Chapter 6:

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Environmental stresses such as salinity and drought have an enormous impact on crop productivity throughout the world. In order to survive under ever-changing environmental conditions, plants should be able to trigger a proper cellular response and that necessitates the plants to be equipped with a precisely controlled gene expression system. The whole work embodied in this thesis pertains to the development of rice transgenic plants with improved tolerance to various abiotic stresses, especially salinity and drought. Important achievements of the present work are described below.

A multiple alignment of amino acid sequences of Rab7 from different organisms such as *O. sativa, P. glaucum, A. thaliana, H. Vulgare* and *H. sapiens* showed an overall similarity among the above organisms ranging from 59% to 92%. The phylogenetic tree analysis showed that PgRab7 is close to AtRab7 and lies on the same branch.

From the rice genome sequence (http://rice.plantbiology.msu.edu/) it was found that there are four orthologs of Rab7 in rice. These are *OsRab7A1, OsRab7A2, OsRab7B1* and *OsRab7B2*. It was noticed that most of the OsRab7 ORFs are about 0.6 kb in length. The chromosome localization and distribution analyses of OsRab7 revealed that they are present only on 2 chromosomes: *OsRab7B1, OsRab7A2* are distributed over chromosome number I while *OsRab7B2, OsRab7A1* are present in chromosome number V. *OsRab7A2* and *OsRab7B1* have been predicted to undergo alternative splicing. *OsRab7A2* can produce three spliced forms and *OsRab7B1* can produce two spliced forms.

A multiple alignment among OsRab7A1, OsRab7A2, OsRab7B1 and OsRab7B2 showed that the overall similarity among four orthologs ranges from 69% to 92% at the amino acid level. The phylogenetic tree showed that OsRab7B1 is close to OsRab7B2 and lies on the same branch.

The amino acid sequence of OsRab7 and PgRab7 proteins shows 92% identity and the protein structures are also highly similar. The phylogenetic tree showed that PgRab7 is close to OsRab7B1 and lies on the same branch.
Publicly available expression data from Rice Oligonucleotide Array Database (ROAD) organ series showed that OsRab7B1 and OsRab7B2 are highly expressed in all the plant parts such as young leaf, mature leaf, shoot apical meristem (SAM), panicle developmental stages (P1-P6), seed developmental stages (S1-S5), 7 days seedling, 7 days root and stress condition like drought, salt and cold. OsRAB7A2 expressed highly in SAM and 7 day root and lowly in other different stages and stress conditions. OsRab7A1 is expressed lowly in all the above stages and stress condition. These data indicate that although not all, OsRab7B1 and OsRab7B2 genes are expressed highly in drought, salt and cold stress conditions.

For plant transformation, PgRab7 cDNA was PCR-amplified and cloned in pCAMBIA 1301 vector with CaMV 35S constitutive promoter. The construct was used for Agrobacterium mediated transformation of PgRab7 in PB1 and IR64 to develop rice transgenic lines. The confirmation of transgenic plants was done by PCR analysis. To monitor the expression of PgRab7 gene in transgenic plants, western blot analyses were performed. In transgenic plants a band of 22.7 kDa corresponding to PgRab7 protein was identified; however, the protein quantity varied between different transgenic lines.

The photosynthetic parameter such as total photosynthesis, Electron Transport Rate (ETR), Fv/Fm ratio, yield of photosystem II, photochemical quenching (qP) and non-photochemical quenching (qN) of WT plants and transgenic lines under control condition showed that in PgRab7 transgenic lines the photosynthetic efficiency was higher as compared to WT plants.

A comparative leaf disc senescence study for 72 h between WT and transgenic lines showed that under control conditions, leaf discs of PgRab7 transgenic lines were equally green like WT plants. However, under NaCl stress, leaf discs of WT plants completely bleached and turned yellow but in case of transgenic lines, the leaf discs remained green. In control condition, the total chlorophyll content in the WT plants and transgenic plants were nearly equal but as the NaCl concentration was increased, WT plants showed less total chlorophyll content as compared to transgenic lines.

The seed germination test of WT plants and different transgenic lines P36, P40.1, P40.2 showed that in control and 100 mM NaCl stress condition, in all transgenic
lines and WT plants there was 100% seed germination. However in 175 mM NaCl stress, WT plants showed 33% and transgenic lines showed 83% to 100% seed germination. In 200 mM NaCl stress, WT plants seeds did not germinate whereas seeds of transgenic lines germinated to the extent of 75% to 92%.

The root and shoot growth comparison of WT plants and transgenic lines showed that, under control conditions, transgenic lines showed 4% to 30% longer shoot length and 1% to 10% longer root length as compared to WT plants. In 100 mM NaCl stress, transgenic lines showed 1% to 2% longer shoot length and 16% to 30% longer root length as compared to WT plants. In 175 mM NaCl stress condition, transgenic lines showed 52% to 74% longer shoot length and 6% to 17% longer root length as compared to WT plants. In 200 mM NaCl stress condition, transgenic lines showed 236% to 333% longer shoot length and 150% to 203% longer root length as compared to WT plants.

It was confirmed that the transgenic lines showed better tolerance to 200 mM NaCl stress, when the NaCl stress was given to the plants throughout life cycle and also to two months old plants upto seed harvest.

Total chlorophyll content measurement in WT plants and different transgenic lines under water control and 200 mM NaCl stress condition showed that the $PgRab7$ transgenic lines have more chlorophyll than WT plants. In control condition, transgenic lines had 12% to 42% more chlorophyll than WT plants. During NaCl stress, there was reduction of 58% in WT whereas as in transgenic lines reduction was 2% to 12%.

Earlier it was shown that higher Gly I and Gly II activity of glyoxalase pathway is related to stress tolerance. To check this in our case, Gly I and Gly II activity was measured in $PgRab7$ overexpressing lines. The Gly I activity of the transgenic lines was found to be 23% to 52% higher than that of the WT plants under control condition. In 200 mM NaCl stress conditions, one transgenic line P24 shows 47.5% higher activity as compared to WT plants whereas other transgenic lines showed 14% to 20% lower activity as compared to WT plants. The Gly II activity of P40.1 was found to be 2% higher than that of the WT plants whereas other transgenic lines showed 2% to 84% decrease in activity as compared to WT plants under control.
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condition. In 200 mM NaCl stress condition, the transgenic lines showed 21% to 168% higher activity than WT whereas, two transgenic lines P24, P27 showed 31% to 63% lower activity than WT plants.

Leaf disc senescence studies were conducted with WT plants and transgenic lines exposed to 200 mM NaCl stress for 120 h. It was observed that transgenic lines retained more chlorophyll than WT plants. Ion measurement data indicated that under control condition, all the plants maintained a basal level of Na$^+$ in the cell of leaf tissue. During 120 h NaCl stress, it was observed that WT plants accumulated 11.7 fold more Na$^+$ as compared to control condition. The increase in Na$^+$ in transgenic lines was significantly less. The K$^+$/Na$^+$ ratio was found to be higher in transgenic lines as compared to WT plants.

To check the photosynthesis efficiency of WT plants and transgenic lines in salinity stress, different photosynthetic parameters such as total photosynthesis, Electron Transport Rate (ETR), Fv/Fm ratio, yield of photosystem II, photochemical quenching (qP) and non-photochemical quenching (qN) were measured by IRGA. From the data it was observed that under control condition, transgenic lines showed 0.32% to 13.40% higher photosynthesis, 12% to 26% higher ETR, 13% to 24% higher PSII, 1% to 3% higher Fv/Fm, 3% to 9% higher photochemical quenching and 7% to 12% lower non-photochemical quenching than the WT plants. During 200 mM NaCl stress, transgenic lines showed 217% to 344% higher photosynthesis, 25% to 40% higher ETR, 16% to 36% higher PSII, 1% to 7% higher Fv/Fm, 14% to 18% higher photochemical quenching and 2% to 9% lower non-photochemical quenching than the WT plants. From the measurement of different parameters it showed that transgenic lines photosynthesis efficiency is much higher in both control and 200 mM NaCl stress as compared to WT plants.

A comparative study of chloroplast ultra structure was carried out between WT plants and two transgenic lines at different time periods at 200 mM NaCl stress. In both WT plants and transgenic lines the chloroplast structure, particularly the grana stacking, was intact upto 24 h of stress but at 72 h, grana stacking of WT plants was completely disturbed whereas in transgenic lines the grana stacking was maintained which probably leads to active photosynthesis.
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A comparative study of ultra structure of rice leaf and root cell was carried out between WT plants and transgenic lines at different time periods following 200 mM NaCl stress to check the effect of salt stress on vacuolar size, if any. In leaf cells, WT plants vacuole size increased up to 12 h and then at 24 h and 72 h it decreased. Also at 72 h the vacuole was completely disturbed. In transgenic lines, on the other hand the vacuole size continued to increase and remained intact up to 72 h. In root cells, at 12 h NaCl stress the vacuole size of WT plants was smaller in size as compared to transgenic lines. As the stress period increased to 72 h, in WT plants the vacuole was enlarged and showed complete disruption whereas in transgenic lines the vacuole size remained intact. From this observation it was found that in transgenic lines vacuole stays intact even under 200 mM NaCl stress.

To see the accumulation of Na\(^+\) in transgenic lines and WT plants at 150 mM NaCl stress for 48 h, CoroNa Green fluorescent dye was used which gives the green coloured fluorescence. PI was also used to stain the nonviable cells. Most of the cells of roots of WT plants showed higher fluorescence with both the dyes indicating that higher amount of Na\(^+\) accumulated inside these cells and there was an increased cell death. However, in transgenic lines the accumulation of Na\(^+\) was significantly reduced compared to WT and they also showed deposition of only a few dead cells in the root tip region.

DAB staining assay of leaf and root tissue was carried out to see the accumulation of superoxide and hydrogen peroxide during NaCl and methyl viologen (MV) stress. Under control condition, leaf tissue of transgenic lines showed 12% to 13% less brown colour than the WT plants. These results suggested that plants do get a basal level of oxidative stress during unstressed conditions. At 24 h of NaCl stress, transgenic lines showed 15% to 20% less brown colour with respect to WT. In 12 h of MV stress, transgenic lines showed 20% to 24% less brown colour with respect to WT plants. These data suggest that during 24 h salt stress or 12 h MV stress, transgenic lines experience less oxidative stress in comparison to WT plants. Similar results were obtained with root tissue also.

The seed weight data from transgenic lines which completed their life cycle in 200 mM NaCl stress showed that WT plants seeds did not grow under these conditions.
and showed 100% yield penalty as compared to their water control. Transgenic lines showed only up to 51% to 67% yield penalty when compared to their respective water controls.

The seed weight data from two months old WT plants and transgenic lines provided with 200 mM NaCl stress showed that WT plants did not set seed in 200 mM NaCl stress and showed 100% yield penalty as compared to their water control. Transgenic lines however showed 33% to 53% yield penalty as compared to their water control.

Total chlorophyll content was measured in WT plants and different transgenic lines under water control and 12 days drought stress condition, and it was observed that the transgenic lines had more chlorophyll than WT plants. In control condition, the transgenic lines have 12% to 42% more chlorophyll than WT plants. During drought stress, the WT plants showed 53% reduction. The transgenic lines showed 2% to 34% more chlorophyll, whereas two transgenic lines showed 1% to 2% reduction in chlorophyll as compared to control condition.

To check the photosynthetic efficiency of WT plants and transgenic lines in drought stress, different photosynthetic parameters such as total photosynthesis, Electron Transport Rate (ETR), Fv/Fm ratio, yield of photosystem II, photochemical quenching (qP) and non-photochemical quenching (qN) were measured by IRGA. From the data it was observed that under control condition, transgenic lines showed 1% to 13% higher photosynthesis, 12% to 26% higher ETR, 13% to 24% higher PSII, 1% to 3% higher Fv/Fm, 3% to 9% higher photochemical quenching and 7% to 12% lower non-photochemical quenching than the WT plants. During drought stress, transgenic lines showed 123% to 190% higher photosynthesis, 54% to 92% higher ETR, 24% to 45% higher PSII, 4% to 7% higher Fv/Fm, 21% to 31% higher photochemical quenching and 8% to 23% lower non-photochemical quenching than the WT plants. From the measurement of different parameters it follows that in \( \text{PgRab7} \) transgenic lines, the photosynthetic efficiency is much higher under both control and drought stress as compared to WT plants.

In 12 days drought stress, WT plants showed 82% yield penalty in terms of seed weight as compared to plants which were irrigated throughout. One transgenic line
showed 3% yield penalty whereas all other transgenic lines showed 3% to 31% higher yield as compared to their water control.

Sortin1, a sorting inhibitor (227 µM) and Brefeldin, a protein secretion inhibitor (20 µg/ml) treatment to WT plants and transgenic lines for 48 h showed that both WT plants and all five transgenic lines died both in Sortin 1 and Brefeldin. These results suggest that both WT plants and transgenic lines, Rab7 follow the same trafficking pathway.

To study the transcriptome level changes in both the WT plants and \textit{PgRab7} transgenic lines under different experimental condition, microarray experiment was performed. The experiment consisted of six samples 1. T\_CONT (transgenic control) 2. WT\_CONT (wild type control) 3. T\_NaCl (transgenic NaCl) 4. WT\_NaCl (wild type NaCl) 5. T\_DROU (transgenic Drought) 6.WT\_DROU (wild type drought) under control, salinity, and drought condition, were chosen for microarray data analysis. The microarray data were analysed in 9 combinations such as 1.WT\_NaCl vs. WT\_CONT, 2. WT\_DROU vs. WT\_CONT, 3. WT\_DROU vs. WT\_NaCl, 4. T\_CONT vs. WT\_CONT, 5. T\_NaCl vs. WT\_NaCl, 6.T\_DROU vs. WT\_DROU, 7. T\_NaCl vs. T\_CONT, 8.T\_DROU vs. T\_CONT, 9.T\_DROU vs. T\_NaCl.

In WT\_NaCl vs. WT\_CONT 625 genes are up-regulated and 432 genes are down-regulated, in WT\_DROU vs. WT\_CONT 980 genes are up-regulated and 903 genes are down-regulated, in WT\_DROU vs. WT\_NaCl 379 genes are up-regulated and 209 genes are down-regulated, in T\_CONT vs. WT\_CONT 442 genes are up-regulated and 1053 genes are down-regulated, in T\_NaCl vs. WT\_NaCl 137 genes are up-regulated and 430 genes are down-regulated, in T\_DROU vs. WT\_DROU 951 genes are up-regulated and 628 genes are down-regulated, in T\_NaCl vs. T\_CONT 7 genes are up-regulated and 12 genes are down-regulated, in T\_DROU vs. T\_CONT 105 genes are up-regulated and 109 genes are down-regulated and in T\_DROU vs. T\_NaCl 394 genes are up-regulated and 308 genes are down-regulated.

To further test the abiotic stress resistance role of \textit{PgRab7} in IR64 rice background and to use the transgenic lines for commercial purpose, IR64 transgenic plants overexpressing \textit{PgRab7} were made. To confirm the gene integration, putative transgenic lines (T\textsubscript{0} plants) were confirmed by PCR. A comparative leaf disc
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A senescence study was carried out between WT plants and *PgRab7* transgenic lines. It was observed that under control conditions, leaf discs of transgenic lines were equally green like WT plants. Under NaCl stress, leaf discs of WT plants completely bleached and turned into yellow color but in case of transgenic lines, the leaf discs remained green. In control condition, the total chlorophyll content in the WT plant was less than transgenic plant. Also when the NaCl concentration increases WT have less total chlorophyll content as compared to transgenic plant. The above data showed transgenic lines retained more chlorophyll as compared to WT plant under stress condition. This indicated that overexpression of *PgRab7* gene provides salt stress tolerance to the transgenic rice in IR64 genetic background also.

Overall Conclusions

Though not much work has been done yet, during recent years components of membrane trafficking have been reported to be involved in protecting abiotic stress induced damage, thus indicating a possibility to explore house-keeping genes as candidate genes for raising crop plants which are protected against multiple abiotic stresses. In this thesis, we have validated function of one of the member of RAB family, involved in trafficking, Rab7 in stress tolerance. Accordingly *PgRab7* whose function was validated in tobacco earlier was overexpressed in rice, a staple crop grown throughout the world. Our results indicate that transgenic rice plants expressing *PgRab7* were protected against salt and drought stress up to the extent where un-transformed plants did not survive at all. Secondly, when evaluated for the seed setting, transgenic lines showed considerable vegetative growth against both the stresses viz. salinity as well as drought. A significant amount of seed setting was maintained in transgenic lines during drought stress; however imposition of salt stress decreased seed setting compared to that of drought stress. Our results indicate that *PgRab7* acts differentially during salinity and drought stress an effect not reported for other plant genes, and could be a more potent candidate for raising drought stress tolerant crops. We also show that *PgRab7* overexpression affects sodium homeostasis, ROS production, vacuole size, overall photosynthesis and affects transcriptomic profile.