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Constitutive Overexpression of the Plasma Membrane Na^+/H^+ Antiporter for Conferring Salinity Tolerance in Rice

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Abstract

At the cellular level, the *Salt Overly Sensitive (SOS)* signaling pathway comprising *SOS3*, *SOS2*, and *SOS1* has been proposed to mediate cellular signaling under salt stress to maintain ion (Na^+) homeostasis. In this regulatory pathway, both *OsSOS1* encoding plasma membrane and *OsNHX1* encoding vacuolar Na^+/H^+ antiporters are regulated by *SOS3-SOS2* protein kinase complex. In the present study, the rice variety BRRI dhan28 - which is popular with farmers and high yielding, but salt sensitive, was transformed with the *OsSOS1* gene isolated from salt tolerant Pokkali rice and driven by the constitutive promoter, *CaMV35S.* The construct was transformed through a tissue culture-independent *Agrobacterium*-mediated *in planta* transformation method that circumvents the problems associated with tissue culture-based *indica* rice transformation methods. Integration of the foreign genes (*OsSOS1*) into the genome of transgenic plants was confirmed by gene-specific PCR and Southern blot analysis. The level of transgene expression (*SOS1*) was also quantified by semi-quantitative RT PCR and real time PCR. Genetic segregation ratio for T_1 progenies was calculated and found to follow the Mendelian law of inheritance in case of positive transformants. The transformants were shown to be salt tolerant compared to wild type in molecular analysis as well as physiological screening. Future work will involve transformation of both the *OsSOS1* and *OsNHX1* genes together; with the expectation for enhancing the tolerance level compared to currently available transgenic rice.

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Introduction

Rice is one of the most important cereal crops in the world, which yields one-third of the population's total carbohydrate source. Three billion people consider rice as their staple food, accounting for 50 - 80% of their daily calorie intake. However, rice is a salt sensitive monocot (Singh et al. 2014). Salinity stress is one of major problems for rice production. It affects about 400 million hectares of land in the world, of which 54 million are found in south and south-east Asia. Rice is sensitive above a soil conductivity of 3 dS/m. Within Bangladesh, greater than 1 million ha of coastal areas are affected by varying degrees of soil salinity. The soil conductivities can vary from 4 - 20 dS/m in coastal areas depending upon the season, distance from rivers originating in upper riparian countries or proximity to the sea. In the monsoon season, the soil salinity levels go down in most areas of the coast and farmers can usually grow one rice crop, whereas the cropping pattern in the rest of the country is two per year. Double cropping of rice for the small and marginal farmers of the coast would give them an advantage, particularly in adverse weather conditions. Production of rice is therefore under pressure: salinity may cause plant demise as well as reduction in growth and development, resulting in up to 50% of a reduced yield (Nozulaidi et al. 2015). BRRI dhan28 is an early-maturing, high-yielding irrigated rice variety, popular with farmers in the coastal areas, suitable for growth in the dry winter season. However farmers cannot grow this rice where soil salinities are greater than 4 dS/m. Improving the salinity tolerance of this rice would therefore be of great benefit to the farmers of the coastal region of Bangladesh.

During salinity stress, several classes of Na^+-transporters have been shown to play central roles in Na^+ homeostasis. Three major Na^+ transporters are involved in plant Na^+ tolerance: namely, the *NHX*, *SOS1*, and *HKT* transporters (Yamaguchi et al. 2013). Several reports have stated that, in roots, the *SOS* proteins may have novel roles in addition to their functions in sodium homeostasis. The *SOS* proteins also play a role in the dynamics of cytoskeleton under stress. These results imply a high complexity of the regulatory networks involved in plant response to salinity (Ji et al. 2013).

In order to produce high yields, producing seeds that give rise to strong, healthy plants, that are resistant to disease and can resist adverse environmental conditions like high salinity, is one of the main concerns nowadays. Therefore, engineering genes encoding Na^+ transporters into seeds can play a crucial role in conferring salinity tolerance.

High salinity stress causes an imbalance in sodium ion (Na^+) homeostasis, which is normally maintained by the coordinated action of various pumps, ions,

Ca^{2+} sensors, and its downstream interacting partners, which ultimately results in the efflux of excess Na^+ ions (Tuteja 2007).

Currently, the *SOS* pathway is one of the most extensively studied mechanisms controlling salt stress response in plants. The *SOS* pathway is responsible for ion homeostasis and salt tolerance in plants. *SOS1* is a plasma membrane Na^+/H^+ antiporter and mediates Na^+ efflux and control long-distance Na^+ transport from roots to shoots, thus protecting individual cells from Na^+ toxicity. *SOS2* is a serine/threonine protein kinase. *SOS3* responds to the Ca^{2+} signal by activating a protein phosphatase or inhibiting a protein kinase that regulates K^+ and Na^+ transport systems. *SOS3* physically interacts with and activates *SOS2* protein kinase. The *SOS2*/*SOS3* kinase complex phosphorylates and activates the *SOS1* protein, resulting in an efflux of Na^+ ions (Ma et al. 2014).

It has been already reported that increased expression of *OsSOS1* results in improved salt tolerance in transgenic *Arabidopsis* (Shi et al. 2003) and tomato (Olías et al. 2009). In order to check its effect in rice, the plasma membrane Na^+/H^+ antiporter gene *OsSOS1,* cloned downstream of the *CaMV35S* promoter (Razzaque et al. 2014), was overexpressed in the high yielding variety BRRI dhan28 in the current study. Being constitutive in nature, the *CaMV35S* promoter expresses the downstream transgenes in all organs and at all the developmental stages. However, high yielding rice varieties are non-responsive to tissue culture methods. So, in the current study, a tissue culture independent transformation method (*in planta)* has been applied to transform farmer popular high yielding but salt-sensitive BRRI dhan28 rice in order to enable higher transformation efficiency (Lin et al. 2009). Since BRRI dhan28 is "farmer popular" due to its high yielding character, conferring salt tolerance to it through the overexpression of this gene may generate a salt tolerant high yielding cultivar.

Materials and Methods

The *OsSOS1* gene in the clone (Fig. 1) used for transformation was obtained from the rice landrace Pokkali as described in detail in Razzaque et al. 2014.

Briefly, the complete cDNA from Pokkali RNA using gene specific primers was prepared using the RT-superscript kit from Invitrogen and cloned into the directional pENTR vector. The direction was ensured by adding CACC in the 5' position to the forward primer. Positive clones confirmed by sequencing were then inserted into the destination vector, *pH7WG2* by LR recombinase following the manufacturer's instructions (In Vitrogen).

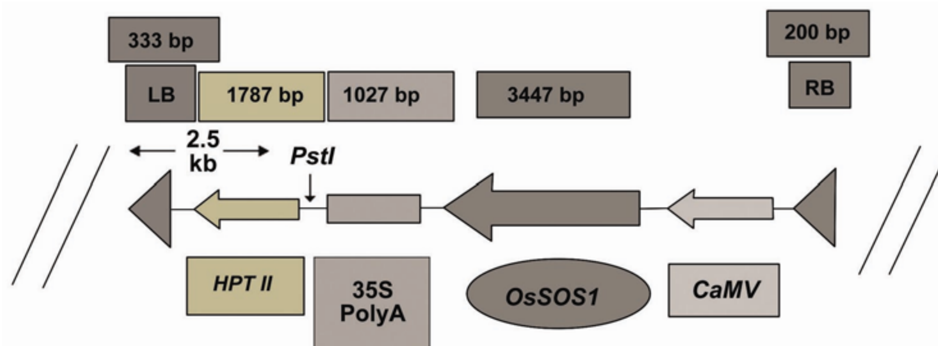

Fig. 1. T-DNA border of *pH7WG2.0_CaMV-OsSOS1* construct.

Transformed *Agrobacterium* strain (LBA4404) containing *pH7WG2.0_CaMV-OsSOS1* constructs was cultured and prepared for transformation following the standard protocol described in Lin et al. (2009) with some modifications. Densely grown bacterial cells were first inoculated into liquid YM (Yeast extract mannitol broth) (Boumahdi et al. 2001) medium and incubated at 28°C overnight, then centrifuged and re-suspended into the liquid AB medium (sucrose, glucose, AB buffer and AB salt) (Hiei et al. 2008). For improving the transformation efficiency, acetosyringone was added to both liquid YM medium and bacterial re-suspension medium (Parvin et al. 2015). *Agrobacterium* was selected using streptomycin (20 mg/l) and spectinomycin (20 mg/l). Finally, bacterial density was measured at OD600 and held at 0.6 (Lin et al. 2009, Parvin, et al. 2015).

Following the protocols described by (Amin et al. 2012), mature seeds of BRRI dhan28 were sterilized. For *Agrobacterium* inoculation, two-day-old germinated seeds were used.

During transformation, the embryonic apical meristem of approximately 40 germinated seeds of BR28 was infected with *Agrobacterium* containing the *pH7WG2.0_CaMV-OsSOS1* construct as described in detail in Lin et al. (2009). Briefly, two-day-old germinating seedlings were inoculated with *Agrobacterium* solution near the plumule with a 0.5 mm injection needle using a 45^0 angle for piercing. These seeds were then vacuum infiltrated while immersed in the *Agrobacterium* inoculum solution. Infected seeds were transferred onto Petri dishes containing wet filter paper and incubated in the dark at 28°C for 6 - 7 days. Then, the seedlings were treated with carbenicillin solution (250 mg/l) for 1 h to kill and remove the remnants of *Agrobacterium*. After that, seedlings were washed well with ddH_2O and transferred to new Petri dishes containing wet

filter paper. Seedlings were then kept in light for 16 hrs and in dark for 8 hrs at 28°C. Seedlings were subsequently transferred to hydroponic solution (Yoshida et al. 1976) when they appeared green and healthy. After 2 - 3 days, the hydroponic pots were transferred to the net house (confined area with cemented floor for growing the rice plants in pots, completely covered in netted mesh, to keep out animals and insects. There is no other rice growing near the vicinity). Mature seedlings of the T_0 line (18 - 21-days-old) were transferred to soil and allowed to pollinate naturally to set seeds (T_1) (Parvin, et al. 2015).

Plants that showed positive results in both leaf disk senescence (LDS) assay and PCR analysis were chosen for generation advancement to T_1. T_1 seeds were sown from 4 panicles whose flag leaves showed good score after senescence tests (P-1-1, P-1-2, P-1-3 and P-2-3).

When the plants were mature, leaf disk senescence (LDS) assays were performed. The flag leaf was taken from T_0 transgenic lines and the 2nd leaf was taken for T_1 transgenic lines for this assay because these are the likely ones transformed according to the original protocol (Lin et al. 2009). Leaf squares of ~1.0 × 1.0 cm were excised from healthy and fully expanded rice leaves of similar age both from the transgenic lines and wild type plants. The disks were floated in a 20 ml solution of NaCl (150 mM or 15 dS/m) for T_0 transgenic lines and 0, 100, 200 mM NaCl (10 and 20 dS/m) for T_1 (the lower concentrations for T_0 transgenic lines was because the transgene is hemizygous) for 3 days and then scored for damage. The treatment was carried out at 25°C. The analysis was carried out with three biological replicates in three independent experiments.

For the evaluation of the sensitivity of seed germination to NaCl, germination tests were conducted using ½ strength MS containing 0, 100 and 200 mM NaCl. The half strength of MS was prepared using macro, micro, organic and Fe-EDTA solutions, and the desired volume was adjusted using ddH_2O. Finally, pH was adjusted to 5.6. Seeds were kept at 50°C for 3 to 4 days to break dormancy followed by placement at room temperature for a few days. After the seeds were dehusked and washed, they were transferred to test MS for 4 to 5 days for germination. ANOVA and Duncan test were performed for the statistical analysis.

For chlorophyll determination, leaf disks of both wild type and transgenic BR28 used in the LDS assays were weighed and kept in a bottle containing 12 ml of 80% acetone. Absorbance of leaf tissue extracts was measured at wavelength 663 and 645 nm after 48 h; 80% acetone was used as blank during measurement. The total amount of chlorophyll was calculated following the protocol by Yoshida et al. (1976) and Chutia and Borah (2012). The chlorophyll content was calculated in the diluted sample using the following equation.

$A = ECd$

A is proportional to C (because E and d is constant)

Here,

A = Observed absorbance

E = A proportionality constant (extinction coefficient) (= 36 ml/cm)

C = Chlorophyll concentration (mg/ml)

d = Distance of the light path (= 1 cm)

Reduction of chlorophyll content was determined using following formula:

Chlorophyll reduction = (Control-stress)/control x 100

For the statistical analysis ANOVA and DMRT were done.

Seedling stage phenotypic screening of T_1 generation transgenic lines was performed. This included the wild type BRRI dhan28 and transgenic lines under 150 mM NaCl stress in hydroponic solution for 15 days until the sensitive controls were dead (Gregorio et al. 1997).

For electrolyte leakage estimates, leaf segments from the seedlings were placed into a bottle containing deionized water and kept in a shaker for 2 hrs. The conductivities (C1) of the obtained solutions were then determined by an electrical conductivity (EC) meter. Subsequently, the leaf segments in deionized water were autoclaved. After being thoroughly cooled to room temperature, the conductivities (C2) of the resulting solutions were determined by EC meter again. The values of C1 to C2 (C1/C2) were calculated and used to evaluate the relative electrolyte leakage by using the following formula:

Relative electrolyte leakage = (C2 – C1)/C1× 100%

Each data point represents average from three independent experiments. The data were subjected to statistical analysis using the *t* test.

Isolation of plant DNA was done by the CTAB (Cetyl trimethylammonium bromide) method (Doyle 1987). An *OsSOS1*-specific primer was used for DNA amplification by the polymerase chain reaction (PCR) to confirm the transformation of the high yielding rice variety BR28 with the *OsSOS1* gene (*OsSOS1* F: 5′-TAAGCAGCAGGCATTCATTG-3′ and *OsSOS1*_R: 5′-GAAGGC ACTCTTGGGTACGA -3′). PCR was carried out with final concentration of 0.15 ng of DNA sample, 0.38% DMSO, 1× PCR buffer, 10 mM dNTPs, 0.4 mM $MgCl_2$, 0.6 ng/µl forward and reverse primers and 1.5 µl Taq DNA polymerase. The reaction was performed in a thermocycler (Eppendorf Master cycler nexus gradient) at 95ºC or 5 min denaturation, followed by 35 cycles of 1 min denaturation at 95ºC, 1 min annealing at 62.8ºC and 1.5 min extension at 72ºC , then a final extension of 10 min at 72ºC. Positive controls included plasmid DNA and negative controls used water instead of DNA.

Survival of the T_1 seedlings was used as an indication of transgene insertion into T_0 and its segregation into the T_1 generation was determined using the Chi square test. Analysis was done using following formula:

The p value is the probability that the deviation of the observed from that expected is due to chance alone (no other forces acting)

P value = $[0.5^{df/2} / \Gamma(df/2)] \times (\chi^2)^{(df/2)-1} \times e^{-\chi^2/2}$

Total RNA was extracted from the shoots of mature T_1 BRRI transgenic and wild-type BRRI dhan28 rice using the Trizol reagent (Ambion, Invitrogen) following the manufacturer's protocol. From 1.5 μg of total RNA, first-strand cDNA was synthesized using the Invitrogen Superscript III reverse transcription (RT)-PCR system according to the manufacturer's protocol (Invitrogen, USA). Elongation Factor-a (EF-a) was used as the normalization control. The1D-Multi tool of the Alpha Ease FC imaging system was used to graphically represent the intensity of the bands in the gel.

For the Southern, genomic DNA (20 micrograms) from both wild-type BRRI dhan28 and PCR positive T_1 plant of BRRI dhan28 rice line was digested with the *Pst*I restriction enzyme, because it cuts within the T-DNA region once (Fig. 1). The digested product was electrophoresed and blotted onto a positively charged nylon membrane (Hybond N^+ membrane, Amersham, UK) and probed using DIG-labeled PCR-amplified product (*hpt* 809 bp) from *hpt* gene following the standard protocol (Roche Diagnostics Inc., Mannheim, Germany). After restriction digestion of the *pH7WG2.0_CaMV-OsSOS1* construct with *Pst*I, the expected band size is 2.5 kb or larger, depending on the cutting position outside the T-DNA (Fig. 1). The number of bands above 2.5 kb would indicate the copy number of transgenes integrated into the genome.

RNA was isolated from wild type and both T_1 seedlings of BRRI dhan28 transgenics under both 0 and 150 mM NaCl stress for 24 hrs. Quantitative Real-time PCR (qPCR) was performed in a 10 μl reaction using SYBR Green (Bio-Rad, USA) with *OsSOS1* internal primers in a CFX96 TM Real-Time PCR detection system (Bio-Rad, USA). PCR efficiency (95 - 105%) was verified. Amplification specificity was validated by melt curve analysis at the end of each PCR cycle. Relative transcript abundance was calculated using the comparative cycle threshold method described by Chen et al. (Chen et al. 2014). Elongation Factor-α (EF- α) was used as the normalization control.

Results and Discussion

The presence of the transgene and its effect was confirmed by leaf disk senescence (LDS) under 150 mM salt stress. During the assay, flag leaf pieces of

non-inoculated BRRI dhan28 plant (control) showed dark brown stripes and necrosis after 7 days (Fig. 2a), whereas the flag leaf of 16 plants out of the 72 infected BRRI dhan28 remained largely green and healthy (Fig. 2b). These 16 plants were assumed to be putatively transformed and used for further analysis.

Wild type BRRI dhan28 (Non-transformed)

Transformed BRRI dhan28 with *pH7WG2.0_CaMV-OsSOS1*

(a) (b)

Fig. 2. Leaf disk senescence assay of flag leaves from T_0 transformants. Transformed plant leaf (BRRI dhan28-transformed) showed healthier and greener appearance (b) compared to control (BRRI dhan28-control, a). Control leaf (BRRI dhan28-control) showed necrosis and dark-brown stripes in 150 mM sodium chloride (NaCl) solution after 3 days.

The transformation efficiency for *indica* rice varieties as reported earlier was only about 6.0 % (Lin, et al. 2009). Due to some modifications of this published method our transformation efficiency was enhanced from the reported 6 to 22% (Table 1). Modifications included the use of acetosyringone in the bacterial culture, and the use of separate infection as well as co-cultivation media. Liquid culture medium was also used to grow the bacteria. Furthermore, instead of the half strength MS used by Lin et al. (2009), the bacteria were centrifuged and re-suspended in bacterial re-suspension media. In centrifugation and subsequent resuspension, double washes in the media ensured removal of trace amount of antibiotic from the *Agrobacterium* used for infection and subsequent co-cultivation.

Table 1. Transformation efficiency of *in planta* transformation at T_0 generation (based on LDS assay).

Gene constructs used for infection	Variety	No. of the germinated seedlings after infection (a)	No. of salt stress positive plants with positive flag leaves (b)	Transformation efficiency (b/a × 100)
pH7WG2.0_CaMV-OsSOS1	BRRI dhan28	72	16	22.22%

Putatively transformed plants which were identified from leaf disk senescence assay were tested for the presence of the *OsSOS1* using gene-specific PCR gene. Desired size bands of *OsSOS1* (849 bp) indicated positively transformed rice (Fig. 3a). Non-transformed BRRI dhan28 was used as a control where no band was found.

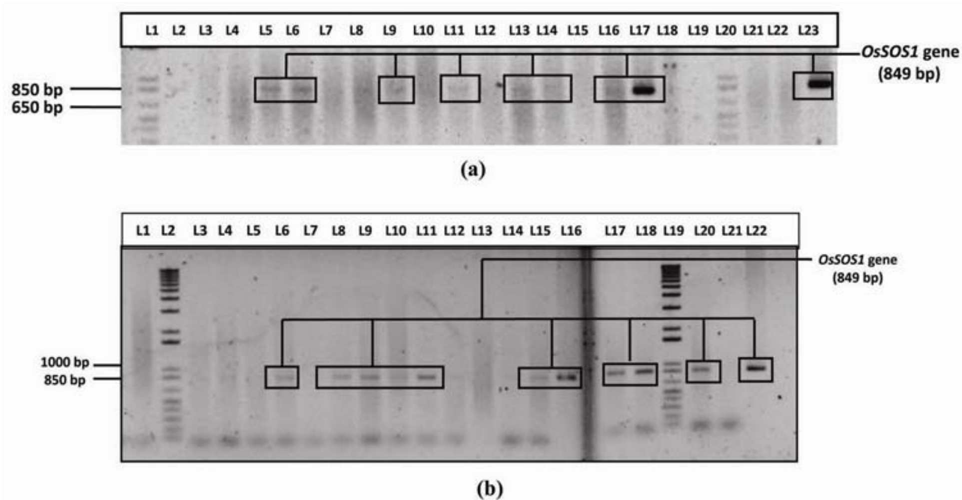

Fig. 3. PCR analysis at T_0 and T_1generation. Detection of *OsSOS1* gene at T_0 and T_1 for transformed plant with *pH7WG2_CaMV-OsSOS1* construct. **a** L1, L19: 1 kb+ DNA ladder, L2: negative control (WT), L3: non-transformed BRRI dhan28; L5, L6, L9, L11, L13, L14, L16: positive transformants; L4, L7, L8, L10, L12, L15, L18, L20, L21: untransformed BR28; L17, L22: positive control. **b** L1: negative control (WT), L2: 1 kb+ DNA ladder, L3: un-transformed BRRI dhan28; L6, L8, L9, L10, L11, L15, L17, L18, L20: positive transformants; L4, L5, L7, L12, L13, L14, L21: un-transformed BRRI dhan28; L16, L22: positive control. The transformed samples showed the correct band size (849 bp).

T_1 transgenic plants were further confirmed by PCR using the *OsSOS1* specific primer. Non-transformed BRRI dhan28 was used as a control. Desired band size (809 bp) for the *SOS1* gene was found in transformed plants (Fig. 3b).

Genetic segregation of *CaMV-OsSOS1* transformed plants was calculated based on the LDS assay at the seedling stage of T_1 (Table 2). Two transformants were found to follow the Mendelian law for inheritance of the transgene. These were subjected to further tests.

Table 2. Segregation analysis of transgenic (resistant) and non transgenic (susceptible) seedlings in the T_1 plants.

Variety	Name of plants showing salt stress positive at T_0 generation	Number of resistant seedlings (T_1)	Number of susceptible seedlings (T_1)	χ^2 value	P-value
BRRI dhan28	P-1-1	6	3	0.333	0.564*
	P-1-2	2	6	10.67	0.001
	P-1-3	9	4	0.231	0.631*
	P-2-3	3	6	8.333	0.004

* Followed the Mendelian law of inheritance (3 : 1).

Positively transformed plants (T_1) showed better performance than the non-transformed BRRI dhan28. Their leaves remained green whereas leaves from non-transformed plants showed necrosis and decolorization (Fig. 4a).

Leaves used in the leaf disk senescence assay were further used for the measurement of chlorophyll content. Chlorophyll content was significantly higher ($p < 0.05$) in transgenic lines compared to wild-type BRRI dhan28 after NaCl stress at 100 and 200 mM (Fig. 4b). The chlorophyll content in wild-type BRRI dhan28 and all transgenic plants at the seedling stage without stress was almost similar ($p < 0.05$). Higher chlorophyll content indicates better performance of the plants under salinity stress.

For germination tests, salinity stress for 4 days at 100 and 200 mM NaCl, showed transformed plants (T_1) to have higher tolerance, better germination rate and lower shoot length reduction compared to wild type. But under normal condition (0 mM NaCl), WT BRRI dhan28 and transformants had similar patterns of germination rate and shoot length (Fig. 4c, d).

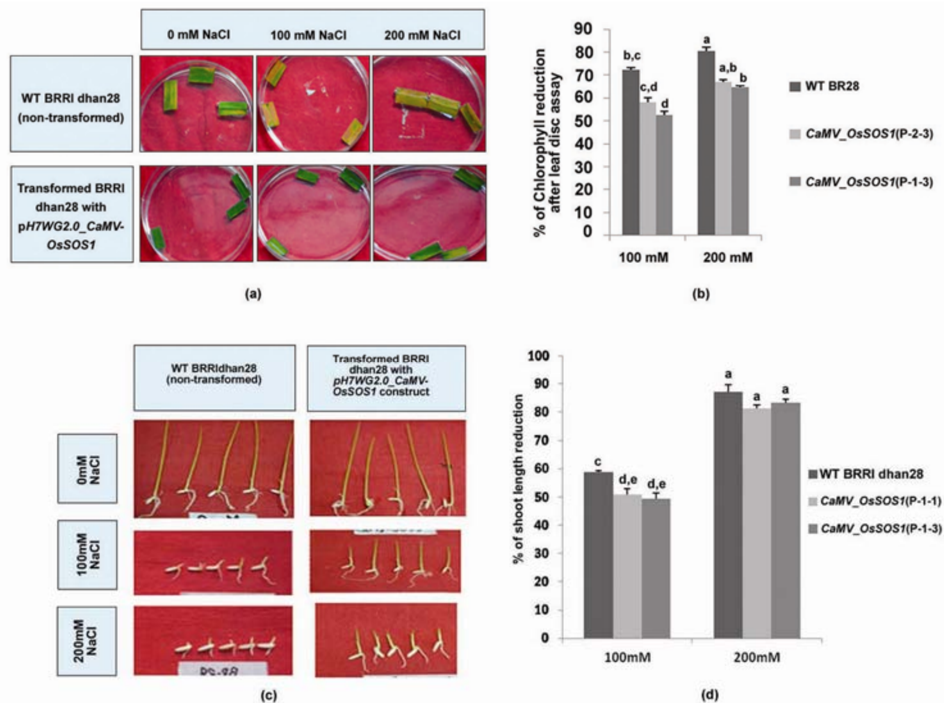

Fig. 4. Physiological screening at T_1 generation. (a) Leaf disk senescence (LDS) assay: Transformed plants remained green even at 200 mM of salt stress (NaCl) (lower two panels), while WT showed necrosis and decolorization after 100 and 200 mM salinity (NaCl) (top panel) stress. (b) Chlorophyll reduction: The leaf disks of transgenic lines continued to show better chlorophyll contents after salinity (NaCl) stress at 100 and 200 mM. Each bar represents the mean ±SE (n = 4). (c) and (d) Germination performance, growth and percent of shoot length reduction of the WT BRRI dhan28 and transgenic lines on MS containing 0, 100 and 200 mM sodium chloride (NaCl) solution. Each bar represents the mean ±SE (n = 4). Different letters in each graph (a-d) indicate significant differences ($p < 0.05$, ANOVA and DMRT).

Physiological screening of 2-week-old T_1 seedlings of BRRI dhan28 transformants showed that they remained green and healthy in hydroponic solution even after 10 days of treatment (150 mM NaCl stress). But non-transformed wild-type BRRI dhan28 plants turned yellow, then brown. Shoot length, root length and fresh weight of the T_1 transgenic line containing *-OsSOS1* gene was higher than the non-transformed WT BRRI dhan28 under stress condition (Fig. 4e, f).

A low percentage of electrolyte leakage was recorded in all plants with no significant difference (Fig. 4) between transgenic and wild-type plants under non-stress conditions (Fig. 4f). The percentage of EL in transgenic T_1 plants was, however, significantly lower (Fig. 4f) compared to wild-type plant BRRI dhan28 after 150 mM salinity treatment for 10 days. Lower electrolyte leakage indicates lower damage of the plants under salinity stress.

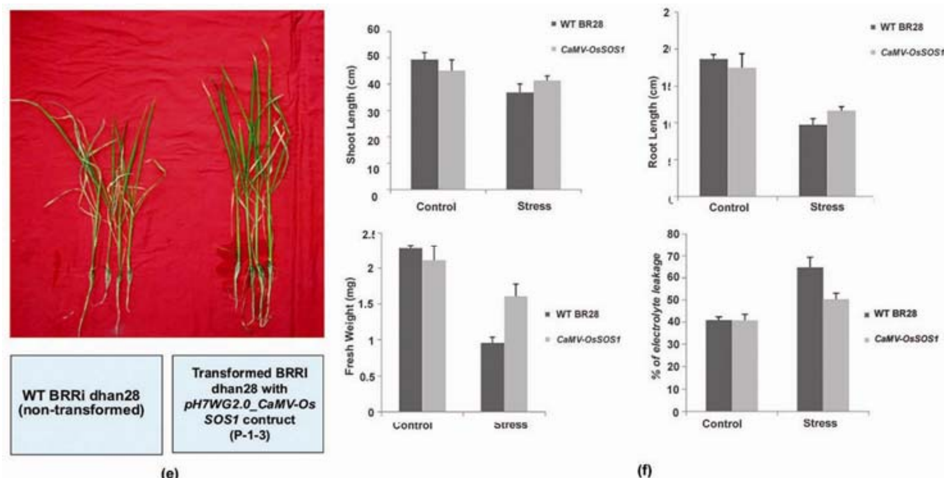

Fig. 4. Physiological screening at T_1 generation. (e) Phenology of the WT BRRI dhan28 and transgenic lines after 150 mM NaCl stress in hydroponics for 14 days (From left to right: WT BRRI dhan28, transgenic line). (f) Shoot length and root length (in cm), and fresh weight of the leaves (in mg) was significantly higher in transgenic rice seedlings in than wild-type after NaCl stress at 150 mM in hydroponics. Percentage of electrolyte leakage was significantly ($P<0.05$) higher in transgenic rice seedlings than wild-type after NaCl stress at 150 mM in hydroponics.

To determine the highest level of constitutive overexpression of the *OsSOS1* gene, RNA was isolated from the four transgenic plants (P-1-1, P-1-2, P-1-3, and P-2-3) at T_1 generation and semi-quantitative RT (reverse transcriptase) PCR was performed with *SOS1* internal primers.

For the optimization of cDNA for all the four plants, a PCR reaction was conducted using primers specific for eEF-1α (eukaryotic elongation factor -1α). Approximately the same level of expression of the housekeeping gene eEF-1α (eukaryotic elongation factor -1α) was found for all the four plants (Fig. 5a)

Semi-quantitative RT (reverse transcriptase) PCR at 28 cycles and 34 cycles was done using *OsSOS1* specific primer. The desired size of 849 bp bands was found. Intensity of the bands was measured using the 1D-Multi tool of Alpha Ease FC imaging system. Among all the four plants, intensity of the band for plant P-1-3 was higher than the plants P-1-1, P-1-2, and P-2-3. Thus among the four plants, plant 1-1-3 had the highest level of constitutive overexpression for *OsSOS1*gene (Fig. 5b, c).

To confirm the stable integration of the transgene, Southern hybridization was done from the pooled DNA of transformed BRRI dhan28 for the T_1 positive transgenic lines. Two copies of transgene were observed for transformed plant (P-1-3) (Fig. 6). Although instability of transgene expression in plants is often associated with multiple copies of transgenes being integrated at the same locus (Yao et al. 2006), insertion of 2 genes is considered to be acceptable (Hu et al.

2006). The double copies of the transgene actually showed considerably better salinity tolerance compared to the non-transformed WT BRRI dhan28.

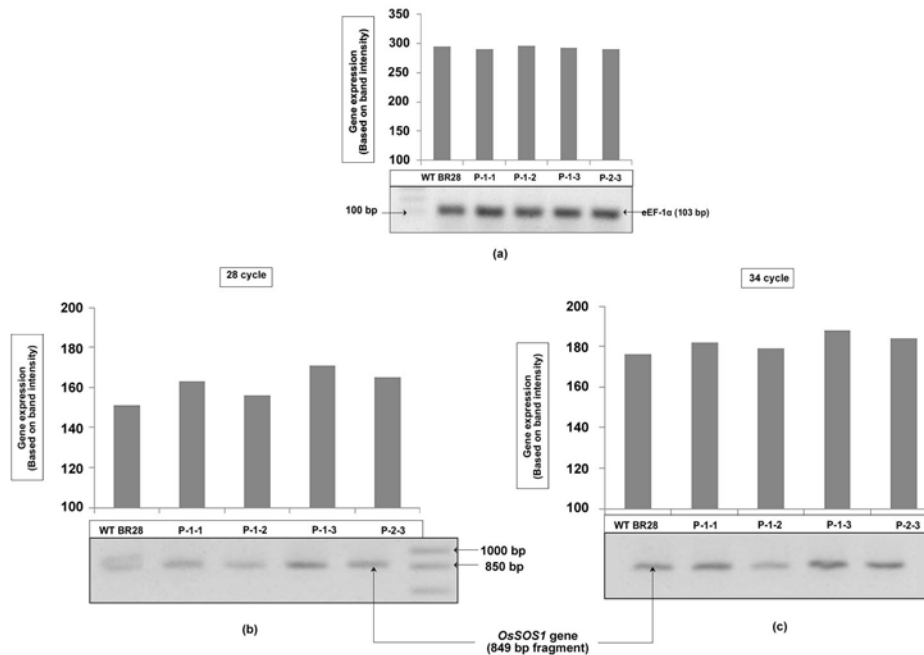

Fig. 5. Expression analysis of *OsSOS1* gene under *CaMV35S* promoter in wild type and T_1 BRRI dhan28 transformed plants by semi-quantitative RT (reverse transcriptase) PCR. (a) PCR amplification of housekeeping gene eEF-1α in wild type and transformed BRRI dhan28 plants. L1: $1Kb^+$ ladder, L2: non-transformed BRRI dhan28, L3: plant-1-1, L4: plant-1-2, L5: plant-1-3, L6: plant-2-3. (b, c) PCR amplification of *OsSOS1* gene in transformed BRRI dhan28 plants. L1: non-transformed BRRI dhan28, L2: plant-1-1, L3: plant-1-2, L4: plant-1-3, L5: plant-2-3; (b) L1: $1Kb^+$ DNA ladder. (a, b, c) Also show graphical representation of the expression level of *eEF-1α* gene and *CaMV-OsSOS1* gene in transformed BRRI dhan28 plants. (a) Expression level *eEF-1α* gene was almost same in all the four plants. At 34 cycles, (c), highest level of *OsSOS1* gene expression is observed in P-1-3 transformed line .

The same plant was shown to follow 3 : 1 Mendelian segregation of the transgene. Although two copy numbers of the gene were observed, these must have been integrated close to each other to show inheritance in the 3 : 1 ratio.

Significant results from this study can be applied to the development of high yielding salt tolerant rice varieties. BRRI has already invented moderately salt tolerant rice varieties (BR40, BR41, BRRI dhan47, BRRI dhan53, BRRI dhan54, BRRI dhan55 and BRRI dhan61), which can withstand moderate level of salinity stress (60 to 140 mM) at seedling stage (Rice knowledge bank, Bangladesh Rice

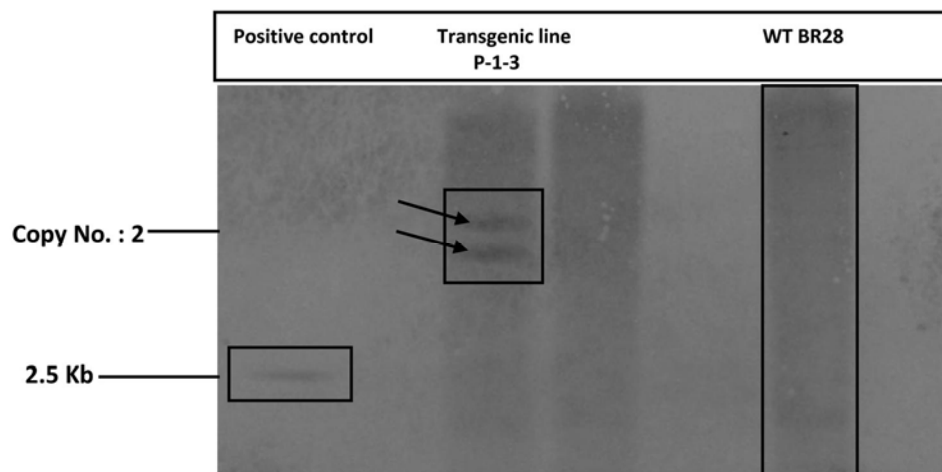

Fig. 6. Southern blot hybridization for transformed BRRI dhan28 containing *CaMV-OsSOS1* construct. L1: positive control, L2: transformed BRRI dhan28 and L5: non-transformed BRRI dhan28. Arrows indicate copy number of the transgene *OsSOS1* insertion in the T_1 transgenic line.

Research Institute). But the present study showed an improved salinity tolerance level of 150 to 200 mM in the high yielding but salt sensitive BRRI dhan28. In the future, comparative studies could be done at reproductive stage to further analyze the effect of *OsSOS1* gene overexpression with respect to constitutive and inducible promoters. Simultaneous overexpression of the plasma membrane and vacuolar Na^+/H^+ antiporter encoding genes might have a cumulative and hence more enhanced effect compared to currently cultivated lines.

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