Overexpression of TSRF1 Improves Salt Tolerance in Sugarcane

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ABSTRACT

Sugarcane is an important industrial crop due to its utilization for sugar and ethanol production. As a glycophytic plant its growth and sucrose contents are severely affected by salinity stress. Genetic engineering offers a rapid solution to improve tolerance level of sugarcane against this stress. Thus, the present study was designed to transform tomato stress-responsive factor 1 (TSRF1) into sugarcane through *Agrobacterium*. Embryogenic callus of sugarcane cultivar Guitang281 was used for transformation with *Agrobacterium* strain LBA4404 harboring pROK2 vector containing TSRF1 gene driven by CaMV35S promoter. Highest transformation efficiency (72%) and PCR positive plants (3.03%) were obtained from the cultivar Guitang281 when it was transformed with TSRF1 gene with inoculum density OD 600 at 0.4 and co-cultivated for 4 days on MS based medium. Under normal condition transgenic lines displayed similar growth status as compared with wild-type plants, in contrast only transgenic plants were able to withstand in salt stress condition, showing tolerance against salinity stress. Physiological and biochemical assay revealed that TSRF1 overexpressing plants not only increased accumulation of proline, soluble sugars and glycine betaine than wild-type plants but also reduced MDA and H_2O_2 content in response to drought and salt stress. The findings of this study demonstrated that TSRF1 conferred salt tolerance to transgenic sugarcane through modulating the accumulation of osmo-protectant and antioxidant metabolism, indicating that these genes may have regulatory roles in response to abiotic stress in sugarcane.

Key words: Overexpression, TSRF1, sugarcane, salt tolerance

INTRODUCTION

Sugarcane is one of the most important industrial crops of the world due to its utilization for sugar and ethanol production. It is widely cultivated in tropical and subtropical countries and accounts for about 80% of sugar production world wide (world sugar production statistics 2011). Sugarcane is a complex polyploid having chromosome number 80 to 120 (D'Hont *et al.*, 1998). Conventional breeding takes 10 to 12 years for introgression and selection of desirable traits. Thus, improvement of sugarcane by conventional breeding is difficult. Therefore, sugarcane improvement through direct insertion of genes via genetic engineering seems a more attractive and practical solutions for improving stress tolerance. Drought and salt tolerance of plants could be achieved through genetic engineering with transcription factors gene, which regulate the expression of a large number of relevant downstream genes (Wang *et al.*, 2008). Since overexpression of transcription factors can lead to the upregulation of a whole array of

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genes under their control, they have been suggested as powerful tools for genetic engineering (Tian *et al.*, 2010). A number of studies demonstrated that the genes encoding transcription factors when overexpress in transgenic plants, greatly improve their tolerance to abiotic stresses such as drought, salinity and cold (Xiang *et al.*, 2008; Song *et al.*, 2011).

Salinity causes non availability of water for plants that severely affect plant growth and developmental processes, resulting yield loss. Plant develops complex mechanisms for their survival to ionic and osmotic stresses caused by drought and salinity (Rivero *et al.*, 2007). When plants are exposed to salinity, accumulation of compatible solutes, such as proline, glycine betaine, sugars and sugar alcohols increase dramatically to prevent water loss by decreasing osmotic potential, thereby keeping water potential at optimal level, resulting tolerance to stress maintaining plasma membrane integrity and protein function (Taiz and Zeiger, 2004).

Stress induced physiological imbalance leads to excess accumulation of reactive oxygen species (ROS) in plant cells (Zhang and Schmidt, 1999). Deceasing intracellular CO_2 levels due to stress cause rapid over-production of H_2O_2, superoxide radical ($O_2^{-\bullet}$) and hydroxyl radicals (OH^-). Their levels depend on the balance between synthesis and degradation, which is facilitated by the ROS-scavenging system of the plant cell (McCann and Huang, 2008).

Ethylene response factor (ERF) proteins are a subfamily of the AP2/ethylene responsive element binding protein (EREBP) transcription factor family which is unique to plants (Singh *et al.*, 2002). Characteristics of the ERF transcription factors are the presence of ERF binding domains that can interact with two similar cis-elements, such as GCC box and the C-repeat (CRT)/dehydration responsive element (DRE) at the promoter of downstream target gene (Xu *et al.*, 2011). The AP2/ERF transcription factors regulate diverse processes of plant development and stress responses, such as vegetative and reproductive development, cell proliferation, abiotic and biotic stress responses (Sharoni *et al.*, 2011).

Abiotic stresses such as drought, salt and freezing activate the expression of ERF genes from clusters II, Va VIb and VII. Overexpression of these ERFs improves tolerance of transgenic plants to drought, salt and freezing (Xu *et al.*, 2008, Zhu *et al.*, 2010). It is reported that several ERFs have been incorporated into crops, resulting improve tolerance to drought and salt. Overexpression of these ERFs have been reported in rice (Gao *et al.*, 2008), tomato, tobacco and soybean (Trujollo *et al.*, 2008; Zhang and Huang, 2010), showing improved tolerance to drought and/or salt in transgenic plants.

The objective of this study was to transform sugarcane with TSRF1 gene through *Agrobacterium tumefaciens* and study the performance of transgenic sugarcane under salt stress condition.

MATERIALS AND METHODS

Construction of plasmid vectors containing TSRF1 gene and bacterial strains

Plasmid pROK2 (provided by Prof. Rongfeng Huang) contained TSRF1 gene, 35S promoter and the nopaline synthase (nos) terminator. Neomycin phosphotransferase resistance gene (npt II) was used as selective marker gene. The plasmid was digested by BamHI and SacI separately. The vector was introduced into *Agrobacterium* strain LBA4404 by freeze thaw transformation methods. The recipient *Agrobacterium* cells were

selected on LB medium solidified with 1.5% (w/v) bacto-agar (SABC) containing 100 mg/L rifampicin and incubated at 28°C in the dark. The transformed *Agrobacterium* were detected by PCR.

Embryogenic callus induction and culture

Embryogenic calli of sugarcane (*Saccharum officinarum* L.) cultivar Guitang281 were initiated from transverse sections (1-2 mm) of immature leaf whorls above the apical meristem of the shoot top. The meristematic explants for callus induction were derived from very young shoots (3 months old) of a healthy sugarcane plant grown in green house condition. Explants were cultured on Murashige and Skoog (MS)-based embryogenic callus induction medium CIM1and CIM2 (Table 1) and left in the dark at 28 ± 1°C for 4-5 weeks with subculturing onto same medium every 2 weeks (Murashige and Skoog1962). The compact, cream coloured nodular, embryogenic callus was subcultured onto fresh M1 medium 4 days prior to inoculation with *Agrobacterium tumefaciens*.

Preparation of *Agrobacterium* suspension

Agrobacterium strain LBA 4404 harboring pROK2 was grown at 30°C, 180 rpm for 48-72 h in YEB medium with addition of 50mg/L kanamycin and 100mg/L rifampicin antibiotic. When the cell density reached OD 600 = 0.7, the cells were collected by centrifuge at 9000 rpm for one minute and bacterial pellets were re-suspended in the 20-fold volume of MS liquid medium. A liquid MS medium reduced the content of macro elements to 1/5 of its original and supplemented amount with 150 µmol/L acetosyringone (AS), 10 mmol/L fructose, 10 µmol/L glucose, pH 5.3, and incubated for 1–4 h at the same conditions, bacterial growth was monitored and adjusted OD 600 at 0.2, 0.4 and 0.6 for infection.

Callus infection, co-cultivation and regeneration of transformed plantlets

Before initiating the infection by *A. tumefaciens*, 4-5 weeks aged calli were transferred onto fresh M1 medium for 4d, and collected on filter paper. Sugarcane embryogenic calli for *A. tumefaciens*-infection were cut into small pieces, and dried for 30-60 min in the flowhood, until the calli dried and begin shrinking. The dried calli were infected by the *A. tumefaciens* suspension and agitated at 100 rpm on a rotary shaker for 30 min, and blotted dry. Infected calli were placed onto MS1, MS2, MS3 and MS4 co-cultivation medium (Table 1) and co-cultured at 23°C in dark for 3, 4 and 5 days. After that, calli were washed for 3-4 times in sterile distilled water containing 500mg/L cefotaxime antibiotic, dried on filter paper, and inoculated on the selective M2 medium (Table 1) plates for 2 weeks, and cultured at 26°C under illumination at 1500 lux with 14 h/d for selection. At the same time uninfected calli were inoculated on M2 medium in the same condition as wild type.

Resistant calli were subcultured in the same fresh M2 selective medium every 2-3 weeks, incubated in the same condition to induce the putative transgenic shoots regeneration. Putative transgenic shoots with 4 -5 cm high were separated and subcultured onto the selective rooting M3, M4 and M5 medium (Table 1).

Table1. Composition of various media used in the experiment

CIM1	MS salts and vitamins, coconut water 10% v/v, casein peptone 0.5 g/l, sucrose 15.0 g/l, 2,4-dichlorophenoxy acetic acid (2,4-D) 3mg/l, Phytagel 3.0g/l, pH 5.6–5.8
CIM2	MS salts and vitamins, sucrose 30.0g/l, 2,4-D 3mg/l, Phytagel 3.0g/l, pH 5.6–5.8
M1	MS salts and vitamins, sucrose 30.0g/l, 2,4-D 1mg/l, Phytagel 3.0g/l, pH 5.6–5.8
M2	MS salts and vitamins, sucrose 30.0g/l, Kinetin 0.5mg/l, 6-BA 1mg/l, Phytagel 3.0g/l, 500 mg/L cefotaxime disodium, 50mg/L kanamycine, pH 5.6–5.8
M3	½MS salts, sucrose 30.0g/l, NAA 1mg/l, Phytagel 3.0g/l, 500 mg/L cefotaxime disodium, 50mg/L kanamycine, pH 5.6–5.8
M4	½MS salts, sucrose 30.0g/l, IAA 1mg/l, Phytagel 3.0g/l, pH 5.6–5.8
M5	½MS salts, sucrose 30.0g/l, 1mg/L IBA+0.3mg/L NAA, Phytagel 3.0g/l, pH 5.6–5.8
MS1	MS salts and vitamins, sucrose 30.0g/l, 2,4-D 1mg/l, Phytagel 3.0g/l, AS 100µmol/l, pH 5.6–5.8
MS2	MS salts and vitamins, sucrose 30.0g/l, Phytagel 3.0g/l, pH 5.6–5.8
MS3	MS salts and vitamins, sucrose 30.0g/l, Phytagel 3.0g/l, AS 100µmol/l, pH 5.6–5.8
MS4	MS salts and vitamins, sucrose 30.0g/l, 2,4-D 3mg/l, Phytagel 3.0g/l, pH 5.6–5.8

Molecular analysis of putative transgenic plants

Polymerase chain reaction (PCR) was performed to confirm the target gene insertion into the genomic DNA of putative transgenic plant. Genomic DNA of putative transgenic and wild type sugarcane was extracted from leaves as described by Aljanabi *et al.*, (1999) with some modification. PCR was carried out in 25 µl reaction volume, one fourth microliter (around 200ng) volume of DNA solution served as template and primer concentration 10µM in the final reaction was used in the PCR reaction. The PCR mixture was initially denatured at 94°C for 5 min followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 53°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min. We used marker gene nptII primers for PCR analyses, the forward and reverse primer sequences were 5′-GAGGCTATTCGGCTATGACTG-3′and 5′-ATCGGGAGCGGCGATACCGTA-3′.The PCR products were resolved on 1 % agarose gel and visualized by gel documentation system.

Salt stress treatment

Three months old transgenic and wild type plants of GuitangTSRF1 sugarcane were exposed to 300mM NaCl solution for 20 days followed by 10 days of recovery with rewatering. Half strength 600 ml Hogland's solution with 300mM NaCl was applied to each plant every two days interval. At the age of eight months GuitangTSRF1 overexpressed lines with their wild-type plants were exposed to 300mM salt stress for 5 days to investigate the modulation of osmolytes, antioxidants and ROS in response to salt stress.

Determination of proline content

Free proline content was determined according to the method of Bates *et al.*, (1973). Briefly, 200mg leaf sample was ground with liquid N_2 and homogenized in 5 ml of 3 % sulfosalicylic acid, the homogenate was filtered through Whatman No. 1 filter paper. Then 2.0 mL of the filtrate was mixed with 2.0 mL ninhydrin reagent (1.25 g ninhydrin in 30 ml of glacial acidic acid and 20 ml of 6M orthophosphoric acid) and 2 ml glacial acetic acid in a test tube. This mixture was incubated at 95°C for 1 hr in a water bath and the reaction was terminated in an ice bath. Finally, 4.0 mL of toluene was added to the solution and mixed vigorously with a stirrer for 10-15 s. The chromphore containing toluene was aspirated from the aqueous phase, warmed at room temperature and the absorbance was recorded at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve using L-proline and expressed as µg g^{-1}FW.

Estimation of total soluble sugars (TSS)

Total soluble sugars (TSS) were estimated following the method described by Watanabe *et al.*, (2000) with some modification using Anthrone reagent (0.06% (w/v) anthrone in 95% H_2SO_4). Leaf samples of 200 mg were homogenized in 10mL of 80% ethanol. The extract was centrifuged at 6000 rpm for 10 min at 4°C. Supernatant (0.25 mL) was mixed with freshly prepared 3 mL Anthrone reagent and the tubes heated at 95 °C for 10 min. The reaction was terminated by quick place on ice. The absorbance was measured at 620 nm and total soluble sugars were quantified from a standard curve prepared using glucose as standard, result expressed as mg g^{-1}FW.

Estimation of lipid peroxidation

Oxidative damage of lipids was estimated by measuring the content of thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA) as per the method of Heath and Packer (1968) with some modifications. Leaf samples (200 mg) was ground with liquid N_2 and homogenized in 5 mL (5% w/v) trichloro acetic acid (TCA). The extract was centrifuged at 10,000 g for 10 min; supernatant was taken to a fresh tube and mixed well with equal volume of 2-thiobarbituric acid (TBA, 0.67% w/v) and the mixture was heated at 95°C for 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation was performed at 5000g for 5min and absorbance was taken at 532nm keeping TBA (0.25% w/v) in TCA (10% w/v) as blank and corrected for non-specific turbidity by subtracting the absorbance at 600 nm.

The malondialdehyde content was calculated using its absorption coefficient (ε) and expressed as nmol g^{-1} fresh weight using the formula:

$$\text{MDA (nmol g}^{-1}\text{ FW)} = \frac{(A523-A600)\times v\times 1000}{\varepsilon\times W}$$

where ε is the specific extinction coefficient (=155 $mMcm^{-1}$), V is the volume of the extract (mL), W is the fresh weight of leaf, A600 is the absorbance at 600 nm wave length and A532 is the absorbance at 532 nm wavelength.

Determination of glycine betaine

Glycine betaine was estimated following the method of Grieve and Grattan (1983). For this, 200mg leaf samples were ground with liquid N_2 and added 3 mL deionized water into it. Thereafter, sample tubes were mechanically shaken at 25°C for 16 hrs. Filtered extract was diluted (1:1) with 2N H_2SO_4 and 500 µl extract of each sample was taken in test tube, cooled on ice for 1hr and mixed with 200 µl of potassium iodide-iodine (KI-I_2) reagent. The samples were incubated again at 4°C for 16 hrs, after incubation tubes were centrifuged at 10,000 rpm for 15 min at 0°C. After centrifugation tubes were kept on ice and supernatant was discarded with micropipette to keep per iodide crystal unchanged. After that the periodide crystals were dissolved in 9 mL of ice cold 1, 2-dichloroethane with vigorous shaking. After 2hrs the absorbance was read at 365 nm, glycine betaine content was quantified from standard curve, prepared with 1N H_2SO_4 and expressed as µmol g^{-1}FW.

Hydrogen peroxide (H_2O_2) estimation

Levels of H_2O_2 were measured according to the method of Sergiev *et al.*, (1997). Leaf samples of 200 mg were ground in liquid N_2 and homogenized with 2 mL of 0.1% (w/v) TCA on an ice bath. The homogenate was centrifuged at 13,000 rpm for 15 min at room temperature. The reaction mixture consisted of 0.5 mL supernatant, 0.5 mL potassium phosphate buffer (KH_2PO_4-10mM; pH-7.0) and 1 mL potassium iodide (KI-1M); mixed by vortex and the absorbance was taken at 390 nm, 0.1% TCA was used as blank. H_2O_2 content was determined from a standard curve and the values were expressed as µmol g^{-1}FW.

Statistics

Data analysis and mean separation were done using R software
(R Foundation for Statistical Computing, Vienna, Austria.URL http://www.R-project.org/).

Statistical differences are referred to as significant when $P < 0.05$.

RESULTS AND DISCUSSION

Effect of inoculum density on transformation

Inoculum density is a crucial factor for efficient *Agrobacterium*-mediated transformation. In our study we have tested three different (OD= 0.2, 0.4 and 0.6) concentrations of *A. tumefaciens* to find out the optimum concentration for experimental cultivars infection. Before initiating the infection by *A. tumefaciens*, 4 - 5 weeks aged callus were transferred onto fresh MI medium (Table 1) for 4d, and collected on filter paper. Sugarcane embryogenic callus for *A. tumefaciens*-infection were cut into small pieces, and dried for 30-60 min in the flowhood, until the callus dried and begin shrinking. The dried callus were infected by the *A. tumefaciens* suspension and agitated at 100 rpm on a rotary shaker for 30 min, and blotted dry. Embryogenic callus infected by *A. tumefaciens* at an inoculum density 0.4 (OD 600) at 23°C temperature produced highest number of regenerated plant from resistant callus of Guitang281TSRF1 (Table 2). Bacterial colony does not appear up to 6 days co-cultivation period when inoculum density was 0.2 (OD 600), in contrast at 0.6 (OD 600) inoculum density, colony appeared on 3rd day. Inoculum density higher than 0.6 favors colonization and cell density increases rapidly and it becomes difficult to make callus free from bacterial lawn after 2-3 times washing by cefotaxime. Resulting slow callus growth, necrosis of callus in shooting medium and low transformation efficiency (Table 2). At low inoculum density (OD= 0.2)

transformation efficiency is lowest due the inability of bacterial strain to infect the callus, as a result most of the callus turned burn and died in shooting media. The results of our study showed (Table 2) that inoculum density of *Agrobacterium* strain invariably affect the transformation efficiency and 68% transformation efficiency is achievable at an inoculum density 0.4 (OD 600).

Effect of co-cultivation media and co-cultivation period on transformation

Co-cultivation media and duration of co-cultivation has been reported to influence the transformation efficiency and subsequent regeneration capacity. In our study, three different co-cultivation periods (3, 4 and 5 days) were applied for co-cultivation of infected callus with four different co-cultivation media MS1, MS2, MS3 and MS4 (Table 1) in order to improve transformation efficiency and plantlet regeneration. Results of this study demonstrate that significantly higher transformation efficiency (Table 3) was observed from Guitang281TSRF1 when callus were co-cultivated on MS4 media for 4 days. Co-cultivation on MS4 media for 4 days showed higher transformation efficiency compare with other three media and two co-cultivation periods. It was observed that co-cultivation for 5 days or more enhanced over growth of bacteria which adversely affected callus growth in shooting media and after 3-4 days most of the callus were died due to rotten. Thus results of this experiment imply that 72% transformation efficiency can be achieved from embryogenic callus of sugarcane with co-cultivation for 4 days on MS4 media.

Table 2. Effect of inoculum density on the transformation efficiency (TE) of sugarcane cultivars after transformation with A. tumefaciens carrying TSRF1 gene

OD of *Agrobacterium* suspension	Regenerated plants (no.)	TE (%)
Guitang281 infected with TSRF1		
0.2	1b	2
0.4	26a	52
0.6	2b	4

For each treatment 50 callus pieces with 2mm diameter were used. The values of regenerated plants are the mean of three replications. TE (%) = Number of resistant shoots/ No. of callus infected with Agrobacterium × 100

Table 3. Effect of co-cultivation medium and co-cultivation period on transformation efficiency (TE) of sugarcane cultivars after transformation with A. tumefaciens carrying TSRF1 gene

Co-cultivation medium	Co-cultivation for 3 days		Co-cultivation for 4 days		Co-cultivation for 5 days	
	Regenerated plants (no.)	TE (%)	Regenerated plants (no.)	TE (%)	Regenerated plants (no.)	TE (%)
Guitang281 transformed with TSRF1						
MS1	20b	40	22c	44	18a	36
MS2	8c	16	28bc	56	12b	24
MS3	22b	44	29b	58	11b	22
MS4	27a	54	36a	72	19a	38

For each treatment 50 callus pieces with 2mm diameter were used. The values of regenerated plants are the mean of three replications. TE (%) = Number of resistant shoots/ No. of callus transformed with Agrobacterium × 100

For the successful transformation of TSRF1 gene in sugarcane cultivar Guitang281 through *Agrobacterium* strain, we have optimized important factors like inoculum density, co-cultivation media and co-cultivation period. Cultivation of bacteria in MS based liquid medium supplemented with acetosyringone, glucose and fructose enhanced vir gene activity and stimulate vir gene expression. Zhangsun *et al.*, (2007) reported that acetosyringone and glucose has increasing effect to each other which enhance transformation efficiency. Our results showed that co-cultivation for 4 days in MS based medium with an inoculum density OD 600 of 0.4 was optimal for achieving highest transformation efficiency of 78% for transgenic sugarcane regeneration using embryogenic callus (Table 2 and 3). This result is consistent with the previous results reported in sugarcane callus based *Agrobacterium*-mediated transformation (Zhangsun *et al.*,2007; Joyce *et al.*, 2010). Thus, our study demonstrated that the transformation system we used for regenerating transgenic sugarcane was efficient.

Plant regeneration

Green shoots were appeared from transformed callus within 2 weeks after inoculation on the selective M2 medium; shoot initiation rate was faster in cream colored friable embryogenic callus compared to whitish brown friable embryogenic callus. No shoots were produced from smooth surfaced hard callus pieces. Wild type callus were continued its growth on selective M2 medium having 25mgl^{-1} kanamycin. But callus growth was inhibited at 50mgl^{-1} kanamycin concentration; subsequently they turned brown and

died. Of the shoot regenerated from transformed resistant callus, some were albino, some exhibited phenotypic alternations, like yellowish leaves, zigzag shaped leaves and retarded growth. Shoots transferred to different rooting medium, started root initiation at 10 days on M3 medium and at 20 days on M5 medium but no root initiation was observed up to 20 days on M4 medium.

Screening of putative transgenic plantlets by PCR

A total of 231 putative transgenic plants were regenerated from embryogenic callus of Guitang281 cultivars of sugarcane (Table 4). These plants were regenerated from sugarcane embryogenic callus after infected with *Agrobacterium* strain at an OD 600 of 0.4 and co-cultivated for 4 days on MS4 medium. To confirm successful integration of transgene (TSRF1 and nptII), we used transgene specific primers of the gene. Fragment of TSRF1 was amplified from both the DNA of transgenic and WT type plants by using gene specific primer. This result indicates there may be homology of TSRF1gene in sugarcane genome. Subsequently we confirmed the presence of nptII gene by PCR using nptII gene specific primer. A fragment of 750bp was amplified from the DNA of transgenic plants; this band was not amplified from the DNA of WT plant. Of the regenerated plants seven from Guitang281TSRF1 contained nptII gene. Thus, our result showing successful insertion of nptII gene into sugarcane genome through *Agrobacterium* mediated transformation method.

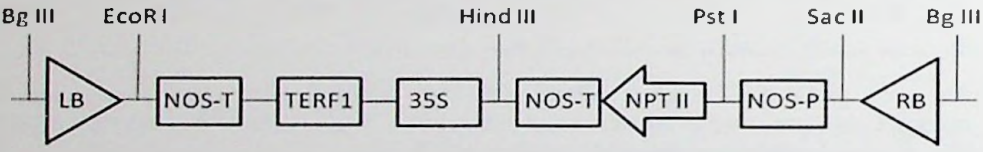

Figure 1. Schematic map of pROK2 vector containing TSRF1 gene and NPT II selectable marker gene, driven under CaMV 35S promoter. RB right border, LB- left border, NOS-T Nos terminator, NOS-P Nos promoter.

Table 4. PCR analysis to confirm transgene insertion in plants regenerated from callus of sugarcane cultivars Guitang281 inoculated with *Agrobacterium* strain containing TSRF1 gene

Cultivar with transgene	No. of plants tested	*nptII* positive	Transformation efficiency (%)
Guitang281TSRF1	231	7	3.03

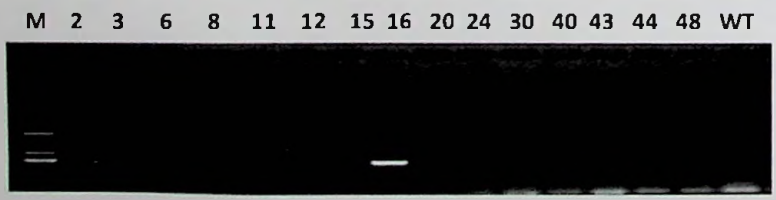

Figure 2. Molecular analysis of transgenic sugarcane plants. PCR amplification of npt-II gene from putative transgenic Guitang281 TSRF1 sugarcane plants. Lanes 2, 3, 6, 16, 20, 24 and 40 are positive transgenic plants; lanes 8, 11, 12, 15, 30, 43, 44 and 48 are negative transgenic plants. M: 2000 bp DNA ladder; WT: Wild- type plant.

Response of transgenic lines under salt stress

From Guitang281 TSRF1 three transgenic lines along with their wild-type plants were subjected to salt stress with 300mM NaCl solution for 20 days. At the end of salt treatment it was observed that two transgenic lines were survived in response to high salt (Fig. 3). All the wild-type plants were died under high salt stress. Ten days after rewatering salt stressed survived transgenic lines were observed to return its normal growth but the phenotype was not as good as normal growth condition. Hence, we postulate that overexpression of TSRF1 confer transgenic sugarcane tolerance to high salt.

Figure 3. Phenotype analysis of OE and WT Guitang281 TSRF1sugarcane plants subjected to salt stress. Control: 3 months old sugarcane plants grew under natural growth conditions. Salt: 3 months old sugarcane plants were exposed to 300mM NaCl solution for 20 days. Recovery: After salt stress transgenic plants were recovered for 10 days with water.

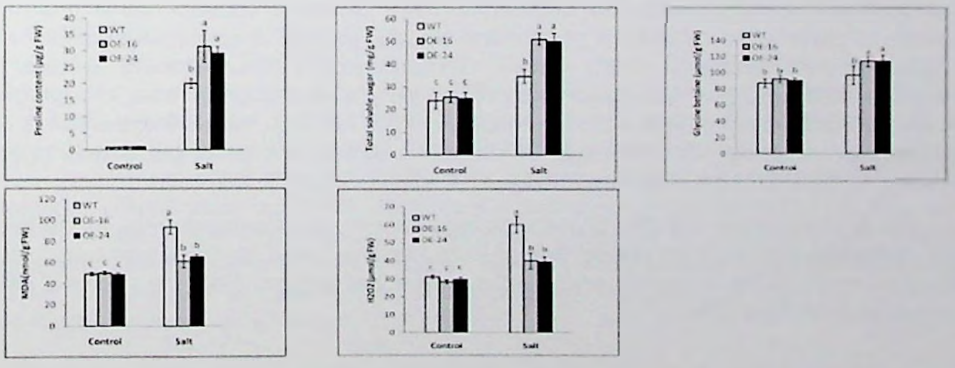

Figure 4. Comparison between control condition and salt induced changes of Proline (A), Total soluble sugars (B), Glycine betaine (C), MDA (D) and H_2O_2 (E) content in Guitang281 TSRF1 overexpressed lines (OE-16 and OE-24) and wild-type (WT) plants. Salt stress was imposed as keeping pots in 300mM NaCl solution for 5 days. Data are mean ± SD with three replicates. Means with different

Overexpression of TSRF1 increases proline, soluble sugars and glycine betaine content in sugarcane under salt stress

Under control conditions, there were no significant differences in proline, soluble sugars and glycine betaine content between transgenic OE lines and WT plants (Fig. 4 A, B, C). After 5 days of salt treatment proline and soluble sugars content were markedly increased in both WT and overexpressed plants. Glycine betaine content of overexpressed lines was found to increase under salt stress but it was similar in wild-type plants as compare to the control condition. However, significant differences were found in accumulation of proline, soluble sugars and glycine betaine content between transgenic and wild-type plants in response to salt stress. Therefore, results of the present study exhibit that TSRF1overexpression might enhance the accumulation of proline, total soluble sugars and glycine betaine content, which play major role for improved tolerance of transgenic sugarcane in response to salt stress.

To know the underlying mechanisms responsible for the enhanced tolerance of TSRF1overexpressing plants to salt stress, we have imposed salt stress to eight month old transgenic and wild-type sugarcane plants and examined the changes in physiological processes associated with plant tolerance to salt stress.

Plants increase accumulation of osmotic substances in response to abiotic stresses to maintain high intracellular osmotic pressure and normal physiological functions of cells (Sharma *et al.*, 2005). Accumulation of compatible solutes such as free proline (Armengaud *et al.*, 2004) and soluble sugars (Gupta and Kaur, 2005) in response to abiotic stress is a common phenomenon of plants. Proline and soluble sugars act as osmolytes to facilitate osmo-regulation, thereby protecting plants from dehydration associated with salt stress by reducing water potential of plant cells (Yang *et al.*, 2012). Free proline and soluble sugars are thought to mainly function for osmotic adjustment by lowering cellular osmotic potential to facilitate water absorption and restore intracellular salt concentrations (Farooq *et al.*, 2009). In response to environmental stress proline accumulates in many plant species and plays osmoregulation and protective role (Szabados and Savouré, 2010). In addition, proline can also function as a molecular chaperone to stabilize the structure of proteins as well as play a role in regulation of the antioxidant system (Szekely *et al.*, 2008). Overproduction of compatible solutes like proline and soluble sugars has been reported to play a predominant role in transgenic plants with enhanced tolerant to abiotic stress (Xu *et al.*, 2008). Moreover, they also play crucial roles in reducing plant damage by oxidative stress to protect plant cells against accumulation of ROS (Apel and Hirt, 2004).

As a compatible solute, glycine betaine plays an important role in adjusting osmotic potential, it is also proposed that glycine betaine could stabilize macromolecular activity and membrane integrity, and had a protective rather than an osmotic effect (Sakamoto and Murata, 2001).

In the present study we found higher accumulation of free proline, soluble sugars and glycine betaine in the TSRF1 overexpressing sugarcane lines relative to WT plants, which may account for the higher tolerance of overexpressing plants to salt stress. In this report, the improved water retention ability in the transgenic sugarcane lines might partially be a result of the increased accumulation of proline and soluble sugars, allowing the transgenic plants for more effective osmoregulation, thus conferring them more tolerant to salt stress by minimizing water loss. Moreover, elevated accumulation of glycine betaine in transgenic lines contributed significantly to maintain photosynthetic activity and cell wall integrity under salt stress.

Overexpression of TSRF1 accumulates less MDA and H_2O_2 in sugarcane under salt stress

It was observed that, under control condition there were no significant differences in MDA and H_2O_2 content between wild-type and overexpressed lines. Upon exposed to 300mM salt stress for 5 days, significant increases were found in H_2O_2 and MDA contents in both WT and transgenic plants (Fig.4 D, E). However, increased accumulation of H_2O_2 and MDA in overexpressed lines due to salt stress was significantly lower than WT plants. About two fold increased accumulation of H_2O_2 and MDA in wild-type plants in response to drought stress were observed compare with control condition. Thus, results of our experiment demonstrate that enhanced tolerance of TSRF1overexpressing sugarcane plants was due to mitigating oxidative damage by suppression of ROS production.

Drought, salinity, extreme temperatures and oxidative stress are often interconnected and produce elevated concentrations of ROS in chloroplasts, resulting damage to the cellular structure of plants (Gechev *et al.*, 2006). MDA is widely recognized as an oxidative stress marker, thought to be involved in denaturation of proteins, metabolic disorder and deterioration of various biological functions in stressed plants (Yamaguchi *et al.*, 2008). When plants are exposed to various stress conditions, the levels of H_2O_2 increase (Foyer *et al.*,1997). Increased accumulation of H_2O_2 causes an overproduction of ROS, which lead to the structural modifications of proteins, lipids and DNA (Halliwell and Gutteridge, 2002). In the present study, salinity caused an increase in MDA and H_2O_2 content in WT plants than TSRF1 overexpressing plants. However, the less amounts of H_2O_2 and MDA in the transgenic plants than in WT plants under salt stress may be due to the higher proline and soluble sugars content in transgenic plants than in WT plants. Since osmolytes have the ability to scavenge ROS, hence, proline and soluble sugars might play a role in ROS scavenging and protected the transgenic plants from oxidative damage by acting as an antioxidant. In this context, some previous report demonstrated that overexpression of TSRF1 in rice increases proline and soluble sugars content under drought stress (Quan *et al.*, 2010). Therefore, TSRF1 transcription factors functions as positive regulator to mediate tolerance of sugarcane plants to salt stress.

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