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Downregulation of the *OsAKT1* K+/Na+ Transporter Gene by CRISPR-Cas9 Mediated Transformation in Sensitive Rice IR29 Makes it Tolerant to Salt Stress

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Abstract

The coastal area of Bangladesh is affected by soil salinity which threatens rice production. Saline soils are mainly caused by excessive Na+ which interferes with the uptake of the essential nutrient K+. Certain K+ transporters may cause leakage of Na+ ions and the OsAKT1 transporter was earlier shown to be downregulated under salt stress in a tolerant rice landrace called Horkuch. We therefore targeted this gene for CRISPR-Cas9 mediated downregulation by *Agrobacterium*-mediated *in planta* transformation in salt sensitive IR29 rice. The gene was shown to be significantly downregulated in at least 2 independent transformants. One of the gene edited IR29 transformants showed significantly high survival, low loss in chlorophyll content, low electrolyte leakage as well as low H_2O_2 after 15-days of salt stress at 120 mM NaCl at the T_3 generation. Therefore the potassium transporter *OsAKT1* gene is a good target for downregulation to produce salt tolerant rice.

Introduction

Rice (*Oryza sativa* L.) is one of the major cereal food crops in the world that supplies about one fourth of the total dietary energy and food supply chain for around three billion people across the globe (Mishra et al. 2022). For sustainable food security of Asia region there is a requirement for further 40% increase of rice production by 2050 (van Dijk et al. 2021). The most significant barriers for increasing rice production in Bangladesh is soil salinity. Bangladesh is already affected by saline stress affecting almost one third of our total cultivable land (Dasgupta et al. 2014). Global warming and

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consequential sea level rise is predicted to cause a further rise in salinity levels and a decline in rice productivity of around 15.6% in the coastal districts, since most commercial varieties are salt susceptible (Dasgupta et al. 2015).

Salinity stress mainly disrupts the Na^+/K^+ balance, which affects all physiological functions in plants and in particular photosynthesis. Rice is particularly salt sensitive. However there are some endemic rice landraces to coastal Bangladesh, like, Horkuch, which has been shown to be tolerant at both seedling and reproductive stage under salinity stress (Lisa et al. 2011, Lisa et al. 2004). Horkuch can grow and complete its life cycle in 100 mM NaCl. Its morphological, physiological and anatomical characteristics help it to survive under moderate salinity. On the other hand, IR29 is a high yielding but salt sensitive variety and so cultivation of this variety in coastal area or area with high salt in soil is not possible. Therefore, a combination of stress tolerance along with enhanced grain yield is a major focus of rice breeding in recent times.

Among fundamental macronutrients for plants, Potassium (K^+) is essential for growth, regulating cellular process, pH control and cell turgor. Potassium (K^+) also regulates many enzymes activity as a cofactor (Chérel and Gaillard 2019). Voltage-gated K^+ channels and voltage-independent K^+ channels are subgroup of K^+ channels (Jegla et al. 2018). Four subgroups of voltage-gated K^+ channels have been identified in plants: the silent rectifying K^+ channel, the weak rectifying K^+ channel, the inward rectifying K^+ channel, and the outward rectifying K+ channel (Dreyer et al. 2019). The Shaker gene family member OsAKT1 produces an inward potassium ion (K^+) channel that is found on the plasma membrane (Li et al. 2014). One of the critical K^+ channels for regulating the K^+ absorption in rice is OsAKT1 (Fuchs et al. 2005). *OsAKT1* is mostly expressed in the epidermis and endodermis of roots, with lower transcript levels seen in cells of the vasculature and cortex. In plants treated with 150 mM NaCl for 48 hours, *OsAKT1* transcripts vanished from the exodermis in salt-tolerant, sodium-excluding types like Pokkali, while OsAKT1 transcription was not suppressed in the salt-sensitive, sodium-accumulating variety IR29 (Golldack et al. 2003). It is believed that such downregulation avoids leaking of the excess Na^+ in the salt-stressed soil into the root cell. RNA-Seq analysis using a 3'tag-seq method and RT-PCR based expression analysis of *OsAKT1* gene in Horkuch and IR29 showed its down-regulation in Horkuch compared to IR29 in 150mM 24 hours salt stress (Razzaque et al. 2019). So, down regulation of *OsAKT1* gene in IR29 may provide salt stress tolerance ability of Horkuch with high yielding characteristics.

In recent times, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated endonuclease Cas9 (CRISPR-Cas9) has become an easy and precise gene editing tool that can selectively down regulate specific gene in plants (Seraj and Haque 2022). In this study, we therefore choose the CRISPR-Cas9 based NHEJ mutation technique to down regulate *OsAKT1* gene in IR29 to see its effect on susceptibility to salt. Single guide RNA against the *OsAKT1* gene of IR29 was designed, cloned in the *Agrobacterium*-compatible pRGBEB32 vector followed by *in planta* transformation. T_2 and

T_3 lines were tested to confirm *OsAKT1* gene down regulation and characterized for salinity tolerance activity at seedling stage. Plants with down regulated *OsAKT1* gave visible salt tolerance activity based on root and shoot morphology, chlorophyll content, Na^+ and K^+ ratio, electrolyte leakage, and H_2O_2 content.

Materials and Methods

Plant material: Seeds of IR29 and Horkuch were obtained from Bangladesh Rice Research Institute.

In silico analysis of candidate genes: To extract the sequence from raw data of Horkuch and IR29 genome (Unpublished work, Zeba I. Seraj) command line (linux) based local BLAST was performed. In BLAST program the reference sequence of *Oryza sativa* Nipponbare was used as a reference to detect the chromosomal location of specific sequences of Horkuch and IR29 for the extraction of the sequences. The Basic Local Alignment Search Tool (BLAST) provided by NCBI was used to identify differences between sequences of Horkuch and IR29 and ORF finder software was used to identify the amino acid sequences.

RNA extraction, cDNA synthesis and quantitative RT-PCR: After being stressed with 120 mM NaCl for 0 and 24 hours, 21-day-old seedling roots were harvested and total RNA was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Total RNA concentration and purity were evaluated using a Nanodrop ND1000 spectrophotometer (Thermo SCIENTIFIC). The cDNA was synthesized from 1.5μg of total extracted RNA (using the Invitrogen Superscript III reverse transcription (RT)-PCR following the manufacturer's protocol (Invitrogen, USA) in a final volume of 20μl. The final cDNA products were diluted 4-fold prior to use in real-time PCR. The Primer3web application (http://bioinfo.ut.ee/primer3) was used to create the qRT-PCR primers. The sequence of the primers were as follows: OSAKT1_RT_F, 5'-TCATCATCTCCCCCTACGAC-3' (62°C) and OSAKT1_RT_R, CCATGCGATCTTCTTTGGAT (61.5°C). In quantitative Real-Time PCR analysis, a total of 3 replicates for the root samples were used. As a control for genomic DNA contamination, an equivalent amount of total RNA without reverse transcription was tested for each sample per gene. Quantitative PCR was done by a 6μl reaction using SYBR Green (Bio-Rad, USA) with gene-specific primers in CFX96 TM Real-Time PCR detection system (Bio-Rad, USA). Using the comparative cycle threshold method, the relative abundance of transcripts was calculated. Elongation Factor- α (EF-α) was used as the normalization control. The thermal profile of the reaction was 95°C for 3 min activation and denaturation, followed by 40 cycles of 95°C for 30 sec, and 62°C for 30 sec. At, 62°C step the amplification was determined by SYBR Green. For statistical analysis mean variables of gene expression between different genotypes and at different time points were compared with the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. The $p < 0.05$ and $p < 0.01$ were considered as significant and highly significant change with respect to control.

Construction of the sgRNA-Cas9 expression vector: Single sgRNA was designed against OsAKT1 (LOC_Os01g45990) using CRISPR-P web base resource (http://CRISPR. hzau.edu.cn/CRISPR2/) (Xie et al. 2014). The PAM sequence was set to be 5′-NGG-3′ (SpCas9 from Streptococcus pyogenes). The guide sequence length was defined to be 20 nucleotides and four nucleotides overhang (forward and reverse) was added to made it compatible for cloning in pRGEB32 multiple cloning site (MCS). In the preliminary step, the sgRNA sequences with an on-score of greater than 0.05, a GC content between (30-80%) and a lower off-target were chosen. The sgRNA sequences chosen were as follows: forward oligo, 5′- GGCAGCTTCATTCCGAAGCCTACG -3′ and reverse oligo, 5′-AAA CCGTAGGCTTCGGAATGAAGC-3. These target exon 2 of the rice *AKT1* gene and start from 371 position of the CDS. *pRGEB32* plant binary expression vector was obtained from Addgene (Addgene plasmid # 63142) (Xie et al. 2015) which contains rice snoRNA U3 (Os U3p) promoter and UBI promoter (UBIp) for simultaneous sgRNA and Cas9 expression respectively. The 20 bp long MCS was removed by *BsaI* restriction enzyme and the designed sgRNA was inserted into the vector by restriction-digestion and ligation reaction according to provider's instructions (Xie et al. 2014). The *sgRNA_pRGEB32* constructs were transformed into *E.coli* using heat shock transformation.

Molecular confirmation of sgRNA constructs by PCR and sequencing: Putative pRGEB32_sgRNA_OsAKT1 plasmids were isolated from *E.coli* using Invitrogen Plasmid Isolation kits following manufacturer's protocol. Confirmation of successful cloning was done by using PCR. Analyses were carried out in a 25 μl reaction mixture containing 50 ng of plant DNA, 100 μM of each dNTP, 1 μl of 10nmol of each primer, 1 unit of Taq DNA polymerase (Invitrogen, USA), 1.5 mM $MgCl_2$,1 × PCR Buffer(-$MgCl_2$) (Invitrogen, USA). The optimized reaction was: Initial denaturation at 98°C for 3 min, 35 cycles of denaturation at 95°C for 45 sec, annealing at 62.0°C for 45 sec and extension at 72°C for 1 min following a final extension at 72°C for 10 min. The PCR-positive colonies were sequenced by First Base DNA sequencing services from Malaysia.

Agrobacterium-mediated in planta transformation: Transformation of pRGEB32_ sgRNA_OsAKT1 into *Agrobacterium tumefaciens* strain LBA4404 was performed by applying standard protocols (Weigel and Glazebrook 2006). Positively inserted colonies were confirmed using gene specific PCR. Following standard protocol for in planta transformation (Ahmed et al. 2018), *Agrobacterium tumifaciens* (LBA4404) containing pRGEB32_sgRNA_OsAKT1 plasmid was cultured and seeds were infected for gene transfer. Mature seeds of IR29 were sterilized and two-days-old, germinated seeds were used for *Agrobacterium* inoculation. Infected seeds were then incubated in the dark at 28°C for 7 days. After treating the seedlings with carbenicillin solution they were kept in light for 16 hours and in dark for 8 hours at 28°C. The seedlings were moved to a hydroponic solution after they turned green and after stabilization planted in pots.

Hygromycin resistance test: was done by dipping cut up leaves in 20 mg/l hygromycin and assessing after 4-5 days following incubation at 25°C.

Leaf disc senescence assay: As plants reached adulthood, the leaf disc senescence (LDS) experiment was conducted to gauge their capacity to withstand stress. From fully established T_0 and T_2 transgenic lines and wild type (WT) plants, flag leaves of about 1.0 cm in size were removed, and they were then allowed to float in a 20 mL solution of 120 mM NaCl for 3 to 7 days at 25°C.

Seed germination test: To compare the germination level under salinity stress, both the non-transformed and pRGEB32_sgRNA_OsAKT1 transformed IR29 seeds were subjected to 0 mM, 60 mM and 120 mM salt-stress at germination. After 5 days, shoot and root length were visually compared.

Putative Gene Editing Confirmation: To confirm on target mutation mediated by CRISPR-Cas9, T7 endonuclease I assay was performed according to manufacturer protocol (NEB, USA) with slight modification of DNA concentration for hybridization along with incubation period and gel documentation was done using Azure™ c200.

Salt-stress screening at the seedling stage: The salt-stress screening of untransformed and sgRNA-transformed IR29 (downregulated *OsAKT1*) was carried out according to the procedure described by Amin and co-workers in hydroponic systems and the salinity stress level was checked by portable conductivity meters (Amin et al. 2012). The 14-day-old seedling received 60 mM of salt stress, which was gradually increased to 120 mM stress. Following the application of salt for 7 days, samples were taken, and several parameters including electrolyte leakage, H_2O_2 concentration, chlorophyll content, root, shoot, and weight measurements were made.

Physiological parameters: Chlorophyll content were tested on both wild type and transgenic leaf samples weighing 0.1 g each were maintained in a container with 12 ml of an 80% acetone solution. After being stored in the dark for 72 hours, the bottles holding the leaf samples underwent 663 nm and 645 nm absorbance analyses. The amount of chlorophyll was determined using the formula $A=ECd$, where A stands for observed absorbance, C for chlorophyll concentration (mg/ml), d for the length of the light path (= 1 cm), and E for a proportionality constant (extinction coefficient) (=36 ml/cm). Relative electrolyte leakage was measured by adding deionized water to a 25 ml falcon tube containing 0.1g of leaf sample, and shaking for 2 hours. After that, a conductivity detector was used to quantify the first electrical conductivity (C1). The leaf segments were then autoclaved in deionized water, cooled to room temperature, and the final electrical conductivity (C2) was assessed. Relative electrolyte leakage was calculated using the values of C1 and C2.

H_2O_2 level was measured in wild-type and transgenic leaf samples by weighing 0.3 grams of each, crushing in liquid nitrogen, and adding 5 ml of TCA solution containing 0.1% (w/v) TCA. The mixture was centrifuged at room temperature for 15 minutes at a speed of 12000 rpm. 0.5 ml of 1 M potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide were added to 3 ml of supernatant. Using 1 ml of 0.1% (w/v) TCA and 1 ml of potassium iodide as a blank control, the mixture's absorbance was measured at

390 nm. The formula used to determine the quantity of H_2O_2 is H_2O_2 (mole.g-1FW) = 1 + (227.8*OD390).

Measurement of Na+ and K+ content: Transgenic and wild type seedling shoots and roots were cleaned before being dried in an oven. According to Yoshida's technique (Yoshida et al. 1976), dried leaves and roots were ground up and subjected to a flame photometer analysis after being extracted for 48 hours with 1N HCL (1976).

Statistical analyses: R studio packages were used to perform one-way ANOVA (analysis of variance) for the data among the different lines and wild type.

Results and Discussion

The OsAKT1 gene sequence showed structural and functional differences in tolerant and sensitive variety: No significant sequence variation in promoter region was found but a significant variation was observed in CDS region of the tolerant and sensitive genotypes with change in amino acid sequence. These substitutions with respect to IR29 and Horkuch are A-C, T-C, A-G, G-A and T-G at positions, 1442, 1794, 2631, 2770 and 2797, respectively. Substitution of these nucleotides in the *OsAKT1* gene between sensitive and tolerant phenotype indicates differential functions of the gene's product in plants under stress conditions. However, among these, three substitutions were found to be non-synonymous at the protein level in IR29 compared to Horkuch as well as the reference genome, which are T481K, N924D and G933C. Effect prediction using SIFT (Sim et al. 2012) indicated the G933C substitution as intolerant and the other two as tolerant. Moreover, the T481K substitution was identified as a potential post translational phosphorylation site which resides in the cyclic nucleotide binding domain. The substitutions, N924D as well as G933C were found in the KHA domain of the C terminal regulatory end of the protein and these together may help in altering the activity of the transporter.

Tissue-specific expression analysis: The expression pattern of *OsAKT1* was analyzed in Horkuch and IR29 root tissue as it has highest transcript abundance in the epidermis, exodermis, endodermis and pericycle cells of the root (Golldack et al. 2003). The result revealed initial upregulation of the genes at 6 hrs after 150mM stress application in both IR29 and Horkuch. However at 24 hours the gene was highly downregulated in Horkuch (Fig. 1a and b).

CRISPR-Cas9 mediated targeted mutagenesis of OsAKT1 in IR29: The expression of *OsAKT1* is down-regulated in salt tolerant Horkuch phenotype. Therefore, to achieve salt tolerance in IR29, *OsAKT1* gene was downregulated to achieve a loss of function of the gene using CRISPR-Cas9 mediated mutagenesis in IR29. Design of the guide RNA, and the confirmation of cloning is shown in Fig. 2a and b. Transformation was confirmed by hygromycin resistant assay which was used as the selection marker (not shown). The transformation rate was (29.16%). For double confirmation, we performed PCR

amplification of the hygromycin positive lines using the primer sets designed to specifically amplify *hptII* (Hygromycin Phosphotransferase gene sequences, not shown).
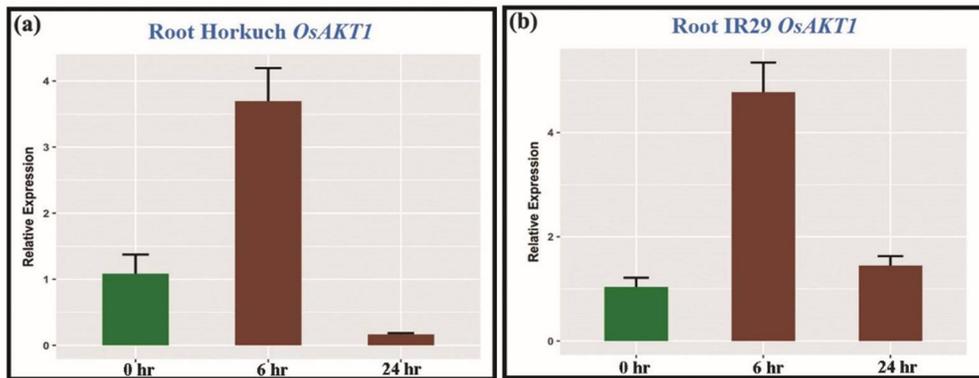

Fig. 1. Gene expression analysis of OsAKT1 by Real-Time quantitative PCR in the root of (a) Horkuch (b) IR29 under 150 mM stress conditions at 6 hours and 24 hours.

Detection of CRISPR-Cas9 mediated Putative OsAKT1 Gene Edited Plant: **p**RGEB32_OsAKT1_sgRNA transformed IR29 lines were advanced from T$_0$ to T$_1$. At the reproductive stage of T$_1$ generation, leaf disks were excised from healthy and fully expanded rice leaves of similar age of IR29 (control) and pRGEB32_OsAKT1_sgRNA transformed transgenic lines to select probable CRISPR-Cas9 mediated OsAKT1 gene-edited lines. LDS assay was performed under 0 mM and 120 mM for 5 days. Out of 15 *hptII* positive lines, 4 lines remained green compared to the visible browning in IR29 wild type (Fig. 3a). These lines are IR29 transformed with pRGEB32_OsAKT1_sgRNA named P-17-1-1, P-17-1-2, P-17-1-3 and P-5-3-2. Lines P-17 are T$_0$ and represent a single transformation event and are likely hemizygous. At T$_1$, these were named, P-17-1-1, P-17-1-2, and P-17-1-3. The plant P-5 represents an independent transformation event and P-5-3-2 is its progeny at T$_1$. At salt concentrations of 60 and 120 mM salt, only the WT IR29 showed poor growth and a significant reduction in root length (Fig. 3b). The positive plants were selected for molecular analysis by T7 Endonuclease I mismatch detection assay. Digested bands (around 600 bp and 200 bp) with an intact band of 801 bp were observed in 2% agarose gel for the four selected plants (Fig. 4a).

OsAKT1 Gene Expression Analysis in Putative Gene Edited Lines vs. Wild Type IR29: In P-17-1-1, P-17-1-2, P-17-1-3 and P-5-3-2, expression of *OsAKT1* in root showed 0.54, 2.66, 2.73 and 2.92 fold lower expression level respectively under 120 mM salt stress at 24 hour compared to control IR29. Therefore a significant down-regulation of *OsAKT1* was observed in P-17-1-2, P-17-1-3 and P-5-3-2. Among them the downregulation was not significant in P-17-1-1 (Fig. 4b).
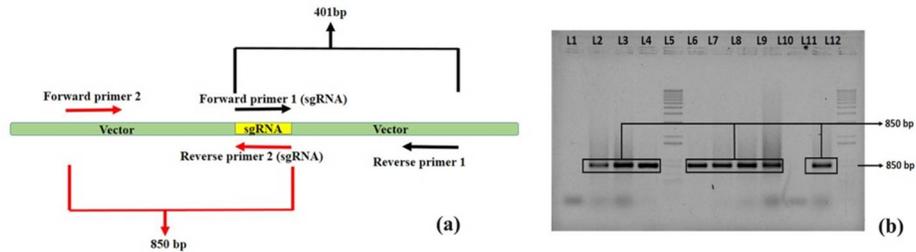
Fig. 2 (a). Design of the guide RNA using pRGEB32 plant binary expression vector from Addgene. (b) Confirmation of the insert after cloning and PCR.

Fig. 3 (a). Leaf disk senescence assay of wild type (IR29) and putative transgenic plants at T_1 generation under 0 mM and 120 mM salt solution. sgRNA transformed plants performed better under salt stress compare to wild type IR29. (b) Germination test of T_2 seeds of IR29 WT and IR29 transformed with pRGEB32_OsAKT1_sgRNA (P-17-1-1, P-17-1-2, P-17-1-3 and P-5-3-2) at 60 mM and 120 mM salt stress. Four lines out of fifteen transformed lines were able to germinate under salt stress where WT IR29 was unable to grow.
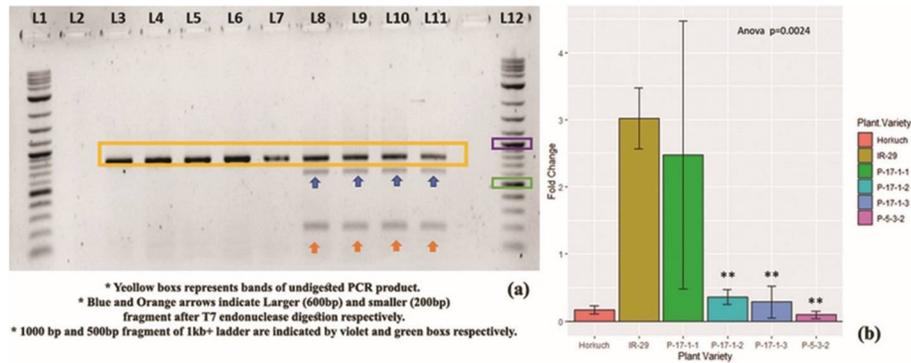
Fig. 4 (a). T7 Endonuclease I mismatch detection assay of T_2 transgenic plant. L1, L12- 1 kb Plus DNA Ladder (NEB #B7025). L2 -Negative Control of PCR. L3-PCR product of IR29 (WT) +T7 Endonuclease. L4, L5, L6, L7- PCR product of P-17-1-1, P-17-1-2, P-17-1-3 and P-5-3-2 respectively + T7 Endonuclease I. L8, L9, L10, L11- Mixture of PCR product of IR29 (WT) with P-17-1-1, P-17-1-2, P-17-1-3 and P-5-3-2 respectively + T7 Endonuclease I. (b) Relative gene expression analysis of OsAKT1 in 120 mM salt stress at 24 hours (n=3). Horkuch is positive control for identification of down-regulation pattern of OsAKT1 gene and P-5-3-2 showed maximum OsAKT1 gene down-regulation under 120 mM salt stress.
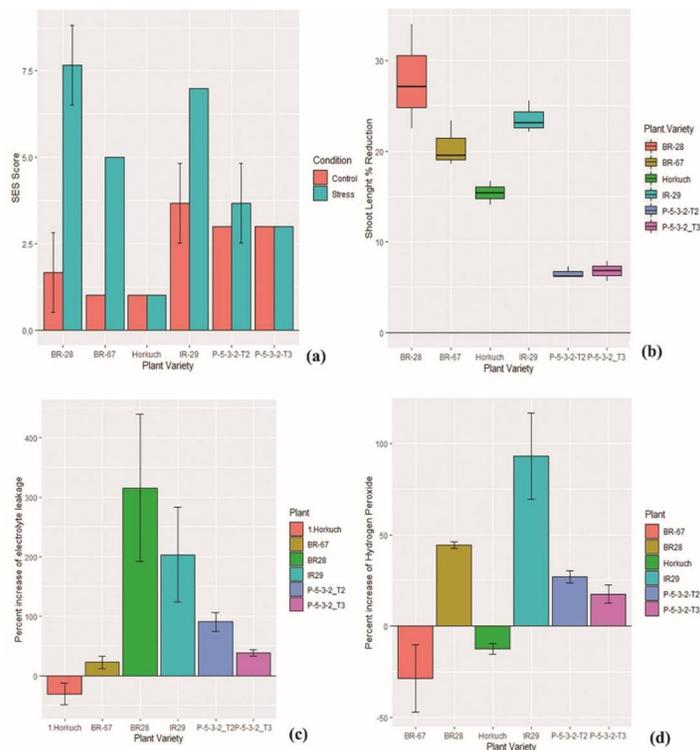

Fig. 5 (a). SES injury proportional score of transgenic plants compared with control plant (n=3). Phenotypic characteristics of salt treated wild type control plants and transgenic plants. Comparison of (b) shoot length (c) electrolyte leakage (d) H_2O_2 content of T_2 and T_3 of P-5-3-2 under 120 mM salt stress.

pREGEB32_OsAKT1_sgRNA Transformed Plants Showed Better Phenotypic Characteristics under Salt Stress: The line which showed maximum *OsAKT1* gene down-regulation was then advanced to T_3 and both T_2 and T_3 lines were selected for salt stress screening at seedling stage. The 21-day-old seedlings of transformed IR29 and wild type were exposed to 120 mM salt-stress for salinity based screening. The score for seedling injury (SES score) was recorded after 14 days. All transgenic plant showed significantly ($p< 0.001$) reduced damaged than IR29 under salt stress (Fig. 5a). The lines showed other salt-resistance phenotypes with respect to several physiological parameters. After 14 days of 120 mM salt-stress, the transgenic T_2 and T_3 of P-5-3-2 plants showed significantly less reduction of shoot length, lower percent increase in electrolyte leakage ($P=0.024$) and less H_2O_2 accumulation ($P=0.002$ and $P< 0.0001$ respectively) compared to wild type indicating the salt tolerance of the edited IR29 (Fig. 5b-d). BRRI dhan28, a salt sensitive variety failed to accumulate chlorophyll whereas Horkuch and BRRI dhan67 (moderately salt tolerant variety released by BRRI) accumulate more chlorophyll under salt stress. Both generations of the P-5-3-2 plants successfully survived during salt stress and accumulated more chlorophyll under stress condition compared to non-stress condition with P value < 0.01 (Fig. 6a).
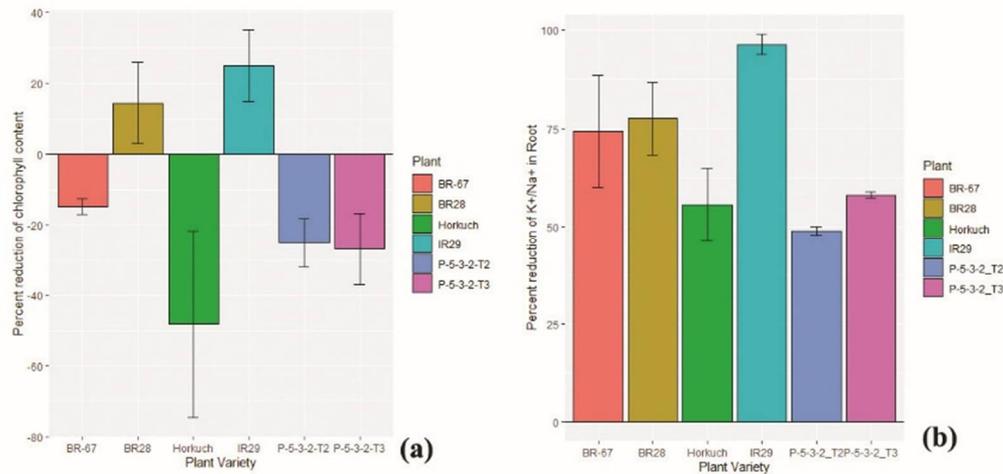

Fig 6. Comparison of (a) chlorophyll content (b) K^+/Na^+ in root of T_2 and T_3 of P-5-3-2 under 120 mM salt stress

Under salt stress, reduction of K^+/Na^+ ratio in root of P-5-3-2 (T_2 and T_3) was significantly lower ($P< 0.0001$ and $P=0.0003$ respectively) than IR29 meaning that the transgenic line accumulated lower Na^+ in root than IR29. Percent reduction of K^+/Na^+ in roots of transgenic lines were similar to Horkuch. Significantly lower reduction of K^+/Na^+ was observed in P-5-3-2 (T_2 and T_3) compared to IR29 ($P< 0.0001$) in salt stress in shoot also. IR29 accumulated maximum amount of sodium in root after salt stress (Fig. 6b).

Most of the commercial rice varieties available in Bangladesh are susceptible to salt stress which makes it impossible to use the coastal region for commercial rice cultivation as salt stress drastically reduces productivity (Dasgupta et al. 2014). Horkuch is an endemic salt tolerant rice landrace of southeast coastal area of Bangladesh which can be used as a resource for understanding salt tolerance strategies. In a previous study, RNA-Seq analysis based on differential gene expression patterns in Horkuch compared to IR29 (salt-sensitive) was carried out (Razzaque et al. 2019). From that analysis, potassium channel protein OsAKT1 was selected and subsequent in-silico analysis along with real-time PCR confirmed the down-regulation of this gene in a tolerant phenotype. Targeting the *OsAKT1* gene for downregulation by CRISPR-Cas9 technology was quite successful in knocking down its expression in at least 2 independent lines of transformed salt sensitive rice. All of the 2 transformed lines showed good tolerance to salt stress in multiple physiological parameters. One of the lines, where the *OsAKT1* was downregulated in a similar manner to Horkuch, showed significant less loss in K+/Na+, low electrolyte leakage, low H_2O_2 as well as low chlorophyll loss after 120 mM salt stress at the T_3 generation. The work therefore unequivocally shows that one of the mechanism by which Horkuch shows a tolerant response to saline stress is by downregulating the *OsAKT1* gene. And if this downregulation is mimicked in a salt sensitive genotype, it shows a tolerant response as well.

Furthermore, bioinformatics analysis showed differences in the coding region in the tolerant and sensitive genotypes, which likely leads to their functional difference under salt stress. Among the three non-synonymous substitutions between Horkuch and IR29, one was found not to result in functional tolerance by the effect prediction tool. This resides at the C terminal end in the KHA domain and presence of Cysteine in IR29 instead of Glycine can help in formation of disulphide bonds and tetramerization. Structural characterization of AKT1 confirmed that formation of a tetramer is required for stable function of the protein (Daram et al. 1997). Hence plants might have adopted to partially inactivate the protein expression in some varieties by substitution of Cysteine to Glycine, disrupting the tetramerization and reducing the activity AKT1 under salt stress. AKT1 has N terminal transmembrane domains involved in potassium transport and a cytoplasmic domain consisting of the cyclic nucleotide binding domain, followed by ankyrin repeats and KHA (K+ channel motif rich in hydrophobic and acidic residues) motif at the C terminal end (Sánchez-Barrena et al. 2020). The cytoplasmic domains are mainly involved in the regulation of this protein. Moreover, the T481K substitution resides in the cyclic nucleotide binding domain of the protein. Lysine residues in this domain can contribute to coordination and stabilization of cyclic nucleotide binding. Substitution of Lysine with Threonine in Horkuch can disrupt the interaction of the protein with cAMP/cGMP leading to reduced or altered binding affinity in which the Lysine residue would play a big role. Plant AKT1 Potassium Channels are activated by CIPK23 kinase which requires the cyclic nucleotide binding domain (Sánchez-Barrena et al. 2020) for interaction. Threonine at site 481 is a potential post translational

phosphorylation site and such phosphorylation can attract the phosphatase enzymes instead of the kinase activity leading to reduced functionality of the protein when necessary, causing adaptation to environmental stress.

Salinity is typically related to relatively little K^+ and high Na^+ in the soil, which usually results in a depolarization of the cell caused by the entry of large amounts of Na^+ (Shabala and Pottosin 2014). Under these conditions, K^+ selective AKT1 channels would mediate K^+ efflux rather than influx (Véry et al. 2014). However, the activation of AKT1-like channels upon hyperpolarization and their deactivation upon depolarization (Véry and Sentenac 2003) provoke a strong reduction of AKT1 activity under salt stress conditions.

The current study could successfully demonstrate that the CRISPR-Cas9 mediated downregulation of the *AKT1* gene can render a plant tolerant to salt. This work was a follow-up of our previous work and mimicked the downregulation of the gene previously shown in tolerant rice landrace Horkuch (Razzaque et al. 2019). In conclusion *OsAKT1* gene is a good target for downregulation in a CRISPR-Cas9 mediated approach for rendering rice plants more salt tolerant.

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