the pGreen plasmid isolated from the transformed agrobacterium confirmed *OVP2* fragment. Lane # 1 is the 1 Kb Plus DNA Ladder; Lane # 2 is the released fragment from the transformed agrobacterium; Lane # 3 is the negative control (without plasmid DNA); Lane # 4 is the positive control; while, Lane # 5 is the negative control (undigested plasmid).

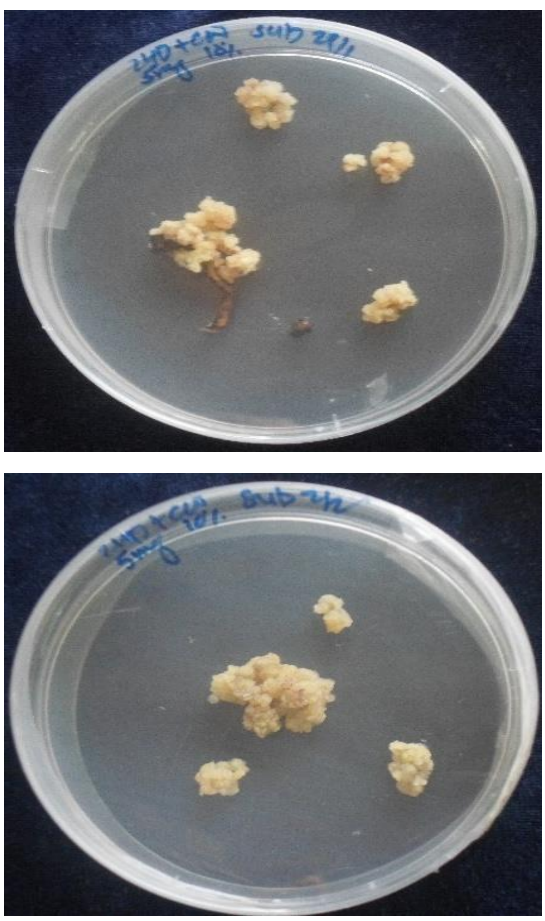

Fig. 3. Callus induction in sugarcane from lower portion of inner immature leaf whorls.

Effect of co-cultivation period: The co-cultivation period showed enormous impact on the transformation efficiencies. Three different co-cultivation periods (2, 3 and 4 days) were used in the experiment (Table 1). Co-cultivating calli for 2 days resulted in minimum transformation efficiencies (14%). Increasing co-cultivation period to 3 days affected the transformation efficiencies (16%) positively. However, the percentage of transformation was still low. Maximum transformation (21%) was observed when co-cultivation period was increased up to 4 days. Further increase in co-cultivation period showed negative impact on the transformation efficiency as it resulted in over growth of bacterial cells and also caused the death of calli.

Effect of acetosyringone and cefatoxime concentrations: Different acetosyringone concentrations i.e., 60 µM, 70 µM and 80 µM were used to investigate its effect on the transformation frequencies (Table 2). Considerable effect of various concentrations of acetosyringone was observed on transformation efficiencies of sugarcane calli. Higher concentration of acetosyringone resulted in early browning and ultimate death of the inoculated calli. Higher concentration of 80 µM resulted in lower transformation frequencies. Concentration of 60 µM acetosyringone was proved to be the optimal and paramount treatment for efficient transformation. Lower transformation efficiencies of 15% was achieved at 80 µM; however at 60 µM, 25% transformation frequencies were observed.

Three levels of cefatoxime (100 mg/l, 400 mg/l and 500 mg/l) were used to eliminate excessive bacteria after co-cultivation (Table 3). Higher concentration of 500 mg/l resulted in immediate death of the calli. At lower concentration of 100 mg/l, over growth of agrobacterium was observed after transfer of the calli to selection media. At 400 mg/l, the calli survived efficiently and also the efficient elimination of excessive agrobacterium was achieved. At 100 mg/l cefatoxime, 20% transformation frequencies were observed. Increasing cefatoxime concentration to 400 mg/l, transformation frequencies increased to 26% and also the calli survival rate was least affected.

Table 1. Effects of co-cultivation period on transformation frequency of sugarcane calli.

Co-cultivation days	Transformation (%)
2	14
3	16
4	21
5	12

Table 2. Effect of various concentrations of acetosyringone on transformation frequency.

Acetosyringone (µM)	Tranformation (%)
60	25
70	18
80	15

Table 3. Effect of cefatoxime concentration on transformation frequency.

Cefatoxime (mg/l)	Transformation (%)
100	20
400	26
500	14

Regeneration and screening for hygromycine: In order to optimize effective dose of hygromycine for selection of putative transgenic plants, different concentrations of hygromycine (50, 100 and 150 mg/l) were used in the regeneration media (Table 4). At 150 mg/l and above, the calli survival rate was affected adversely. Higher concentration caused browning and ultimate death of the calli. Therefore, 50 mg/l hygromycine was augmented in the regeneration medium that efficiently eliminated the non-transformed calli from those of transformed calli. Primary shoots emerged from the transformed calli within 16-20 days. After emergence of shoots, these were transferred to the same shoot induction media for multiplication (Fig. 4). The regenerated shoots were then transferred to root induction media. Genomic DNA was extracted from individual transgenic and control lines and PCR was conducted with specific primers i.e.,*Ubi-F1* and *OVP2-R*. A fragment size of approximately 2.4 KB correspondent to the size of *OVP2* cDNA was amplified from the transgenic lines (Fig. 5). Further, RT-PCR was conducted to analyse expression of the *OVP2* gene in the non-transgenic control and the individual transgenic sugarcane lines (Fig. 6). Our results revealed expression of the *OVP2* gene in all the tested transgenic lines. No expression was observed in the non-transgenic control sugarcane line. Three transgenic lines designated as T2, T5 and T7 showed normal plant phenotype and were therefore, used for further salt stress tolerance and sugar analysis.

Table 4. Effect of hygromycine on transformation frequency.

Hygromycine (mg/l)	Transformation (%)
50	20
100	14
150	6

Table 5. Soluble sugar contents [µmol/mg fresh weight (FW)] extracted from leaves of non-transgenic and independent transgenic lines harbouring the pGreen+*OVP2* construct.

Lines	Glucose	Fructose	Sucrose
NT	0.60 ± 0.1[a]	0.23 ± 0.06[a]	0.93 ± 0.15[a]
T2	0.70 ± 0.1[a]	0.40 ± 0.10[a]	1.06 ± 0.29[a]
T5	0.77 ± 0.15[a]	0.63 ± 0.15[b]	1.53 ± 0.45[b]
T7	0.53 ± 0.15[a]	0.43 ± 0.14[a]	1.07 ± 0.15[a]

Values are means of three biological replicates per line ± standard deviations (SD). Student t-test was used to compare means and the similar letters in a column indicate not significantly different among lines at a confidence level of $p \leq 0.05$. The experiment was repeated thrice

From regeneration to soil acclimatization: Previously, the transgenic sugarcane calli were regenerated on MS media containing 50 mg/l hygromycine as the selectable antibiotic (Fig. 7A). Independent shoots, emerged from separate calli were excised and were transferred to glass jars containing MS media with appropriate concentrations of auxins, cytokinins and hygromycine (Fig. 7B). Transgenic lines produced roots on the selective media (Fig. 7C). After proper growth on MS media in glass jars and test tubes, the *In vitro* plantlets were transferred to soil conditions and acclimatized at the growth room conditions. For this purpose, small plastic bags were used to retain moisture content and protect the fragile plants from wilting (Fig. 7D). The bags with the plants were covered with saren rape to retain moisture and were water sprayed with regular intervals. The transgenic and non-transgenic plants were kept in these plastic bags for two weeks. After this period, the plants were transferred to small soil pots. Transgenic and non-transgenic lines were vegetatively propagated by transferring the individual tillers to small pots with compost soil (Fig. 7E). At least three individual plants were kept for each transgenic and control lines in the soil pots. The pots were arranged in a completely randomized design. The plants were regularly irrigated with normal tap water for one month, after which the salt stress was started.

Soluble sugar analysis: After one month of soil acclimatization, the transgenic and control lines were evaluated for total soluble sugars. Contents of soluble sugars were determined enzymatically (Table 5). Variable concentrations of soluble sugars were recorded for transgenic and control lines. Glucose content slightly varied among all lines but no significant differences were observed between the control and transgenic lines. In case of fructose, the transgenic lines showed comparatively high concentrations than that of the control. Particularly, the T5 showed significantly high fructose content (0.63±0.15 µmol/mg fresh weight) than the other transgenic and non-transgenic control lines. The sucrose content was also varied among all lines. Transgenic line, T5 showed significantly high ($p \leq 0.05$) sucrose content (1.53±0.45 µmol/mg fresh weight) as compared to that of non-transgenic control as indicated by the student t-test. These results need to be verified on comparatively higher plants under greenhouse conditions. It may be possible that larger plants grown under greenhouse conditions may show significant variation in soluble sugar content. The greenhouse level studies may clearly show the impact of the transgene on sucrose accumulation in transgenic lines.

Salt stress evaluation: The soil acclimatized plants of both transgenic and control lines were weak and fragile compared to the normal field grown sugarcane plants. Therefore, a medium salt concentration of 100 mM (NaCl) was selected to test the tolerance response of the transgenic lines against that of the non-transgenic control line. The plants of both genotypes were watered with 100 mM NaCl concentration for a period of four weeks. At the end of this period, slight wilting and chlorosis was observed in the non-transgenic control line. Salt stress negatively affected the growth of control line. However, the transgenic lines were still green and showed moderate salt tolerance, which was evident by the comparatively higher leaf chlorophyll and proline contents.

Measurement of chlorophyll content: The salt stress impact on leaf chlorophyll content was analysed in the transgenic and non-transgenic control lines subjected to control and salt stress conditions. Leaf chlorophyll contents were measured after four weeks of the start of salt stress application. The cholorophyll contents were taken as SPAD values (Fig. 8). The data showed a decline in the chlorophyll content in all transgenic and control lines after four weeks of 100 mM salt stress application. However, the differences for chlorophyll content between

control and salt stress were non-significant ($p \leq 0.05$). All transgenic and control lines showed chlorophyll reduction at 100 mM NaCl stress. But the reduction was slightly lower, although non-significant in transgenic lines than that of the control line. It means the transgene expression in transgenic lines conferred stress tolerance which enabled the plants to show high chlorophyll content even at high salt stress condition. It is anticipated that if these transgenic and non-transgenic lines are grown under greenhouse condition for prolonged growth, more prominent differences in the chlorophyll content may be observed at medium to high salt stress applications.

Measurement of proline content: Plants accumulate proline in order to compete with the salt stress that results adverse effects on its growth condition and viability of the cells. At control condition, there were non-significant differences for proline content between the non-transgenic and transgenic lines (Fig. 9). However, as a response to salt stress (100 mM), the proline content increased in non-transgenic control and all transgenic lines. All transgenic lines showed high proline content as compared to that of control at 100 mM NaCl stress. Among the transgenic lines, high proline content (above 35 µmol g^{-1} FW) was observed in the T2 line. This proline content was significantly higher than that accumulated in the non-transgenic control (28 µmol g^{-1} FW). It means that the *OVP2* gene expression in transgenic lines increased proline content as compared to that of the non-transgenic control line. The proline accumulation and its potential impact on salt tolerance in transgenic and control lines should be further evaluated on comparatively mature plants under greenhouse conditions.

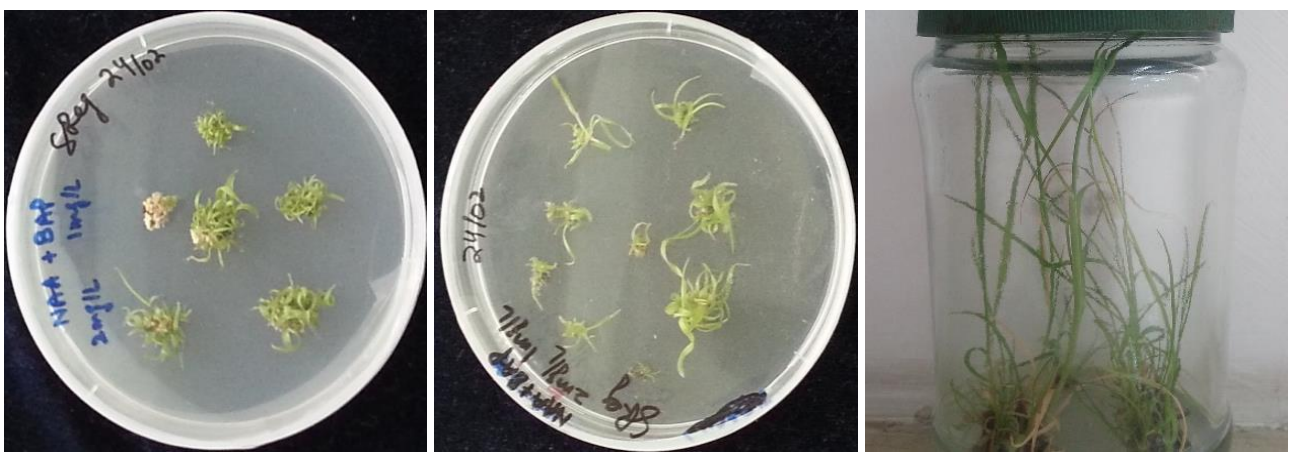

Fig. 4. Regeneration of transformed calli on regeneration media containing 50 mg/l Hygromycine.

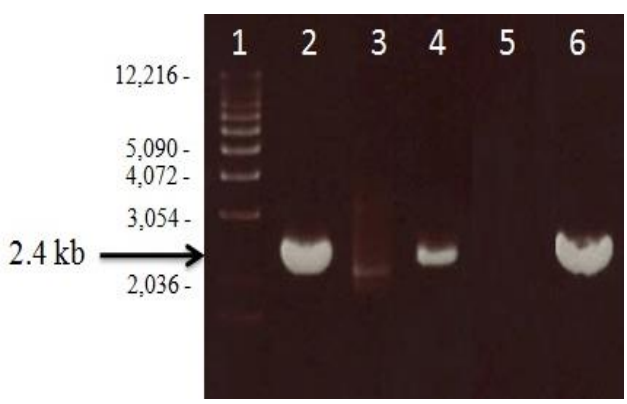

Fig. 5. PCR confirmation of the *OVP2* gene fragment with specific primers (*UBI* forward 1 and *OVP2* R). (Lane#1 is 1 Kb DNA Ladder; Lanes# 2-4 are the individual transgenic lines; Line# 5 is the negative control (Non-transgenic line); Lane#6 is the positive control (Amplification from the plasmid).

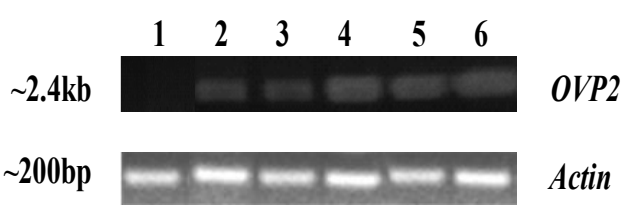

Fig. 6. RT-PCR analysis of the *OVP2* gene in individual transgenic lines. Lane # 1 is the non-transgenic control line; Lanes # 2-6 are the transgenic lines. *Actin* gene was used as an internal control in the lower section.

Discussion

The modern day sugarcane cultivars are difficult to be exploited for improvement through conventional breeding methods due to complex genetic characteristics and low fertility (Santosa *et al.,* 2005). The only promising technique that allows the genetic improvement of sugarcane varieties is genetic engineering. One of the pre-requisites for successful insertion of foreign gene cassette into the plant genome is to have an optimized transformation protocol. In our previous work, we successfully achieved sugarcane transformation of the variety CP 77/400 (Ullah *et al.,* 2016).

Initially, the target *OVP* genes were amplified from the rice genomic DNA through specific primers; however due to large size of the gene, both PCR amplification of the gene and cDNA synthesis were met with problems. Therefore, full length cDNA clone was ordered from KOME, Japan Gene Bank. The open reading frames (ORF) of the *OVP2* was then amplified using specific set of primers for each cDNA. After copy multiplication and purification, the plasmid carrying the *OVP2* gene was cloned in pGreen expression vector. Agrobacterium strain EHA 105 was transformed with the pGreen plasmid. The EHA 105 was previously used as an appropriate strain for sugarcane transformation (Kumar *et al.,* 2014). Established calli were infected with proper agrobacterium concentration. The transformation efficiency depends on a number of factors which collectively contribute to

productive agrobacterium mediated transformation in sugarcane. These factors include co-cultivation period, acetosyringone concentration, hygromycine and cefatoxime concentration. In the present experiment, remarkable increase in transformation efficiency was observed when co-cultivation period was increased to 4 days. Co-cultivation period below 4 days was proved to be insufficient for agrobacterium to affect maximum calli. Despite the fact that increases in co-cultivation period resulted in efficient transformation, some extra overgrowth of bacterial culture was observed. Furthermore, prolonged co-cultivation of calli exerted pressure on the calli cells because much energy is spent to compete with pathogen by excreting its phytoelexins and secondary metabolites. This caused the exhaustion of the calli cells and as a result, browning and death of the cells occurred. Death of the calli cells in response to overgrowth and other environmental pressures during prolonged co-cultivation was previously reported (Kumar *et al.,* 2014; Trifonova *et al.,* 2001; Dong *et al.,* 1991). On the contrary, our results suggest 2 days as the optimum duration of co-cultivation for efficient transformation.

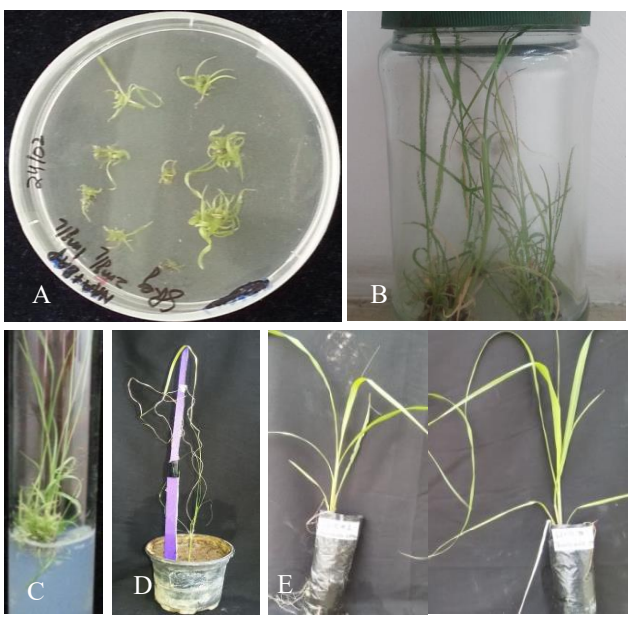

Fig. 7. From regeneration of transformed calli to plantlets acclimatization to soil condition. (A) Regeneration of transformed calli on regeneration media containing 50 mg/l Hygromycine. (B) Culturing of transformed shoots on rooting media. (C) Rooting from transformed shoots. (D) Soil acclimatization. (E) Transferring of individual shoots to soil pots.

Acetosyringone concentration showed inverse relationship with transformation frequencies i.e., the lower the acetosyringone concentration, the higher the transformation efficiencies were observed. Higher concentration of acetosyringone also caused overgrowth of bacterial cells which subsequently caused browning and death of the calli. The present research suggests 60 mM acetosyringone as an optimum dose for efficient transformation events and caused no bacterial overgrowth. Inverse relation between acetosyringone and transformation efficiency has also been reported earlier (Kumar *et al.,* 2014).

Following the co-cultivation, washing off the calli with cefatoxime to remove excessive bacterium was a laborious task. Cefatoxime being a broad spectrum antibiotic was effective to eliminate the bacterial cells but it exerted environmental stress on the developing transgenic calli. Higher cefatoxime concentration was proven to be extremely lethal for the developing transgenic calli. The present research suggests 400 mg/l of cefatoxime to be an optimum dose of antibiotic for eliminating agrobacterium. Lower concentration of cefatoxime may not be effective for eliminating accesses of bacterial cells. Hygromycine antibiotic was used as plant selectable marker (depending upon the antibiotic gene in the T-DNA). A concentration of 50 mg/l of hygromycine resulted in optimal survival of the developing transformed calli.

Vacuolar accumulation of solutes has been a strategy adopted by plants not only to store important solutes but also to cope with excessive Na^+ content (Ahmed *et al.*, 2016). The sugarcane production and yield has been affected by various factors such as genetic diversity and environmental stresses. One of the main reasons of low sucrose accumulation in the sink tissue/vacuoles is the lower efficiency of the tonoplast sucrose antiporters. The function of these antiporters is dependent upon the vacuolar H^+-pyrophosphatases which establish H^+ gradient across the vacuolar membrane (Krebs *et al.,* 2010). It is proposed that improving the activity of H^+-pyrophosphatses may result into efficient sucrose flow from the source tissues to the sink tissues and subsequent accumulation in the vacuole. As these H^+-pyrophosphatases provide the same proton gradient for the Na^+/H^+ antiporters, improving function of these pyrophosphatases may further increase the accumulation of Na^+ into the vacuole resulting high salt tolerance (Ahmed *et al.,* 2016). It means manipulation of the H^+-pyrophosphatses may increase the potential of sugarcane plants to accumulate more sucrose into the vacuole and also it may confer increased salt stress tolerance.

In the present study, the rice H^+-pyrophosphatse gene (*OVP2*) was expressed in sugarcane (CP/77-400). The *In vitro* grown transgenic and control lines were acclimatized to soil condition and then were subjected to total soluble sugar analysis under growth room conditions. Glucose, fructose and sucrose were determined in the leaves samples of both transgenic and non-transgenic lines. Glucose content did not differ between transgenic and non-transgenic lines. Fructose content somehow differed between the two genotypes. Transgenic lines showed comparatively high fructose content. The important and required sugar, sucrose was found highly accumulated in all transgenic lines as compared to the control lines. However, one transgenic lines i.e., T5 showed significantly high sucrose content than that accumulated in the control line. Apparently, it seems that expression of the *OVP2* gene has positively affected the activity of the tonoplast sucrose transporters resulting comparatively high sucrose accumulation in one of the transgenic lines. However, to further determine the effect of transgene expression on the overall sugar accumulation, much larger plants of transgenic and control lines should be evaluated under greenhouse conditions.

As a response to salt stress, plants trigger induction of several defence mechanisms including production of osmoprotectants such as proline (Khan *et al.,* 2015). Maintenance of high chlorophyll content is a signal of improved response to salt stress. High chlorophyll content has been observed with plants adaptation to salt stress (Saleh, 2012). In addition to chlorophyll content, proline accumulation has been observed as one of the preferred strategies adopted by plants subjected to salt stress condition (Khan *et al.,* 2015; Huang *et al.,* 2013). Several transgenic plants were previously reported with high proline accumulation that protected the plants against salt stress negative effects (Guerzoni *et al.,* 2014; Reddy *et al.,* 2015). In the present study, we observed non-significant changes in the leaf chlorophyll content between transgenic and non-transgenic plants watered with normal tap water. Chlorophyll content was reduced in control and transgenic lines when watered with 100 mM NaCl. However, compared with control, the transgenic lines particularly, T2 and T5 maintained high chlorophyll content even at 100 mM salt stress. Similarly, the plants subjected to 100 mM salt stress showed high proline accumulation in all transgenic and non-transgenic lines. Two of the transgenic lines exhibited significantly high proline content than that of the control line. On the whole, expression of the *OVP2* gene conferred transgenic plants salt tolerance as indicated by the comparatively high chlorophyll and proline contents. These results should be verified at higher plants grown under greenhouse condition.

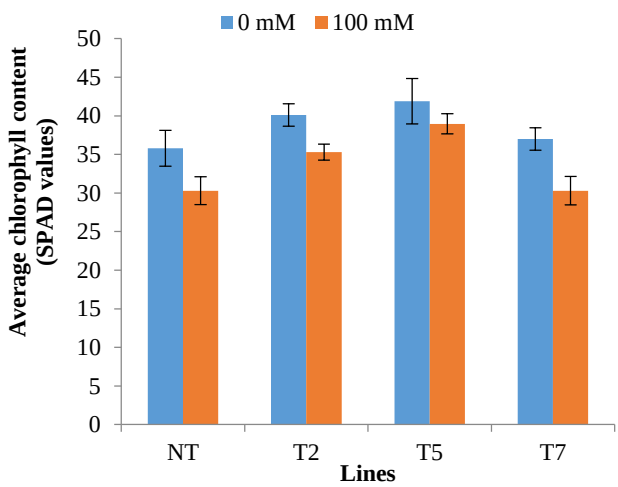

Fig. 8. Average chlorophyll data of non-transgenic control and transgenic lines four weeks of salt stress application in the growth room. Values are averages of three replicates ± SD.

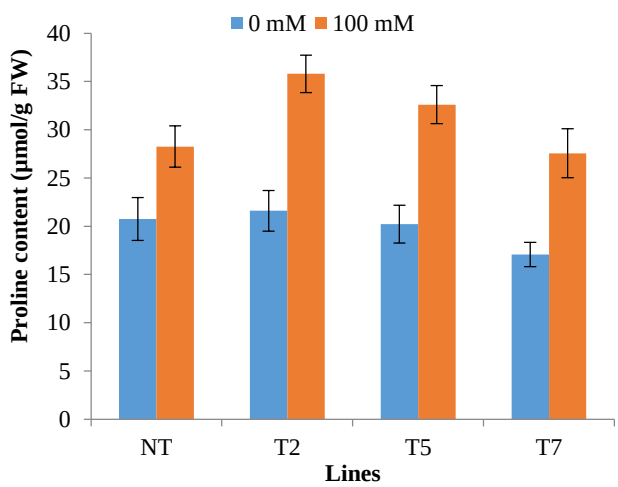

Fig. 9. Average proline data of non-transgenic control and transgenic lines for four weeks of salt stress application in the growth room. Values are averages of three replicates ± SD.

Conclusions

Callus induction and regeneration of the selected sugarcane varieties were optimized. The best performing CP-77/400 sugarcane variety was successfully genetically engineered with the rice tonoplast *OVP2* gene in order to increase salt tolerance and sugar content. The transformed shoots were confirmed through PCR and Rt-PCR. Individual transformants were then multiplied and were acclimatized to soil condition. The potted plants were subjected to salt tolerance evaluation and sugar content analysis. Our results revealed that some transgenic lines showed improved salt tolerance in terms of high chlorophyll and proline contents. Sucrose content was also increased in one of the transgenic lines. These results need further confirmation through evaluation of the mature transgenic lines under green house and confined field condition.

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