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Research article

Overexpression of heterotrimeric G protein beta subunit gene (OsRGB1) confers both heat and salinity stress tolerance in rice

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ABSTRACT

Constitutive overexpression of the rice heterotrimeric G protein beta subunit gene (*RGB1*) in the commercial rice cultivar BRRI Dhan 55 resulted in improved tolerance to heat or salinity or their combination. Two independently *in planta* transformed plants with the gene confirmed to be integrated at T_2 by Southern hybridization and showing high expression at the T_3 seedling stage showed better physiological performance after 8 days in 120 mM salt stress than the wild type. The plants had significantly lower electrolyte leakage and malondialdehyde production, while showing higher levels of chlorophyll. Significantly higher germination at 48 °C or with combined stresses of 42/40 °C day/night stress in the presence of 120 mM salt for 2 days was also observed. Stress responsive genes such as *OsAPX1*, *OsSOD*, *OsHKT1*, *OsHSP1*, *OsHSP2* and *OsCOR47* showed higher expression in the RGB1 positive plants. These RGB1 transgenic plants can likely provide a strong defense against climate change.

1. Introduction

Fulfilling the escalating global food demand of a rapidly growing population requires that the yields on existing cropland be increased by using marginal lands, such as coastal areas (Licker et al., 2010). However, there has been a gradual increase in soil salinity commensurate with the increase in environmental temperature and expanding sea water volumes. This poses a grave threat to adequate crop production particularly for subsistence farmers in coastal regions (Rengasamy, 2010). Rice is foremost among the staple crops, which feeds half the world population and it's affected by different degrees of salinity. Salt stress causes damage to the rice plant when it is 2–4 weeks old and again at flowering and seed-setting stages (Gerona et al., 2019). Moreover, rice genotypes may differ in their level of sensitivity, and some salt stress tolerant popular rice varieties have been characterized like Pokkali and Horkuch but they are low yielding (Razzaque et al., 2017).

Increase in environmental temperature exacerbates prevailing abiotic stresses such as salinity and multiple stresses severely affect rice yields (Shah et al., 2011). According to a report of the Intergovernmental Panel on Climatic Change (IPCC), global mean temperature will rise 0.3 °C per decade reaching approximately 1 and 3 °C above the present value by the years 2025 and 2100, respectively (Wahid et al., 2007). It is estimated that a 1 °C increase in temperature will reduce rice yield by 10% (Peng et al., 2004). Spikelet sterility results above 33 °C and rice is already facing temperatures higher than this critical threshold in South Asian countries like Bangladesh and India (Jagadish et al., 2007). Although a small number of salt tolerant commercial lines have been released in South and South-East Asia, reports on heat tolerant rice varieties is insignificant (Manigbas et al., 2014). Therefore, incorporation of salinity and heat tolerance traits in rice have become a key objective for researchers. Keeping this in mind, the RNA sequencing of a naturally heat tolerant rice variety called N22 has recently been done to identify valuable candidate genes that can be used for molecular breeding that are associated with heat tolerance (González-Schain et al., 2016). QTL mapping in rice was also done for heat tolerance during the most sensitive flowering stage (Ye et al., 2012). But there are no reports on conferral of a combination of salinity and heat tolerance in plants.

Plants indigenous to a particular environment have evolved adaptive mechanism over hundreds of years to prevailing stresses such as salinity and heat. Cascades of molecular networks are likely involved in dealing with the stresses, such as perception, signal transduction, and the expression of specific responsive genes and corresponding

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metabolite production (Zhu, 2016). Therefore, in order to engineer a similar defense response to stress in commercial cultivars, it is necessary to use regulatory genes that can respond in a way to cause as less metabolic perturbation as possible and plant cells and tissues can still function adequately. Evolutionarily conserved plant heterotrimeric G protein is one of the regulatory genes that was found to be involved in playing a crucial role in signaling, growth and development as well as, the effect of stress on these processes (Colaneri et al., 2014). Though both animals and plants share similar heterotrimeric core elements, plants have developed a distinct regulatory system for heterotrimeric G proteins (Trusov and Botella, 2016). Heterotrimeric G protein signaling and its downstream effectors in plants are not as extensively explored as in the animal system. But recently the emerging attention to the role of heterotrimeric G protein in abiotic stress signaling has made it a potential target to improve plant survival and increase yield under various stress conditions (Urano et al., 2016).

Heterotrimeric G proteins have three different subunits α, β, γ which couple external stimuli to signal transmission (Perfus-Barbeoch et al., 2004). Both Arabidopsis and rice have single Gα and Gβ subunits. However the former has three Gγ subunits while rice has five (Trusov et al., 2012). Function of the three Heterotrimeric G protein subunits is still under investigation. The different subunits are individually being used to improve stress tolerance in plants. In Arabidopsis, maize and rice, Gα is involved in cell cycle regulation and mediating senescence during salt stress (Urano et al., 2014). Under salt stress, overexpression of Gα subunit in *Pisum sativum* and tobacco has shown its up-regulated transcript levels and a salt tolerant phenotype (Misra et al., 2007). Recently it has been shown that Gγ subunit can contribute to increase in grain yield as well as efficiency of nitrogen-use in rice (Sun et al., 2014; Xu et al., 2016).

Utsunomiya et al. (2011) conducted a study where rice heterotrimeric G protein beta (*RGB1*) knocked-down transgenic rice plants were found to be shorter in height with browning of lamina joint region and nodes. *RGB1* mutant plants showed reduced number of panicles with slight reduction in seed size and an increased number of sterile seeds. The authors therefore suggested that RGB1 may play a role in cellular proliferation and seed fertility (Utsunomiya et al., 2011). In Arabidopsis, Heterotrimeric G protein beta subunit (*AGB1*) was found to be involved in leaf, flower and seed development (Lease et al., 2007). The *agb1* null mutants also had shorter primary roots, with an expanded lateral root structure, as well as lower Na^+, K^+ and antioxidant enzyme activity in Arabidopsis (Ullah et al., 2003). The *agb1* null mutants have been shown to be hypersensitive to salt and Gβ was identified as essential for its survival under salt stress. AGB1 is involved in the regulation of Na^+ fluxes in the root, and in root to shoot Na^+ translocation (Colaneri et al., 2014). Moreover, there was up-regulation of the *Pisum sativum* Gβ transcript under heat stress (Bhardwaj et al., 2011). Although both rice and Arabidopsis Gβ subunit share similar characteristics, the homologue to the regulator of heterotrimeric G protein signaling present in the Arabidopsis plasma membrane, At-*RGS1*, is missing in rice. Some monocot species however possess genes which are highly homologous to Arabidopsis *RGS1* (Hackenberg et al., 2017). It is therefore worthwhile to investigate if the stress tolerance response is similar or different in rice.

For the current work, Swarna rice, a popular cultivar of Eastern India and bordering Bangladesh, was used to isolate the rice heterotrimeric G protein beta gene (*RGB1*). The *RGB1* gene was over-expressed in the modern indica rice cultivar called BR55 using a tissue culture free, *in planta* transformation technique (Parvin et al., 2015). Earlier work on over expression of the Arabidopsis heterotrimeric G protein beta subunit in tobacco, conferred heat tolerance (Misra et al., 2007). We undertook the work with the aim to determine whether the transgenic plants could show both heat and salt tolerance. The transgenic rice in the current work grew better with relatively higher content of chlorophyll, but with lower electrolyte leakage and accumulation of MDA and H_2O_2. Leaf damage was also low under both stresses at the seedling stage than wild type plants. The transgenic plants were also shown to exhibit higher expression of genes reported to be important in plant defense against multiple stresses, including those encoding a heat shock protein (*HSP*) and *HKT1*, implicated in heat and salt tolerance respectively (González-Schain et al., 2016; Platten et al., 2013) when compared to wild type.

2. Materials and methods

2.1. Plant material

The indica rice cultivar BRRI Dhan-55 (BR55) used in this study was selected based on its high yield (~7.0 tons/hectare), medium duration growth in Boro and Aus season, and slight salinity, drought and cold-stress tolerance. In our lab, BR55 was previously transformed with *SNAC1* transcription factor by *in planta* transformation method and which had conferred a higher level of both salt and drought tolerance (Parvin et al., 2015).

2.2. Vector design and confirmation

Rice heterotrimeric G protein beta (*RGB1*) subunit gene (HM768320.1) was isolated from *Oryza sativa* indica cultivar, Swarna. This gene is located on chromosome 3. The cDNA (1143 bp) of *OsRGB1* was amplified; gel extracted and cloned into Multiple Cloning Site (MCS) of the cloning vector pRT100 by using *Xho*I and *Eco*RI restriction enzymes for generating the CaMV35S-*RGB1*-poly A fragment. The CaMV35S-*RGB1*-polyA fragment (1.8 kb length) was cut out with *Hind*III and incorporated into the MCS destination vector pCAMBIA1302 which contains both the Hygromycin phosphotransferase (hpt) selectable marker and the reporter *GFP* gene (Supplementary Fig. 1). The vectors (pRT100/CAMV35S-*OsRGB1* and pCAMBIA 1302/CaMV35S-*OsRGB1*) were confirmed by gene specific PCR, restriction digestion and sequencing (Supplementary Fig. 2). The recombinant plasmid pCAMBIA 1302/CaMV35S-*OsRGB1* was transferred to electro-competent *Agrobacterium tumefaciens* (LBA4404). The insertion in the vector was confirmed by its isolation from transformed bacteria and subjecting it to PCR followed by restriction digestion (data not shown).

2.3. In planta transformation and confirmation

Transformed *Agrobacterium* strain (LBA4404) containing the *OsRGB1* construct was used to infect high yielding cultivar, BR55 using *in planta* methods established previously (Parvin et al., 2015). Rice seedlings surviving after infection were grown in potted soil to produce T_0 plants. Flag leaves of T_0 plants which tested positive for presence of *hpt* gene were allowed to grow and set seeds. Hygromycin resistance assay and PCR were performed to confirm the presence of the transgene in surviving plants and their advanced generations.

2.4. Hygromycin resistance assay of RGB1 transformants

To check for gene insertion, flag leaves were cut into 3–4 pieces and soaked in petri dishes containing 50 mg/L hygromycin solution in ½ strength MS media. The petri dishes were incubated under both dark and light condition (16 h day/8 h night) at 25 ± 2 °C. After 7 days of selection the leaf pieces showing dark brown strips and necrosis were considered non transformed, while those remaining largely green and healthy were selected as transformants (Lin et al., 2009).

3. Molecular analysis of the transformed plants

3.1. PCR analysis

Genomic DNA was isolated from flag and second leaves below the

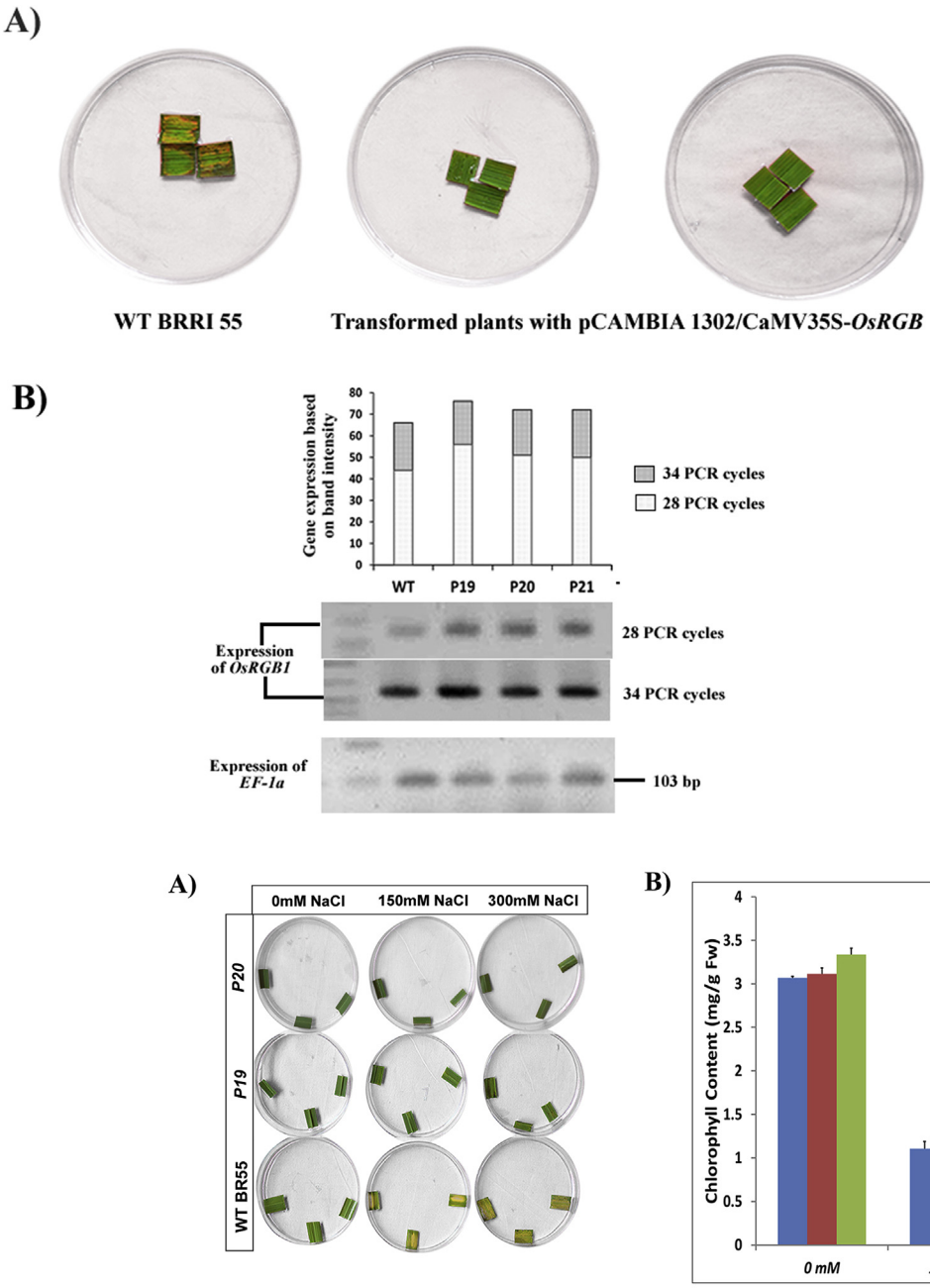

Fig. 1. A) Hygromycin resistance assay at T_0 generation. Flag leaves of transformed plants showed healthier and greener appearance compared to WT (Non-transformed BR55), which showed necrosis and dark-brown color in 50 mg/l hygromycin solution. B) Expression analysis of *OsRGB1* gene in T_1 BR55 transgenic lines: three transgenic plants (P19, P20 and P21) showed higher expression of *OsRGB1* gene compared to WT BR55 at both 28 and 34 PCR cycles. EF-1a (reference gene) was used as a control in the semi-quantitative reverse transcriptase PCR. Equal expression of EF-1a was found in both wild type BR55 and transgenic lines. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. Leaf senescence assay under salt stress. Leaf discs from fully expanded leaves of 10 week-old WT and each transgenic line were incubated in different concentrations of NaCl (0 mM, 150 mM and 300 mM NaCl). A) Transformed plants remained green even at 300 mM of salt stress (NaCl), while the non-transformed leaves showed necrosis and decolorization at 150 and 300 mM salinity (NaCl) stress. B) Chlorophyll content was measured from the leaf discs of transgenic and WT plants. The leaf discs of transgenic lines showed better chlorophyll content even after exposure to salinity (NaCl) stress at 150 and 300 mM for 5 days. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

panicles from positively transformed plants using the CTAB method (Doyle and Doyle, 1987). Presence of *RGB1* was confirmed by PCR using specific primers homologous to the *RGB1* gene. Genomic DNA was used for amplification with the following primers: *RGB1*_flank_F and *RGB1*_int_R (Supplementary Table 1). Conditions of the amplification reaction was as follows: 95 °C for 5 min followed by 35 cycles of denaturation, annealing and extension for 1 min at 95 °C, 1 min at 64 °C and 1 min at 72 °C, respectively. Finally, a single extension of 10 min at 72 °C was done.

3.2. Southern blot hybridization

Wild type BR55 and positively transformed T_2 BR55 rice lines (P19 and P20) were used to isolate 20 µg of genomic DNA, which was digested with *Sac*I, electrophoresed and blotted onto a positively charged nylon membrane (Hybond N^+ membrane, Amersham, UK). The membrane was probed using DIG labeled PCR amplified product (809 bp) with *hpt* gene specific primers (Supplementary Fig. 3) using the manufacturer's protocol (Roche Diagnostics Inc., Mannheim, Germany). The *Sac*I enzyme cuts once inside the integrated DNA and elsewhere and is therefore expected to show a band greater than the insert of ~3 kb (Supplementary Fig. 3). The number of bands obtained is expected to indicate the total number of insertions in the genome.

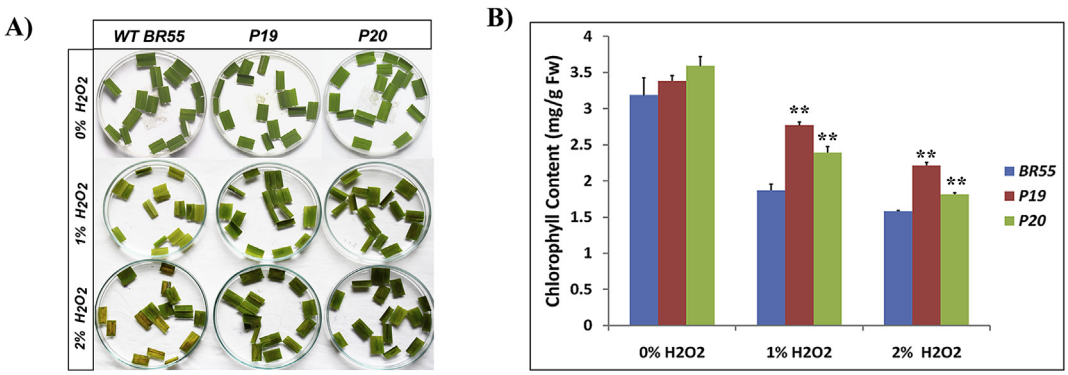

Fig. 3. Oxidative stress tolerance assays. Leaf sections from fully expanded leaves of 10 week-old WT and each transgenic line were incubated in different concentrations of H_2O_2 (0%, 1% and 2%, respectively) under continuous white light for 48 h. A) Leaf sections of transgenic plants are more tolerant to H_2O_2 stress. B) Chlorophyll content analysis in the leaf sections of WT and transgenic plants after treatment with H_2O_2 for 48 h. Leaf sections floated in water served as control. Throughout, error bars represent SD from three independent experiments.

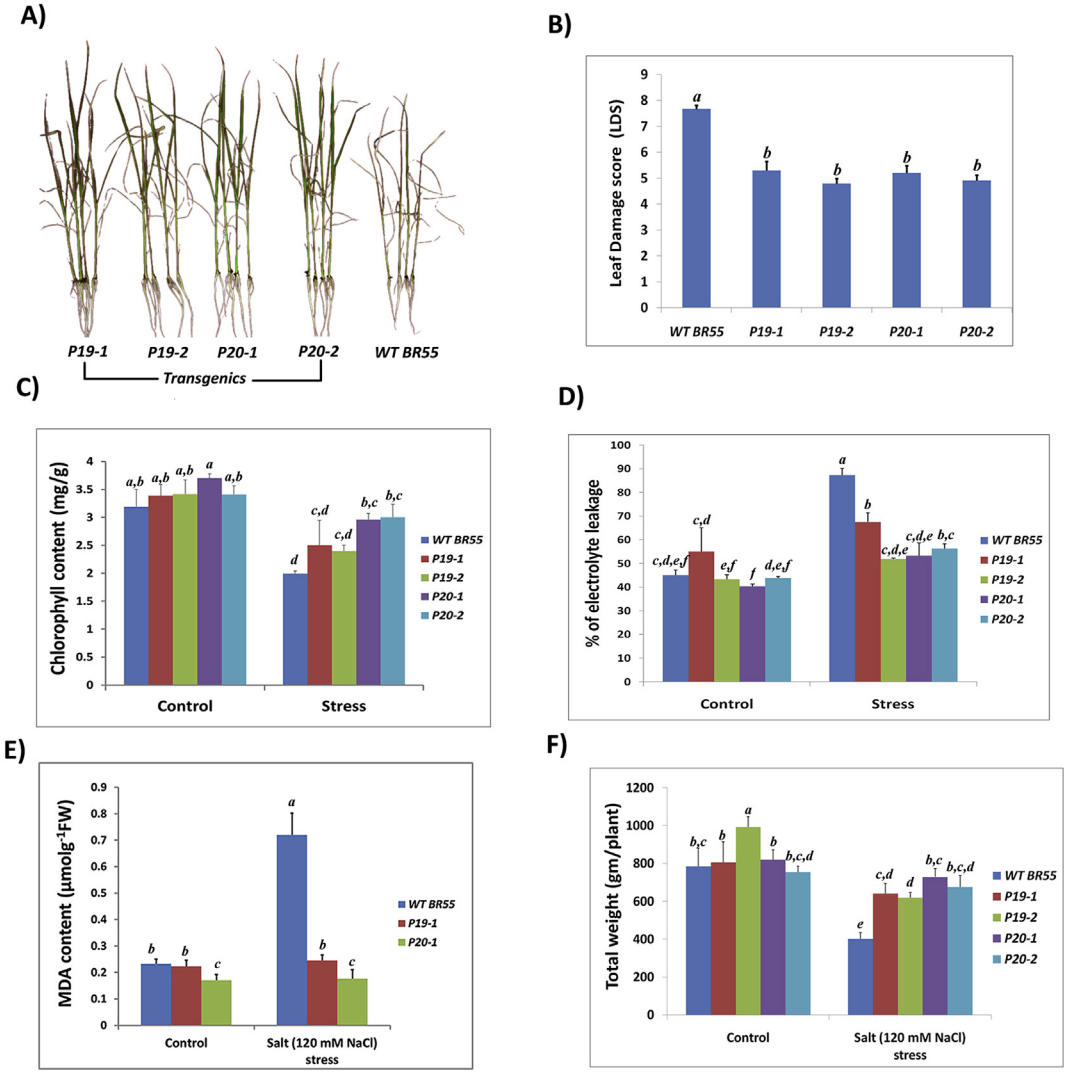

Fig. 4. Physiological testing under salt stress at the T_3 stage. A) Phenotypic view of all WTs and both transgenic lines after 8 days at 120 mM NaCl stress in hydroponics. The growth of all transgenic lines was better compared to their corresponding WTs; B) Leaf damage score (LDS), C) Percent of electrolyte leakage D) chlorophyll content, E) MDA content and F) Total weight in all transgenic lines compared to their WT plants under control and NaCl stress conditions. All transgenic plants showed significantly better performance compared to the WTs under salt stress. Each bar stands for the mean ± SE ($n = 5$). Different letters in each graph (a-f) indicate significant differences ($P < 0.05$, ANOVA and Duncan's Multiple Range test).

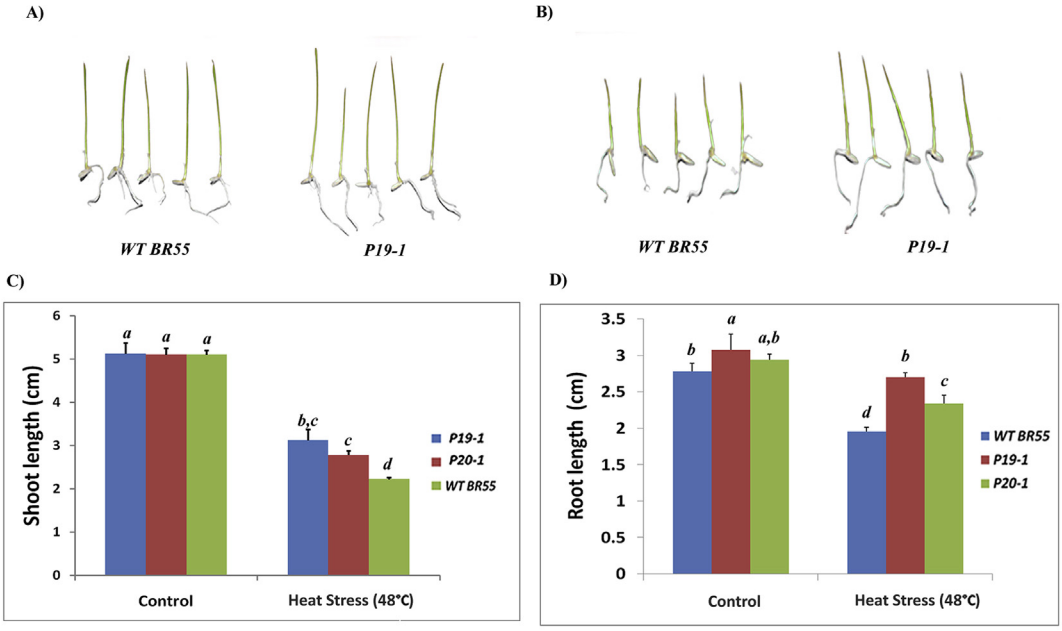

Fig. 5. Thermotolerance assay of transgenic rice plants at the germination stage. A) WT BR-55 and transgenic line without heat stress B) Germinating test of wild type BR-55 and transgenic rice plants with heat stresses at 48 °C (4 h) with heat acclimation at 42 °C (4 h), photos were taken after a recovery for 2 days at 37 °C. The growth of WT was seriously inhibited compared to non-stress condition, while RGB1transgenic lines showed much better growth and vigor during recovery. C) and D) Measurement of shoot and root length after the heat stress. The root and shoot length was significantly higher in all the transgenic plants than wild type BR55. Each bar stands for the mean ± SE (n = 5). Different letters in each graph (a-f) indicate significant differences ($P < 0.05$, ANOVA and Duncan's Multiple Range test).

3.3. RNA and real time PCR

The Trizol® reagent (Ambion, Invitrogen) was used to isolate total RNA from 14 days old seedlings of transgenic and wild type (WT) BR55 rice obtained from T_2 and T_3 seeds according to the manufacturer's instructions. The Invitrogen Superscript III reverse transcription RT-PCR system was used to synthesize cDNA from 1.5 µg total RNA according to the manufacturer's protocol. Semi-quantitative RT-PCR was used to confirm expression of the *OsRGB1* gene using *RGB1* specific primers (*RGB1*_int_F and *RGB1*_int_R2) (Supplementary Table 1) at the T_2 generation in order to select transgenic plants with the highest expression, according to Amin et al. (2012).

The expression of 6 salt-responsive genes was checked at the T_3 generation by quantitative PCR using SYBR Green (Bio-Rad, USA) in a CFX96™ detection system (Bio-Rad, USA). Primers are listed in Supplementary Table 1. The elongation Factor-1a (*EF-1a*) was used to normalize the reactions and the specificity of amplification was validated by a melt curve analysis at the end of each PCR cycle. The comparative cycle threshold method was used to calculate the relative transcript abundance (Livak and Schmittgen, 2001).

3.4. Leaf senescence in salt and H_2O_2

Pieces of leaves were cut from mature T_3 plants of the same age (WT BR55 and BR55_*RGB1*). Leaf sections were floated in petri dishes with solution containing 0, 150, 300 mM NaCl and different concentrations of H_2O_2 (0%, 1%, and 2% and left at 25 °C for 3 days (Negi et al., 2015; Parvin et al., 2015). The dried leaf pieces were weighed and used for chlorophyll measurement. Pieces of leaves (100 mg fresh weight) from control and stressed seedlings were soaked in a bottle containing 12 ml of 80% acetone for 48 h and absorbance of the leaf-imbibed acetone was measured at 663 and 645 nm. Chlorophyll content was estimated according to the method with adaptations from Chutia and Borah, 2012.

3.5. Thermo-tolerant test during germination stage

Mature seeds of transgenic and WT BR55 were dehusked and placed in MS medium for 3 days at 37 °C for sprouting. The germinating seeds were then stressed at 48 °C for 4 h with heat acclimation at 42 °C for 4 h (and not 2 h as recommended) followed by recovery of 2 days at 37 °C (Qin et al., 2015). The plants were photographed after retrieval and shoot and root length measured.

3.6. Evaluation of seedlings for salt and heat-tolerance

The *OsRGB1*-BR55 transgenic T_3 and WT BR55 seedlings were subjected to salt stress screening along with Pokkali and IR29 which served as tolerant and salt sensitive controls respectively (Amin et al., 2012). Germinated seeds were placed in nets supported by styrofoam and floated in PVC trays containing 10 L Yoshida solution. In each tray, there were 11 rows with 9 randomly planted seeds consisting of 3 replicates each of transformed, non-transformed and tolerant or sensitive controls. After 14 days of growth, in a net house (30–35 °C/25–28 °C, day and night temperature and ~65% humidity), the seedlings were subjected to gradual NaCl stress starting from 40 mM to 120 mM, with increments of 20 mM at 24 h intervals. Stress was applied until 90% of the leaves of the sensitive control were damaged, usually after a period of 2 weeks. Traits related to measure of tolerance, leaf damage score (LDS), chlorophyll content, electrolyte leakage, Malondialdehyde (MDA) content and total weight of all control and stressed plants were determined.

Seedlings (4–5 leaf stage) were subjected to heat treatment by transferring the plants to a 45 °C growth chamber. Replicates were exactly as described above for screening under salt stress. After heat stress treatment for 48 h with 72% humidity, the seedlings were allowed to recover in normal growth conditions for 7 days. Leaf samples were then collected for analyzing heat stress tolerance-related traits including chlorophyll and hydrogen peroxide content as well as electrolyte leakage.

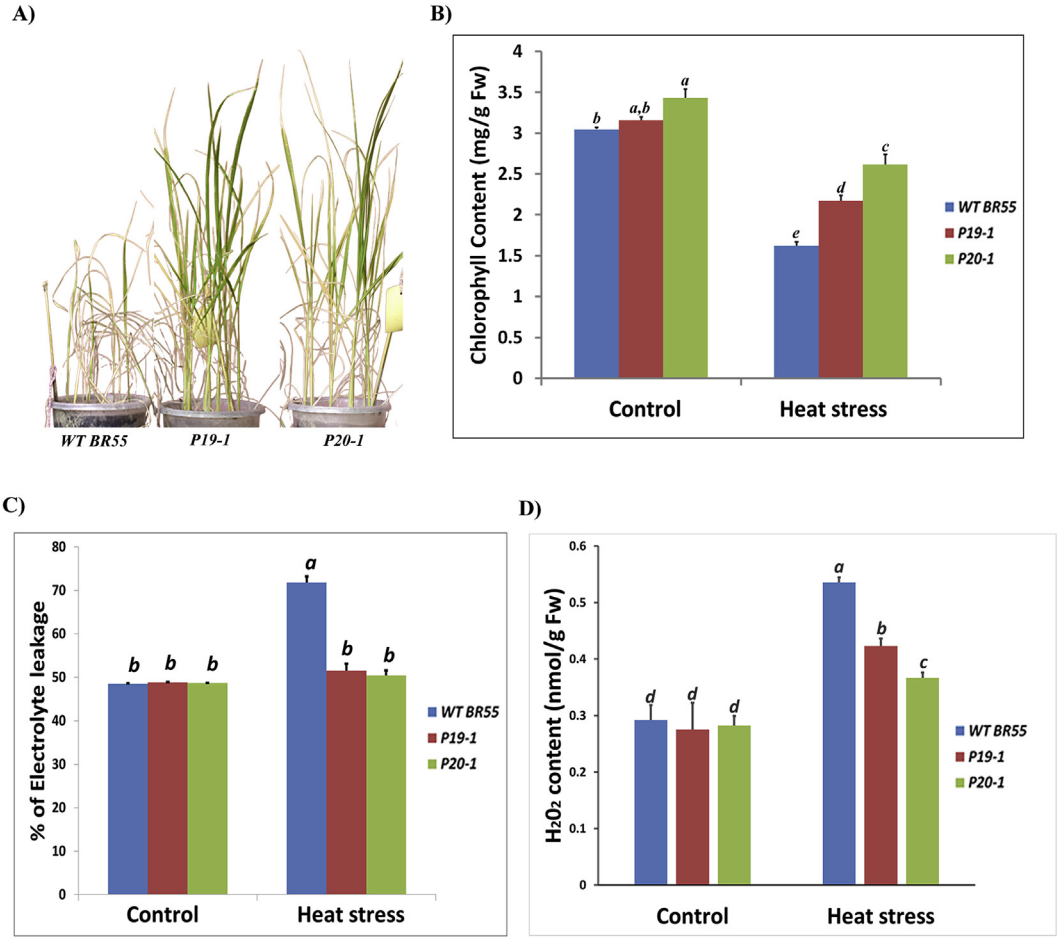

Fig. 6. Thermotolerance assay of transgenic rice plants at the seedling stage. A) Growth Vigor analysis of WT BR55 and transgenic rice plants after cultivation at heat stress of 45 °C/40 °C (day/night, 14 h/10 h) for 1 day and after 7 days recovery. Measurement of chlorophyll content (B), electrolyte leakage (C), H_2O_2 content (D) after 7 d of recovery after the heat stress. Each bar stands for the mean ± SE (n = 5). Different letters in each graph (a-f) indicate significant differences ($P < 0.05$, ANOVA and Duncan's Multiple Range test).

3.7. Combined heat and salinity screening at seedling stage

Seedlings were exposed to combined heat and salinity stress (4-leaf stage) by treatment in 120 mM NaCl salt solution for 3 days, followed by transfer of the plants to a 42 °C growth chamber for a further 48 h at 72% humidity. Control and stressed transgenic and WT plants were then measured for electrolyte leakage, chlorophyll, hydrogen peroxide and MDA content.

3.8. Estimation of leaf damage

The level of salt tolerance was evaluated using a leaf damage score (LDS) based on methodology established by IRRI and described elsewhere (Amin et al., 2012). Scores comprised of: 1) highly tolerant (10% or less damaged tissue); 2) tolerant (10–30%); 4) moderately tolerant (30–50%); 5) moderately susceptible (50–70%) and 6) susceptible (70%).

3.9. Relative electrolyte leakage measurement

The relative electrolyte leakage was measured as described by Parvin and colleagues (Parvin et al., 2015). Leaf samples (100 mg) from stressed and control experiments were immersed in 25 ml deionized water, shaken for 2 h at 80 rpm and the resulting solution measured for electrical conductivity (C1). The leaf samples were subsequently boiled in deionized water at 120 °C for 10 min in order to release all the electrolytes and the electrical conductivity (C2) was measured again. Electrolyte leakage percentage was calculated using the formula: (C1/C2) × 100.

3.10. Estimation of malondialdehyde (MDA) content

Malondialdehyde (MDA) was used as an indicator of lipid peroxidation mediated cell membrane damage under stressed conditions (Heath and Packer, 1968). Homogenized leaf (10 mg) in 10 ml of 10% (w/v) trichloroacetic acid (TCA) was centrifuged at 4000 g for 10 min and 2 mL of supernatant added to 2 mL of 0.6% thiobarbituric acid (in 10% TCA) followed by boiling for 20 min at 95 °C. The tubes were then placed quickly on ice and the absorbance of the supernatant measured at 532 nm. Adjustment for non-specificity of the reaction was done by another reading at 600 nm. Lipid peroxidation was expressed as μmol/g fresh weight using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

3.11. Estimation of H_2O_2 content

Leaf H_2O_2 amount was estimated under different stress conditions (Negi et al., 2015). Frozen leaf tissue in liquid nitrogen (300 mg) ground to a fine powder was mixed with 3 ml of 0.1% (w/v) TCA. After centrifugation (12,000 × g) for 20 min at room temperature, 0.5 ml of 1 M potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide (1 M) were added to the supernatant and absorbance at 390 nm noted. The content of H_2O_2 was calculated using the equation: H_2O_2

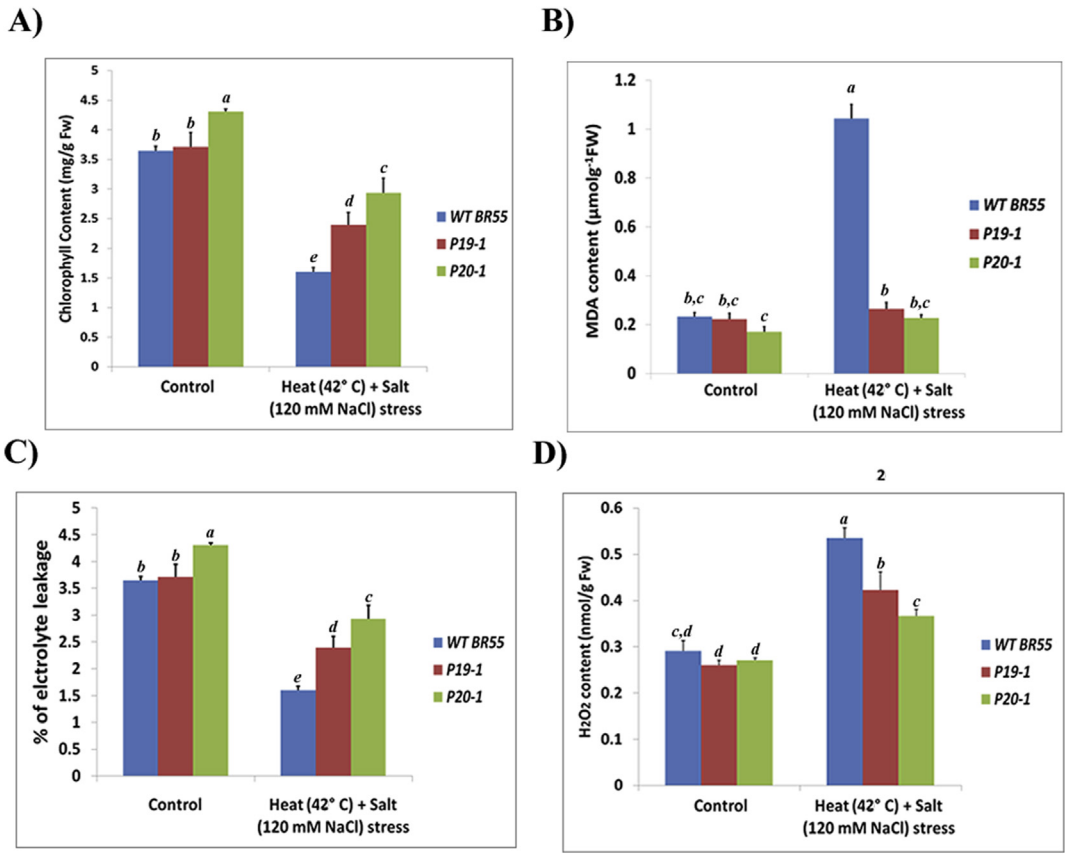

Fig. 7. Combined tolerance to salt and heat stress of transgenic rice plants at seedling stage. Chlorophyll content (A), MDA content (B), electrolyte leakage (C), H_2O_2 content (D) after 24 h combined heat stress treatment at 42 °C and salt stress at 120 mM NaCl. Transgenic plants showed significantly better performance compared to the WT after combined stress. Each bar stands for the mean ± SE (n = 5). Different letters in each graph (a-e)- indicate significant differences ($P < 0.05$, ANOVA and Duncan's Multiple Range test).

$(\mu molg^{-1}FW) = 1 + 227.8 \times O.D.390.$

3.12. Statistical analysis

All data analysis and figures were generated by using Microsoft Office Excel 2013 and R programming language was used to analyze data, apply statistics and generate figures. Significant differences ($P < 0.05$) between the transgenic lines and the WT were determined using T-test, ANOVA and Duncan's Multiple Range test.

4. Results

A third of the plants subjected to transformation were considered putatively transformed due to the resistance of their flag leaves to hygromycin (Fig. 1A). The DNA of the flag and second leaves of these plants when subjected to PCR with *RGB1* gene specific primers, showed the correct sized band (Supplementary Fig. 3A). These selected plants were used for quantitative PCR at T_1 for locating plants having the highest expression of the RGB1 gene. In both 28 cycles and 34 cycles of PCR, the expression in three transgenic plants P19, P20 and P21 than the WT was consistently higher (Fig. 1B). Southern hybridization of the corresponding T_2 plants (Supplementary Fig. 3B) showed the transgenic line P19 to have a single copy of the gene, while P20 had two. These 2 lines were selected for further salinity screening at seedling stage to test their level of tolerance. The transgenic lines P19 and P20 were confirmed to have the lowest LDS score and were advanced to T_3 for further screening.

4.1. Effect of salt stress on leaf sections of transgenic plants

Cut leaf pieces from WT BR55 and transgenic T_3 plants (P19 and P20) were subjected to different concentrations of NaCl solution for 72 h. At the high salt concentration of 150 and 300 mM leaf sections from the transformed plants remained green without any observable necrosis or spots unlike those of WT (Fig. 2A). The former also retained a significantly higher content of chlorophyll (Fig. 2B). However, there was no difference between leaf sections of WT and transgenic plants and all remained green without salt stress.

4.2. Leaf sections in H_2O_2 stress

Cut leaves from wild-type and transgenic plants responded differently to increasing concentrations of H_2O_2 for 48 h. As shown in Fig. 3, transgenic plants remained unaffected. At both 1.0% and 2% H_2O_2, leaf sections from wild type plants were bleached at the end of the stress treatment, the leaf pieces of the transgenic plants (lines P19, P20) remained green without any signs of H_2O_2 induced damage (Fig. 3A). Moreover, chlorophyll loss was significantly delayed in *RGB1* expressing plants in the presence of high concentrations of H_2O_2 (1% and 2.0%) compared to WT (Fig. 3B).

4.3. Physiological index analysis under salt stress

The *OsRGB1* gene was shown to protect P19 and P20 at the T_3 generation under 120 mM NaCl salt stress as shown by several physiological tests. Physiological parameters including the LDS score, chlorophyll content, electrolyte leakage, MDA content and total weight after 8 days of salinity stress indicated the tolerant response of the

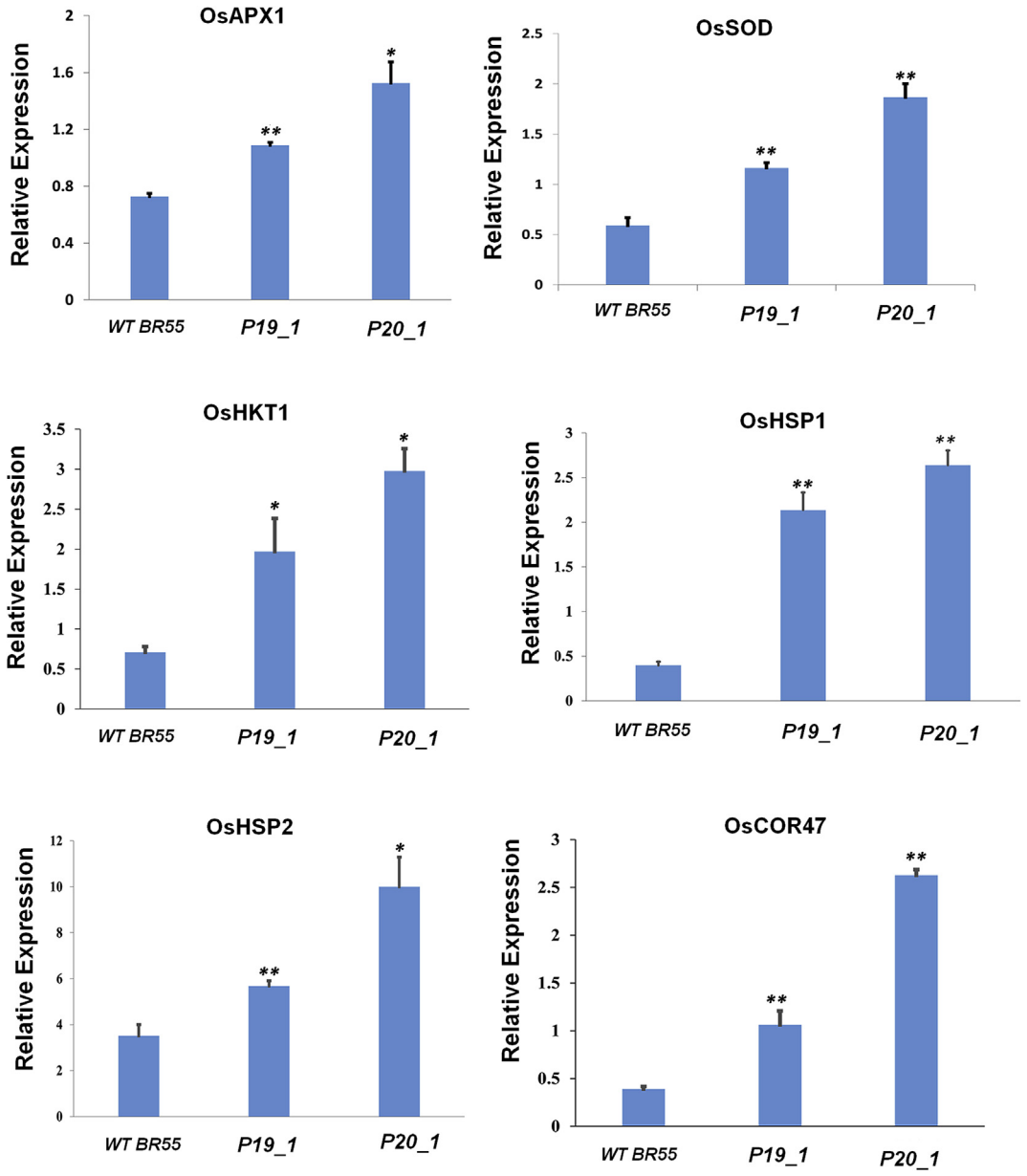

Fig. 8. Expression analysis of six stress related genes in wild type BR55 and transgenic plants under normal conditions by real time PCR (RT-PCR) analysis. Elongation factor-1a (EF-1a) was used as the internal standard and the $2-\Delta\Delta CT$ method was used to calculate relative expression of respective genes in all WTs and transgenic plants. Values are means ± standard errors from three independent measurements. **Shows statistical significant difference between wild-type and transgenic variety at $P < 0.01$. ***Shows statistical significant difference between wild-type and transgenic variety at $P < 0.001$.

transgenic T_3 seedlings in contrast to WT (Fig. 4A-F). In addition to WT, IR29 was used as the sensitive and Pokkali as tolerant control. Under control conditions, there was no significant difference between wild type and transgenic plants (Fig. 4C-F).

4.4. Thermotolerance assay of OsRGB1 in rice

The effect of *OsRGB1* in positive, homozygous progenies of the P19 and P20 transgenic lines was investigated for heat tolerance. Heat treatment of germinating seeds was inhibitory only for the WT plants, where slight sprouting occurred, whereas both P19 and P20 seeds emerged normally and grew steadily after stress withdrawal. After germination under heat stress at 48 °C for 24 h, the shoots and roots of the transgenic lines were significantly longer compared to WT BR55. There was no significant difference in transgenic and wild type without stress (Fig. 5). After application of heat stress at 45 °C/40 °C (day/night, 14 h/10 h) for 1 day followed by 7 days recovery, the transgenic lines (P19, P20) remained green with higher chlorophyll, whereas non-transformed BR55 became yellow (Fig. 6A and Fig. 6B). The tolerance of the transgenic lines was further characterized by their lower H_2O_2 content and electrolyte leakage after heat stress (Fig. 6C and D).

4.5. Simultaneous and combined tolerance to salt and heat stress conferred by RGB1

Four leaf stage RGB1 transgenic plants showed lower electrolyte leakage, higher chlorophyll content, lower MDA content and lower H_2O_2 content (Fig. 7) after 24 h at 42 °C and salt stress at 120 mM NaCl in a growth chamber. After the combined heat/salt treatment, the *OsRGB1* transgenic lines showed a lower leaf wilting phenotype and each seedling was healthy (data not shown).

4.6. Expression analysis of some stress marker genes in RGB1 transgenic plants

There may be alteration in transcription of certain genes under stress. Examples of these include *HSP1* genes, which are upregulated in heat tolerant lines (González-Schain et al., 2016). *COR47* provides protection from oxidative stress besides providing cold stress protection (Graether and Boddington, 2014). *SOD* and *APX* have been reported to be upregulated under heat (Shah and Nahakpam, 2012) and salt stress (Yan et al., 2016). *HKT1* has been found to be related to low Na^+/K^+ ratios under salt stress (Platten et al., 2013). These genes were therefore tested for their expression levels in the transgenic plants. The transcription levels of *OsAPX1, OsSOD, OsHKT1, OsHSP1, OsHSP2* and *COR47* were found to be significantly upregulated in RGB1 transformed BR55 compared to WT (Fig. 8). Moreover for all the genes, P20 line plants showed higher expression compared to P19 lines. This is likely due to the observation that it has 2 copies of the *OsRGB1* gene.

5. Discussion

Overexpression of the beta subunit of Rice heterotrimeric G protein, beta subunit, *OsRGB1* isolated from the commercial cultivar Swarna and integrated into rice cultivar BR55 clearly makes the latter tolerate both salt and heat stress separately as well as when the stresses were applied together at the seedling stage. Two independently transformed plants at the T_3 generation showed significantly better performance in terms of survival, leaf damage scores, electrolyte leakage, chlorophyll and malondialdehyde content. The higher expression of *OsRGB1* in the current work, allowed the seedlings to recover after the heat stress was removed. On the other hand, the WT showed very poor growth (not shown).

The roles of heterotrimeric G protein alpha and beta subunits under salt, heat and H_2O_2 have been shown to upregulated in Pea (Misra et al., 2007). Both the proteins are also transcriptionally activated by other abiotic and biotic elicitors like ABA, methyl jasmonate, brassinosteroids and pathogens (Bhardwaj et al., 2012). Arabidopsis mutants without the heterotrimeric G protein beta subunit (*AGB1*) are hypersensitive to ABA compared to those lacking the alpha subunit (At-*GPA1*). Therefore, *AGB1* is likely the main regulator of ABA signaling and At-*GPA1* probably fine tunes the efficacy of *AGB1* execution (Pandey et al., 2006). The Arabidopsis gpa1 allele was observed to decrease the number of stomata. In contrast, the Arabidopsis agb1 null mutant showed a 25% increase in stomatal density (Zhang et al., 2008). Tobacco plants constitutively over-expressing At-*GPA1* showed tolerance to salinity and heat, while *AGB1* overexpressors showed only heat tolerance (Misra et al., 2007). The ectopic expression of *AGB1* increased tillers and reduced leaf length in rice (Sun et al., 2014). In the current study, we obtained both salt and heat tolerance, when we over-expressed the *OsRGB1* in rice. The G-beta proteins from Arabidopsis and rice have a homology of only about 76%. And rice and other cereals lack the regulator of heterotrimeric G protein (At-*RGS1*) present in Arabidopsis and other dicots. Arabidopsis and rice mutants therefore show species, tissue and development specific phenotypes (Perfus-Barbeoch et al., 2004). Arabidopsis and rice can both combine three to five different heterotrimers (such as one alpha, one beta and any one of 3 gamma subunits for Arabidopsis and 5 for rice). While Na^+ induced senescence is enhanced in *agb1-2* mutants, both At-*GPA1* and *AGB1* promote shoot cell proliferation. Thus, inactivation of plant heterotrimeric G proteins under stress can slow down the pace of growth (Colaneri and Jones, 2014). *AGB1* was also shown to negatively regulate cell death. Thus, proper regulation of the heterotrimeric *AGB1* ratio may be required to balance death and growth under stressed conditions.

Plant heterotrimeric G proteins may function in signaling circuits as a variable resistor rather than a switch when responding to flow of information under different metabolic states (Colaneri and Jones, 2014). Also there is evidence that alternative mechanisms may activate/deactivate heterotrimeric G protein alpha and beta subunits even without being part of the heterotrimeric state. Heterotrimeric G proteins can be activated by glucose amounts and their duration such as, a burst of high glucose or a prolonged exposure to its low concentration. In addition, second messengers can transmit signals both downstream and upstream of the heterotrimers. For example, variable stomatal opening and closure under different conditions are controlled by different arrangements of environmental signals, heterotrimeric G proteins, hormones and second messengers (Colaneri and Jones, 2014).

In Arabidopsis, the regulator of heterotrimeric G protein signaling is located within the plasma membrane (PM) and called At-RGS1 (Urano et al., 2016). On the other hand, some monocot species possess gene(s) highly homologous to At-*RGS1* but this is missing in rice (Hackenberg et al., 2017). Therefore caution needs to exercised, when predicting function through comparison. Presence of At-*RGS1*on the PM, decreases the active pool of At-*GPA1*. However, when At-RGS1 is endocytosed, the ratio of At-*GPA1* with respect to the former increases (Colaneri and Jones, 2014). Interestingly, At-*RGS1* internalization occurs roughly ~13 h after exposure to Na^+ and coincides with the recovery of growth in coincidence with increase in glucose levels (Urano et al., 2016). Therefore, glucose may actually be the molecule that causes RGS internalization when subjected to salt stress. Changes in the carbon metabolic energy due to stress induced by salt may also cause a change in the level of At-*RGS1* (Hill et al., 2013). Therefore it seems that when the system reaches homeostasis, growth can resume, and the changed level of internalized RGS will result in a new ratio of active/inactive heterotrimeric G protein. The latter would then likely determine an adjusted pace of growth. This relationship between heterotrimeric G proteins, metabolism and its perturbation caused by salt stress is observed in the heterotrimeric G protein interactome (Colaneri and Jones, 2014). The effect on carbon energy metabolism could be because many enzymes in glycolysis and the tricarboxylic acid cycle can partner with Arabidopsis heterotrimeric G proteins (Hill et al., 2013). Therefore, under specific stresses, the level of active alpha and beta subunits of the heterotrimeric G protein may be controlled by the energy state of the cell. This state is in turnsensed by the glucose level while being mediated by At-RGS1. The altered metabolic state can in turn connect with other related signaling pathways to produce an integrated response. Due to the absence of a transmembrane RGS1 protein in the monocot lineage, the signaling and networking in rice is likely to be different. Therefore an alternative mechanism or additional components for plant heterotrimeric G protein activation and signaling can be envisaged in rice and other monocots.

The mechanism by which over-expression of *OsRGB1* is causing not only tolerance to salt but to heat as well in our transformed rice plants is unclear. But both salt and heterotrimeric G proteins are known to cause increase in cellular calcium levels (Ismail et al., 2014; Qu et al., 2013). In case of salt stress, increase in calcium activates the SOS1 pathway ultimately resulting in reducing sodium levels in cellular cytoplasm (Ismail et al., 2014). Under heat stress, the levels of PA (phosphatidic acid) and PIP2 (phosphatidyl inositol 4,5-bisphosphate) are enhanced, while heterotrimeric G proteins activate PIPK (phospho inosititide phosphate kinase) (Qu et al., 2013). This pathway eventually produces IP3 followed by IP6, which in turn causes the increase of calcium in cytoplasm. The increase in calcium in turn induces the production of HSP. The insertion of *OsRGB1* caused the higher induction of *HSP1* as well as *HSP2* in the transgenic plants. OsHSP2 has also been shown to play a role in protection of plants against heat stress (González-Schain et al., 2016). We also showed the over-expression of ROS scavenging enzymes which would reduce the oxidative stress occurring during multiple stresses, like salt and heat (Shah and Nahakpam, 2012). It can be envisaged that the enhanced activity of these enzymes as a result of *RGB1* overexpression can protect the transgenic BR55 plants from heat as well as salt stress.

The protection accorded by overexpression of *OsRGB1* in rice has

been shown convincingly in the current study at the seedling stage. However to be commercially useful, all the performance tests need to done at the reproductive stage and the effect of RGB1 in protecting the rice plants from yield loss under heat, salt and combined stress will need to be investigated in the future.

Author contributions statement

SB, MNI and ZIS designed the study. SB and MNI conducted all physiological, molecular and data analysis. ZIS wrote the manuscript with some help from SS and SB and served as PI for funds for the project from the Ministry of Science and Technology and University Grants Commission (UGC), Bangladesh. NT provided the OsRGB1 construct in the *pCAMBIA1302* vector.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.005.

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