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Introgression, Generational Expression and Salinity Tolerance Conferred by the Pea DNA Helicase 45 Transgene into Two Commercial Rice Genotypes, BR28 and BR47

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Abstract

DNA helicase (*PDH45*) from the pea plant (*Pisum sativum*) is a member of the DEAD box protein family and plays a vital regulatory role in saline stress tolerance in plants. We previously reported that over-expression of *PDH45* gene confers both seedling and reproductive stage salinity tolerance to a Bangladeshi rice landrace, Binnatoa (BA). In this study, transgenic BA-containing PDH45 (♂) was crossed with two different farmer-popular BRRI rice varieties (♀), BR28 and BR47, in a contained net house. F_1 plants positive for the transgene and having recipient phenotype were advanced from F_1 to F_5. Expression of the *PDH45* gene was detected in all generations. The expression level of *PDH45* was 200-fold higher in the donor compared to the two recipient genotypes but without any effect on their salt stress tolerance ability in various assays. Under 120 mM NaCl stress at seedling stage, all rice genotypes showed vigorous growth, higher chlorophyll content, lower electrolyte leakage and lower LDS (Leaf Damage Score) compared to their corresponding wild types. At the reproductive stage under continuous salinity stress at 80 mM NaCl, the cross-bred lines BR28 and BR47 showed significantly better spikelet fertility and yield per plant, which were two- and 2.5-folds, respectively, than their corresponding wild types. The *PDH45* transgene was observed to increase the expression of 6 salt stress-related downstream genes at 150 mM NaCl stress to similar differential degrees in the donor and recipient genotypes. However, the expression of *OsLEA* was significantly higher in transgenic BR28 compared to transgenic BR47, where the latter shows comparatively higher salt tolerance. The study shows stability of transgene expression across generations. It also demonstrates that there may be an effect of background genotype on transgene expression. Moreover, some downstream effects of the transgene may also be genotype-specific.

Keywords Commercial rice genotypes · Transgenic BA · Spikelet fertility · Yield · Expression pattern

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Introduction

Rice is the staple food of Asia and constitutes about 43% of the global food grain production [1–3]. However, the rice plant is inherently sensitive to salt stress and is currently listed as the most salt-sensitive cereal crop with a threshold of 3 dS/m for most cultivated varieties [4]. Soil salinity produces devastating effects on rice growth and yield. It is estimated that 45 Mha of irrigated and 32 Mha of dry land area is globally affected by salt stress [5, 6]. So, development of salt-tolerant varieties with high yields is considered to be one of the most economical and effective ways to increase crop production in saline lands.

Tolerance towards salinity stress in rice is a complex physiological mechanism [7], regulated by many quantitative trait loci (QTLs) [8, 9] and ultimately by a host of genes.

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A number of critical genes involved in salinity stress tolerance have been identified and validated, which are generally classified into two types: functional genes and regulatory genes [10]. The former can encode important enzymes and metabolic proteins, for example, a detoxification enzyme [11], a water channel protein [12], an ion transporter [13], a heat shock protein [14] and a late embryogenesis abundant (LEA) protein [15], which are directly involved in functions which protect cells from salt stress. The second group includes various regulatory proteins including transcription factors [16], transcriptional and translational activator-like protein kinases [17], protein phosphatases [18] and helicases [19] which regulate signal transduction and gene expression in response to stress.

In general helicases are motor proteins which catalyse the unwinding of stable duplex DNA or RNA molecules by using the energy of ATP hydrolysis. DNA helicases therefore play important roles in replication, recombination, repair and transcription as well as in maintenance of chromosome stability [20]. RNA helicases unwind local secondary RNA structures, act as RNA chaperones during RNA cellular movement through the nucleus and help in transcription, ribosome assembly, RNA processing and translation. RNA helicases may also function in oligomerization or as RNPases to displace protein from structured or unstructured RNA [21]. Helicases belonging to the DEAD box group (having the DEAD motif or Asp–Glu–Ala–Asp) play a pivotal role in stress management in plants. The effect of DEAD box helicases in plant stress has been validated in mutants [22, 23], in multiple transgenic plants [24–28] as well as microarray and other transcriptional assays [29].

Previous work with the Pea DNA helicase (*PDH45*) with over-expression in tobacco, rice, peanut, sugarcane and chili has shown that the transgene confers strong salinity tolerance in multiple plants, like tobacco [30], rice [19], sugarcane [31] while also providing drought tolerance in peanut [32]. Moreover, *PDH45* showed a greater level of salinity tolerance when co-transformed with another gene *DREB2* in sugarcane [33].

Interestingly, *PDH45* is a unique member in that it contains DESD and SRT instead of DEAD/H and SAT domains in motifs V and VI, respectively. *PDH45* is localized in both nucleus and cytosol and exhibits 3′–5′ directional unwinding activity in an ATP-dependent manner. Moreover, it shows both DNA as well as RNA helicase activity and is highly homologous to the eukaryotic initiation factor eIF4A as are most other DEAD box helicases [34, 35]. Na^+, Ca^{2+} and ROS-specific dyes showed the gradual reduction of all three of these molecules with time only in over-expressing IR64 rice lines [36]. They also hypothesized that in roots of over-expressing *PDH45* transgenic rice, up-regulation of O*sSOS1,* i.e., salt overly sensitive (SOS) pathway may lead to less Na^+ accumulation via exclusion. Such low Na^+ accumulation results in low ROS (H_2O_2) generation and ultimately cell viability followed by balanced Ca^{2+} homeostasis under salinity stress. Further, up-regulation of cation transporters such as *OsCHX11*, *OsCAX*, *OsTPC1* and *OsCNGC1* collectively contributed to provide tolerance to the over-expressing *PDH45* transgenic lines. These are all cationic transporters with rice accession numbers as *CHX11* (Os05g31730), *OsCAX* (Os02g04630), *OsTPC1* (Os01g48680) and *OsCNGC1* (Os06g33600) [36]. Limited success has been achieved for obtaining rice plants with salinity tolerance beyond 6 dS/m using conventional breeding or marker-aided backcrossing of single QTLs into elite varieties. This is likely due to the need for selection and pyramiding of multiple QTLs for salt tolerance in addition to high yields [37]. Since extensive work has shown that Pea DNA helicase (*PDH45*) confers strong tolerance to salinity, it is an ideal gene to target for genetic transformation into elite rice lines. Success, however, is not guaranteed due to the reported low efficiencies of transformation and regeneration of indica rice, the subspecies most popular in Bangladesh and South Asia [38, 39]. Thus, it may be necessary to insert the transgene into the genetic background of multiple farmer-popular modern indica genotypes having high yields by conventional crossing after successful transformation of a responsive genotype. There are limited reports about the functional efficacy of transgenes after their introgression into different genetic backgrounds by such backcrossing. In the work of [42], the transferred *OsNHX1* gene from transgenic *Binnatoa* (BA) to high-yielding commercial BR28 genetic backgrounds showed similar level of gene expression but conferred considerably lower levels of salinity tolerance at both seedling and reproductive stage compared to the donor. In this study, we tested the performance of the *PDH45* transgene after its transfer by crossing from highly salt-tolerant BA-*PDH45*, used as a pollen donor [19], to two high-yielding varieties, BR28 and BR47. These genotypes are Boro varieties which can be grown in the coastal areas in the dry winter season. The paternal parent BA (Binnatoa) is highly efficient in transformation and regeneration but is a low-yielding traditional rice. On the other hand, both maternal recipients are farmer-popular and while BR28 is salt-sensitive BR47 is a moderately salt-tolerant variety at both seedling and reproductive stages developed from Pokkali, a highly saline-tolerant traditional indica low-yielding rice. The objective here was to introduce salt tolerance to sensitive BR28 rice and to further enhance the tolerance capacity of BR47 for targeting its growth to soils with higher salinity levels than 6 dS/m. Accordingly, the expression of the transgene *PDH45* and its ability to confer tolerance in two different genetic backgrounds across several generations was quantified at both seedling and

reproductive stages until the F_5 generation. The effect of the transgene on expression of 6 genes previously reported to be influenced by *PDH45* was also quantified.

Materials and Methods

Plant Materials

BRRI dhan28

BRRI dhan28 (BR28) is a high-yielding, early-maturing boro-season (dry season) variety, released by BRRI in 1994. It produces 5.5–6 t/ha and is popular all over Bangladesh. It was derived from across of IR28 (from IRRI) and Purbachi (IRTP 18013) and is sensitive to salt stress [40].

BRRI dhan47

BRRI dhan47 (BR47) is a high-yielding boro variety and is moderately saline tolerant at both seedling and reproductive stages [41]. It is an IR line with the well-known salt-tolerant rice Pokkali in its pedigree (IR 63307-4B-4-3).

BA-*PDH45*

Transgenic Binnatoa (BA-*PDH45*) at T_3 is a tissue-culture-responsive traditional rice containing the Pea DNA helicase gene (*PDH45*) as a single copy and well-characterized as saline tolerant at both seedling and reproductive stage [19]. So, BA-*PDH45* was used as a pollinator to transfer the PDH45 gene into the two elite BRRI varieties, BR28 and BR47 by conventional crossing.

Conventional Crossing

Crossing was performed as described previously by Biswas et al. [42]. All three rice varieties were allowed to grow until panicle maturity. Immediately prior to flowering, the panicles of BA–*PDH45* plant were cut at their ends and dipped in water for 3 h (between 11 a.m. and 2 p.m.). Emasculation of the maternal genotypes (BR28 and BR47) was performed at the same time. Emasculated panicles were covered with oil paper to prevent external pollination. Pollen were collected in a petri plate by smoothly sliding one panicle with another. The pollen were then dusted on to the stigma of the recipient plant by a small brush. The panicles were then covered again for about 5–10 days until seeds developed. After harvesting, naked seeds of both BR28 and BR47 were sterilized and germinated in semi-solid MS medium (1/4 strength) [43]. After a few days, the seedlings were transferred to hydroponics [44] for 15 days and then transplanted in soil. Plants were grown to maturity and the hybrids which were morphologically similar to the respective recipient parent were tested for presence of the transgene. Maternal-type plants positive for the presence of the transgene were selected at each generation and eventually advanced up to F_5.

Molecular Screening of Transgenic Plants

PCR

Transgenic cross-bred rice plants were screened by PCR analysis using 50 ng rice genomic DNA as template at F_1–F_5 generations with different *PDH45* (Accession No. Y17186)-specific primers for amplification of short 358-bp region and full length 1.2 kbp *PDH45* (Supplementary Table 1). DNA was extracted from rice plant leaves using the CTAB method [45, 46]. For amplification of the short fragment, the reaction was performed with initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 60.4 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 7 min.

For full length *PDH45* amplification, the PCR reaction was performed at 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 61 °C and 1.5 min at 72 °C, then a final extension of 10 min at 72 °C. Both PCR analysis was carried out in a 25 µl reaction mixture containing 50 ng of plant DNA, 100 µM of each dNTP, 2.4 ng each of primers (Supplementary Table 1), 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$, DMSO 2.4% and 1 × PCR Buffer-$MgCl_2$ (Invitrogen).

Quantitative RT-PCR

Total RNA was extracted from salt-treated (150 mM NaCl, 24 h) leaves of 3-week-old WTs (BA, BR28 and BR47) and different transgenic lines over-expressing *PDH45* (BA_*PDH45*, BR28-*PDH45* and BR47-*PDH45* at F_2–F_5 generations) using Trizol (Invitrogen) and used for reverse transcription reactions. The complementary DNA (cDNA) was synthesized from total RNA (1 µg) using a cDNA synthesis kit (Invitrogen).

Quantitative real-time PCR was performed in a 10 µl reaction using SYBR Green (Bio-Rad, USA) with *PDH45* specific primers (Supplementary Table 1) in CFX96™ Real-Time PCR detection system (Bio-Rad, USA). For *PDH45* gene expression, we used leaves of 3-weeks-old transgenic lines of both cross-bred BR28-*PDH45* and BR47-*PDH45* (F_2–F_5 generations) and donor, BA-*PDH45*, which were salt-treated (150 mM NaCl, 24 h).

For expression profiling of salt-responsive genes, all wild types (BA, BR28 and BR47) and their respective transgenic lines, BA-*PDH45*, BR28-*PDH45* and BR47-*PDH45,* were used. Primers for the 6 salt-responsive rice genes are listed in Supplementary Table 1. PCR efficiency of all primers was

between 95 and 105 %. 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 [47]. Elongation Factor-α (EF-α) (Supplementary Table 1) was used as the normalization control.

Physiological Analysis

Leaf Disc Senescence Test Under Salt (NaCl) Stress

Leaf discs were excised from healthy and fully expanded rice leaves of similar age from both WTs (BR28 and BR47) and cross-bred transgenic lines (BR28-*PDH45* and BR47-*PDH45*) at F_5 generation. The discs were floated in a 20 ml solution containing 0, 100, 200 mM NaCl for 3 days as described in detail in Parvin et al. [48]. Discs floated in water served as control. The treatment was carried out at 25 °C. After the treatment and scoring, the leaf pieces were blotted with tissue paper and weighed before measuring their chlorophyll content.

Salinity Stress Screening at Seedling Stage

Phenotypic screening for salinity tolerance at the seedling stage was done following the method described by Amin et al. [19]. Screening was done on BA-*PDH45*, BR28-*PDH45* and BR47-*PDH45* at the F_5 stage. WTs (BA, BR28 and BR47), salt-tolerant control Pokkali and salt-sensitive IR29 were also used in the screening. Sprouted seeds were sown in netted styrofoam and floated in PVC trays containing 10 L Yoshida solution [44]. The germinated seeds (9 for each line in 11 rows) were allowed to grow for 14 days. Then, NaCl stress was applied gradually starting from 4 to 12 dS/m at 24 h increments of 2 dS/m. After 7–10 days, when the sensitive variety IR29 was nearly dead, the tolerance-related traits (LDS, root length, shoot length and leaf width) of all stressed plants were measured. The level of salinity tolerance was evaluated mainly based on the value of LDS, which is based on the percentage of leaf damage. The plants were scored according to the protocol mentioned by Amin et al. [19]. Here the score of 1–9 corresponds from highly tolerant to extremely sensitive, respectively. The chlorophyll content and electrolyte leakage of the stressed and control transgenic shoots as well as all wild types were measured at this stage [49].

Measurement of Chlorophyll Content and Electrolyte Leakage

Fresh leaves were cut into pieces and 100 mg put into a bottle containing 12 ml of 80% acetone and kept in dark for 2 days. After 48 h, absorbance of leaf tissue extract was measured at wavelength 663 and 645 nm. The total amount of chlorophyll content was calculated using the formula: $[\{-(0.00802 \times A_{663}) + (0.0202 \times A_{645})\} \times V/W]$; A = absorbance, V = volume, and W = weight [50].

Relative electrolyte leakage was measured as described in Parvin et al. [48]. The plant leaf segments were weighed (0.1 g) and taken in a falcon tube with 25 ml deionized water. The tubes were shaken on a gyratory shaker at room temperature for 2 h. The initial electrical conductivity ($C1$) of the solution was measured by using a conductivity detector. The leaf samples were then boiled in deionized water at 120 °C for 10 min to release all the electrolytes from the tissues completely. The final electrical conductivity ($C2$) of the resulting solution was recorded. The percentage of electrolyte leakage was calculated according to the formula: $(C1/C2) \times 100$.

Salinity Stress Screening at Reproductive Stage

Reproductive stage screening under continuous salinity was performed according to the protocol followed in [19]. After 18 days in hydroponic system in Yoshida solution, 30-day-old wild types and transgenic lines were transferred to soil contained in perforated pots of 6″ diameter and 5.5″ height. Six pots were placed in a bowl of slightly greater height, with the perforations ensuring equilibrium of the soil with added water or salt water. Each pot contained two plants (replicates) of any specific genotype. Each bowl contained WT, sensitive control, tolerant control and three transgenic plants. After 10 days, the pots were taken out of the water and allowed to drain for 24 h and then transferred to large bowls filled with 8 dS/m NaCl solution in 5 bowls or 5 biological replicates. Water instead of NaCl solution was used in three more bowls to serve as control. The experiment was set up in a net house where the average temperature and humidity were 29 °C and 72%, respectively. At the end of reproductive stage, seeds were collected from transgenic and non-transgenic plants and the weight of total filled grains (g/plant) was measured to determine the grain yield. Other yield-related traits (tiller number, panicle number, panicle length, spikelet number, and spikelet fertility in percent) were also measured.

Data Analysis

Statistical analyses were done using the Data Analysis ToolPak of Microsoft Office Excel 2007 and Cropstat 7.2. The F test was performed to verify equal variance of the independent set of samples and Student's t test. ANOVA was performed, assuming equal variance or unequal variance as applicable, to compare significant differences ($P > 0.05$) between the transgenic and the WT lines.

Results

Crossing with BR28 and BR47 and Generation Advancement

BR28 and BR47 were selected as females for crossing with the BA-*PDH45* plant to transfer the *PDH45* gene into the former plants. F_1 plants were allowed to grow and plants which looked phenotypically like their mothers were selected and screened by PCR with internal *PDH45*-specific primers (Supplementary Table 1 and Supplementary Fig. 1) and *hygromycin* assay (data not shown). Six PCR-positive plants for BR28-*PDH45* (P-31, P-35, P-42, P-45, P-65 and P-77) and 5 PCR-Positive plants (P-3, P-8, P-20, P-21 and P-22) for BR47-*PDH45* were advanced to F_2 and later to F_3 by selfing. Presence of the transgene in the F_2 and F_3 progenies (those that had the maternal parent phenotype) was confirmed by *PDH45*-specific PCR and *hygromycin* assay. Seedling stage screening under salinity stress was done (Supplementary Fig. 2) on all cross-bred transgenics at the F_3 generation. Then two independently cross-bred lines (P-42 and P-65 lines for BR28 and P-20 and P-21 lines for BR47) from F_3 with better phenotypic data under salinity stress were advanced to F_4 and later F_5 generation by selfing. Presence of the target gene, *PDH45,* at F_5 generation was also confirmed by PCR with *full length PDH45 primers* (Fig. 1). All further molecular and physiological analysis was performed at the F_5 generation for both the BR28-*PDH45* and BR47-*PDH45* cross-bred lines.

Expression of *PDH45* in Transgenic Plants

Expression of *PDH45* was analysed in both cross-bred transgenics (BR28-*PDH45* and BR47-*PDH45*) in different generations (F_2–F_5) and BA-*PDH45* by quantitative RT-PCR after 24 h of salt stress at 150 mM. It was found that the expression of the transgene *PDH45* was similar in different generations of both the cross-bred transgenics (Fig. 2). However, BR47 cross-bred transgenics showed higher level of expression (3–3.5) compared to the BR28 cross-bred lines (2–2.5). But in the pollinator genotype, the *PDH45* gene was very highly expressed which was approximately 100 times higher than both cross-bred transgenic plants (Fig. 2c).

Effects of Salt Stress on Matured Leaf Disc

We analysed the salt tolerance of leaf discs from both BR28-*PDH45* and BR47-*PDH45* transgenic plants. Different concentrations of NaCl solution were applied to leaf discs of both WTs and transgenic cross-bred plants at the F_5 generation for 4–5 days. No significant difference was found between WTs and transgenic plants under control condition (no salt). But in the presence of high salt (100 and 200 mM NaCl), leaf discs from transgenic plants were more green and healthy (Fig. 3a, c) and maintained a relatively higher content of chlorophyll compared to corresponding WTs (Fig. 3b, d).

Effects of Salt Stress on Seedling Stage

Seedling stage screening was performed with all WTs (BA, BR28 and BR47,) and all types of transgenics (BR28-*PDH45*, BR47-*PDH45* and BA-*PDH45*). All transgenic lines showed significantly better phenotypic characteristics compared their corresponding WTs under salt stress (Fig. 4a, c). However, BR47-*PDH45* transgenic lines and BA_*PDH45* had lower score (~ 4–4.5) compared to BR28-PDH45 transgenic lines (~ 5) (Fig. 4b, d).

Under control condition, all transgenic plants (both BA-*PDH45* and cross-bred lines) had significantly higher shoot length and fresh weight compared to their wild-type plant, but no significant difference was found for root length between the wild types and transgenics. Interestingly after salt stress at 12 dS/m for 7 days, transgenic plants performed

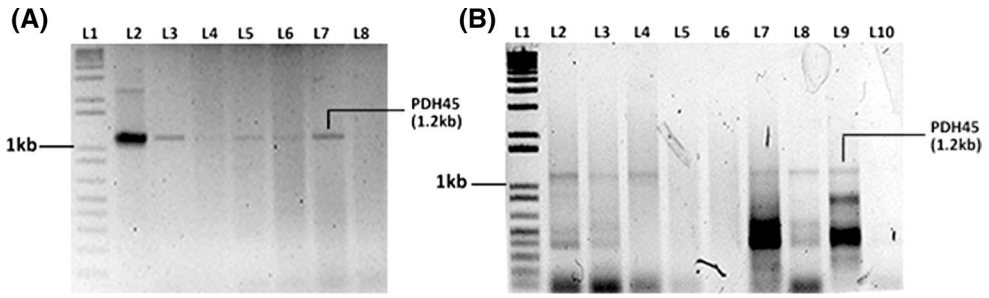

Fig. 1 PCR confirmation of full length *PDH45* gene in transgenic BR28-*PDH45* (**a**) and BR47-*PDH45* (**b**) plants at F_5 generation. For **a** L1: 1 kb$^+$ ladder, L2: positive plasmid control, L3, L5, L6: transgenic BR28-*PDH45* plants (P-42-1, P-42-2 and P-65-1) and L8: negative control; **b** L1: 1 kb$^+$ ladder, L2: positive plasmid control, L3, L4, L7-L9: transgenic BR47 plants (P-20-1, P-20-2, P-21-1 and P-21-2), L10: negative control

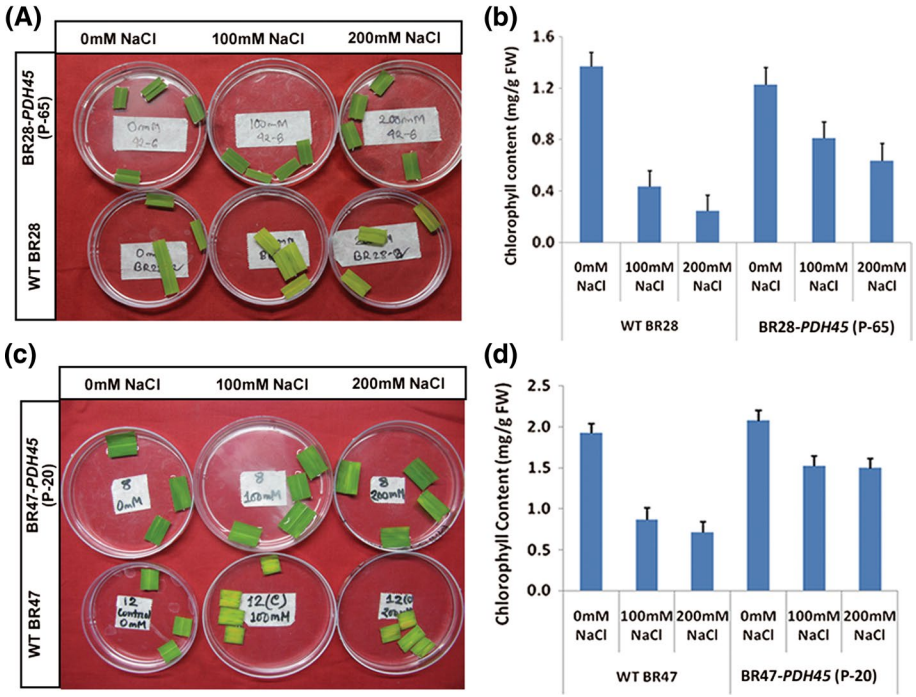

Fig. 2 Quantitative expression analysis of *PDH45* gene in cross-bred transgenic BR28-*PDH45* (**a**), BR47-*PDH45* (**b**) at F_2–F_5 generations and also in donor transgenic BA-*PDH45* (**c**) using RT-PCR. Total RNAs were extracted from the leaves of all transgenic plants after 24 h 150 mM NaCl stress. All mRNA levels were normalized with respect to an internal control gene elongation Factor-α (EF-α). The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of *PDH45* gene in all transgenics. The data bars represent the mean ± SD of triplicate measurements

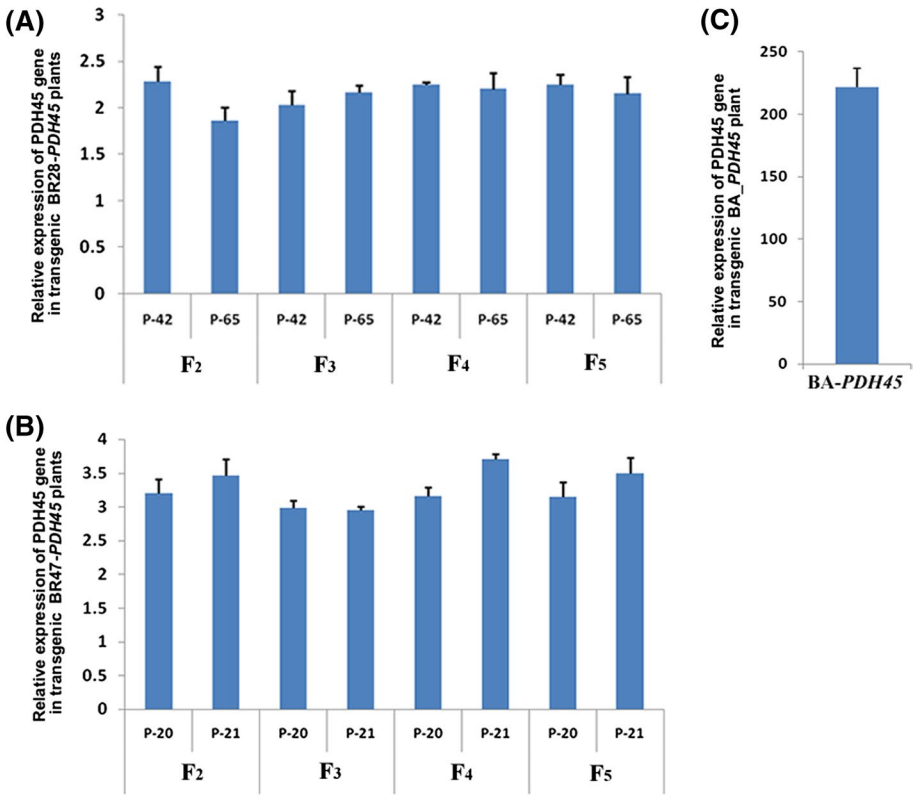

Fig. 3 Leaf disc senescence (LDS) assay and chlorophyll content measurement of both BR28-*PDH45* and BR47-*PDH45* transgenic lines at the F_5 generation. **a**, **c** Transformed plants remained green even at 200 mM of salt stress (NaCl) (top panel), while the non-transformed leaves showed necrosis and decolorization after 100 and 200 mM salinity (NaCl) (lower panel) stress. **b**, **d** Chlorophyll content was measured from the leaf discs of transgenic and WT plants. The leaf discs of transgenic BR28-*PDH45* and BR47-*PDH45* lines continued to show better chlorophyll contents even after salinity (NaCl) stress at 100 and 200 mM

significantly better result in all these physiological parameters like shoot length, root length and fresh weight (Supplementary Fig. 3).

Electrolyte Leakage and Chlorophyll Content at Seedling Stage

Electrolyte leakage is a hallmark of stress response in intact plant cells. The percent of electrolyte leakage in

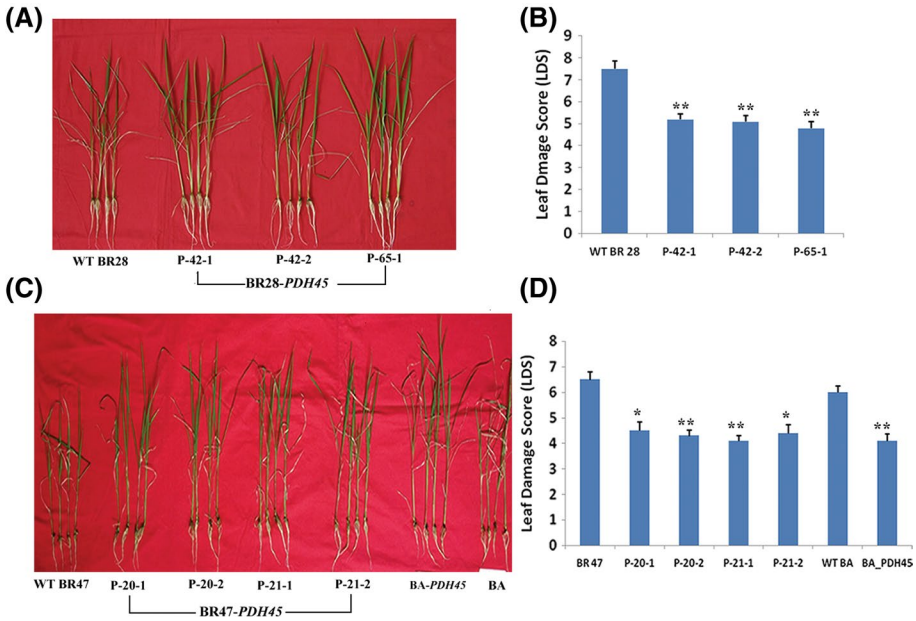

Fig. 4 Seedling stage salt stress screening of *BR28-PDH45, BR47-PDH4 5 and BA-PDH45* transgenic lines. **a**, **c** Phenotypic view of all WTs and both transgenic lines after 7 days at 12 dS/m NaCl stress in hydroponics. The growth of all transgenic lines was better compared to their corresponding WTs. **b**, **d** LDS in wild-types and transgenic rice seedlings after 7 days of NaCl stress at 12 dS/m in hydroponics. *Each bar* represents the mean ± SE ($n = 5$). It is to be noted that LDS was significantly higher in transgenic plants compared to their corresponding wild types. ** or * indicates significant difference between wild-type and transgenic variety at $P < 0.01$ or $P < 0.05$, respectively

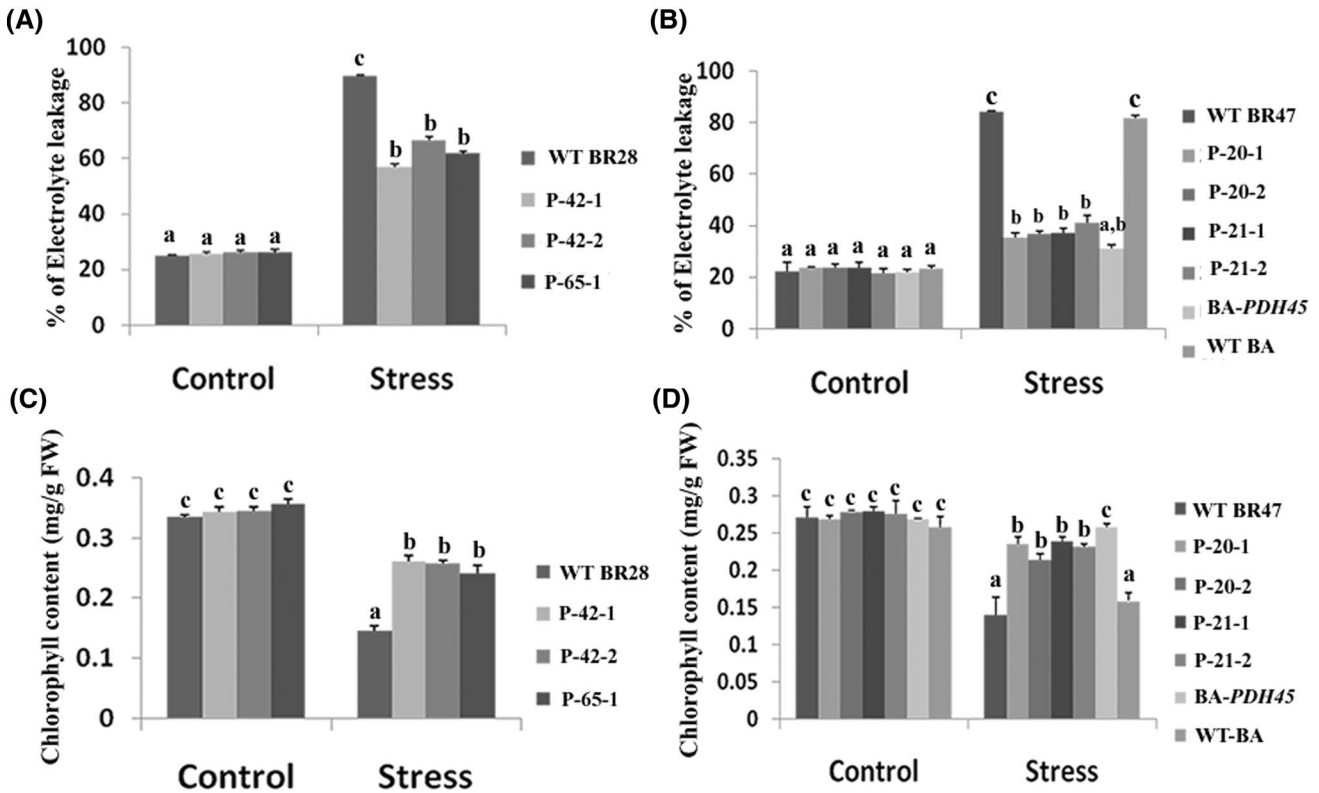

Fig. 5 Percent of electrolyte leakage (**a**, **b**) and chlorophyll content (**c**, **d**) in all BR28-*PDH45*, BR47-*PDH45* and BA-*PDH45* transgenic lines compared to their WT plants under control and stress (NaCl) condition. All transgenic plants showed significantly better performance compared to the WTs under salt stress. Each bar represents the mean ± SE ($n = 5$). Different letters in each graph (**a**–**c**) indicate significant differences ($P < 0.05$, ANOVA and Duncan test)

all transgenic plants was significantly lower compared to corresponding WTs after 12 dS/m NaCl stress for 7 days (Fig. 5a, b). However, BR47-*PDH45* lines and BA-*PDH45* showed much lower electrolyte leakage (~ 30%) than BR28-*PDH45* lines (60%). But under control condition (no salt stress), there was no significance difference found between all WTs and transgenics in respect to electrolyte leakage (Fig. 5a, b).

Chlorophyll is a fundamental component of photosynthesis, and chlorophyll content acts as an index of leaf senescence under salt stress. Chlorophyll content was also significantly higher in all transgenic lines compared to corresponding WTs after NaCl stress at 12 dS/m in hydroponics (Fig. 5c, d). Moreover, under control condition (No salt stress) the chlorophyll content between wild-types and all transgenic plants (both donor and recipient plants) at the seedling stage was almost similar.

Effects of Salinity Stress in Reproductive Stage

Under control conditions without stress, different agronomic traits like tillers per plant, panicle length, filled grains per panicle, number of grains per panicle, spikelet fertility and yield were similar between all transgenics (BA_*PDH45,* BR28-*PDH45* and BR47-*PDH45* transgenic lines) and their corresponding WTs (BA, BR28 and BR47) (Supplementary Table 2 and Fig. 6). Moreover, seed structure of all transgenic lines also looked like their corresponding WTs (Fig. 6b, e). But under continuous salinity stress at reproductive stage, all transgenic plants showed lower panicle damage, (Fig. 6c, f) better

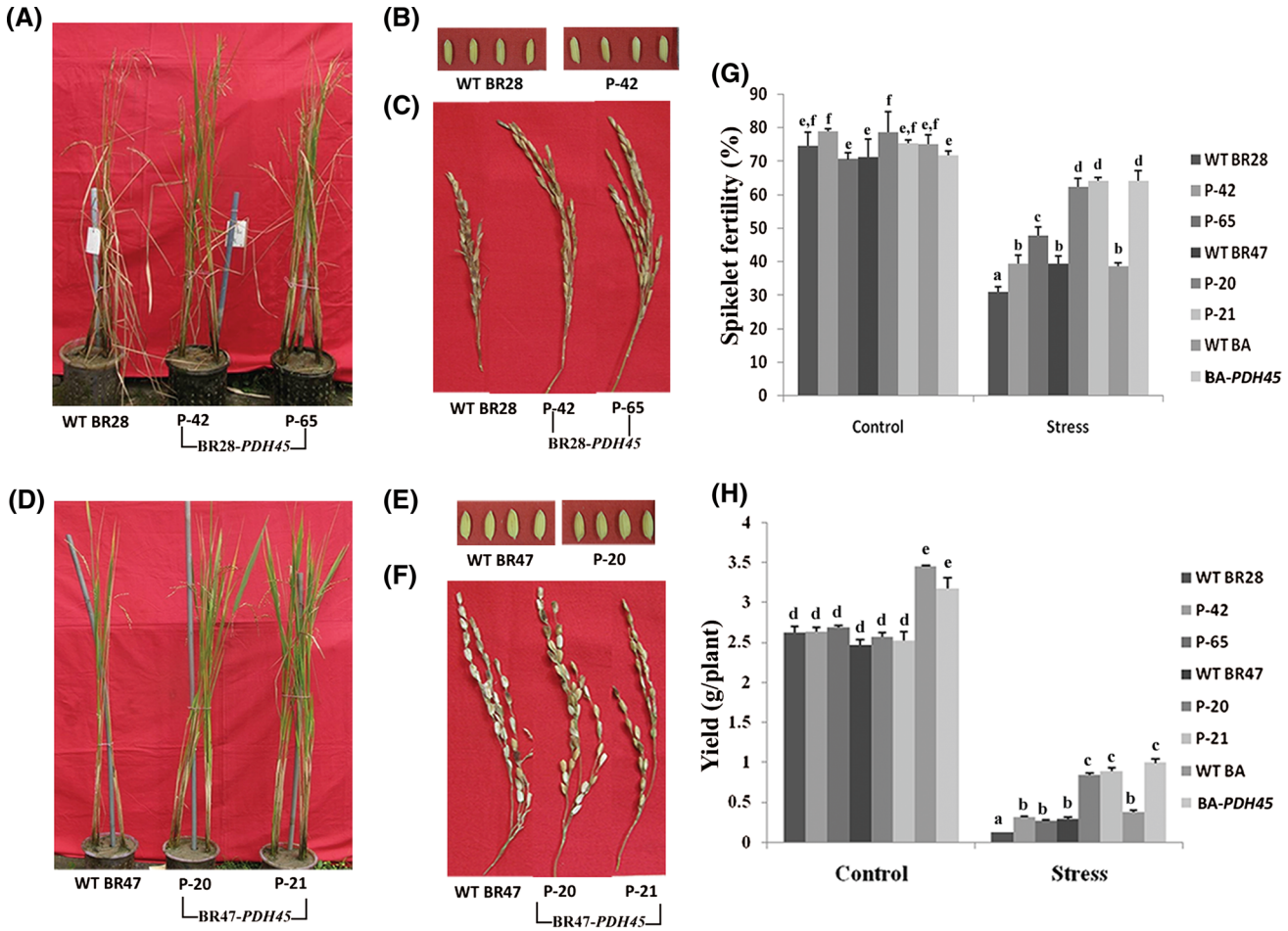

Fig. 6 Physiological screening data at reproductive stage under continuous salinity stress at 8 dS/m. **a**, **d** Phenotype of BR28-*PDH45* and BR47-*PDH45* transgenic lines and corresponding WTs under stress condition at reproductive stage. **c**, **e** Pictorial view of seeds of BR28-*PDH45* and BR47-*PDH45* transgenic lines and corresponding WTs under control condition. **d**, **f** Picture of Spikelet damage of BR28-*PDH45* and BR47-*PDH45* transgenic lines and corresponding WTs after continuous salinity condition. **g**, **h** The percent of spikelet fertility and yield of all transgenic lines (BR28-*PDH45*, BR47-*PDH45* and BA-*PDH45*) and corresponding WTs under both control (no stress) and continuous salinity condition. Different letters in each graph (**a**–**f**) indicate significant differences ($P < 0.05$, ANOVA and Duncan test)

phenology (Fig. 6a) and higher performance in some agronomic parameters (Supplementary Table 2) compared to WTs (Fig. 6a). In respect to both spikelet fertility and yield, transgenic plants performed better than their WTs. However, the yield in BR28-*PDH45* transgenic lines was twofolds, while in BR47-*PDH45* lines and BA-*PDH45* approximately 2.5-folds higher than their corresponding WTs (Fig. 6g, h).

Expression Analysis of Salinity-Responsive Genes in all PDH45 Containing Transgenic Lines and Their WTs

To examine the molecular function of the *PDH45* gene, expression of 6 salinity-responsive genes were performed in all transgenic lines and their WTs after 150 mM salinity stress for 24 h. We checked the expression of *OsSOD* (superoxide dismutase), *OsLEA* (late embryogenesis abundant protein), *OsAPX2* (ascorbate peroxidase 2), *OsDREB2* (DREB2 transcription factor), *OsGRC* (glutathione reductase) and

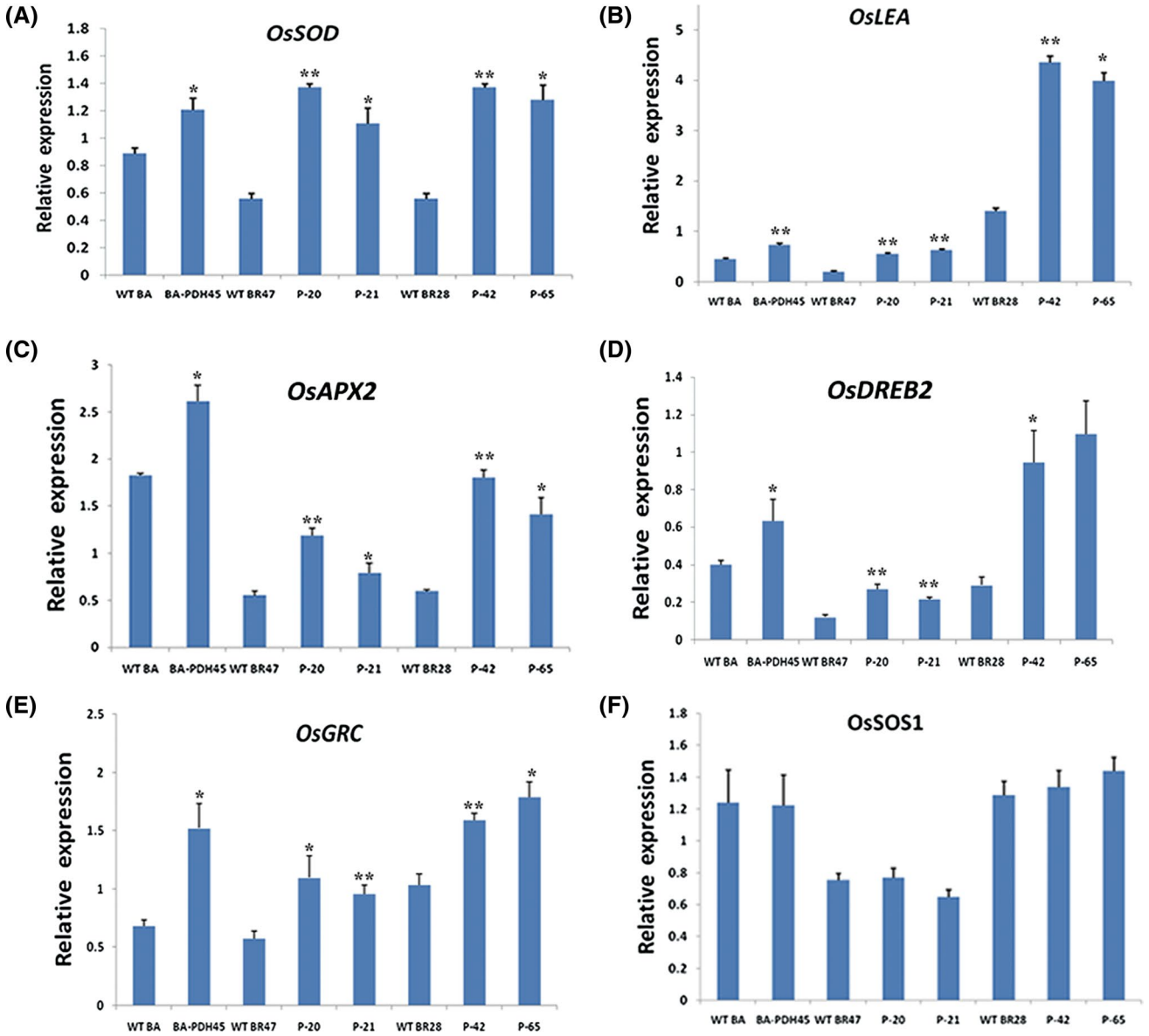

Fig. 7 Expression profiling of salt-responsive genes **a** *OsSOD*, **b** *OsLEA*, **c** *OsAPX2*, **d** *OsDREB2*, **e** *OsGRC* and **f** *OsSOS1* in all wild types (BA, BR28 and BR47) and *PDH45*- containing transgenics (BA_*PDH45*, BR28-*PDH45* and BR47-*PDH45* after 150 mM NaCl stress for 24 h. The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression of respective genes in all WTs and transgenics. All mRNA levels were normalized with respect to an internal control gene elongation factor-α (EF-α). Values are means ± standard errors from three independent measurements

OsSOS1 (salt overly sensitive 1) through quantitative RT-PCR (Fig. 7). After 150 mM salt stress for 24 h, expression of most of these genes in the transgenics were significantly higher than their corresponding WTs (Fig. 7a–e). BR28-*PDH45* lines showed higher expression for *OsLEA* and *OsDREB2* genes (Fig. 7b, d). On the other hand, BA-*PDH45* showed higher expression for the *OsAPX2* gene compared to the other transgenic lines (Fig. 7c). BA-*PDH45* and BR47-*PDH45* showed higher expression of the *OsGRC* gene compared to the BR28-*PDH45* lines (Fig. 7e). However, *SOS1* expression was found almost similar in all transgenic lines and their corresponding WTs (Fig. 7f). However, interestingly the level of *SOS1* expression was higher in the BR28 WT and corresponding cross-bred transgenic lines in comparison with BR47 and their corresponding transgenics.

Discussion

In the backdrop of the current global climate change and increasing levels of salinity in coastal areas, high-yielding commercial rice varieties which also have tolerance traits have become an important necessity. Transgenic rice engineered for ectopic expression of regulatory genes which can influence multiple downstream genes causing plants to adapt to salt stress and be able set grains are therefore highly desirable. Among a few genes which fit this description, Pea DNA helicase (*PDH45*) has been shown to be very successful with examples of salinity stress tolerance in many crops, including rice [19, 36]. There are no reports, however, of the inheritance, stability of expression and performance of the gene in terms of gain in yield, particularly in staples like rice compared to WT in homozygous popular commercial varieties, which could be targeted for eventual release. Assessment of the yield of rice varieties like BR28 and BR47 has been done in the coastal saline zones of Bangladesh where total yields have been estimated with no determination of the quantitative reduction compared to control non-saline soils. Moreover, the evaluation of these genotypes was done in fields with highly variable range of salinities like 2–16 dS/m [51, 52]. The yield of the WT genotypes BR28 and BR47 and their reduction under continuous stress of 8 dS/m in the current study was determined as grams per plant. While these yield estimates are not comparable with the former studies, the gain in yield in the transgenic plants over WT in the current set of experiments provides a good indicator of the effect of the presence of the *PDH45* gene. The salinity stress tolerance of the BR28 and BR47 transgenic plants was tested at both the seedling and reproductive stages and were shown to set significantly more amount of grains than their corresponding wild type at 8 dS/m (Figs. 5, 6). The lines were shown to express the transgene stably across generations and to continue to confer similar levels of tolerance up to the F_5 generation and set seeds indistinguishable from the corresponding WT mother parent. Since the lines have the genetic background of their high-yielding parents, they are photoperiod insensitive and can set grains at any time of the year, unlike common salt-tolerant landraces which have poor agronomic properties and set grains only once annually. The transgenic lines were produced by crossing over of a single well-characterized transformation-friendly genotype [19] into the genetic background of the two dry season farmer-popular high-yielding rice varieties, BR28 and BR47. BR28 is a most popular high-yielding rice variety with an acreage of 6 t/ha, grown all over in Bangladesh with moderate blast resistance, but which is very sensitive to saline stress. BR47 is a traditionally bred salt-tolerant variety with donor genes from Pokkali, a photoperiod-sensitive landrace. It is slowly gaining popularity in the Southern coastal areas of Bangladesh but shows a significant loss in yield beyond salinity levels of 6 dS/m. So by introgressing *PDH45* into *BR28*, we *made* it saline tolerant. On the other hand, by inserting *PDH45* into BR47, we succeeded in augmenting its salt tolerance to a higher level and our results show yield gain at continuous stress level of 8 dS/min in comparison with WT.

This work also clearly demonstrated that the transgene (*PDH45*) continues to be expressed and produce almost similar levels of salinity tolerance after transfer by crossing from donor BA-P3 into two rice genetic backgrounds (BR28 and BR47). In this study, we did not follow the back crossing technique, because it is very tedious and time-consuming. According to Biswas et al. [42], after crossing, positive plants with morphology similar to their respective recipient parents were selected through presence of transgene at successive generations of F_1–F_5. Under control conditions, different agronomic traits and seeds structure of all transgenic plants were similar to their recipient parent♀ (Supplementary Table 2 and Fig. 6).

At the seedling and reproductive stages, under salt stress, all transgenic plants showed significantly better results compared to their corresponding wild types. After exposure to salinity stress, reduction in chlorophyll content has been reported in various crop plants [53]. However, it was well established that the *PDH45* overexpressing transgenic plants retained more chlorophyll than WT under salinity stress, which has strong correlation with the salinity tolerance potential [31]. The larger amounts of chlorophyll could be responsible for the increased yields in transgenic plants both in control and stressed conditions [19]. In this study, we also found that under both leaf disc assay and seedling stage screening under salinity stress, both BR28-*PDH45* and BR47-*PDH45* transgenic lines contain significantly higher chlorophyll content than their wild types. Moreover, in other physiological parameters like LDS score, electrolyte leakage, shoot length and root length, transgenic plants showed better performance. After continuous salinity stress

at reproductive stage, both BR28-*PDH45* and BR47-*PDH45* transgenic lines performed better under physiological tests, like spikelet fertility and yield. Among them, BR28-*PDH45* transgenic lines gave twofolds more in yield, whereas BR47-*PDH45* produced 2.5-folds higher yield than their corresponding wild types. Therefore, these results also clearly show that by introducing *PDH45*, we successfully increased and augmented further the salinity tolerance characteristics of both BR28 and BR47 varieties.

However, we found lower *PDH45* expression in both BR28 and BR47 transgenics compared to donor parent BA-*PDH45*. Perhaps, the lower expression may be due to the different genetic backgrounds of the traditional rice Binnatoa (BA) and the high-yielding genotypes BR28 and BR47. But interestingly the salinity tolerance did not vary much between donor and recipient parents. PDH45 is a regulatory protein and its over-expression may have a direct or indirect role in the expression of downstream stress-related genes. Such effects may be due to its involvement in cellular processes such as DNA replication, transcription, translation, recombination, DNA repair and ribosome biogenesis [54]. Moreover, PDH45 is involved in regulation of Na^+, ROS, cytosolic Ca^{2+} contents and cell viability in rice under salinity stress [36]. Due to the different expression of *PDH45* in donor and recipients, we tried to investigate the expression pattern of some downstream genes shown previously to be affected by the *PDH45* gene [5, 36]. Six abiotic stress-responsive genes, *OsSOD*, *OsLEA*, *OsAPX2*, and *OsDREB2*, *OsGRC* and *OsSOS1* were evaluated in all three transgenic lines (both donor and recipients). Except for *OsSOS1*, all genes were up-regulated and fold of expression varied only slightly among all transgenics. Differences among the genotype of the transgenics and their respective WT were not, however, evident. This lack of difference was remarkable, given that the donor BA-*PDH45* showed more than 200-fold higher activity of the *PDH45* gene. It is likely that being a regulatory gene, low but sufficient expression is enough to turn on downstream genes. One difference between the highly tolerant transgenic BR47-*PDH45* and moderately tolerant BR28-*PDH45* was found in the relative expression of the *OsLEA* gene, which was significantly higer in the latter. OsLEA proteins are extremely hydrophilic and have a chaperone function after being induced under water and salt stress. In a salt-tolerant rice cultivar called Bura Rata, recovery from salt stress was consistently accompanied by degradation of the salt stress-induced LEA proteins. The authors therefore suggested that LEA proteins accumulated during the salinity-triggered growth arrest of young Bura Rata seedlings but were mobilized during recovery of the seedlings from salinity stress. Therefore, for tolerant plants that can recoverfrom stress, LEA proteins need to be broken down for the plants to recover and function with their adjusted metabolism [55].

Further confined field studies are required to determine yield potential of these HYV rice transgenics under field conditions and studies are in progress to assess the field tolerance of some of the promising transgenic events. If the trends for enhanced salinity tolerance are maintained, transgenic rice varieties could clearly have a significant positive impact on world food production.

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