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Transformation of *SNAC*1 under Stress Inducible Promoter rd29A Confers High Yield and Stress Tolerance in Rice

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Keywords: High yielding rice variety, *in planta* transformation, Transcription factor *SNAC*1, stress inducible promoter rd29A.

Abstract

Abiotic stress tolerance in plants is often induced by activation of transcription factors. An increased stress tolerance was observed in *indica* rice cultivar BRRI dhan55 after transforming with the transcription factor *SNAC*1 (stress responsive *NAC*1) under stress inducible promoter rd29A that minimized unexpected phenotype and metabolic burden in transgenic lines. The lines were produced by tissue culture independent *Agrobacterium*-mediated *in planta* transformation. Molecular analyses confirmed the successful integration of the *SNAC*1 gene and significantly higher gene expression level in the transgenic lines. The transgenic lines showed 3:1 segregation ratio at T_2 generation following the Mendelian law of inheritance. Assays for leaf disk senescence and chlorophyll content at 100 mM and 200 mM salt and survival rates at 200 mM salt and drought conditions at 12 days of water withdrawal and recovery showed significantly increased stress tolerance in the transgenic lines at seedling stage compared to the wild type. Enhanced yield, spikelet fertility, and 1000-grain weight were observed at the reproductive stage compared to wild type under both salinity and drought stress conditions. Thus, *SNAC*1 expression in plants under inducible promoter is a better choice to enhance stress tolerance and yield under salinity and drought conditions.

Introduction

Drought and salinity are two major abiotic stresses which limit crop productivity. Plant adaptation to these environmental stresses depends on the activation of cascades of molecular networks like stress perception, signal transduction and the expression of

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specific stress-related genes and metabolites (Oh et al. 2009, Tang et al. 2019). Plants show tolerance to abiotic stress through activating regulatory genes like transcription factors. *SNAC*1 (Stress responsive NAC1), is one of the members of a large plant transcription factor family of NAC proteins. The NAC transcription factors are widely distributed in plants such as *Arabidopsis,* cotton, wheat, soya bean, and rice (Puranik et al. 2012). These proteins have been confirmed to have important roles in plant growth, development, yield and stress tolerance (Nakashima et al. 2012). *SNAC*1 has already been reported to increase salt and drought tolerance when overexpressed using the *CaMV*35S constitutive promoter in rice plants under field stress conditions compared to wild type in japonica rice (Hu et al. 2006). There was a report about better performance in *indica* rice seedlings (Parvin et al. 2015) but none of improved yield using a stress-inducible promoter.

In Bangladesh, drought and salinity are of great concern due to poor irrigation facilities and lack of infrastructure to store water in the monsoon season. Moreover, a huge area in the southern part of Bangladesh suffers from high salinity, particularly in the dry winter season. The northern and southwestern districts of the north-west region of Bangladesh are more drought prone (Kamruzzaman et al. 2019). So, salinity and drought are well-documented as a limiting factor in crop productivity. In this study farmer popular high yielding variety BRRI dhan55 which is moderately drought tolerant (source: Bangladesh Rice knowledge Bank), was selected for transformation with the *SNAC*1 gene. Farmers will be benefitted if popular commercial rice varieties become more stress tolerant. High yielding *indica* rice varieties are less tissue culture responsive having poor regeneration potency after transformation (Kumar et al. 2005, Nishimura et al. 2006). Hence, *Agrobacterium* mediated *in planta* transformation (Supartana et al. 2005, Lin et al. 2009, Ahmed et al. 2018) method was used which is easier and less time-consuming. With some modification in *in planta* transformation method (Parvin et al. 2015), BRRI dhan55 was successfully transformed with the *SNAC*1 gene.

In *Arabidopsis*, rd29A (responsive to desiccation), is a stress-responsive downstream gene in the ABA signalling pathway and induced under abiotic stress. As both DRE and ABRE elements are found in the rd29A promoter region, the gene is readily induced by high salinity, dehydration, high and low temperatures. It was reported that minimal negative effect on plant growth was found when DREB1A expression was driven by the stress-inducible rd29A promoter and showed greater tolerance, as compared with the *CaMV*35S promoter (Kasuga et al. 1999). Use of the rd29A promoter in rice also showed enhanced yield in transgenic plants (Datta et al. 2012). In transgenic tomato (*Solanum lycopersicum*), the *miR399d* gene, the IPT (isopentyl transferase) gene in transgenic tobacco and the *AtCBF1-3* gene in potato (*S. tuberosum*) were transformed under rd29A promoter (Pino et al. 2007, Qiu et al. 2012, Gao et al. 2015) showing improved stress tolerance without adverse effect on growth and development, and any negative effects on yield. The rd29A promoter from *Arabidopsis* was cloned and characterised in *indica* rice and showed increased expression in leaves in an inducible manner (Sarker et al. 2016). In this

study the rd29A promoter was used as a stress inducible promoter for transgene expression.

Methods and Materials

Plant materials and vectors: BRRI dhan55 is an Aus and Boro season variety with white, long and slender grain. It has an early maturation of about 105 days and possesses yields of about 5.0 M. ton/hectare. In the Boro season, its life span is 145 days with high yield of about 7.0 M. ton/hectare. It is reported to be moderately drought tolerant (source: Bangladesh Rice knowledge Bank).

The pH7WG2 is a binary destination vector of GATEWAY™ system for easy insertion of genes in plants through transformation. The stress inducible promoter rd29A (597bp) was isolated from *Arabidopsis* and inserted into pH7WG2 binary vector by removing the constitutive promoter *CaMV*35S from the vector, producing a new rd29A_pH7WG2 binary vector. The coding region of the *SNAC*1 gene (1051bp) was isolated from salt tolerant variety *O. sativa* cultivar Pokkali and first cloned into pENTR vector (Parvin et al. 2015). After LR recombination *SNAC*1 gene was transferred to pH7WG2 downstream of rd29A promoter, producing pH7WG2_rd29A_*SNAC*1 vector (Fig. 1).

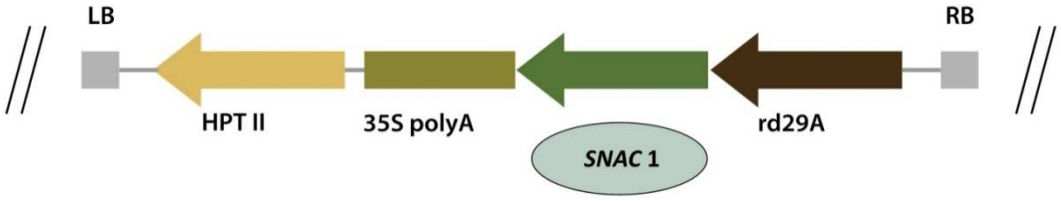

Fig. 1. T-DNA border of pH7WG2_rd29A_*SNAC*1 construct.

In planta transformation: Agrobacterium tumefaciens strain LBA4404 containing pH7WG2_rd29A_SNAC1 construct was used for plant transformation. In this study, the modified procedure of *in planta* transformation method (Parvin et al. 2015, Ahmed et al. 2018) was used. *Agrobacterium* inoculum was prepared in bacterial resuspension media with the final absorbance of 0.6 at OD_{600}. Mature seeds were treated with 99% ethanol followed by 30% chlorox with 1 drop of tween 20 and washed thoroughly. Two days after incubation at 37°C the embryo region of the seeds turned white. *Agrobacterium tumefaciens* was then inoculated into the embryonic apical meristem of the soaked seed by piercing to a depth of 1-1.5 mm with a needle that was dipped in the bacterial solution. Vacuum infiltration of the pierced seeds was done while being soaked in the *Agrobacterium* inoculum suspension in a reagent bottle. The inoculated seeds were transferred onto Petri dishes with wet filter paper and kept in the dark for 6-7 days at 28°C temperature. After 7 days the seedlings were treated with 250 mg/l carbenicillin

solution. The seedlings were washed with ddH_20 and kept in light and dark for 16 and 8 hours, respectively. When the seedlings turn green after 5 days, they were transferred to hydroponic solution in the net house, where the temperature were maintained between 30-32°C in the day and 24-25°C during the night, with humidity around 72%. After another 7-10 days, the seedlings were mature enough to transfer to soil.

Hygromycin resistance assay: The flag leaf pieces from both wild type and T_0 putative transformed plants were taken and placed in the hygromycin solution (50 mg/l). Leaf disk from wild type plants and non-transformed flag leaf began to show necrosis and dark-brown spots, but transformed flag leaf pieces remain green (Parvin et al. 2015).

Molecular analysis of transformants: The pH7WG2_*rd29A*_*SNAC*1 construct contains both the rd29A promoter region and *SNAC*1 gene. Primers for these genes were used for DNA amplification by standard PCR reactions to confirm the transformation. PCR program was carried out as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 1.30 min at respective annealing temperature and 1.30 min at 72°C, then a final extension of 10 min at 72°C.

For Southern blot hybridization, genomic DNA (20 µg) from both wild-type and transgenic plants of T_2 generation was digested with B*amH*I restriction enzyme. The digested products were electrophoresed and blotted onto a positively charged nylon membrane (Hybond N+ membrane, Amersham, UK) and probed using DIG-labelled PCR-amplified product from *SNAC*1 gene following DIG Luminescent Detection Kit standard protocol (Roche Diagnostics Inc., Mannheim, Germany). Expression analysis of transgenic lines was done by real time (qRT) PCR. Total RNA was extracted from the transgenic lines and wild type BRRI dhan55 at T_2 generation. The Trizol method was used for RNA isolation. From 1.5 µg of total RNA, first-strand cDNA was synthesized using the Thermo Scientific Revert Aid H Minus First Stand cDNA synthesis kit following the manufacturer's protocol. Quantitative Real-time PCR was performed in a 15 µl reaction using Power SYBR® Green PCR Master Mix of Applied biosystems with *SNAC*1 internal primers in Applied Biosystems 7500 Fast Real-Time PCR System**.** Elongation Factor-α (EF-α) was used as the normalization control. Relative transcript abundance was calculated using the comparative cycle threshold method described by (Chen et al. 2014).

Leaf disk senescence (LDS) assay and chlorophyll content measurement: At T_2 generation, the leaf disks were taken from both transgenic lines and wild type plants of approximately the same size (~1.0 cm). The disks were floated in a Petri-dish with 20ml solution of NaCl with 100 mM or 200 mM salt concentration or water (for control) for 5-7 days and the temperature were maintained at 25°C (Yasmin et al. 2016). Three independent experiments were done with three biological replicates. Chlorophyll contents were measured from the leaf disks of both wild type and transgenic lines used in leaf disk senescence (LDS) assay. The samples were weighed and kept in a bottle containing 12.5 ml of 80% acetone. After 48 hrs, chlorophyll a and chlorophyll b were measured at wavelength 663 and 645 nm, respectively by spectrophotometer from leaf

tissue extract. The protocol mentioned by (Yoshida et al. 1971) and (Chutia et al. 2012) was used to calculate the total amount of chlorophyll.

Measurement of relative electrolyte leakage: Relative electrolyte leakage was measured by using the protocol of (Yasmin et al. 2016). The leaf pieces from the seedlings of transgenic and wild type plants were taken into deionized water and kept in shaker for 2 hours. After measuring the conductivities (C1) of the solutions, the leaf pieces were autoclaved. Again, the conductivities (C2) of the solutions were measured. The values of C1 to C2 (C1/C2) were calculated and used to estimate the relative electrolyte leakage. Results represent average from five replicates.

Survival assay in salt and drought condition: Survival assay in salinity and drought condition were done at T_3 generation in the year 2017. The germinated seeds of transgenic lines and wild type plants were grown in netted Styrofoam floater in PVC tray containing 10L Yoshida solution (Yoshida et al. 1971). At four-leaf stage (14–18 days from germination) of seedlings, NaCl stress was applied gradually starting from 6 dS/m to 20 dS/m at 24 h increments of 2 dS/m. After 7-10 days, when 90% of wild type plants about to die, the stress was stopped, and the floater were kept in Yoshida solution without salt for recovery. After one week of recovery, the number of survived plants were counted.

For survival assay in the drought condition both T_3 transgenic and wild type plants were grown in the same pot. The pot was filled with same weight of thoroughly mixed soil. 12 plants of each type were grown for two weeks (containing four leaves). Drought stress was applied by withholding water for 12 days until wild type plants looked almost dead. The plants were then re-watered for 14 days. After recovery, the survived wild type and transgenic plants were counted (Hu et al. 2006). Both the above experiments on salinity and drought were repeated three times.

Seedling stage stress tolerance for salinity and drought: The phenotypic screening for salinity tolerance at seedling stage was done on T_3 transgenic lines and wild type plants. Pokkali and IR29 rice varieties were used as salt tolerant and salt sensitive control, respectively in the screening experiment. Germinated seeds were sown in netted Styrofoam and floated in PVC trays containing 10L Yoshida solution (Yoshida et al. 1971). At four-leaf stage (14-18 days from germination) of the seedlings, NaCl stress was applied gradually from 6 dS/m to 12 dS/m in increments of 2 dS/m every 24h. Two trays were used as control without salt stress. After 8-10 days, when 90% of IR29 (sensitive control) were almost dead, tolerance-related traits (Standard Evaluation System (SES) score, root length, shoot length, shoot weight) were measured from all stressed and control plants. The level of salinity tolerance was calculated based on the percentage of leaf damage and score (Gregorio et al. 1997). The chlorophyll content and electrolyte leakage of the stressed and control transgenic plants as well as WT were measured at this stage (Amin et al. 2012).

For seedling stage drought screening, the T_3 transgenic lines were grown in the same pot with wild type. Ten of each type of plants were grown in the same pot until four leaf

of stage (about two weeks of age) (Fukao et al. 2011). Drought stress was applied by the total withdrawal of water for 8 days and then re-watering for 14 days. Fresh weight, shoot length, root length and chlorophyll content of both wild type and transgenic lines were measured after recovery (Hu et al. 2006).

Reproductive stage stress tolerance at salinity and drought condition: Two weeks old seedlings of T_4 transgenic lines and wild type were transferred into soil-filled perforated pots. Each pot contained a single plant. The pots were placed in bowls of water, with six pots in each bowl. Tolerant and sensitive controls (Pokkali and IR29), wild type and three transgenic lines were placed in each bowl. At 6-week of age, near booting stage, 8 dS/m NaCl in Yoshida solution was applied in bowls. Some bowls with water served as controls without stress. Water was kept at the same level by topping up throughout the experiment until harvest. When 80% grain were matured, some physiological parameters were recorded such as flowering date, plant height, number of total tillers, effective tiller per plant, panicle length and flag leaf length. At the end of reproductive stage screening, seeds were collected from transgenic and wild type plants as well as tolerant and sensitive control plants. Other yield-related traits such as spikelet per panicle, spikelet fertility, yield (g/plant) and 1000-grain weight were also measured.

For reproductive stage drought stress, two-week old T_4 transgenic lines and wild type plants were transferred into individual soil-filled pots of the same size, containing 8 kg of thoroughly mixed soil with fertilizer. After 30 days, at booting stage (panicle initiation stage) drought stress were individually applied (Yue et al. 2006) to each plant. Unplugging three holes at the bottom of the pot allowed the water drain out. During drought stress the leaves were rolled during day but opened at night. When all leaves of a stressed plant became fully rolled without opening at night (a point corresponding to the relative water content in the range of 72-75%), water was re-applied to the full capacity of the pot. The plants were allowed recovery for flowering and seed maturation (Xiao et al. 2009). All the phenotypic parameters and yield related traits were recorded after seed harvest.

Data analysis: Chi-square test (χ^2) was done for segregation analysis to examine the inheritance pattern of T_1 transformants. It is a statistical test performed to determine the difference between the observed and the expected data under the specific hypothesis (Yasmin et al. 2016). The F test was performed to verify equal variance of the independent set of samples and based on that results the student's t test was done (*, **, *** at $P<0.05$, $P<0.01$, $P<0.001$).

Results and Discussion

In planta Transformation: For plant transformation tissue culture is a commonly practiced technique. But not all plants response well in tissue culture. High yielding rice varieties showed lower regeneration potential in tissue culture in normal condition. After infection they become recalcitrant and did not regenerate at all (Kumar et al. 2005). In this

study *in planta* transformation technique (Supartana et al. 2005, Lin et al. 2009, Ahmed et al. 2018) with some modifications (Parvin et al. 2015) was used which is a simple and efficient transformation method for any rice variety. In this method a lot of plants need to be screened, but being genotype independent, any recalcitrant plant can be transformed. After transformation by *in planta* method, putative plants were grown in soil filled pot for maturation (Fig. 2).

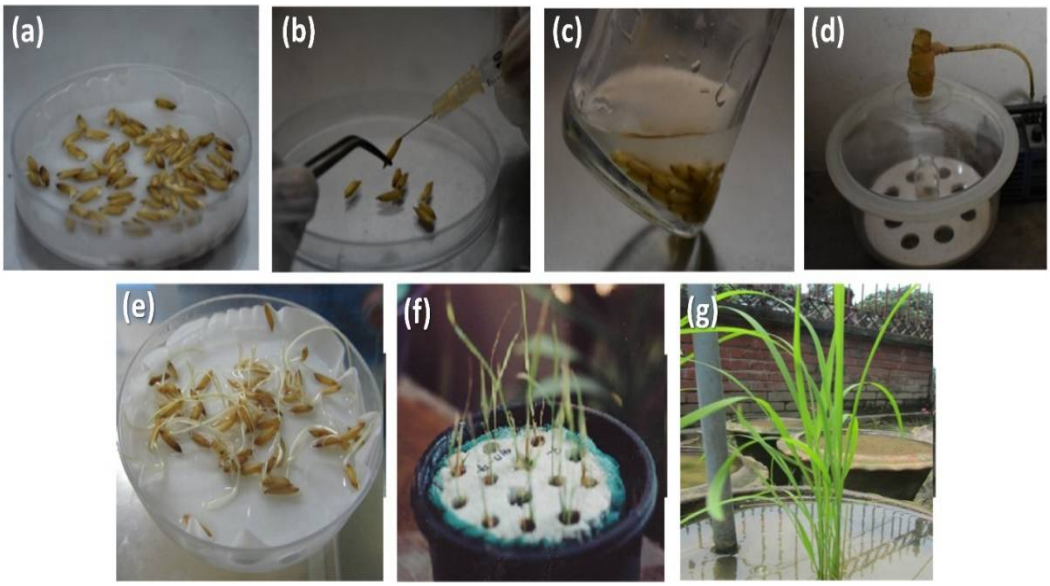

Fig. 2. Transformation of high yielding rice varieties through *in planta* transformation method. (a) Seeds soaked in water. (b) Inoculation of a seed with a needle. (c) The pierced seeds soaked in *Agrobacterium* inoculum. (d) vacuumed for infiltration. (e) Incubation of infected seeds. (f)Transfer to hydroponic solution. (g) Transformed plant in soil.

In this method, the T_0 plants might be chimeras. Hence, large number of plants were planted, and selection took place by checking flag leaves in T_0 generation by hygromycin resistance assay as well as in T_0 and T_1 generation by molecular analysis (PCR). Hygromycin resistance assay was done with flag leaf samples when the T_0 plants were mature, and grains were set. After confirmation, seeds (T_1) only from panicle of the positive flag leaf were collected. Transformation efficiencies were also calculated for transgenic lines. The pH7WG2_rd29A_*SNAC*1 construct contains hygromycin phosphotransferase (HPT) gene under the *CaMV*35S promoter in the T-DNA region. This gene was used as marker gene to ensure the transfer of transgene into transformed plants. Hygromycin phosphotransferase (HPT) gene helps transformed plants remain healthy even in presence of the antibiotic hygromycin. During hygromycin resistance analysis, leaf disk from wild type plants and non-transformed flag leaf began to show necrosis and dark-brown spots after 7 days (Fig. 3). Flag leaves which remained green

and healthy (hygromycin resistant) were primarily selected and T_1 seeds collected only from those flag leaf panicles.

Transformation efficiency of the in planta method: The transformation efficiency of *in planta* method for *indica* rice varieties was reported as 6.0 % (Lin et al. 2009). With some modifications such as adding acetosyringone during infection, transformation efficiency for high yielding *indica* rice varieties were enhanced up to 25 % (Parvin et al. 2015, Ahmed et al. 2018). Here, in three individual experiments, after hygromycin resistance assay 6, 4, 4 out of 30, 22, 23 respectively germinated seeds of BRRI dhan55 were found tolerant in hygromycin solution. The transformation efficiency was measured as 20%, 18% and 17% for these experiments (Table 2) which is close to the previously reported transformation efficiency of this method.

Molecular analysis of the transformants: Molecular analysis confirmed the T_1 transformants to be transgenic by distinguishing them from the wild type. PCR analysis was performed with rd29A promoter specific primers. Only transformed plants showed the rd29A-specific 597 bp band and were selected for generation advancement. No band was found in wild type plants and in water control (Fig. 4a). Among all positive plants three lines (line P4, P7, P8) were selected for further T_2 generation advancement. Since rd29A is a stress inducible promoter from *Arabidopsis*, this sequence served as a specific molecular marker for the transgenic lines.

Fig. 3. Hygromycin resistance assay of flag leaves of T_0 transformants. Transformants remained healthy and dark green (left) than the non-transformants which were light green with brown stripes (right) in 50 mg/l hygromycin solution.

At T_2 generation, Southern blot hybridization confirmed the stable integration of transgene in genomic DNA (Fig. 4b). *SNAC*1 gene specific, intron spanning, PCR amplified product was used as probe which should bind only with the transgene but not the endogenous one. Three transgenic lines were found positive. The

pH7WG2_rd29A_*SNAC*1 construct was used as positive control. After stable integration analysis it is necessary to quantify the expression of the *SNAC*1 transcription factor in different transgenic lines. Quantitative real-time PCR was done with the transgenic lines as well as the wild type plants. Transgenic plants showed significantly higher *SNAC*1 gene expression in quantitative RT-PCR compared to wild type BRRI dhan55. *SNAC*1 transcription factor was significantly more expressed in line P7 among the transgenic lines (Fig. 4c).

Table 1. The primers are used in Molecular analysis.

Name	Sequence	Annealing temperature
*SNAC*1_F	5' AGAAGCAAGCAAGAAGCGAT 3'	57°C
*SNAC*1_R	5' CCGAGCCATCTCTTGAC 3'	
rd29A_F	5' CACCTGAGGAATATTCTCTAGTAAGATA 3'	63°C
rd29A_R	5' GTAATCAAACCCTTTATTCCTGATGATTG 3'	
*SNAC*1_P_F (internal)	5' GCCGAGGTGGATCTCTACAA 3'	63°C
*SNAC*1_P_R (internal)	5' GTTGTCCACGATCTCCGACT 3'	
eEF_1α_F	5' TTTCACTCTTGGTGTGAAGCAGAT 3'	63°C
eEF_1α_R	5' GACTTCCTTCACGATTTCATCGTAA 3'	

Table 2. Transformation efficiency of *in planta* transformation at T_0 generation (based on hygromycin resistance assay).

Construct name	Variety	No. of the germinated seedlings after infection (a)	No. of hygromycin positive plants tested on flag leave (b)	Transformation efficiency (b/a*100)
pH7WG2_rd29A_*SNAC*1	Experiment 1	30	6	20%
	Experiment 2	22	4	18.18%
	Experiment 3	23	4	17.39 %

Leaf disk senescence assay: T_2 seeds were collected from PCR positive T_1 plants. Leaf disk senescence assay was done in mature T_2 plants to confirm the inheritance of genes. The damage caused by salinity stress was reflected in the degree of bleaching observed in the leaf tissue. The leaves of the control plants bleached, whereas the leaf disks of transformed plants remained healthy and retained more chlorophyll (Fig. 5). Plants having the transgenes showed resistance in salt water and remained greener than the wild type plants. Chlorophyll content was measured and found that under salt stress, the

percent reduction of chlorophyll content was significantly lower in the transgenic plants than wild type. Percent reduction of chlorophyll content at 200 mM salt condition was much higher than 100 mM salt condition (Fig. 5). Since Chlorophyll content is an important measure indicating photosynthesis ability of the plant, it is a good indicator of enhanced tolerance under salt stress.

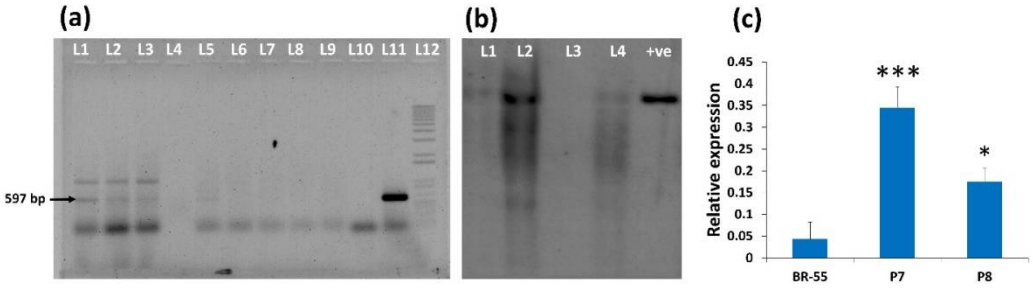

Fig. 4. Molecular analysis of rd29A_*SNAC*1_BR-55 transgenic lines. (a) PCR amplification of rd29A promoter in T_1 transgenic lines. L1-L8: Transgenics; L9: wild type BRRI dhan55; L10: water control; L11: positive control; L12: 1kb$^+$ ladder. (b) Southern blot analysis of rd29A_*SNAC*1_BR-55 transgenic lines with *SNAC*1 gene specific probe. L1-L4: transgenic lines P4, P7, empty, P8 respectively, +ve: plasmid pH7WG2_rd29A_*SNAC*1. (c) Relative expression analysis of *SNAC*1 gene. Transgenic line P7 showed significantly higher gene expression among transgenics as well as wild type BR-55. Student's *t* test (*, *** P<0.05, P<0.001).

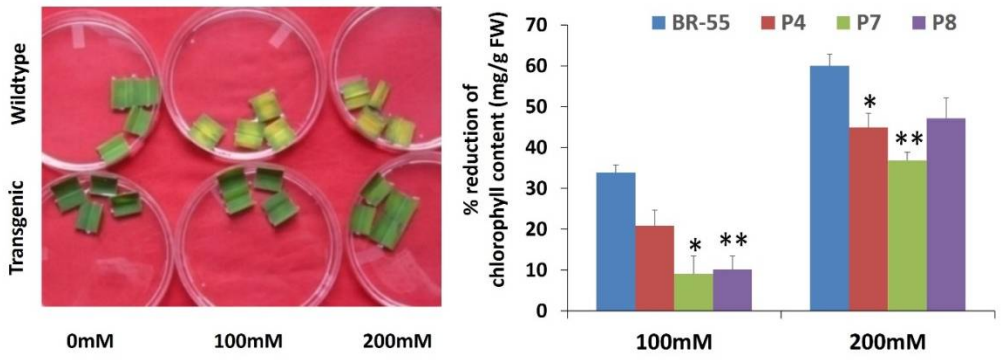

Fig. 5. Leaf disk senescence assay and measurement of chlorophyll content of T_2 plants. Chlorophyll content reduced significantly in wildtype plants than transformed plant at 100 mM and 200 mM NaCl salt solution. Student's *t* test (*, ** P<0.05, P<0.01).

Segregation ratio at T_2 generation: Segregation analysis of transgenes was done based on the result of leaf disk senescence assay of the T_2 seedlings. In tissue culture independent transformation, Mendelian inheritance (3:1) is usually followed by the positive transformants. At T_2 generation all 3 previously selected lines showed (3:1) segregation ratio or followed Mendelian inheritance of *SNAC*1 gene (Table 3).

Expression analysis of SNAC1 gene under salinity stress: The induced expression of *SNAC*1 gene was analyzed under 150 mM salt conditions in both wild type and transgenic lines by real time PCR for two weeks old seedlings. Relative expression of *SNAC*1 gene in transgenic lines were calculated along with the gene expression in wild type plants under salt stress. At 150 mM salt stress for 24 hours, transgene expression was significantly increased in transgenic lines than wild type plants under stress condition (Fig. 6). Real time PCR was performed using *SNAC*1 internal primers compared with reference housekeeping genes, thus confirming the induced expression of this gene in the transgenic plants.

Table 3. Segregation analysis of transformed (resistant) and non-transformed (susceptible) seedlings in the T_2 plants.

Transgenic line	Name of plants	Number of resistant seedlings (T_2)	Number of susceptible seedlings (T_2)	*chi-square test* or χ^2 test = (observed-expected) ^{2}/ (expected)	P-value
	P4	4	1	0.066667	0.796353*
rd29A_*SNAC*1_BR-55	P7	5	2	0.047619	0.827293*
	P8	6	3	0.333333	0.563722*

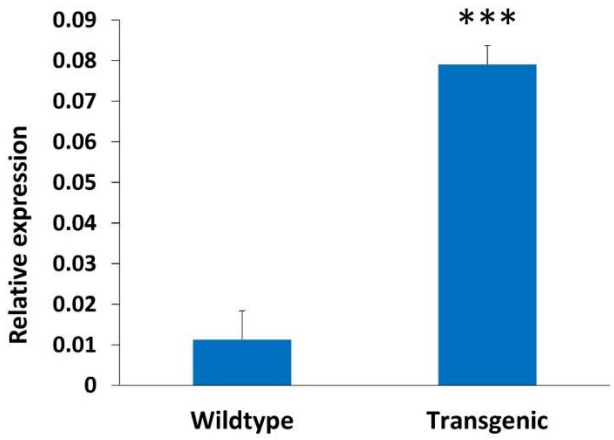

Fig. 6. Relative *SNAC*1 gene expression under 150 mM salt condition by real time PCR. After 24h of 150 mM salt stress, transgenic lines showed significantly higher gene expression compared to their wild type plants. Student's *t* test (*** P<0.001).

Survival assay after salinity and drought stress in T_3 plants: Among the three lines, line P7 and P8 were selected as they performed consistently better compared to the wild type plants. Survival assay in 200 mM salt was performed with these two lines along with

wild type. Survival rate for wild type was found 52% whereas for transgenics 60-68% (Fig. 7a). For survival assay in drought condition, after withholding of water for 12 days, and then 14 days of recovery in water, survival rate was measured. In both salinity and Drought conditions, survival rate of transgenic lines was significantly higher than wild types (Fig. 7b). It was reported that transgenic lines showed higher survival rate at both salinity and drought condition when *SNAC*1 was overexpressed in japonica rice (Hu et al. 2006). Here, similar results were found for *indica* rice when *SNAC*1 was induced.

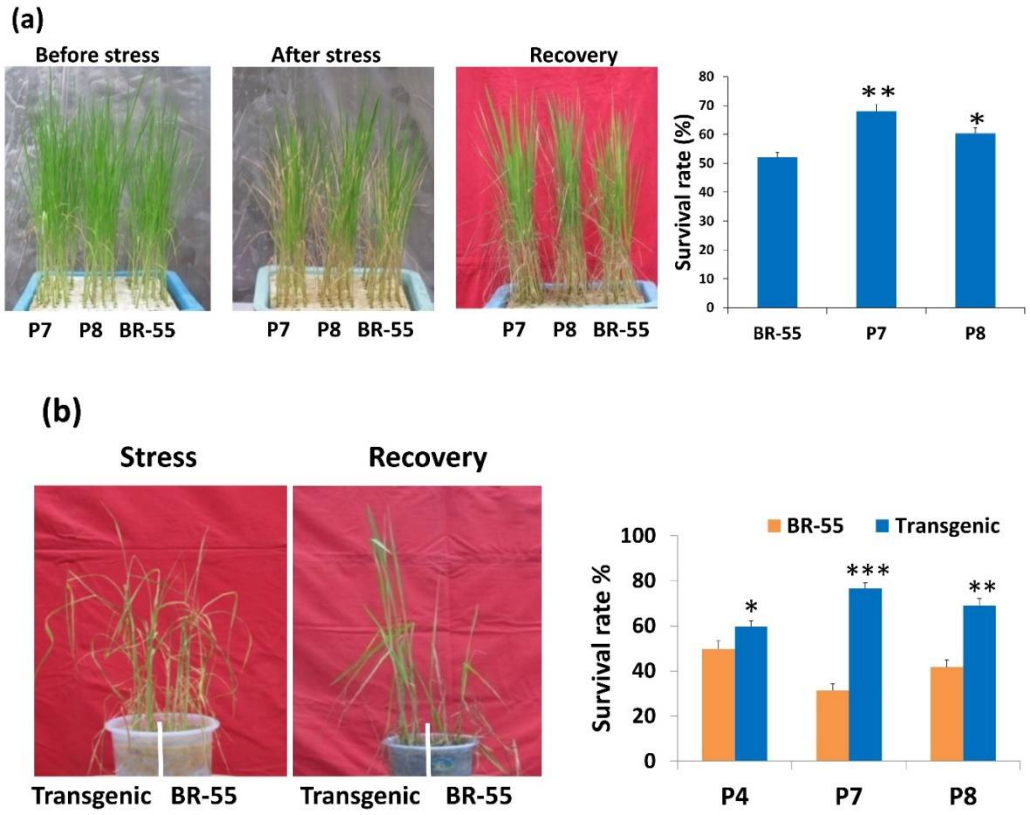

Fig. 7. (a) Survival assay at 200 mM salinity stress. transgenic lines showed better survival rate compared to wild type BRRI dhan55. (b) Survival assay at drought stress. Transgenic lines showed better survival rate at drought condition than wild type plants Student's *t* test (*, **, *** $P < 0.05$, $P < 0.01$, $P < 0.001$).

Seedling stage salinity screening: T_3 plants were selected for seedling stage salinity screening. Under stressed condition, transgenic lines showed significantly lower SES value than their parent. The stability of cell membrane under salt stress was also measured by relative electrolyte leakage. More chlorophyll content indicates plants remained greener in stressed condition. Lower reduction in shoot length and differences in root length are also important parameters indicating stress tolerance of the plants. Transgenic lines showed significantly lower percent reduction of chlorophyll content and

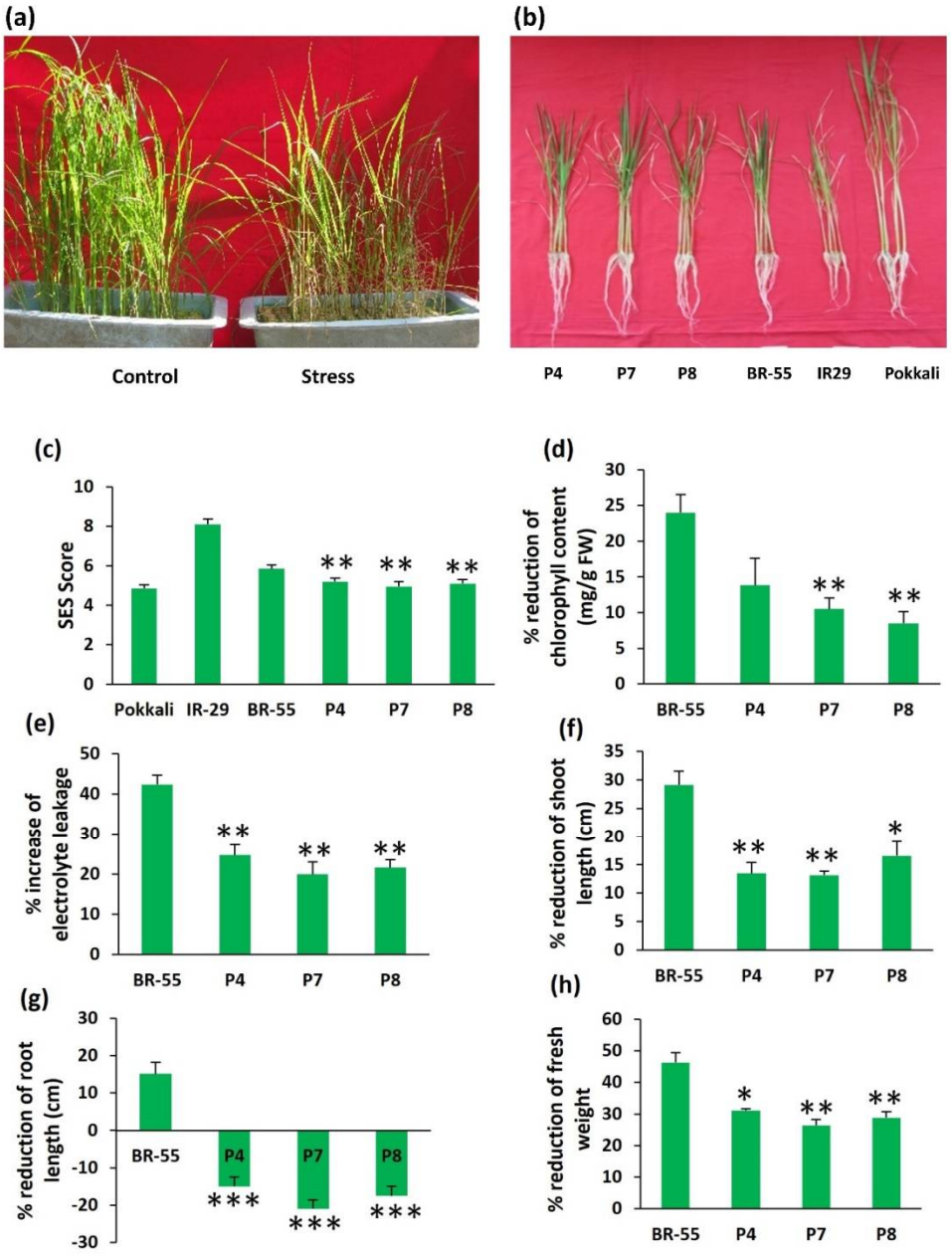

Fig. 8. Seedling stage salinity screening at T_3 plants of rd29A_*SNAC*1_BR-55 lines. (a) Control and stressed plants. (b) Phenotype of transgenic lines and wild type plants after stress. (c) Standard Evaluation System (SES) Score. (d) Percent reduction of chlorophyll content. (e) percent increase of electrolyte leakage. Percent reduction of (f) shoot length (g) root length and (h) fresh weight. In all parameters transgenic plants showed better salt tolerance than wild types BRRI dhan55. Each bar represents the mean ± SE (n = 5). Student's t test (*, **, *** $P < 0.05$, $P < 0.01$, $P < 0.001$).

shoot length than wild type (Fig. 8 d and f). Wild type plant showed higher percent increase of electrolyte leakage in salt stress condition. At stress the wild type plants showed reduced root length, but transgenic lines showing increased root length which is a tolerance characteristic of plant under stress (Liu et al. 2014). In non-stress condition there were no significant differences in all these parameters between wild type and transgenic lines (Fig. 8).

Seedling stage drought screening: Two transgenic lines and wild type plants were subjected in drought stress at T_3 generation. After water withdrawal, transgenic lines showed better tolerance than wild type. After 8 days of stress, plants were re-watered for 14 days for recovery. Chlorophyll content and shoot length were measured after recovery. Drought stress recovered transgenic plants contained 4.3- 3.6 mg/g chlorophyll content whereas the wild type had 2.1 mg/g chlorophyll content. Transgenic lines contained significantly more chlorophyll and had longer shoot length compared to wild type (Fig. 9).

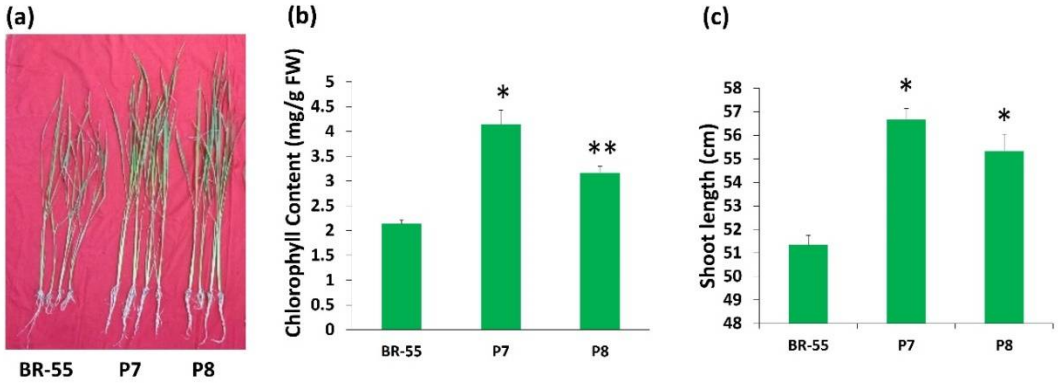

Fig. 9. Seedling stage drought screening at T_3 generation. rd29A_*SNAC*1_BR-55 showed (a) phenotype, (b) chlorophyll content and (c) shoot length after drought stress recovery. Transgenic lines showed significantly better chlorophyll content and more shoot length compared to their wild types. *, ** significant differences between wild type and transgenic lines at $P<0.05$, $P<0.01$ respectively.

Reproductive stage screening at salinity stress: Reproductive stage salinity screening was done at the T_4 generation. Transgenic lines and wild type plants were subjected to 8 dS/m salt (NaCl) at booting stage until harvest. Tillering, panicle formation, flowering and seed setting were done under the influence of salt. During harvest all phenotypic and yield related parameters were recorded (Fig. 10). Yield related traits were measured for both wild type and transgenics after salt stress and in non-stressed plants. Wild type and transgenic plants did not differ in these parameters in non-stressed condition. Percent reduction of these parameters was calculated under stress. A reduction of 16% in plant height was measured for wild type whereas this was 5-9% for the transgenics. Wild type plant showed 24% reduction of spikelet fertility and 47% reduction in yield whereas

transgenics showed 8-10% reduction of spikelet fertility and 31-35% in yield reduction. Wild type and transgenics showed 20% and 8-10% of 1000 grain weight reduction respectively. Transgenic lines showed significantly lower percent reduction in panicle length and flag leaf length than wild type (Fig. 10).

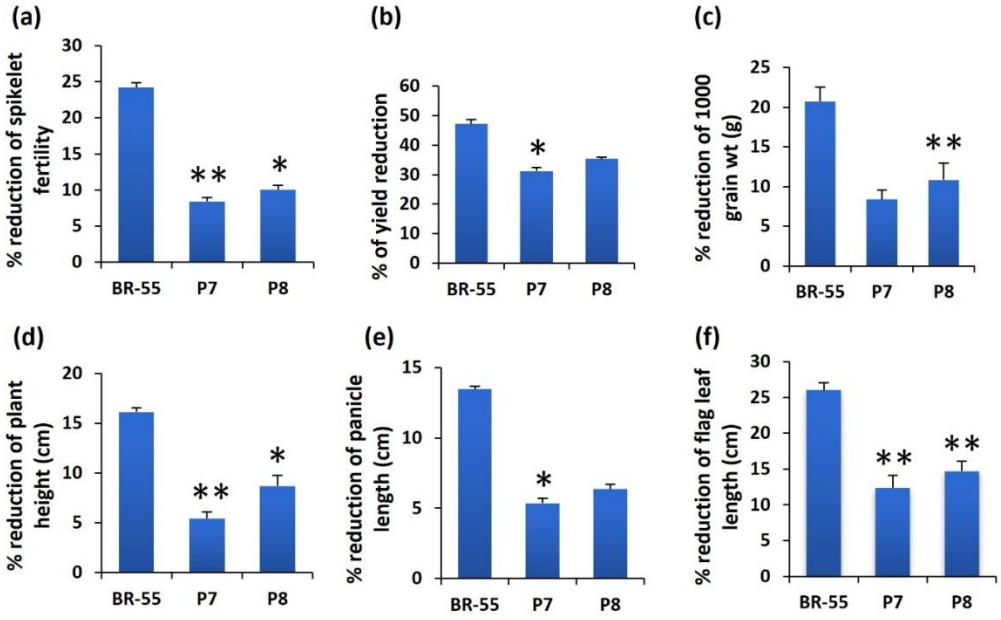

Fig. 10. Yield related parameters at reproductive stage salinity screening. (a) % reduction of spikelet fertility, (b) % of yield reduction and (c) % reduction of 1000 grain weight, (d) % reduction of plant height, (e) % reduction of panicle length and (f) % reduction of flag leaf length. All transgenic lines showed significantly good results in above parameters than wild type. Student's *t* test (*, ** P < 0.05, P < 0.01).

Reproductive stage screening under drought stress: Yield performance at reproductive stage was assessed under drought stress. Both wild type and transgenic lines were kept in drought stress by withdrawing water at panicle initiation stage. Plants were stressed until total leaves were rolled, indicating 70-72% of water content. After stress all plants were kept in water until seed maturation. This stress level provides a proper comparison in yield related parameters between wild type and transgenic lines (Fig. 11). After completion of the drought stress application, percent reduction of plant height, spikelet fertility, yield per plant and 1000 grain weight were calculated. Transgenic lines showed 3.5-6% reduction in plant height whereas wild type was reduced by 9.2%. About 19% of spikelet fertility was reduced for wild type whereas 7-9% for transgenic lines (Fig. 11). Therefore, transgenic lines therefore showed better tolerance in drought stress.

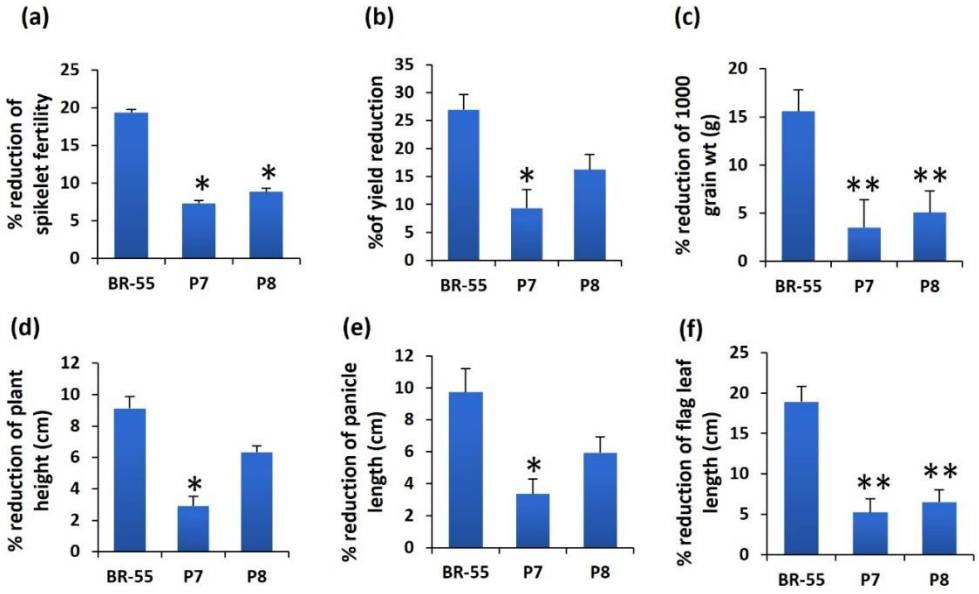

Fig. 11. Yield related traits at reproductive level drought stress. (a) % reduction of spikelet fertility, (b) % of yield reduction (c) % reduction of 1000 grain weight, (d) % reduction of plant height, (e) % reduction of panicle length and (f) % reduction of flag leaf length. All transgenic lines showed significantly good results in above parameters than wild type. Student's *t* test (*, ** P < 0.05, P < 0.01).

Thus, the multidimensional screening under separate salt and drought stress events confirmed that the *SNAC*1 transformed BRRI dhan55 showed enhanced tolerance to salt stress as well as drought stress compared to the wild type. BRRI dhan55, which is a farmer popular moderately drought tolerant variety, showed enhanced yield under stress when transformed with *SNAC*1 under a stress inducible promoter. Hence, the differences between wild type and transgenic was not visible without stress but was significant under stressed conditions.

Conclusion

The main purpose of this work was to increase the stress tolerance level of a farmer-popular high yielding *indica* rice variety. Being less responsive to tissue culture, high yielding variety BRRI dhan55 was transformed by *Agrobacterium* mediated *in planta* transformation method (Lin et al. 2009). Higher rate of transformation efficiencies indicates that the *in planta* method is quite an efficient method and applicable to any rice variety. Transgenic lines, transformed with *SNAC*1 under the stress inducible promoter rd29A, showed normal morphology and growth. Best transgenic lines were selected through molecular analysis and seedling level screening. Stable *SNAC*1 insertion was identified by Southern blot hybridization and transgenic lines showed higher expression

of transgene under salt stress. Induced expression of *SNAC*1 helps to give transgenic plants higher survival rate in both salt and drought conditions. They also showed significantly better stress tolerance at seedling level at both salinity and drought stress and gave better tolerance at reproductive level. Therefore, the tolerance level achieved due to induced expression of *SNAC*1 integration suggests that this transcription factor shows great promise for the genetic improvement of stress tolerance in *indica* rice. In future, other farmer popular high yielding rice varieties can be transformed with *SNAC*1 gene under stress inducible promoter by *in planta* method for the development of salt and drought tolerant varieties.

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