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Expressional analysis of *OsNHX1, OsNHX2, OsSOS1* and *Os DREB* transporters in salt tolerant (FR13A) and salt sensitive rice (brri dhan29) induced by salinity stress

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Abstract: *Here we study the expression of OsNHX1, OsNHX2,OsSOS1 and OsDREB transporter genes under different time points (control, 1h, 6h, 24h and 72h) of salinity stress (150mM NaCl). Upon NaCl stress, OsNHX1 and OsNHX2 transcripts were up-regulated till 1h up to 72h of salt stress, respectively in FR13A.Whereas, at BRRIdhan-29, expression of OsNHX1 was down regulated till 24h only up-regulated in 72h salt stress; and OsNHX2 expression was detected only control condition and undetected till 72h salt stress. Stable and higher expression of OsSOS1was found till 72h of salt stress in FR13A. Whereas, at BRRIdhan-29, expression of OsSOS1 was found in oh, 24h and in another time points was totally undetectable. The expression of OsDREB was detected at control condition and up regulated to 72h of salinity stress and remained stable in FR13A. The highest expression of OsDREB was observed at 6h up to 72h of salinity stress. In case of BRRI dhan29 the expression was found at control condition, 6h to 72h and undetectable observed at 1h of salinity stress. The uptake of Na^+ and K^+ under 60mM and 120mM NaCl stress was measured using atomic absorption spectrophotometer. It was found that BRRI dhan29 accumulated higher amounts of Na^+ in roots, leaf sheath and leaf blade than that of FR13A. On the other hand, in roots, leaf sheath and leaf blade accumulated higher amounts of K^+ in FR13A than that of BRRI dhan29. FR13A maintains cytosolic K^+/Na^+ homeostasis by increasing the K^+-Na^+ coupled uptake through the induction of these genes, as well as by increasing the compartmentalization of cytosolic Na^+ into the vacuole. But BRRI dhan29 could not maintained cytosolic K^+/Na^+ homeostasis due to down-regulation of transport proteins. We conclude that simultaneous induction and up-regulation of transporters found to be an effective factor to control Na^+ translocation and less accumulation in FR13A. This mechanisms were almost absent in BRRI dhan29 and could not maintain effective K^+/Na^+ homeostasis and long term salinity tolerance.*

Keywords: *Salinity stress, Transport proteins, Plasmamembrane, Tonoplast, Compartmentation, Homeostasis*

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I. Introduction

Salt stress is one of the most important abiotic stresses affecting natural productivity and causes significant crop loss worldwide. Rice is one of the most important cereal crops in tropical and temperate regions of the world. Under the current climate change context attaining food security, especially in rice, is being considered as a serious issue. However, for the saline-affected coastal area these high-yielding rice cultivars are not suitable since they are very sensitive to salinity stress. On the other hand, rice dominates the cropping pattern in the coastal region of Bangladesh due to its suitability with other agro-climatic conditions such as water stagnation. For plants, the sodium ion (Na^+) is harmful whereas the potassium ion (K^+) is an essential ion. The cytosol of plant cells normally contains 100–200mM of K^+ and 1–10mM of Na^+ (Taiz and Zeiger, 2002); this K^+/Na^+ ratio is optimal for many metabolic functions in cells.Soil salinity affects plant growth in two different phases. In the first phase, called osmotic phase, high concentration of salts in the soil leads to lower soil water potential and consequently reduced plant ability to take up water. This phase starts rapidly, within minutes, upon root exposition to high salt concentration. Such phase, which is independent of ion accumulation, leads to a reduced cell expansion in root tip and young leaves, and causes stomata closure (Roy et al. 2014). Salt stress caused by these changes in Na^+ and K^+ may be the main reason of severe reductions of photosynthetic pigment and the net photosynthetic rate and a sharp increase in membrane permeability (Yang *et al.* 2009). Additionally, when Na^+ enters cells and accumulates in high levels, it becomes toxic to enzymes. Therefore, it is believed that the maintenance of K^+ and Na^+ homeostasis is crucial for salinity tolerance. To prevent growth cessation or cell death, excessive Na^+ must be extruded or compartmentalized in the vacuole (Zhu, 2003). Many

transporters of K^+ and Na+ have been identified to date. In addition, the regulatory mechanisms that control the expression and activity of the transporters are beginning to be elucidated (Munns and Tester, 2008). At saline conditions, Na^+ competes with K^+ for uptake through common transport systems, since Na^+ and K^+ are physico-chemically similar monovalent cations. Thus, elevated levels of cytosolic Na^+, or in other way high Na^+/K^+ ratios, exert metabolic toxicity by a competition between Na^+ and K^+ for the binding sites of many enzymes (Tester and Davenport, 2003; Munns and Tester, 2008). Though the mechanism of Na^+ entry into plant roots is largely unidentified, it is believed that Na^+ enters via both symplastic and apoplastic pathways using various ion channels/transporters. Several classes of cation channels including outward- and inward-rectifying K^+-selective channels (Maathius and Sanders, 1995), and non-selective cation channels, NSCCs (Kader and Lindberg, 2005), high affinity potassium transporters have been proposed to mediate substantial Na^+ entry into plant roots (Horie *et al*. 2001; Golldack *et al*. 2002). NSCCs are, however, the dominant pathways for Na^+ influx into root cells (Demidchik *et al*. 2002; Kader and Lindberg, 2005). Furthermore in rice, it has been observed that the rate of Na^+ uptake into shoots mediated by the intrusive apoplastic ion transport is considerably high under salinity stress (Ochiai and Matoh, 2002).

The candidate proteins *OsNHX1* and *OsNHX2* for compartmentalizing Na^+ in the vacuole is the tonoplast Na^+/H^+ antiporter, which is energized to do so by the vacuolar H^+ ATPase (VATPase or VHA). Like other salt-tolerant species, the salt-tolerant rice cv. FR13A contains less Na^+ both in its roots and shoots under salt stress than do salt-sensitive rice cultivars. Several Na^+/K^+ -transporters could be involved in conferring the ability to maintain a low cytosolic Na^+ level in FR13A. *Arabidopsis* contains eight members of NHX type antiporters family belonging to three subclasses with distinct localizations: two in the plasma membrane (SOS1/*AtNHX7* and *AtNHX8*) and six intracellular members that are either in the tonoplast, *AtNHX1* up to *AtNHX4*, or in the prevacuolar compartment (Golgi, *trans*-Golgi network and prevacuolar compartments), *AtNHX5* and *AtNHX6* (Reguera *et al.* 2015). It was also reported that NHX1 and NHX2 are essential for K^+ homeostasis (Andrés *et al*. 2014). The transport protein *OsSOS1*catalyzes electro neutral Na^+/H^+ exchange at the plasma membrane. SOS1 appears to be highly specific for Na+ and does not transport other monovalent cations, such as K^+ or Li^+. Based on the expression pattern of *OsSOS1*and the characterization of SOS1 controls both Na^+ efflux in the root and long-distance Na^+ transport via xylem to partition this ion among root and shoot (Oh *et al.* 2009). In addition to Arabidopsis, the SOS1gene has been identified in other plants like rice (Martínez-Atienza *et al*. 2007), wheat (Feki *et al*. 2011), tomato (Olías *et al.* 2009) and *Thellungiella salsuginea* (Oh *et al.* 2009). Despite the demonstrated role of some SOS1 genes in ion homeostasis and in the partitioning of the toxic ion Na^+ between plant organs (Olías *et al.* 2009), only experimental functional analysis of *Arabidopsis* SOS1 and *Salicorniabr achiata SbSOS1* promoter was performed in transgenic Arabidopsis and tobacco plants, respectively (Goyal *et al.* 2013). In Arabidopsis, the salt overly sensitive protein (SOS1) functions in Na^+ exclusion from root epidermal cells into the rhizosphere, which also plays a role in retrieving Na^+ from the xylemstream under severe salt stress (Shi *et al.* 2002). Dehydration responsive element-binding (DREBs) proteins are a large subfamily of the AP2/EREBP super family that plays crucial roles in plant response to adverse environmental factors (Yamaguchi-Shinozaki and Shinozaki 2006). DREB binds to dehydration responsive cis-acting elements in gene promoters and activates transcription of downstream genes. Therefore, DREB proteins play important roles in regulating abiotic stress-related gene expression and conferring stress tolerance to plants (Gutha and Reddy, 2008). Numerous studies have demonstrated that DREB proteins can activate a series of stress related genes and induce tolerance to different abiotic stresses such as drought, salt, cold, and heat (Peng *et al.* 2011; Kudo *et al*. 2014).

In a recent study, it was also demonstrated that vacuolar compartmentalization is evident under salt stress in the salt-tolerant rice, cv. FR13A, whereas apoplastic sequestration of cytosolic Na^+ is dominant in the salt-sensitive cv. BRRI Dhan29. To clarify the regulatory mechanisms involved in maintaining cytosolic Na^+ homeostasis in rice, the expression of *OsNHX1, OsNHX2,OsSOS1 and OsDREB* were compared in both a salt-tolerant (FR13A) and salt-sensitive (BRRI dhan29) rice at different time points of NaCl stress. The functions of different transport proteins responsible for conferring salinity tolerance in FR13A still remain to be understood. The intensity of salinity stress is expected to increase in the coastal area of Bangladesh over the years due to climate change impact. Therefore, clear understanding of the tolerance mechanisms rice cultivar FR13A is important for generating the scientific knowledge demonstrating the cellular mechanisms of salinity tolerance. This will facilitate to make the platform for developing more salt tolerant high yielding rice cultivar in the future for improving the livelihood of resource-poor farmers living in the coastal area of Bangladesh.

II. Materials And Methods

Hydroponic plant culture:

The experiment was conducted at glass house and Biotechnology laboratory in Bangladesh Institute of Nuclear Agriculture (BINA). Seeds of rice (Oryza sativa L. indica cvs FR13A and BRRI Dhan29) were provided by the Bangladesh Rice Research Institute (BRRI, Gazipur, Bangladesh).Rice cultivars FR13A

(salt/drought tolerant) and BRRI dhan29 (salt susceptible) were used in this study. Rice seeds were kept in oven to break the dormancy and soaked with distilled water in the Petridis. The radical of the pre-germinated rice seeds were carefully sown and inserted in nylon mesh in each hole of the Styrofoam seeding float, then placed in the water. The water was replaced with nutrient solution (Yoshida solution and Ferrous sulphate) after three days. The salinity level was measured through electrical conductivity (EC) using the EC meter. New solution was added every eight days and the pH was monitored everyday and maintained at pH 5.2. Seedlings were grown in a controlled environment chamber (Glass house) with day/night temperatures of 25/21°C under 14 h of light ($300\mu Em^{-2}s^{-1}$); humidity was approximately 50%. Afterwards, the plants were stressed by adding NaCl at a final concentration of 150 mM to the nutrient solution for control, 1h, 6h, 24h and 72h. On the other hand, the plants were stressed by adding NaCl at the rate of 60mM and 120mM to the nutrient solution for 72h for ion estimation from root, leaf sheath and leaf blade. Non-stressed control plants were grown concurrently and harvested at the same time. After harvesting, all samples were stored at a temperature of -80^0C before being subjected to RNA isolation.

Ion Estimation:

The dried samples were ground into powder using a pestle and mortar. Different weight (g) dried samples was digested with 15 ml of an acid mixture (HNO_3: $HClO_4$:H_2SO_4¼10:4:1) for about 1h at 350^0C on a hot plate. The suspension was filtered and diluted with distilled water to a final volume of 20 ml. The Na^+, K^+ contents were measured using atomic absorption spectrophotometer (Z-8000, Hitachi, Tokyo, Japan) according to **Wang and Zhao (1995)**.

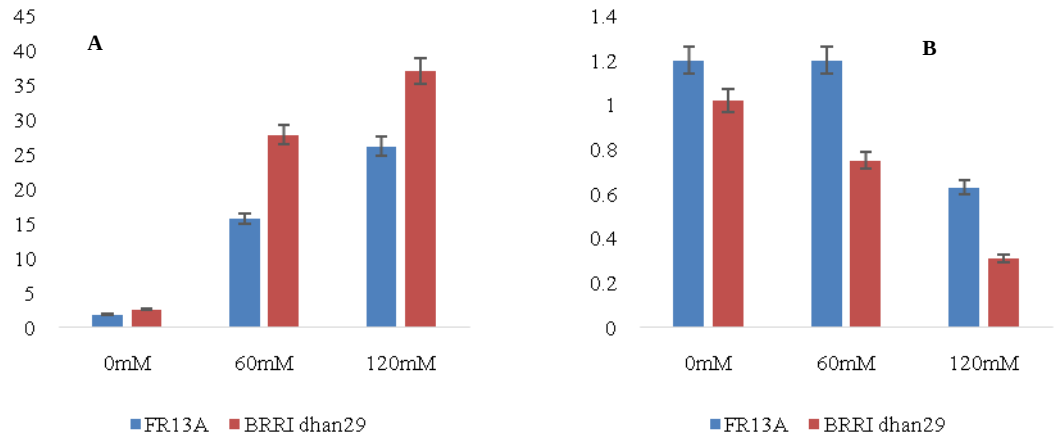
RNA isolation and cDNA synthesis:

RNA was isolated from the leaf of rice cvsFR13A and BRRI Dhan29. For RT-PCR, total RNA was isolated using the RNA mini kit and First-Strand cDNA synthesis Using Superscript" III reverse transcriptase (Invitrogen) kit according to manufacturer's protocol. For PCR amplification, the following sequence-specific forward and reverse oligo nucleotide primers were used: 5'-GTTCAAGAGTTACAACAAAGCACG-3' and 3'-CAGCGGGAATACAAAAGCAG -5' (*OsNHX1*), 5'- TAACCAAGACGAAACACCCCTA -3' and 3'-AACCCAGCAACTACTCCAAGAA -5' (*OsNHX2*) and 5'- CTCCGTGCTCATAGAATCGC -3' and 3'-ATACTCACTCAAGTGGGTCAATACC -5' (*OsSOS1*). 5'-TGGGTCAGGAAGAAGAGAAC-3' and 3'-ATTTTCCGGACCTCCTTTCCC-5' (*OsDREB*).The following conditions were used for the PCR reactions: 1 cycle consisted of 30sec at 98^0C, 10sec at 98^0C, 51^0C (for *OsNHX1*), 52^0C (for *OsNHX2*), 53^0C (for *OsSOS1*) and 50^0C (for *OsDREB*) 30sec at 72^0C, and a final extension of 1min at 72^0C. *OsNHX1*, *OsNHX2*, *OsSOS1* and *OsDREB* were amplified for 34 cycles for samples from both cultivar of rice. The PCR products from RT-PCR amplifications were separated on 1.5% (w/v) agarose gels and stained with ethidium bromide. Photographic documentation was performed using a gel documentation system.

III. Results

Uptake of Na^+ and K^+ ion by root, leaf blades and leaf sheath

In root, leaf blades and leaf sheath of both cultivars FR13A and BRRI dhan29 at control condition were uptake almost similar amount of Na^+, but in case of K^+,FR13A uptake highest amount of K^+ then BRRI dhan29. At 60mM and 120mM salinity stress conditions, less Na^+ was uptake by root, leaf blades and leaf sheath in FR13A compared with BRRI Dhan29. In case of K^+, FR13A uptake highest amount then BRRI dhan29 in all salt stress condition.



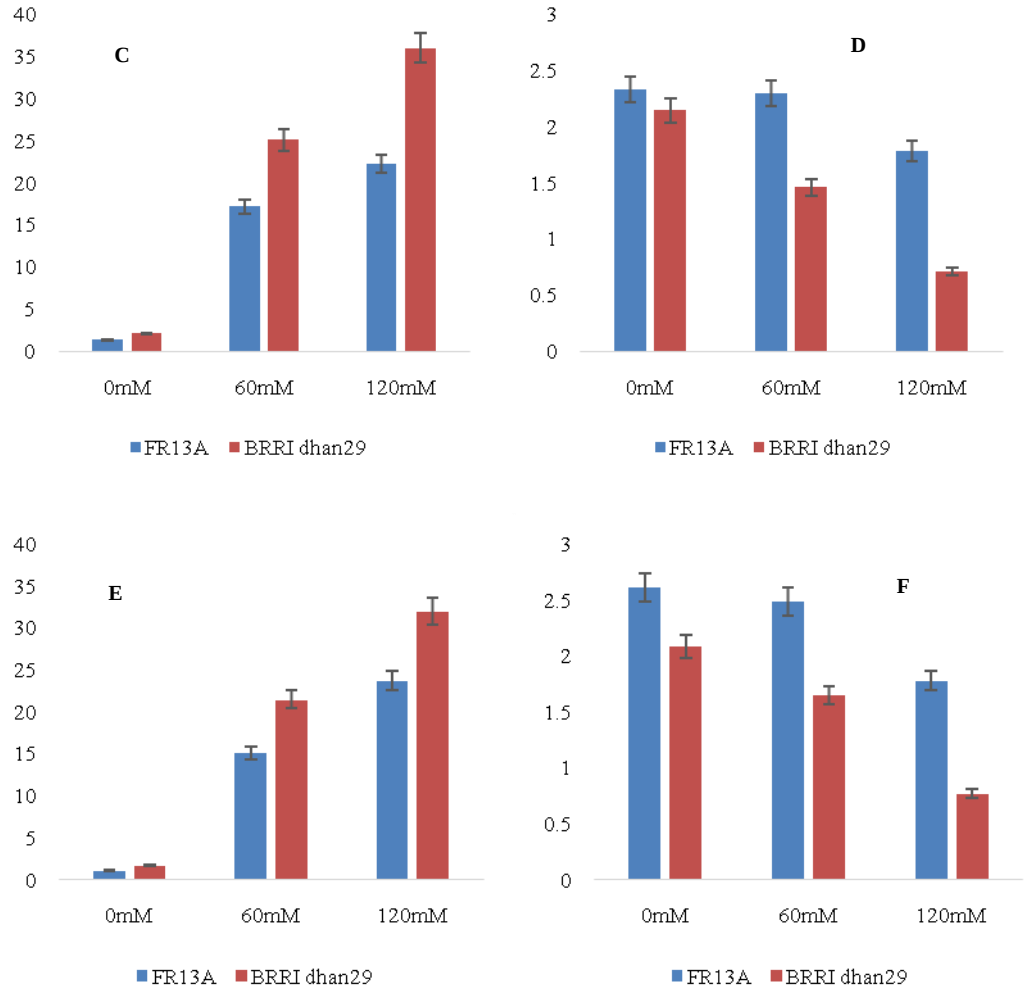


Figure 1: Cation contents of FR13A and BRRI dhan29 (A) Na^+ uptake by root (B) K^+ uptake by root (C) Na^+ uptake by leaf sheath (D) K^+ uptake by leaf sheath (E) Na^+ uptake by leaf blade (F) K^+ uptake by leaf blade. Samples were taken from control (0mM) and 72h after 60mM and 150mM NaCl stress application. Vertical bars represent the SE of the mean for triplicate determinations.

Expression analysis of genes

To assess the effect of salt on the expression pattern of *OsNHX1, OsNHX2, OsSOS1*and *OsDREB*total RNA from leaf of NaCl treated rice plants (cvs. FR13A and BRRI dhan29) were isolated. The transcript levels of *OsNHX1, OsNHX2, OsSOS1* and *OsDREB* (a Na^+ transporter) were quantified using semi-quantitative RT-PCR in the salt-tolerant rice cv. FR13Aand the salt-sensitive rice cv. BRRI dhan29 after 0h, 1h, 6h, 24h, and 72h of salt stress with 150mM NaCl.

Expression pattern of *OsNHX1* gene under salinity stress

The results indicated that *OsNHX1* was undetected at control condition and up regulated 1h up to 72h in FR13A. Higher expression was detected at 1h salinity stress and continued up to 72h salinity stress in FR13A. On the other hand, in BRRI dhan29 the expression was undetectable at control condition to 24h salinity stress. But at 72h salinity stress the expression of *OsNHX1* was detected in BRRI dhan29.

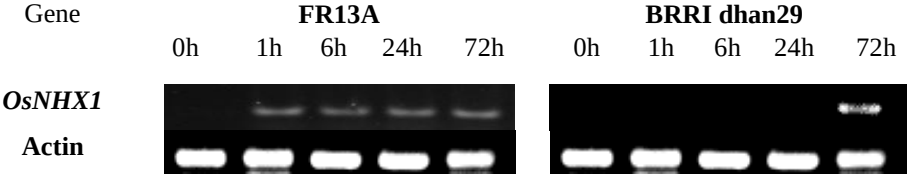


Figure 2: Expressional analysis of *OsNHX1* in the salt-tolerant rice cv. FR13A and salt-sensitive rice cv. BRRI dhan29 after different time points under salinity stress of 150mM NaCl. ACTIN was used as an internal control
Expression pattern of *OsNHX2* gene under salinity stress

The*OsNHX2* transcript levels was detected at all-time point in FR13A,the expression was up-regulated from control to 72h salinity stress and remained stable. Higher expression was detected at 1h salinity stress and continued up to 72h salinity stress in FR13A. In case of BRRI dhan29 the expression was detected at control condition butat 1h to 72h of salinity stress the expression was totally undetectable.

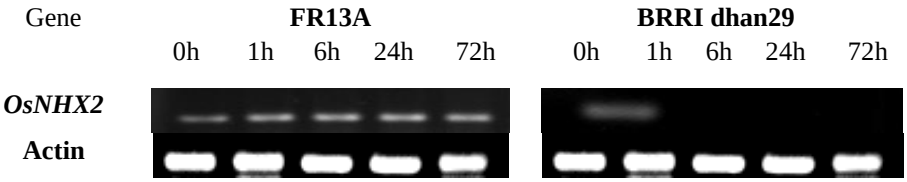


Figure 3: Expressional analysis of *OsNHX2* in the salt-tolerant rice cv. FR13A and salt-sensitive rice cv. BRRI dhan29 after different time points under salinity stress of 150mM NaCl. ACTIN was used as an internal control

Expression pattern of *OsSOS1* gene under salinity stress

The expression of *OsSOS1* was detected at control condition up to 72h. The up regulated and stable expression observed at all time point but highest expression was found 72h in FR13A. In case of BRRI dhan29 the expression was detected at control condition and 24h of salinity stress but undetectable in other time points.

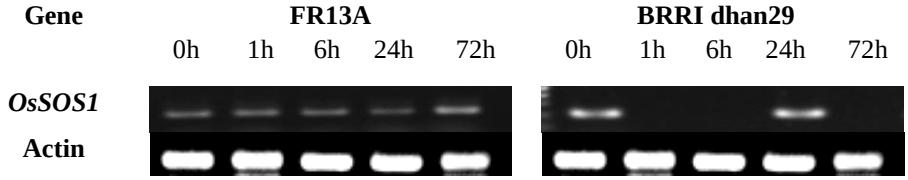


Figure 4: Expressional analysis of *OsSOS1* in the salt-tolerant rice cv. FR13A and salt-sensitive rice cv. BRRI dhan29 after different time points under salinity stress of 150mM NaCl. ACTIN was used as an internal control

Expression pattern of *OsDREB* gene under salinity stress

The expression of *OsDREB* was detected at control condition and up regulated to 72h of salinity stress and remained stable in FR13A. The highest expression of *OsDREB* was observed at 6h up to 72h of salinity stress. In case of BRRI dhan29 the expression was found at control condition, 6h to 72h and undetectable observed at 1hof salinity stress.

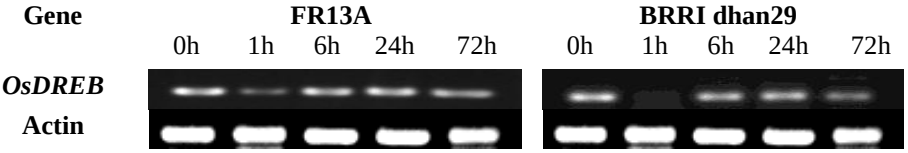


Figure 5: Expressional analysis of *OsDREB* in the salt-tolerant rice cv. FR13A and salt-sensitive rice cv. BRRI dhan29 after different time points under salinity stress of 150mM NaCl. ACTIN was used as an internal control

IV. Discussion

The present study detected the tissues (root, leaf sheath and leaf blade) in response to salinity stress in a salt tolerant rice cultivar FR13A and salt sensitive cultivar BRRI dhan29. As indicated by our results, along with increasing sodium content in tissues dramatically increased at 60mM and 120mM salinity stress to get rid of excessive Na^+ions in BRRI dhan29 than FR13A. This phenomenon indicated the important role of salt glands and protection of plant tissues against toxic ions, without losing indispensable nutrients in FR13A cultivar. Significant up-regulation of *OsNHX1, OsNHX2, OsSOS1* and *OsDREB* gene under salt stress was shown in our experiments in salt tolerant rice cultivar FR13A and non-significant expression in salt sensitive cultivar BRRI dhan29. Free proline usually accumulates by DREB at high concentrations when plants are in abiotic stress. It can protect cells from damage by acting as an osmotic agent **(Kishor *et al.* 2014)**. In the present study, the proline content in FR13A under salt stress was significantly higher than salt sensitive cultivar BRRI dhan29. The inter-relationship observed between*OsNHX1, OsNHX2, OsSOS1* and *OsDREB* gene under salt stress whereas, there has no inter-relationship between genes were detected when upon imposed salinity stress in BRRI dhan29. The response to stress condition in the tissues of FR13A was stronger than the tissues of BRRI dhan29 and higher transcript levels of all gene were observed in FR13A cultivar. Transcript abundance of *OsNHX* gene in response to salinity can trigger high activities of tonoplast Na^+/H^+antiporter in FR13A.

Induction of expression of different isoforms of *OsNHX* gene under NaCl treatment including *AtNHX1*, 2 and 5 in *A. thaliana* **(Yokoi *et al.* 2002)** and *PeNHX1*, 2, 3, 5 and 6 in *Populus euphratica* **(Ye *et al.* 2009)** was shown. Accumulation of sodium ions and expression level of *OsNHX1,2* in FR13A tissues might be correlated with each other. The up-regulation of *OsNHX1,2* gene expression might diminish Na^+ translocation from root to shoot via Na^+ accumulation in the vacuoles. As explained by **(Fukuda *et al.* 2004)** alkalization of vacuolar lumen might regulate the H^+ pump gene expression and its acidification induces Na^+/H^+ antiporters. Over expression of H^+ pumps in coordination with Na^+/H^+ antiporter may govern salt tolerance mechanisms in plants. Transcriptional levels of *OsSOS1* in the FR13A cultivars significantly increased in response to salinity. High abundance of *OsSOS1* transcript level in the FR13A was accompanied with a lower sodium accumulation in comparison with BRRI dhan29. Parallel activity of *OsNHX1,2*, *OsSOS1* and *OsDREB* that were induced by salinity may result in compartmentalization of sodium ions in the vacuoles of salt tolerant rice cultivar FR13A. It appears that simultaneous induction of *OsSOS1*, *OsNHX1,2* and *OsDREB* in FR13A tissues is determinant and effective factors to control Na^+ translocation and accumulation in FR13A but this mechanisms were absent in BRRI dhan29 tissues as indicated.

V. Conclusion

With respect to *OsNHX1*, *OsNHX2*, *OsSOS1* and *OsDREB* expression data, the regulatory mechanism of cytosolic K^+/Na^+ homeostasis seems to be an important salt-tolerance determinant in the salt-tolerant rice cv. FR13A. This mechanism is less efficient in the salt-sensitive cv. BRRI Dhan29. At the onset of NaCl stress, FR13A increases the expression of above gene tissues. FR13A also induces the expression of these genes at the onset of high NaCl conditions, most likely to compartmentalize cytosolic Na^+ into the vacuole. This might occur either because of K^+ deficiency in cells (caused by Na^+ competition at transport sites), or by interruption of the cytosolic Na^+/K^+ ratio, which cells might sense as a K^+-deficiency. However, at a certain stage later on, FR13A down-regulates the expression of these genes. It is concluded that, at the onset of high NaCl conditions, FR13A maintains cytosolic K^+/Na^+ homeostasis by increasing the K^+/Na^+ coupled uptake through the induction of these genes, as well as by increasing the compartmentalization of cytosolic Na^+ into the vacuole. FR13A might also maintain a low influx of cytosolic Na^+ either by means of a conformational change of the transport proteins and/or any post-transcriptional changes of above genes. On the other hand, FR13A maintains cytosolic K^+/Na^+ homeostasis by down-regulating transport proteins. However, to understand the mechanism of K^+/Na^+ homeostasis in rice fully, the cell- and tissue-specific expression patterns of these genes need to be investigated under conditions of NaCl stress.

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