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Cloning and Transformation of the Transcription Factor *SNAC1* from Rice (*Oryza sativa* L.) Landrace Pokkali

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Abstract

The two major environmental factors that currently reduce plant productivity are drought and salinity. It has been reported that the plant-specific NAC (NAM, ATAF1/2, CUC2) transcription factors play diverse roles in plant development and stress responses. *Stress-specific NAC1 or SNAC1*-overexpressing transgenic rice plants showed significantly improved drought resistance under field conditions and strong tolerance to salt stress. So the purpose of this study was to clone the *SNAC1* gene from the salt tolerant rice landrace Pokkali for transformation into a salt sensitive rice variety. *SNAC1* cDNA was isolated and successfully cloned in the pENTR vector. Cloning was confirmed by PCR, restriction digestion and sequencing analysis and subsequently mobilized into the *Agrobacterium* compatible destination vector pH7WG2 for rice transformation. Transgenic status of T₀ plants of *SNAC1* was confirmed by PCR analysis. T₀ plants were advanced to T₁ transgenic lines and further confirmed by PCR analysis.

Introduction

Rice is the most important staple food for a large part of the world's human population, especially in tropical Latin America, the West Indies, East, South and Southeast Asia. In Bangladesh the dominant food crop is rice, accounting for about 75 per cent of agricultural land use. In Bangladesh a large part of cultivable area lies in the coastal saline zone, and rice as well as other crop cultivation is largely hindered by the salinity (Karim and Iqbal 2001). Seven per cent of the land's surface and five per cent of cultivated lands are affected by salinity (Flowers et al. 1997) and it is one of the most serious environmental factors limiting the productivity of crop plants (Ashraf 1999). Despite advances in increasing plant productivity and resistance to a number of pests and diseases, improving salt tolerance in crop plants remains elusive, mainly because salinity simultaneously affects several aspects of plant physiology. Saline conditions reduce the ability of plants to absorb water, causing rapid reductions in growth rate, and

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induce many metabolic changes similar to those caused by water stress (Yeo 1983). So there is an obvious need to improve salt tolerance of crops (especially rice) to meet up the emerging demand of foods in order to ensure food security. Two basic genetic approaches that are currently being used to improve stress tolerance include: (i) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci (Foolad 2004 and Lindsay 2004) and subsequent marker-assisted selection and (ii) generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance (Seki 2003 and Chinnusamy 2005).

Previously it has been reported that some transcription factors, such as CBF1/DREB1B (Jaglo-Ottosen et al. 1998), OsbZIP72 (Lu et al. 2009) and AtMYB44 (Jung et al. 2008) to be involved in plant stress responses. Transgenic plants over-expressing these genes could enhance their tolerance to various stresses. Transcription factors play essential roles in stress responses by regulating their target genes through binding to the cognate cis-acting elements (Tran et al. 2004). The NAC family, is one of the largest plant transcription factor families which are characterized by a highly conserved DNA binding domain, known as the NAC domain in the N-terminal region. In contrast, the C-terminal region of NAC proteins, usually containing the transcriptional activation domain, is highly diversified both in length and sequence (Fang et al. 2008, Ooka et al. 2003). Recently, the NAC gene, *SNAC1* (*STRESS-RESPONSIVE NAC1*) was isolated and characterized in rice (Hu et al. 2006). This gene was found to be specifically induced in the guard cells of rice under drought stress conditions. Over-expression of this gene in rice resulted in significantly increased stomatal closure and drought resistance in the field conditions. The *SNAC1*-overexpressing rice plants also showed improved salt tolerance, further emphasizing the usefulness of this gene in stress tolerance improvement (Honghong et al. 2006). A comparative transcriptome analysis between *SNAC1*-overexpressing plants and WT suggested that a set of 80 genes are up regulated in *SNAC1*-overexpressing plants. Forty of these genes are related to drought stress protection mechanisms such as specific signal transduction pathway, osmolyte production, detoxification and redox homeostasis, protection of macromolecules and stomatal closure. These data showed that *SNAC1* controls a complex gene regulatory network leading to drought and salt stress tolerance in rice (Khong et al. 2008).

Since *SNAC1* plays an important role in salt tolerance, ~~therefore~~ the objectives of the present study was to clone *SNAC1* cDNA from the salt tolerant land race Pokali into pETNR/D-TOPO vector and subsequently transfer it into ~~into~~ the Destination vector (pH7WG2) by LR recombination for *Agrobacterium* mediated transformation of the Bangladeshi indica rice landrace Binnatoa to induce enhanced salt tolerance.

Materials and Methods

For cloning, the 1051bp *SNAC1* cDNA (Gene ID- DQ394702.1 in original work) was reverse transcribed from mRNA (Total RNA was extracted from 7d old Pokkali using TRIZOL reagent. The extracted RNA was quantified using a Nanodrop, and the corresponding cDNA was synthesized by using SuperScript™ First-strand Synthesis System of Invitrogen. *SNAC1* cDNA was amplified by PCR to generate template with 5' end CACC overhang for directional cloning into pENTR/D-TOPO vector with the forward (5'-CACCAGAAGCAA GCAAGAAGCGAT-3') and reverse primer (5'-CCCGA GCCATC TCTTGAC-3'). PCR reactions were carried out in 15 µl of 10× PCR buffer with 10 mM dNTP, 1mM MgCl₂, 10 µM of each oligonucleotide primer, 20% DMSO and 1 µl of Taq DNA polymerase. PCR conditions were initial denaturation at 95°C for 5 min and 35 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 7 min. The resulting *SNAC1* cDNA was cloned into pENTR/D-TOPO vector by TOPO cloning reaction of Invitrogen. The pENTR_ *SNAC1* plasmid construct was transformed into *E. coli* DH5α competent cells through heat shock using standard protocols (Sambrook et al. 1989). Cloning was then confirmed by PCR of pENTR_ *SNAC1* plasmid, restriction digestion of the isolated plasmid with *Eco* RV, *Not* I restriction enzymes (NEBr inc) and further confirmed by direct sequencing of the vector and gene specific primers.

SNAC1 gene was ~~was~~ transferred from the pENTR_ *SNAC1* construct into the gateway destination vector pH7WG2 by LR recombination reaction (Gateway® Cloning, PREMIER Biosoft International). The LR recombination reaction was confirmed by PCR using insert specific primers (SNAC-F: 5' CACCAGAAGCAAGCAAGAAGC GAT 3' and SNAC-R: 5'-CCCGAGCCATCTCTTGAC-3') and primers for hygromycin phospho transferase (HPT_600F: 5'GATGTTGGCGACCTCGTATT 3' and HPT_600R: 5'GCGAAGAA TCTCGTGCTTTC3'). The PCR conditions were an initial denaturation at 95°C (5 min), 35 cycles of: denaturation at 95°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 7 min. PCR reactions were carried out in 15 µl of 10× PCR buffer, for 10 mM dNTP, 1 mM MgCl₂, 10 µM of each oligonucleotide primer, 20% DMSO and 1 µl of Taq DNA polymerase. Then further confirmed by restriction digestion with restriction endonuclease BamH1 and Nde1 (NEBr inc.) by comparing the digested product with undigested plasmid after agarose gel electrophoresis.

Agrobacterium tumefaciens (LBA 4404) was electroporated with the construct SNAC1-pH7WG2 using standard protocols (Sambrook et al. 1989). Twenty-day-old rice calli were transferred by using the modified Khanna and Raina protocol (Rasul et al. 1997, Khanna and Raina 1999). Transformation responsive calli of Bangladeshi rice Binnatoa were regenerated to produce T₀ plants.

T₀ plants were allowed to grow up in a net house and transgenic status was confirmed by PCR analysis. The primers used in PCR for SNAC1 and the PCR conditions were exactly as above. After molecular confirmation T₀ plants were advanced to T₁ generation and tested by PCR using the same conditions.

Results and Discussion

Before cloning, total RNA was isolated from salt tolerant landrace Pokkali and then further reverse transcribed for isolation of SNAC1 cDNA. The isolated RNA is shown in Fig. 1.

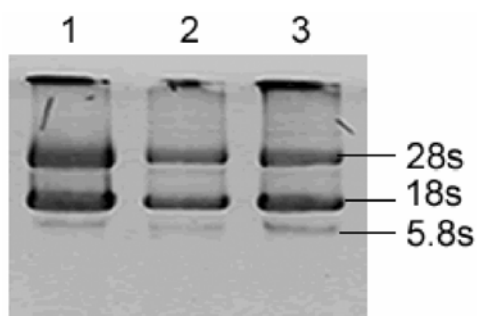


Fig. 1. Gel view of RNA in 1.5% agarose gel. (Total RNA was extracted from Pokkali leaf using TRIZOL reagent (Invitrogen). The samples were run in 1 X MOPS at 30-60 V).

For cloning of SNAC1 gene, its RNA was transcribed by RT-PCR kit (Invitrogen) to produce cDNA, which was amplified with gene specific primers. The amplification product is shown in Fig 2. The expected size of SNAC1 cDNA was 1051bp. From the gel it was observed that the product size ~ 1051 bp perfectly matched with the expected size.

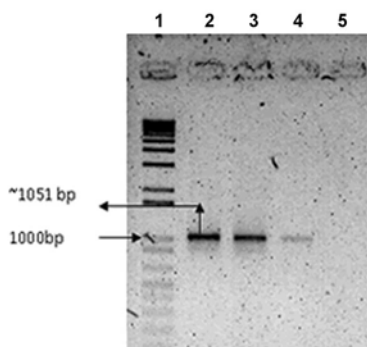


Fig. 2. RT-PCR amplification of SNAC1 mRNA from Pokkali. (Lane 1: 1kb plus ladder (Invitrogen). Lanes 2-4: Amplified product of SNAC1 gene Lane 5: negative (water) control).

The PCR product was cloned in the pENTR/D-TOPO cloning vector according to the manufacturer's instructions. Following cloning and transformation on the next day, four colonies were observed on the LB plate containing the antibiotic kanamycin. To confirm the cloning, plasmids were isolated from the four colonies. Out of four colonies only 1 colony showed the expected result, which is shown in Fig 3.

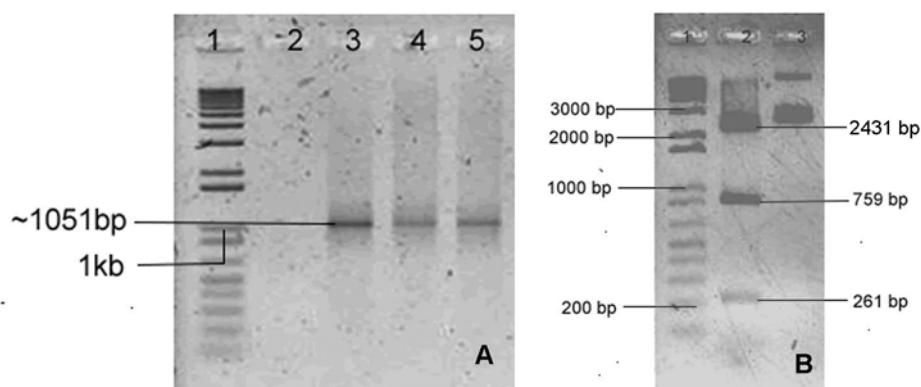


Fig. 3. PCR amplification of pENTR-SNAC1 (A), Lane 1: 1kb+ ladder, Lane 2: negative control, Lanes 3-5: PCR product of Pentr-SNAC1 in different concentrations. Cloning was further confirmed by restriction digestion of the pENTR-SNAC1 construct with the restriction enzymes *EcoRV* and *NotI*, Lane 1: 1 kb+ ladder, Lane 2: restriction digests with *EcoRV* and *NotI*, Lane 3: plasmid without digestion.

The clone was further confirmed by restriction digestion of the pENTR_SNAC1 plasmid with *EcoRV* and *NotI*. By digestion with *EcoRV* and *NotI* four products were expected. They were 2431bp, 759bp, 261bp and 70bp. These bands were all observed (Fig. 3B). The pENTR plasmid containing with the insert (*SNAC1*) was sequenced using gene specific and M13 primer pairs. All the sequence contigs were obtained (1315bp) using the online alignment tool (<http://mobylye.pasteur.fr/cgi-bin/portal>). The contigs were then aligned with the database deposition using the BLAST tool of NCBI and found to be identical with the original sequence.

SNAC1 cDNA was transferred into the gateway destination vector pH7WG2 from the entry clone pENTR_SNAC1 by LR recombination reaction. This recombination reaction creates an expression clone for plant transformation and contains the selectable marker for transformation into both bacteria and plants and is compatible as a vector for *Agrobacterium*. LR recombination reaction was confirmed by PCR using insert specific primers and primers for hygromycin phospho transferase (*hpt*, vector specific). Notably after PCR analysis accurate bands of 1051 bp (Fig. 4A) and 600bp (Fig. 4B) were found respectively corresponding to the *SNAC1* and *hpt* product sizes. This was further confirmed by restriction digestion with restriction endonuclease *BamHI* and *NdeI* (Fig. 4C).

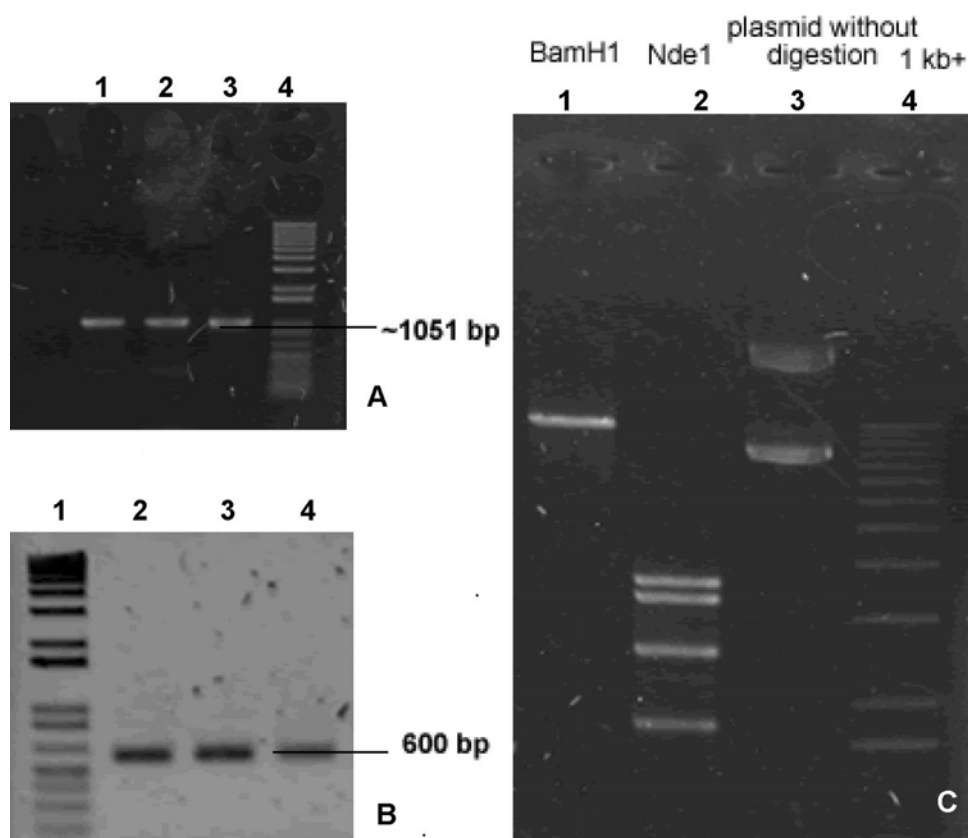


Fig. 4. LR amplification of pH7WG2.0-SNAC1 with insert specific primer (A), Lane 4: 1kb+ ladder, Lanes 1-3: PCR product of pH7WG2.0-SNAC1 and then by vector specific primer (B) lane 1: 1kb+ ladder, Lanes 2-4: PCR product of pH7WG2.0-SNAC1. LR was further confirmed by restriction digestion of the pH7WG2.0-SNAC1 construct with the restriction enzymes *Nde1* and *BamH1*. Lane 1: restriction digests of Bam H1, lane 2: restriction digests of *Nde1*, Lane 3: plasmid without digestion, Lane 4: 1kb+ ladder.

For rice transformation, standard *Agrobacterium* mediated transformation methods were applied (Rasul et al. 1997, Khanna and Raina 1999). Around 250 Binnatoa rice seeds were infected by transformed *Agrobacterium* strain (LBA 4404) (Fig. 6) and were subsequently subjected to Hygromycin stress in successive selection media.

After selection the desired calli for more than one month, these were subcultured to regeneration shooting and rooting media (Fig. 5A, B). Up to regeneration the plants were kept in controlled temperature (28°C) and photoperiod (in tissue culture room). So before transplanting into soil the temperature and humidity were gradually changed and the plants were kept in hydroponics with nutrient for acclimation and then finally the plants were transferred to soil.

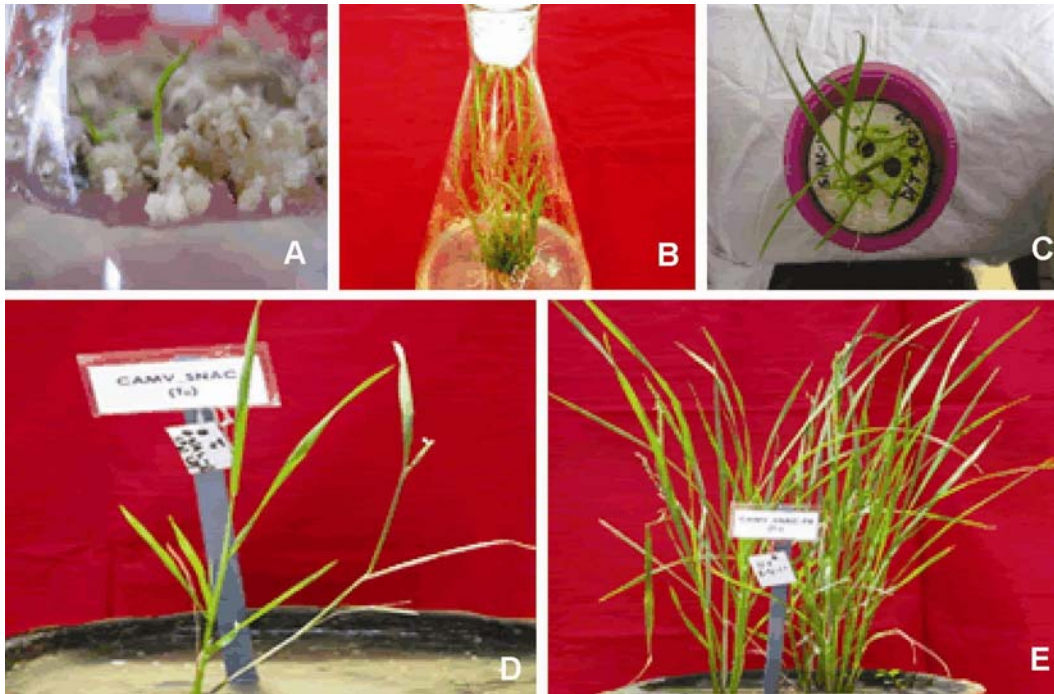


Fig. 5. Shoot (A) and root (B) regeneration. Plants were subsequently transferred to hydroponics (C) and then to soil at T_0 stage (D) and then advanced to T_1 stage (E).

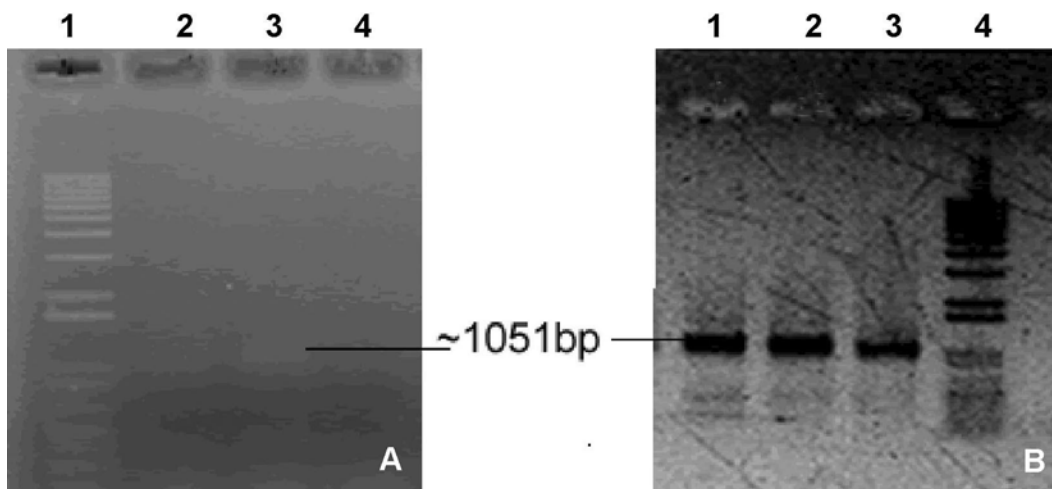


Fig. 6. PCR confirmation of the transgenic plants at T_0 and T_1 stage. In fig 6A, Lane 1: 1kb+ ladder, Lanes 2 and 3: SNAC 1 amplified product from T_0 plants having size of 1051 bp, lane4: negative control. In Fig 6B, Lanes 1-3: SNAC 1 amplified product from T_1 plants having size of 1051 bp, Lane 4: 1kb+ ladder.

Six transgenic T₀ plants were obtained and confirmed by PCR reaction using insert specific primers (SNAC-F and SNAC-R) and the precise band of 1051 bp was found. For control and to determine the presence of any *SNAC1* background in parent lines Binnatoa plants were used as negative control and no band was found for negative control (Fig. 6A).

T₀ plants were advanced to T₁ stage and the transgenic level was tested by PCR analysis and 3 lines at this stage were found positive for *SNAC1* transgene (Fig. 6B)

Conclusion

Rice is one of the most important crops in the world. The growth and productivity of rice are often threatened by environmental factors, such as drought, salt, cold, and biotic stresses. Many efforts have been undertaken to generate stress tolerant rice by manipulating the expression of stress-responsive genes (Umezawa et al. 2006). It was previously been reported that NAC gene *SNAC1* gene over-expression in transgenic rice plants showed significantly improved drought resistance under field conditions and strong tolerance to salt stress (Honghong Hu et al. 2006). Therefore, the purpose of this study was to clone the *SNAC1* gene from a salt tolerant rice landrace, Pokkali, for *Agrobacterium* mediated transformation into salt sensitive rice variety of Bangladesh to produce salt tolerance rice.

In the cloning process of *SNAC1*, Gateway cloning technology was used as it was easy and less time consuming process. From the results it was concluded that *SNAC1* cDNA successfully cloned into pENTR vector. Cloning was confirmed by PCR, restriction digestion and further sequencing of the insert. LR recombination between entry and destination vector was done and confirmed by PCR and restriction digestion of pH7WG2.0_ *SNAC1* plasmid. This construct was further transformed into *Agrobacterium* and the *SNAC1* gene successfully inserted into the traditional rice variety Binnatoa. Transgenic status of the six T₀ plants were confirmed by PCR and then these plants were advanced to T₁ transgenic lines out of the six plants, three plants were found to be positive for *SNAC1* gene Therefore it can be concluded that stable integration of the *SNAC1* gene was confirmed after molecular analysis in the three transgenic lines at T₁ stage. In future these T₁ transgenic plants will be advanced to T₃ stage and further tested for salt tolerance.

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