DNA MARKER-ASSISTED BREEDING AND GENETIC TRANSFORMATION FOR PRODUCING SALT-TOLERANT RICE FOR BANGLADESH

ZEBA I. SERAJ, U.S. MAHZABIN AMIN AND M. SAZZADUR RAHMAN[1]

Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka 1000, Bangladesh
[1]Plant Physiology Division, Bangladesh Rice Research Institute, Gazipur, Bangladesh

ABSTRACT

Bangladesh is very small fertile country with a total land area of 14.8 Mha and cultivable area of 9.1 Mha (62%). The total irrigated land area is 52% with a cropping intensity of 181%. The share of agriculture in the GDP is 19.95%. The paddy production has so far been almost adequate for the growing population of rice eaters at 33.5 Mt (~150 million people), with the exception of import of ~1 Mt to mitigate floods or similar disasters. Another 100 million is likely to be added to the population, before stabilizing by the middle of this century. Therefore rice production has to nearly double. Abiotic stresses like salinity affects 1.2 Mha in the South. Drought affects 2.7 Mha in the North. In addition there is 1 Mha of flash flood-prone areas and 3.2 Mha of rainfed low-lying areas in Bangladesh.

Use of biotechnological tools like DNA marker-assisted breeding with the help of IRRI and currently funded by the Gates Foundation with the project STRASA (Stress tolerant Rice for Africa and South Asia) has resulted in the release of submergence tolerant rice like BRRIdhan 51 and 52. Some traditionally bred drought tolerant varieties as well as IR64 introgression lines with QTLs from drought tolerant donors have been produced and are in the pipeline. Our own work at Dhaka University helped incorporate salinity tolerance QTLs (*Saltol*) into farmer-popular BR11 and BRRIdhan 28, which are currently undergoing field trials in the South of Bangladesh. STRASA is also funding for pyramiding of multiple traits into farmer-popular rice through marker-assisted breeding. For example, the submergence tolerance trait, *Sub1* is being combined with each of stagnant flood tolerance (SF), anaerobic germination (AG), drought tolerant yield (DTY) and salinity tolerance (*Saltol*). Marker-assisted breeding for introgression of the kinase gene, *Pstol1*, which confers tolerance of phosphorous deficiency and drought and results in 20% increase in yield has just been initiated for Bangladesh.

Rice genetic transformation for incorporation of genes with multiple downstream effects has been shown to be effective in producing field level tolerance to drought, salinity and providing water-use efficiency. Moreover, tolerant traits coded for by different mechanisms can be pyramided providing more durable tolerance. Some of the target genes are those for antiporters, helicases and transcription factors like, HARDY and SNAC1 These genes are being cloned and incorporated into rice at Dhaka University. Some of these are collaborative efforts like those of Pea DNA Helicase with ICGEB, India.

INTRODUCTION

Tolerance to abiotic stresses such as drought, salinity or flooding is controlled by quantitative trait loci (QTLs) or multiple genes. In order to produce tolerant crops, for example, rice, these genes need to be combined with those responsible for good agronomic performance resulting in high yielding abiotic stress-tolerant genotypes suitable for cultivation. Breeding for high yield in rice has been in practice for several decades now and with help and inputs from the International Rice Research Institute (IRRI), the Bangladesh Rice Research Institute (BRRI) has produced at least 50 high yielding rice cultivars [(1)]. Climate change scenarios and scarcity of water resources has however now made introgression of stress tolerance genes necessary into these high-yielding cultivars.

One approach is the use of DNA markers associated with the tolerance genetic loci to guide introgression of the latter into the background of a high yielding cultivar. This is referred to as DNA-marker assisted backcrossing and involves the crossing of a donor genotype (having the tolerance genetic locus) with the high-yielder or the farmer-popular. This is followed by 2-3 backcrosses with the high-yielder, while selecting progenies having the tolerance loci aided by DNA markers. The backcross with the high-yielder ensures retention of its genetic background and the good agronomic traits associated with it. One very successful example of this approach was the introgression of the submergence tolerance locus or *Sub1A* from the rice landrace FR13A into the agronomically superior Swarna and BR11 [(2, 3)]. These two genotypes were released as BRRIdhan 51 and 52 respectively in Bangladesh [(4)]. One important factor in the success of this approach is that the targeted tolerant genetic locus has to be responsible for a high proportion of the phenotype of interest, since multiple loci may be responsible for the latter. For the *Sub1A* locus, this was precisely the case as it turned out to encode a transcription factor called ERF1 which in turn affected many downstream genes [(5, 6)]. It is likely that the *Pstol1* locus encoding a protein kinase gene will also affect multiple downstream genes and be a good target for introgression of drought tolerance as well as efficient phosphorous absorption in a DNA marker-assisted backcross approach [(7)].

Knowledge of suitable DNA markers associated with abiotic traits of interest such as stagnant flooding (SF), anaerobic germination (AG) and drought tolerant yield (DTY) is essential for the success of the backcross breeding approach. Breeding programs for introgression and pyramiding of these traits are already underway [(8)]. A major salinity tolerance locus, *Saltol*, was described in rice Chr 1 [(9, 10)] and has been the focus of salinity tolerance marker-assisted backcross breeding at IRRI, BRRI and Dhaka University (DU) and is briefly described in this paper.

Genetic transformation is another approach to producing abiotic stress tolerant crops such as rice. Initial efforts were made to overexpress single genes encoding rate limiting enzymes of osmolyte production. This resulted in limited improvement in tolerance at the vegetative state of several crops (reviewed by [(11)]. Overexpression of the Na/H antiporter which sequesters Na in the

vacuole provided good tolerance to dicots [12, 13] but produced less dramatic effect in monocots like wheat [14] and limited in rice [15]. Our own experience with the vacuolar Na/H antiporter transformation in rice has shown that its regulation is complex because it can produce 3 alternate transcripts from the single gene. We have also shown that inclusion of the 5′ and 3′ UTRs increases the level of tolerance compared to the cDNA only. Even so the vacuolar Na/H antiporter provides only moderate salt tolerance to rice (Seraj Z. I. unpublished results). Transformation with regulatory molecules like transcription factors and regulatory enzymes was more successful. These include the SNAC1 [16] and HARDY [17] transcription factors as well as the RNA and DNA unwinding helicase enzyme) [18, 19]. Work at Dhaka University, involving the cloning and transformation of these transcription factors and regulatory enzymes in rice, some of it in collaboration with the International Center for Genetic Engineering and Biotechnology (ICGEB, India) is described in this paper.

DNA MARKER-ASSISTED BACKCROSSING

Marker-assisted backcrossing (MABC) is now frequently used to introgress favorable alleles and major effect QTLs (Quantitative Trait Loci) in to mega varieties and elite genotypes for the improvement of complex abiotic and biotic stress tolerance traits specifically where conventional breeding fails to introgress stress tolerant traits to elite genotypes. *Saltol*, a major effect QTL for salinity tolerance was identified in rice chromosome 1, which was targeted for introgression to improve seedling stage salinity tolerance of two Bangladeshi mega rice variety BR11 (*Transplated Aman* or monsoon season) and BRRI dhan28 (BR28) (*Boro* or winter season). FL378, a tolerant F_8 Recombinant Inbred Line (RIL) was used as the donor to introgress *Saltol* alleles conferring seedling stage salinity tolerance into BR11 and BRRI dhan28 by MABC. Three-step marker aided selections i.e. Foreground, Recombinant and Background selection were employed to select progenies having precise QTL within a clean background of recurrent parent. In foreground selection 3 most tightly linked and robust SSR marker i.e. RM1287 (10.90 Mb), RM3412 (11.50 Mb) and RM493 (12.20 Mb) were used to locate the *Saltol* QTL in backcross progenies [10, 20-22]. Two to four SSR markers i.e. RM3627 (10.31 Mb) at the distal end of the QTL and RM10825 (13.30 Mb), RM10864 (14.20 Mb), RM562 (14.60 Mb), RM7075 (15.10 Mb) at the proximal end were used in recombinant selection to precisely limit the QTL segment (to reduce negative linkage drag). For the background selection 87-103 SSRs, InDel and gene-based markers were used to recover the recurrent genome [23]. Three backcrosses and two selfs were done to transfer positive alleles of *Saltol* from FL378 into a clean and/or minimum background of BR11 and BRRI dhan28.

PROGENY SELECTION FOR TESTING AND RELEASE

Two and six Near Isogenic Lines (NILs) at BC_3F_3 stage were selected having 1.3-3.7 Mb introgression at the *Saltol* region with 97 to 99% recurrent parent genome for BR11 and BRRI dhan28 respectively. Fig 1 illustrates the MABC scheme for the development of introgression lines (BR11-*Saltol*) in a nutshell. Introgressing 1.3-3.7 Mb regions of target loci in to BR11 and

BRRI dhan28 slightly improved their overall tolerance at seedling stage (SES score) (Fig 2). All introgression lines however showed improved agronomic performance i.e. showed good stature, long panicles, high and dense grains, etc. The NILs looked very similar to the recurrent parents (BR11, BRRIdhan28), some of which however, showed greater yield potential in both saline and non-saline conditions. Tolerance to salinity is highly polygenic in nature; three different types of stresses (Osmotic, Ionic and Oxidative) are associated with the trait. So, in all likelihood a single QTL is not enough to significantly increase the tolerance level. Pyramiding of multiple QTL controlling different physiological mechanisms with different genetic background could help to achieve a higher level of salt tolerance.

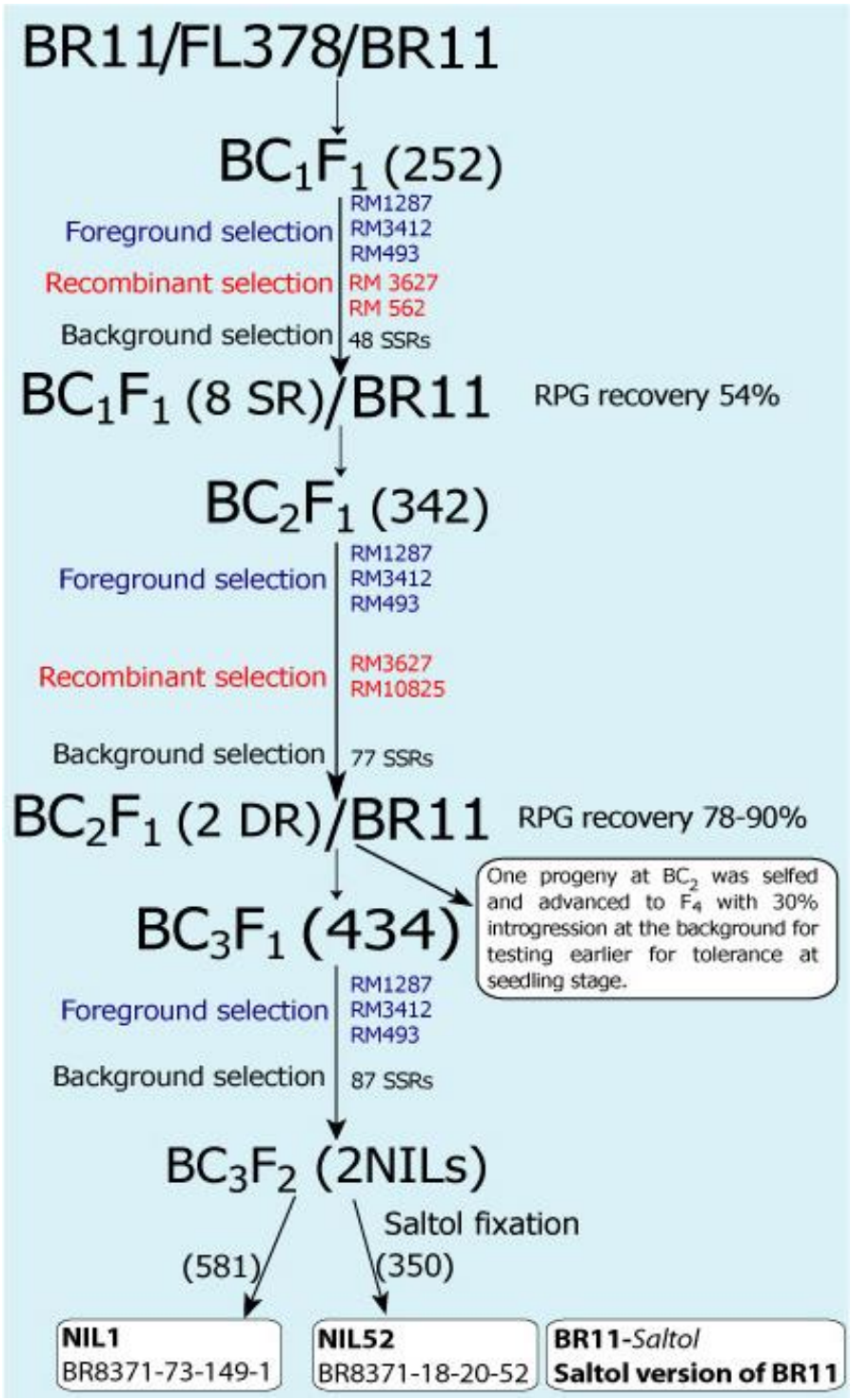


Fig 1. Flow chart of 3-backcross step MABC scheme for the development of BR11-*Saltol* introgression lines with DNA markers used and the numbers of progenies genotyped in different backcross generation.

Fig 2. Photograph showing tolerance of introgression lines at seedling stage in hydroponics culture with stress @12 dSm^{-1} in Net house condition. From L-R, Rows 1-3 are the negative controls without *Saltol*, rows 4-6 are lines with *Saltol* (show increased vigor). Rows 7, 8, 9 and 10 are respectively, the donor FL378, the sensitive parent BR28, the sensitive control IR29 and the tolerant control Pokkali

GENETIC TRANSFORMATION

The *Helicase* gene (*Pea DNA Helicase* or *PDH45*) construct was obtained from Dr. Narendra Tuteja of ICGEB under an MTA. This was transformed into *E.coli*, confirmed and subsequently transformed into *A. tumefaciens* for rice transformation as described in detail including testing of the transgenic rice, Binnatoa, for salt tolerance in Amin *et al.* [19]. Since Binnatoa is a traditional cultivar with poor agronomic traits, the gene was backcrossed into three farmer-popular Bangladeshi rice cultivars, BRRIdhan 28, 29 and 47 as described below. Progenies showing good tolerance score at the seedling stage were advanced and stable inheritance of the transgene was shown upto the F_5 generation. These F_5 progenies are now being tested for reproductive stage stress tolerance.

The *SNAC1* RNA was isolated from the salt tolerant rice landrace Pokkali, converted to cDNA and cloned into the InVitrogen directional cloning ENTR vector, confirmed by sequencing and restriction digestion before recombining it into the Gateway *Agrobacterium*-compatible Binary Destination vector, pH7WG2 [24]. Standard rice transformation and

regeneration protocols were done, gene insertion and expression confirmed, plants advanced to T_2 and seedling salinity tolerance assays conducted as described in Amin et al. [19].

Similar protocols were used for cloning of the HARDY gene, except that the RNA was isolated from *Arabidopsis thaliana* Landsberg. All these transformations were done in the tissue culture responsive rice variety Binnatoa which is a traditional cultivar and hence not high-yielding. In order to transform the transcription factor genes into high yielding cultivars, we performed co-transformations with *Helicase* and *SNAC1* and *Helicase* and *HARDY* using an *in planta* transformation protocol according to Lin *et al*. [25] (Please see below). The genes were co-transformed into BRRIdhan-27, BRRIdhan-29, BRRIdhan-43, BRRIdhan-49, BRRIdhan-52 and BRRIdhan-55. Transgenic T_2 progenies are being assessed for their seedling salinity tolerance as described in Amin *et al*. [19].

AGROBACTERIUM –MEDIATED TRANSFORMATION (TISSUE-CULTURE DEPENDENT*)*

Rice calli were transformed with Agrobacterium tumefaciens (strain LBA4404) containing the selected gene construct. The transformation method was carried out according to the method described by Khanna and Raina [26] with some modifications [27]. The Bangladeshi tissue culture responsive cultivar Binnatoa, was used for transformation [28].

AGROBACTERUM-MEDIATED TRANSFORMATION (TISSUE-CULTURE INDEPENDENT OR IN PLANTA)

Mature rice seeds of individual variety were sterilized with 99% ethanol, 30% chlorox, Tween 20 and kept at 37ºC. After 2 days the embryo region turned white. The embryonic apical meristem in the seed was pierced to a depth of 1-1.5 mm with a needle that had been dipped in the bacterial solution to inoculate gene construct in *Agrobacterium* into plumule where a shoot would later emerge as described in Lin et al. [25] with the exception that 0.4% acetosyringone was added to the bacterial culture media. For co-transformation, a mixture of *Agrobacteria* solution, containing two gene constructs was used. The pierced seeds were then placed in a conical flask and soaked in *the Agrobacterium* inoculums and vacuum applied. The inoculated seeds were transferred onto petri dishes containing wet filter papers and incubated in the dark at 28°C for 6-7 days. The emerged seedlings were treated with 250mg/L carbenicillin solution, washed with ddH_20 and transferred to new petri dishes containing wet filter papers. Seedlings were then kept in light for 16 hours and in dark for 6 hours. When the seedlings turn green, they were transferred to hydroponic solution. After 2-3 days the hydroponic pots were transferred to net-house. When the seedlings were mature, they were transferred to soil. T_1 seeds only from those panicles whose flag leaf was resistant to hygromycin solution were germinated again in hygromycin. Seedlings which survived in hygromycin and were PCR-positive for the transgene were advanced to the T_2 generation and salinity tolerance tests performed.

SALT STRESS SCREENING FOR SELECTING TRANSGENICS WITH GOOD TOLERANCE SCORES

Transgenic plants were checked for tolerance level by seedling stage screening as described in detail in Amin *et al.*[19] . The germinated seeds of WT, transgenic lines and sensitive controls (IR29) were transferred to Hydroponics in netted, floating styrofoam in a completely randomized design, allowed to grow for 2 weeks and then salt stress up to 12 dS/m was applied gradually. [29]. The EC of the solution was maintained at 12 dS/m until the end of the experiment. The pH of the non-aerated Yoshida [30] culture solution was adjusted to 5.0 every day and the culture solution was changed every 2 days. After 8–10 days, when the sensitive control IR29 in stress were dead, tolerance-related traits (Leaf Drying score, root length, shoot length) of all stressed as well as control plants were measured. Data for percent survival and total leaf area affected was recorded according to the standard evaluation system of rice at IRRI [31; 32]. The level of salinity tolerance was evaluated based mainly on the value of LDS, which is based on the percentage of leaf damage. The plants were scored according to the following scale: 1: highly tolerant (10%); 3: tolerant (10–30%); 5: moderately tolerant (30–50%); 7: moderately susceptible (50–70%) and 9: susceptible (70%) [31]. The chlorophyll content of the stressed and control transgenic shoots as well as WT was measured at this stage [33], as well as their dry weight after keeping them for 72 h at 70^0C in a hot-air circulating oven (Honeywell, UK model DT200).

INTRODUCING THE HELICASE TRANSGENE INTO FARMER-POPULAR CULTIVARS BY BACKCROSSING AND SELECTION OF THE BEST TOLERANT LINES

The *Helicase* gene was backcrossed from the Binnatoa genetic background into three farmer-popular Bangladeshi rice cultivars, BRRIdhan 28, 29 and 47. The F_1 plantlets containing the *PDH45* transgene were selected by PCR and allowed to self up to the F_5 generation. The presence of the transgene was confirmed at each generation. Seedling stage screening was carried out at both F_3 and F_4 generations. Some transgenic progenies showed better tolerance than their sensitive parent (Fig 3). Significantly tolerant plants of each variety were selected and will be screened at reproductive stage to check the grain yield under salt stress.

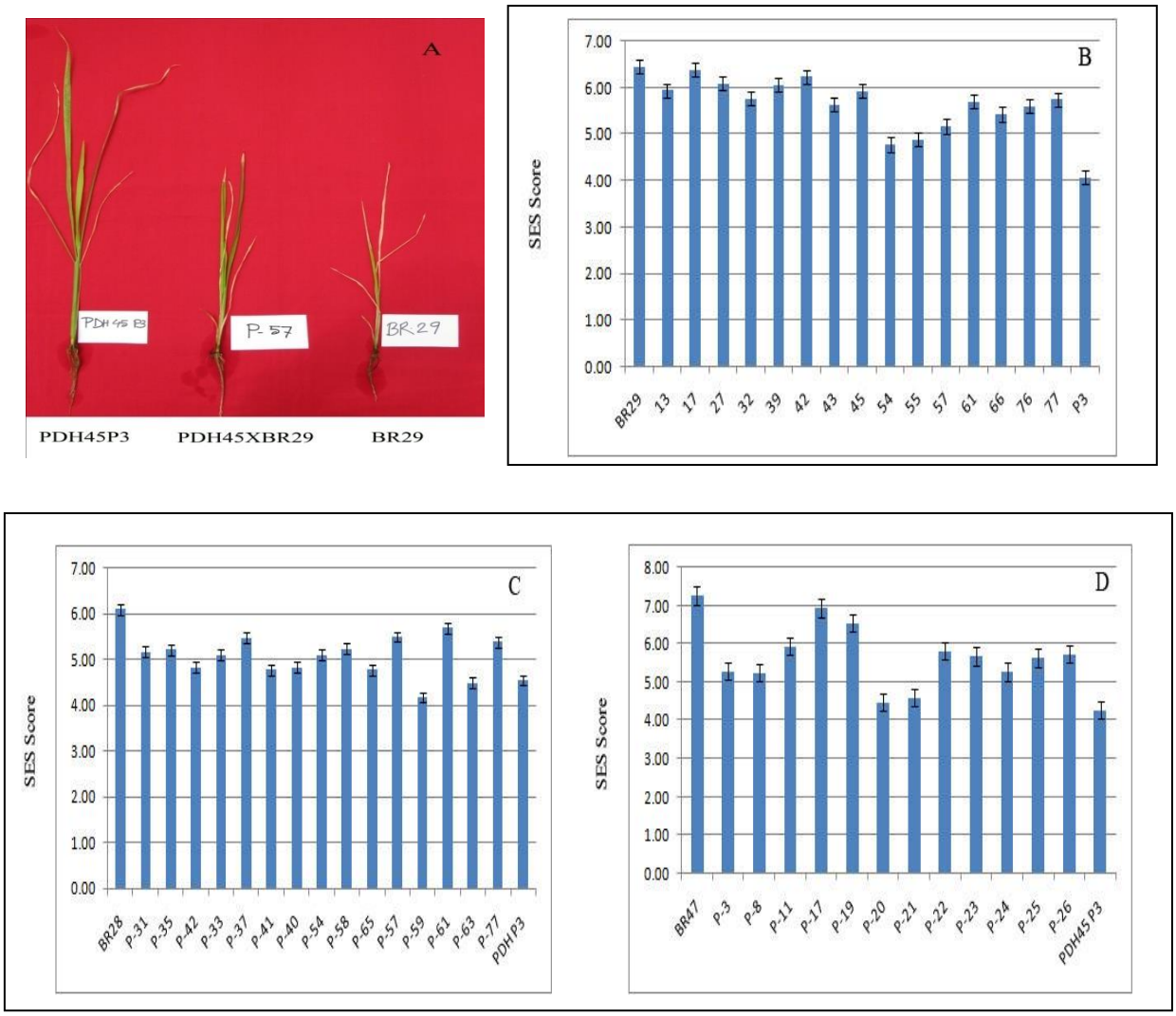


Fig 3 : Transgenic rice with PDH45 performed better at physiological screening.(A) Seedlings of PDH45, PDH45×BR29 and BR29 after 12 dS/m salt stress in a hydroponic system. PDH45×BR29 seedling is more vigorus than the Wild type BR29. (B,C,D) SES Score of wild-type and transgenic rice seedlings after NaCl stress at 12 dS/m in hydroponics. Each bar represents the mean ± SE (n = 18); $P<0.05$

SELECTION OF THE BEST-PERFORMING LINES TRANSFORMED WITH THE TRANSCRIPTION FACTORS SNAC1 AND HARDY

Six transgenic rice lines containing the SNAC1 gene were selected by PCR and advanced to the T_3 generation. The presence of the transgene was confirmed at each generation. Depending on the expression levels of the transgene by RT-PCR, four lines have been selected for physiological screening. Among these four lines, progenies of one line showed better tolerance than the wild type (Fig 4).

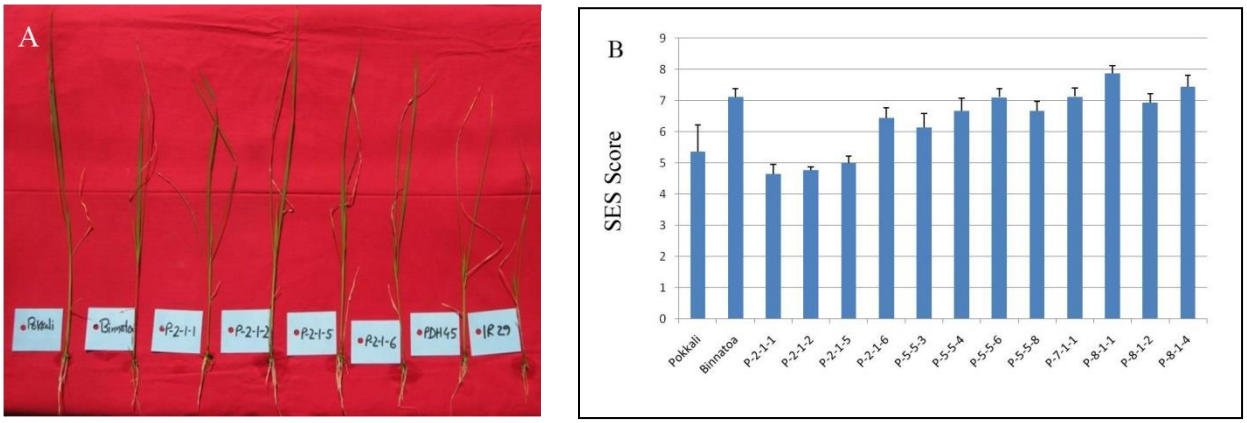
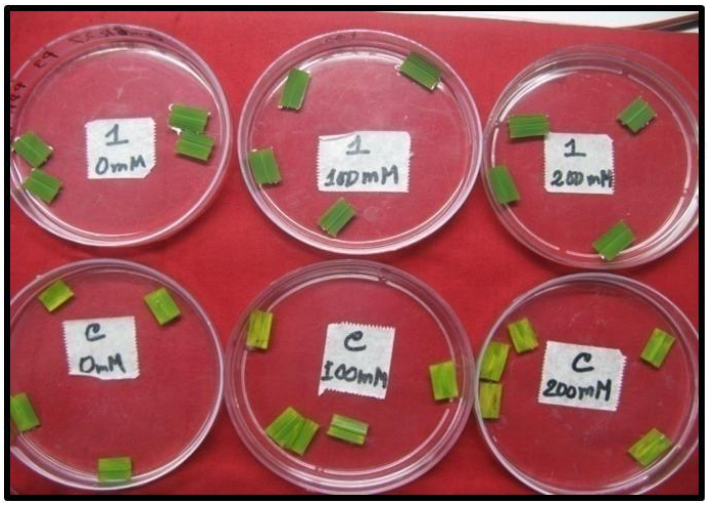


Fig 4: Transgenic rice containing SNAC1 gene showed better tolerance. (A) SNAC1 gene containing transgenic seedlings, Wild Type (Binnatoa), Sensitive control IR29 after 12 dS/m salt stress in hydroponic system. (B)) SES Score of wild-type and transgenic rice seedlings after NaCl stress at 12 dS/m in hydroponics. Each bar represents the mean ± SE (n = 18); $P<0.05$

The presence of HARDY gene in the transgenic rice plants was confirmed by PCR and their expression checked by RT PCR. One of the three transgenic plants had higher expression of HARDY gene and was thus selected for further analysis. The co-transformed seedlings were screened by Hygromycin resistance assay for the presence of respective transgenes at the T_0 generation and further confirmed by PCR. T_1 seeds were collected from the hygromycin resistant PCR positive plants. The transgenic plants were further characterized by leaf disc senescence assay (Fig 5).



Transgenic

Wild Type

Fig 5: Leaf Senescence Assay at T_1 generation for the HARDY transformants

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