Chapter 4:

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Results

Rab7 is a member of a small guanosine triphosphate (GTP)-binding protein family involved in intracellular vesicle trafficking from late endosome to the vacuole. Previously involvement of Rab7 was demonstrated in different abiotic stresses in model plants such as A. thaliana and N. tabaccum (Mazel et al., 2004; Agarwal et al., 2008). The present study centered around functional validation of Pennisetum glaucum Rab7 (PgRab7) in rice for abiotic stress tolerance such as salinity and drought stress. The functional aspects were addressed by raising rice transgenics by ectopically expressing PgRab7 through Agrobacterium mediated transformation.

Similarity Among Rab7 from Different Organisms

A multiple alignment of Rab7 was performed among different organisms such as O. sativa (Acc no. AY226827), P. glaucum (Acc no. AY829438), A. thaliana (Acc no. NP001031161), H. vulgare (Acc no. DQ154923) and H. sapiens (Acc no. BC013728). The deduced amino acid alignment of PgRab7 showed a strong similarity to several other plants and animals such as H. sapiens Rab7. Overall similarity among the above organisms ranged from 59.5% to 92.3% at the amino acid level (Fig. 3A). The Rab7 from different organisms showed the presence of functionally important four guanine nucleotide-binding domains and an effector-binding domain. The G1 (P-loop; GDSGVGKT) involved in Mg\(^{2+}\) and phosphate binding, G3 (Switch II; WDTAGQ), where DTAG interacts with \(\gamma\)-phosphate of GTP, G4 (GNKXD) where NKXD is the guanine specificity region and G5 (ETSAK) where ETSA interacts with the D residue in the NKXD. The G1 and G3-G5 motifs are collectively involved in nucleotide binding and hydrolysis. The effector region G2 (Switch I; YKATIGADF) contains information for individual function and its TIGADF motif interacts with specific GTPase-activating GAP proteins. The role of YRG motif conserved in Rab7 protein is not yet established. The phylogenetic tree analysis showed that PgRab7 was close to AtRab7 and lies on the same branch (Fig. 3B).
Figure 3: (A) Clustal W analysis of Rab7 amino acid sequence of O. sativa (Acc no. AY226827), P. glaucum (Acc no. AY829438), A. thaliana (Acc no. NP001031161), H. vulgare (Acc no. DQ154923) and H. sapiens (Acc no. BC013728). The black shade indicates the identical amino acids. The gaps indicate regions introduced to maximize the sequence homology between the two sequences. (B) Phylogenetic tree analysis of Rab7 amino acid sequence among different organisms.
Chromosomal Localization of Rice *Rab7* Genes

From the rice genome sequence it was found that there are four orthologs of *Rab7* in rice. These are *OsRab7A1*, *OsRab7A2*, *OsRab7B1* and *OsRab7B2*. The list of *OsRab7A1*, *OsRab7A2*, *OsRab7B1* and *OsRab7B2* genes along with their TIGR IDs, locus name, coordinates, nucleotide sequence, amino acid length identified from the TIGR rice database (http://rice.plantbiology.msu.edu/) is given in Fig. 4A.

<table>
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<th>Protein</th>
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<th>Coordinate (5' - 3')</th>
<th>Nucleotide</th>
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<td>OsRAB7B1</td>
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<tr>
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<td>OsRAB7A2</td>
<td>13101.m05396</td>
<td>LOC_Os01g51700.1 Alternative Splice Form: LOC_Os01g51700.2 LOC_Os01g51700.3</td>
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<td>621</td>
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</tr>
</tbody>
</table>

**Figure 4**: Rice *Rab7* genes and their genomic distribution on chromosomes. (A) List of *OsRab7* genes along with their co-ordinates, ORF and protein length. (B) Genomic distribution of *Rab7* genes on rice chromosomes. White ovals on the chromosomes (vertical bars) indicate the position of centromeres. Chromosome numbers are indicated at the bottom of each bar. The position of first exon of genes (in Mb) has been marked in the parentheses along with their names at the same location on chromosomes. Arrow marks the direction of the ORF specific to the gene encoding RAB7 protein. Among 12 chromosomes of rice, only those chromosomes having *Rab7* genes are shown.
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 out of 12 chromosomes in rice. OsRab7B1, OsRab7A2 are distributed over chromosome number I and OsRab7B2, OsRab7A1 are present in chromosome number V (Fig. 4B).

Out of four orthologs of Rab7 in rice, OsRab7A2 and OsRab7B1 have been predicted to undergo alternative splicing. OsRab7A2 can produce three spliced forms and OsRab7B1 can produce two spliced forms (Fig. 5).

**Figure 5:** Gene structures including alternative spliced forms of OsRab7A1, OsRab7A2, OsRab7B1 and OsRab7B2 (from TIGR version 5).

**Similarity Among Different Rice Rab7s**

A multiple alignment was performed among the OsRab7s to determine the percentage similarity among themselves. Overall similarity among four orthologs ranged from 69.2% to 92.8% (Fig. 6A) at the amino acid level. The phylogenetic tree showed that OsRab7B1 was close to OsRab7B2 and lies on the same branch (Fig. 6B).
Figure 6: (A) Clustal W analysis of different orthologs of OsRab7 (OsRab7A1, OsRab7A2, OsRab7B1, OsRab7B2) amino-acid sequence. The black shade indicates the identical amino acids. The gaps indicate regions introduced to maximize the sequence homology between the two sequences. (B) Phylogenetic tree analysis of different orthologs of OsRab7 amino acid sequence.

Similarity of *Pennisetum glaucum* Rab7 and Rice Rab7

A multiple alignment was performed among the *Pennisetum glaucum* Rab7 and rice Rab7 to determine the percentage similarity among them. Overall similarity among *Pennisetum glaucum* Rab7 and rice Rab7 four orthologs ranged from 69.2% to 99.5% (Fig. 7A) at the amino acid level. The phylogenetic tree showed that PgRab7 was close to OsRab7B1 and lies on the same branch (Fig. 7B).
Figure 7: (A) Clustal W analysis of different orthologs of OsRab7 (OsRab7A1, OsRab7A2, OsRab7B1, OsRab7B2) and PgRab7 amino acid sequence. The black shade indicates the identical amino acids. The gaps indicate regions introduced to maximize the sequence homology between the two sequences. (B) Phylogenetic analysis of different orthologs of OsRab7 and PgRab7 amino acid sequence.
Comparison of Different Rab7 Protein Structures and their Binding Sites

To compare the protein structure and binding site of different orthologs of OsRAB7 proteins and OsRAB7 protein with PgRab7 protein, three-dimensional (3D) structures were constructed from amino acid sequence of the different proteins with the help of I-TRASSER (Roy et al., 2010) software (http://zhanglab.ccmb.med.umich.edu/I-TASSER). Though there was 69.2% to 92.8% identity of amino acid sequence of four different orthologs of OsRAB7 proteins, their protein structure and binding sites were found to be quite different from each other (Fig. 8A). When compared, the protein structure and binding site of OsRAB7 protein with PgRAB7 protein, it was found that the three-dimensional (3D) structures of both the proteins are highly similar to each other (Fig. 8B, C). The amino acid sequence of both the proteins showed 92.3% identity.

Figure 8: Protein structure with binding sites, Clustal W and phylogenetic analysis of different orthologs of OsRAB7 proteins. (A) Comparison of different OsRAB7 protein structure and their binding sites. (B) Comparison of PgRAB7 and OsRAB7 protein three dimensional structure. (C) Comparison of PgRAB7 and OsRAB7 protein predicted binding site with binding site residue. The structure was determined through I-TASSER site.
Development Specific Expression of OsRab7 Genes

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 days root and lowly in other different stages and stress conditions. OsRab7A1 is expressed lowly in the all the above stages and stress condition. These data indicate that although not all, OsRab7B1 and OsRab7B2 genes expressed highly in drought, salt and cold stress condition (Fig. 9).

**Figure 9:** Heat map analyses of OsRab7 genes using microarray data obtained from Rice Oligonucleotide Array Database (ROAD). The microarray data for various abiotic stresses and vegetative stage such as cold, drought, salinity, young leaf, mature leaf, shoot apical meristem (SAM), panicle developmental stages (P1-P6), seed developmental stages (S1-S5), 7 days seedling and 7 days root were retrieved from the Rice Oligonucleotide Array Database (ROAD).

**Construction of Plant Transformation Vector**

For plant transformation, PgRab7 cDNA (Fig. 10) was PCR-amplified using forward (5’ TCGGGATCCCGGATCCGCCTCCCATG 3’) and reverse (5’ GCTCT AGAGCACCCTATAGGACCAAACCG 3’) primers containing BamHI and XbaI sites, respectively. The digested fragment was then cloned in pRT 101 vector (To¨pfer et al., 1987); thereafter full cassette containing CaMV 35S
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constitutive promoter with \textit{PgRab7} gene was digested with PstI and cloned in pCAMBIA 1301 vector (Agarwal et al., 2008).

\begin{tabular}{l}
\texttt{cgcggtccggtccggtccgatccgctccgctcccctcctcccccagatccgccgcgacctcccc} \texttt{ATG} \\
\texttt{AGG CGAATG CTC CTC AAG GTC ATC ATC CTC GGC GAC AGC GGG GTC GGC AAG} \texttt{M} \texttt{A} \texttt{T} \texttt{R} \\
\texttt{R R ML L K V I I L G D S G V G K} \\
\texttt{ACC TCG CTG ATG AAC CAG TAC GTG AAC AAAAAG TTC AGC AAC CAG TAC AAG} \texttt{S} \texttt{L} \texttt{M} \texttt{N Q Y N K K F S N Q Y K} \\
\texttt{GCC ACC ATC GGC GCC GAT TTT CTC ACC AAG GAG GTC CAG ATC GAC GAG CGC} \texttt{A T I G A D F L T K E V Q I D D R} \\
\texttt{CTC TTC ACC TTG CAG ATA TGG GAT ACG GCA GAA GAG CCG TTG CAG AGT} \texttt{L F T L Q I W D T A G Q E R F Q S} \\
\texttt{CTT GTG GTG GCA TTT TAT CGG GGA GCT GAC TCG TGT GTT CTT GTA TAT GAT} \texttt{L G V A F Y R G A D C C V L V Y D} \\
\texttt{GTC AAT GTC ACC AAG TCA TTT GAG AAG CTC AAT AAC TGG CGT GAG GAA TTC} \texttt{V N V T K S F E K L N N W R E E F} \\
\texttt{CTA CTT CAA GCT AGC CCA TCT GAT CCA GAG AAT TTC CTG GCG GAT AGC GTC AA T GTC ACC AAG TCA TTT GAG AAG CTC AA T AAC TGG CGT GAG GAA TTC} \texttt{V N V T K S F E K L N N W R E E F} \\
\texttt{GGAAAC AAG ATT GAC GTT GAT GGT GGT AAT AGC CGG ACA ATT TCG GAG AAA} \texttt{G N K I D V D G G N S R T J I S E K} \\
\texttt{AAAGCT AAA GCA TGG TGT TGT TCC AAG GGG AAT ATC CCC TAT TTT GAG ACG} \texttt{K A K A W C A S K G N I P Y F E T} \\
\texttt{TCG GCT AAG GAA GCC TTC AAT GTG GAA GCT TCT TTT GAG TGC GTA AGG S A K E G F N V E A A F E C I A R} \\
\texttt{AAT GCT ATC AAG AAT GAC CAA GAA GAT GAC ATG TAT CTT CCT GAT ACC ATT} \texttt{N A I K N E P E D D M Y L P D T I} \\
\texttt{GAT GTG GGG GGT GGA GCG CAA CAA CCG TCG TCA GGC TTC GAA TG CG TAG D V G A G R Q Q R S S G C E C *} \\
\end{tabular}

\texttt{aatatagt gaaggccat tttc cggcgttttaaggg ggatggctgattaggg} \texttt{a} \texttt{a}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10}
\caption{Complete nucleotide sequence of \textit{PgRab7} cDNA. Nucleotides marked in red indicate the 5'UTR, green: 3'UTR, black: complete ORF with the start and stop codons underlined, blue: amino acid sequences.}
\end{figure}

\textit{Agrobacterium} Mediated Transformation of Rice (\textit{Oryza sativa cv PB1}) with \textit{PgRab7} for Development of Transgenic Plants

The construct as mentioned above was used for \textit{Agrobacterium} mediated transformation of \textit{PgRab7} in to rice PB1 to develop the transgenic lines (Fig. 11).


**Results**

![](image)

**Figure 11:** Schematic representation of strategy used for cloning \textit{PgRab7} gene in the plant transformation vector pCAMBIA 1301. The \textit{PgRab7} ORF was cloned as a \textit{Bam}HI/\textit{Xho}I fragment into pRT101 vector, following which the entire cassette (35S promoter + \textit{PgRab7} + terminator) was taken out as a \textit{Pst}I fragment and cloned in pCAMBIA 1301 vector.

The construct was simultaneously transferred to \textit{Agrobacterium} strain LBA4404 and \textit{E. coli} strain DH5\(\alpha\). The transformation was confirmed by colony PCR by using \textit{hpt} \textit{II} forward and reverse primers (Fig. 12A) and \textit{Rab7} gene specific primers (Fig. 12B). The confirmed colony (No. 2) was further confirmed by restriction digestion of the DNA with enzyme \textit{Pst}I for confirmation of the presence of 35S promoter + \textit{PgRab7} + terminator in both LBA4404 Col. 2 (Fig. 12C) and DH5\(\alpha\) Col. 2 (Fig. 12D).

![Colony PCR and restriction digestion confirmation](image)

**Figure 12:** Colony PCR and restriction digestion confirmation. The confirmation of transformation of pCAMBIA+\textit{PgRab7} in two colonies (1 and 2) of LBA4404 and DH5\(\alpha\) respectively done by PCR using (A) \textit{hpt} \textit{II} forward and reverse primers (B) \textit{Rab7} gene specific primers. Restriction digestion confirmation of the presence of 35S promoter + \textit{PgRab7} + terminator by digested with enzyme \textit{Pst}I in (C) LBA4404 Col. 2 (D) DH5\(\alpha\) Col. 2.

The embryogenic calli were developed from mature dehusked seeds in MS callus induction medium containing 2, 4-D (2.5 mg/l) and BAP (0.2 mg/l) in the presence of
casein hydrolysate and L-proline, kept under darkness for three to four weeks. The callus induction frequency was found to be very high i.e. 95%. Embryogenic calli were sub-cultured into 2-3 small pieces and kept on same callus induction medium for another 4-5 days in darkness. The calli were infected with *Agrobacterium* and selected using hygromycin antibiotic (50 mg/l). After first selection, most of the cream coloured calli were transferred to the second selection stage where the brownish or black coloured calli were discarded. After the second selection, minute outgrowth were coming from the selected calli and these microcalli were then transferred to the first regeneration medium and kept in darkness. During this phase, somatic embryos were seen developing from the globular round calli. During the second phase of regeneration, the microcalli growing on first regeneration medium under darkness were shifted directly to light for 5 days without any change of medium. In the second phase, new shoots came out from the somatic embryos within two weeks. The regenerated shoots were transferred to rooting medium containing hygromycin (40 mg/l). The average transformation efficiency of PB1 was found to be approximately 20% using LBA4404 *Agrobacterium* strain. When roots developed properly under hygromycin selection, the putative transgenic lines were shifted to vermiculite and subsequently to soil pot having garden soil for hardening (Fig. 13A-H).

Figure 13: *Agrobacterium*-mediated transformation of pCAMBIA- *PgRab7* construct in PB1 calli. (A-H) Various steps of transformation and regeneration of PB1 rice. (A) PB1 seeds on callus induction medium. (B) Callus induction. (C) Callus sub culture. (D) Callus co-cultivated with *Agrobacterium*. (E) Calli in selection medium. (F) Micro calli regenerated. (G) Regenerated explants are on rooting medium. (H) Putative transgenic lines in soil.
Confirmation of Putative Transgenic Lines by PCR Analysis

To confirm the gene integration, putative transgenic lines (T₀ seeds) were selected on MS medium containing hygromycin (50 mg/l) (Fig. 14A). The hygromycin resistant plants were further confirmed by PCR by using three pairs of primers. The first pair is PgRab7 gene specific. The forward primer was designed from the junction of 35SCaMV promoter and 5’ end of PgRab7 gene and reverse from 3’ end of PgRab7 gene. This primer set yielded ~ 700 bp amplification product (Fig. 14B). For the second pair, forward primer was designed from the 5’ end of 35SCaMV promoter and the reverse primer was designed from 3’ end of PgRab7 gene. This primer set led to ~ 1.1 kb amplification product (Fig. 14C). The third pair, hptII forward and reverse primer. This primer set generated ~ 1 kb amplification product (Fig. 14D). All the transgenic lines showed single product of expected size in all the three cases (Fig. 14B, C and D) and there was no such band in the WT plants. The PCR positive plants were subsequently transferred to vermiculite pots for hardening and finally transferred to soils in the earthen pot.

Figure 12: Hygromycin selection and confirmation of Rab7 putative transgenic PB1 plants by PCR. (A) WT and T₀ transgenic seeds were selected on MS medium having hygromycin, WT seeds growing without hygromycin. Transgenic confirmation of hygromycin resistance plants were done by PCR using (B) Rab7 forward and reverse primers (C) 35S forward and Rab7 reverse primers (D) hptII forward and reverse primers.
Confirmation of Putative Transgenic Lines by Western Blot Analysis

To monitor the expression of \(PgRab7\) gene in transgenic rice, western blot analyses were performed with PCR positive transgenic lines (Fig. 15A). For this purpose total protein was isolated from green leaves of WT plant and transgenic lines and quantified by Bradford assay. About 50 \(\mu\)g protein was loaded per lane in 12.5% SDS-PAGE gel (Fig. 15B) to check the equal loading. Protein samples from the gel were transferred to nitrocellulose membrane and immunoblot analysis of \(PgRab7\) proteins was done by using anti-\(PgRab7\) antibody. In transgenic plants a band of 22.7 kDa was identified which correspond to \(PgRab7\) protein; however, the protein quantity varied between different transgenic lines (Fig. 15C).

**Figure 15:** Phenotypic features of transgenic PB1 plants overexpressing \(PgRab7\) and Immunoblot analysis of \(PgRab7\). (A) Putative transgenic lines (B) 12.5% SDS-PAGE protein profile of WT and \(PgRab7\) plants showing equal loading. 50 \(\mu\)g protein was loaded in each lane. (C) Protein samples from the gel were transferred to nitrocellulose membrane and immunoblot analysis of \(PgRab7\) protein (22.7 kDa) was done as described in materials and methods.
Measurement of Different Photosynthesis Parameters of *PgRab7* Transgenic lines by IRGA

To examine the photosynthesis efficiency of WT plants and T₁ transgenic lines under control condition, different photosynthetic parameters, such as total photosynthesis, Electron Transport Rate (ETR), Fv/Fm ratio, yield of photosystem II, photochemical quenching (qP), non-photochemical quenching (qN) were measured by Infrared gas analyzer (IRGA) (Fig.16A-F).

\[ \text{Net assimilation (µmol m}^{-2} \text{s}^{-1}) \]

\[ \text{Electron Transport Rate (ETR)} \]

\[ \text{Fv/Fm ratio} \]

\[ \text{Yield of PSII} \]

\[ \text{Photochemical quenching (qP)} \]

\[ \text{Non-photochemical quenching (qN)} \]

\[ \text{Non-photochemical quenching (qN)} \]

Figure 16: Measurement of different photosynthesis parameters of transgenic lines over-expressing *PgRab7* and WT in control condition by IRGA (A) Rate of photosynthesis, (B) Electron transport rate (C) Fv/Fm (D) Photosystem II (E) Photochemical quenching (qP) (F) Non-photochemical quenching (qN)

From the data it was observed that transgenic lines P24, P27, P36, P40.1 and P40.2 showed 13.41% to 30.78% higher photosynthesis, 27.74% to 33.45% higher ETR, 0.65% to 2.11% higher Fv/Fm ratio, 30.87% to 44.41% higher yield of PS II, 1.59% to 24.27% higher photochemical quenching and 32% to 42.94% lower non-photochemical quenching than the WT plants (Fig. 16A-F). From the measurement of
different parameters it showed that *PgRab7* transgenic lines photosynthesis efficiency was higher as compared to WT plants.

**Leaf Disc Senescence Assay of *PgRab7* Transgenic Rice Under Different Concentration of Salinity Stress**

A comparative leaf disc senescence study was carried out between WT plant and *PgRab7* transgenic lines. Equal sized (1.2 cm) segments of leaves were floated on water and 100, 200, 400 mM NaCl solution and kept in light under greenhouse condition for 72 h. It was observed that under control conditions, leaf discs of *PgRab7* transgenic lines were equally green like WT plants. Under NaCl stress, leaf discs of WT plants completely bleached and turned into yellow but in case of transgenic lines, the leaf discs remained green (Fig. 17A). In control condition, the total chlorophyll content in the WT plant was 1.568 mg/g of tissue where as in transgenic plant P36 the value was 1.57 mg/g of tissue and in P40.1 the value was 1.6 mg/g of tissue. In 100 mM NaCl stress, the total chlorophyll content in the WT plant reduced to 0.32 mg/g of tissue where as in transgenic plant P36 the value was 0.78 mg/g of tissue and P40.1 the value was 0.6 mg/g of tissue.

Under 200 mM NaCl stress, the total chlorophyll content in the WT plant reduced to 0.3 mg/g of tissue where as in transgenic plant P36 the value was 0.54 mg/g of tissue and in P40.1 the value was 0.45 mg/g of tissue. In case of 400 mM NaCl stress, the total chlorophyll content in the WT plant reduced to 0.085 mg/g of tissue where as in transgenic plants P36 the value was 0.5 mg/g of tissue and in P40.1 the value was 0.425 mg/g of tissue. A histogram of the above data shows transgenic lines retained more chlorophyll as compared to WT plant under stress condition (Fig. 17B). This indicated that overexpression of *PgRab7* gene provides NaCl stress tolerance to the transgenic lines.
Figure 17: Determination of NaCl stress tolerance of transgenic lines over-expressing \textit{PgRab7} by leaf disc senescence assay. (A) Leaf discs of WT and transgenic lines P36, P40.1 of \textit{PgRab7} were subjected to 100, 200, 400 mM NaCl treatments as indicated. As control, leaves were treated in water. (B) Total Chlorophyll estimation of the leaf discs 72 h following treatment.

\section*{T$_2$ Seed Germination Assay of Transgenic Lines Under Different Concentration of NaCl Stress}

To check the seed germination of WT plants and different transgenic lines, seeds of \textit{PgRab7} transgenic lines P36, P40.1, P40.2 and WT plants were germinated and grown in MS medium without or with 100, 175, 200 mM NaCl. For each treatment 12 seeds were taken from each line. After 15 days of germination data were recorded. From the data it was observed that in control and 100 mM NaCl stress condition, there was 100\% seed germination in all transgenic lines and WT plants (Fig. 18A, B). However in 175 mM NaCl stress, WT plant shows 33.33\% seed germination where as in P36 it was 83.33\%, in P40.1 it was 100\% and P40.2 it was 91.66\% (Fig. 18C). In 200 mM NaCl stress, WT plant seeds did not germinate where as seeds of P36,
P40.1, P40.2 germinated up to the levels of 91.66%, 91.66% and 75% respectively (Fig. 18D).

**Figure 18:** Seed germination test of transgenic lines overexpressing *PgRab7* and WT under different concentrations of NaCl. WT and transgenic lines P36, P40.1, P40.2 were under (A) 0 mM NaCl. (B) 100 mM NaCl. (C) 175 mM NaCl. (D) 200 mM NaCl.
Root and Shoot Proliferation Under Different Concentrations of NaCl Stress

To compare the root and shoot growth of WT plant and transgenic lines seeds from WT, P36, P40.1 lines were surface sterilized and put on square plates containing MS medium having 0, 100, 175 and 200 mM concentrations of NaCl. In control and 100 mM NaCl stress condition, all the three seeds of WT, P36, P40.1 germinated and grew well (Fig. 19A, B). In control conditions, transgenic line P36 showed 3.9% and P40.1 showed 29.69% longer shoot length and 0.6% and 9.89% longer root length as compared to WT plants (Fig. 19A). In 100 mM NaCl stress, transgenic lines P36 showed 1.55% and P40.1 showed 1.03% longer shoot length and 29.36% and 15.75% longer root length as compared to WT plants (Fig. 19B). In 175 mM NaCl stress condition, transgenic lines P36 showed 52.2% and P40.1 showed 73.9% longer shoot length and 6% and 16.86% longer root length as compared to WT plants (Fig. 19C). In 200 mM NaCl stress condition, all the three seeds of WT did not germinates where as transgenic lines seeds germinates and seedlings comes but growth is slower as compared to control condition. In 200 mM NaCl stress condition, transgenic lines P36 showed 236% and P40.1 showed 333% longer shoot length and 203% and 150% longer root length as compared to WT plants (Fig. 19D).
Figure 19: Root and shoot proliferation under different NaCl concentrations. WT and transgenic lines P36, P40.1 were under (A) 0 mM NaCl. (B) 100 mM NaCl. (C) 175 mM NaCl. (D) 200 mM NaCl.
Transgenic Lines Completed Life Cycle in 200 mM NaCl Stress

To check the grain productivity of different transgenic lines under stress, plants were grown under 200 mM NaCl stress (both transgenic lines and the WT plants were germinated in MS medium under control, 200 mM NaCl stress). In control condition all the transgenic lines and WT seeds germinated and grew well (Fig. 20A). However in 200 mM NaCl stress condition, WT seeds did not germinate whereas seeds of all the transgenic lines germinated and grew well (Fig. 20B). The germinated seedlings were allowed to continue their growth till tillering and seed setting stage in water and 200 mM NaCl stress condition (Fig. 20C). Morphologically, the transgenic plants in 200 mM NaCl stress showed shorter height in comparison to the transgenic lines growing in water (Fig. 20D). After completion of the life cycle the total seeds weights were compared (Fig. 32A).

Figure 20: Completed life cycle of *PgRab7* transgenic lines in 200 mM NaCl stress condition. (A) WT and transgenic lines are growing in MS medium. (B) WT growing in MS medium and transgenic lines are growing in MS medium having 200 mM NaCl. (C) WT plants are growing in soil pot and transgenic lines are growing in soil pot having 200 mM NaCl in green house. (D) Comparison of growth of WT and transgenic lines grown on water control and 200 mM NaCl stress condition.
Transgenic Lines Show Better Tolerance to 200 mM NaCl Stress

To once again confirmed the transgenic lines show better tolerance to 200 mM NaCl stress, two month old WT plants and transgenic lines were provided with 200 mM NaCl stress upto seed harvest (Fig. 21A, B). The total seeds weights were compared between water control and 200 mM NaCl stress of WT plants and transgenic lines (Fig. 32B).

Figure 21: *PgRab7* transgenic lines show better resistance to 200 mM NaCl stress. (A) WT plant and transgenic lines are growing in soil pot in greenhouse. (B) Comparison of growth of WT and transgenic lines grown on water control and 200 mM NaCl stress condition.

Comparison of Total Chlorophyll in WT and *PgRab7* Transgenic Lines in Salinity Stress Condition

Total chlorophyll content was measured in WT and different transgenic lines in water control and salinity stress condition. As shown in Fig. 22, the *PgRab7* transgenic lines had more chlorophyll than WT plants. In control condition, P24 have 30.7%, P27 have 12.27%, P36 have 12.47%, P40.1 have 15.45%, P40.2 have 41.95% more chlorophyll than WT plants. During salt stress, there was reduction of 57.86% in WT plants where as P24, P27, P36, P40.1, P40.2 reduction of chlorophyll was 6%, 5.96%, 4.23%, 2.45%, 12.22% respectively (Fig. 22).

Figure 22: *PgRab7* transgenic lines showed high quantity of total chlorophyll in salinity stress condition as compared to WT plants.
Glyoxalase I and Glyoxalase II Activity in PgRab7 Transgenic Lines

Earlier it was shown that higher Gly I and Gly II activity of glyoxalase pathway is related to stress tolerance. Gly I and Gly II activity was measured in PgRab7 over expressing lines. For enzyme assay, total protein was extracted in native condition from transgenic and WT plants grown under control and salt stress condition. The specific activity of Gly I was measured (see materials and methods) by taking equal quantity of proteins from each line. The Gly I activity of the transgenic lines was found to be 22.6% to 51.6% higher than that of the WT plant under control condition. Under 200 mM NaCl stress conditions, the transgenic line P24 shows 47.45% higher activity as compared to WT plants whereas transgenic lines P27, P36, P40.1, P40.2 showed 20.33%, 13.56%, 16.57%, 19.52% lower activity as compared to WT plants (Fig. 23).

![Glyoxalase I activity of WT and PgRab7 overexpressed transgenic lines in control and salinity stress condition. Total proteins were isolated from various transgenic lines, WT and Glyoxalase I activity was determined under control and 200 mM NaCl stress condition.](image)

The specific activity of Gly II was also measured in transgenic lines both in control and under 200 mM NaCl stress condition (see materials and methods). In Fig. 24, it was found that the Gly II activity of the transgenic line P40.1 was found to be 1.75% higher than that of the WT plants whereas transgenic lines P24, P27, P36, P40.2 showed 84.21%, 64.91%, 1.75%, 47.36% respectively decrease in activity as compared to WT plants under control. In 200 mM NaCl stress conditions, the transgenic lines P36, P40.1, P40.2 showed 126.31%, 168.42%, 21.05% higher

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activity than WT plants whereas, the transgenic lines P24 and P27 shows 63.15%, 31.57% lower activity than WT plants.

**Figure 24:** Glyoxalase II activity of WT and *PgRab7* overexpressed transgenic lines in control and salinity stress condition. Total proteins were isolated from various transgenic lines, WT and Glyoxalase II activity was determined under control and 200 mM NaCl stress condition.

**Leaf Disc Senescence Assay of Different Transgenic Lines and Measurement of Different Ions in Control and NaCl Stress**

Leaf disc senescence studies were conducted with WT and transgenic lines exposed to 200 mM NaCl stress for 120 h. It was observed that the transgenic lines retained more chlorophyll than the WT plants (Fig. 25A). Potassium (K⁺) to sodium (Na⁺) ion ratio was measured in all the transgenic lines along with the WT plants both under control and stress condition (Fig. 25B). Accumulation of Na⁺ in the leaf tissue under control and stress condition was also measured. These data indicate that in control condition, all the plants maintained a basal level of Na⁺ in the cell of leaf tissue. During 120 h salt stress (Fig. 25C), it was observed that WT plants accumulated 11.7 fold more sodium as compared to control condition. The increase in Na⁺ in transgenic lines was significantly less.
Results

Figure 25: Leaf disc senescence assay and measurement of $K^+$ to $Na^+$ ratio in control and 200 mM NaCl stress. (A) A comparative leaf disc senescence assay were made for $PgRab7$ transgenic lines and WT plants under 200 mM NaCl stress after 120 h. (B) $K^+/Na^+$ ratio were measured in control and 200 mM NaCl stress condition. (C) Histogram showing accumulation of sodium ions inside the leaf tissue under control and 200 mM NaCl stress in $PgRab7$ transgenic lines and WT plants.

Measurement of Different Ions in WT and Transgenic Lines in Control and NaCl Stress

Seeds from WT plants and transgenic lines P36 and P40.1 were germinated in MS medium having 0 mM and 200 mM NaCl. In 200 mM NaCl stress WT seeds did not germinate (Fig. 26A). Different ions were measured in 30 days WT and transgenic lines under control and 200 mM NaCl stress condition. Potassium ($K^+$) to sodium ($Na^+$) ion ratio was measured in the transgenic lines along with the WT plants both under control and stress condition (Fig. 26B). Accumulation of sodium ions in the leaf
Results

tissue under control and stress condition was also measured (Fig. 26C). These data also indicated that in control condition, all the plants maintained a basal level of sodium ion in leaf tissue.

Figure 26: \(PgRab7\) overexpressed transgenic lines and WT seed germination at control and 200 mM NaCl stress and measurement of different ions from shoot tissue after 30 days. (A) \(PgRab7\) transgenic lines and PB1 WT plant seeds germinated in control and 200 mM NaCl stress for 30 days. (B) \(K^+/Na^+\) ratio were measured in control and 200 mM NaCl stress condition. (C) Histogram showing accumulation of \(Na^+\) inside the leaf tissue under control and 200 mM NaCl stress in \(PgRab7\) transgenic lines and WT plants.

**Measurement of Different Photosynthesis Parameters of \(PgRab7\) Transgenic lines Using IRGA in NaCl Stress**

To investigate the photosynthesis efficiency of WT plants and transgenic lines under 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 (Fig. 27A-F).
Figure 27: Measurement of different photosynthesis parameters of transgenic lines overexpressing *PgRab7* and WT plants in control and salinity stress condition by IRGA (A) Rate of photosynthesis, (B) Electron transport rate (C) Photosystem II (D) Fv/Fm (E) Photochemical quenching(qP) (F) Non-photochemical quenching (qN).

From the data it was observed that in control condition transgenic lines P24, P27, P36, P40.1, P40.2 showed 0.32% to 13.4% higher photosynthesis, 12.4% to 26.16% higher ETR, 13.31% to 24.18% higher PSII, 1.31% to 3% higher Fv/Fm, 3.55% to 8.92% higher photochemical quenching and 7.33% to 12.25% lower non-photochemical quenching than the WT plants (Fig. 27A-F).

During 200 mM NaCl stress, transgenic lines P24, P27, P36, P40.1, P40.2 showed 217.59 % to 344.36% higher photosynthesis, 25.46% to 40% higher ETR, 16.23% to 36.37% higher PSII, 0.34% to 6.96% higher Fv/Fm, 14.54% to 18.67% higher photochemical quenching and 1.68% to 8.86% lower non-photochemical quenching than the WT plants (Fig. 27A-F). From the measurement of different parameters it showed that *PgRab7* transgenic lines photosynthesis efficiency is higher under both control and 200 mM NaCl stress as compared to WT plants.
**Results**

Comparison of Ultrastructure of Chloroplast of WT and *PgRab7* Transgenic Lines at 200 mM NaCl Stress

A comparative study of chloroplast structure 8000X (Fig. 28A) and 25000X (Fig. 28B) was carried out between WT and two transgenic lines P36, P40.1 at different time periods when grown under 200 mM NaCl stress.

![Figure 28: Transmission electron micrographs of chloroplast of PB1 WT and *PgRab7* transgenic plants at 200 mM salinity stress at different time intervals (A) 8000X. (B) 25000X.](image-url)
In both WT and P36, P40.1, the chloroplast structure, especially grana stacking was intact up to 24 h of stress but at 72 h, grana stacking of WT plants was completely disturbed whereas in P36, P40.1 the grana stacking was maintained intact which probably sustains active photosynthesis (Fig. 28A, B).

**Measurement of Vacuolar Volume of Rice Leaf and Root Cells in Plants Growing at 200 mM NaCl Stress**

A comparative study of ultra structure of rice leaf and root cells were carried out between WT plant and transgenic line P36 at different time periods following 200 mM NaCl stress to check the effect of salt stress on vacuolar size if any.

![Vacuolar Volume Graph](image)

**Figure 29:** Increase in the vacuolar volume of leaf (A, C) and root (B, D) cells of PB1 WT and *PgRab7* transgenic lines under 200 mM salinity stress at different time intervals.
In leaf cells, WT vacuole size increased up to 12 h followed by a decrease at 24 h and 72 h. Also at 72 h the vacuole was completely disturbed. But in P36, the vacuole size continued to increase and stayed intact up to 72 h (Fig. 29A, C). In root cells, at 12 h NaCl stress the vacuole size of WT plant was smaller in size as compared to transgenic lines P36. As the stress periods increased to 72 h, in WT the vacuole was enlarged and showed complete disruption whereas in transgenic lines P36, the vacuole size remained intact (Fig. 29B, D). From this observation it is clear that in transgenic lines vacuole stays intact in 200 mM NaCl stress.

Confocal Microscopy to Monitor Sodium Levels in the Root Tip of Transgenic Lines and WT with CoroNa Green and PI Stain Under 150 mM NaCl Stress

Seeds of PgRab7 transgenic lines and WT plants were germinated and grown in hydroponics under control conditions for 7 days in growth chamber followed by 150 mM NaCl stress for 48 h. To see the accumulation of sodium ions, CoroNa Green fluorescent dye was used which gives green coloured fluorescence. PI was also used to stain the nonviable cells. The confocal images were taken at 10X magnification at fixed focal length and constant filter intensity. Most of the cells of roots of WT plants showed high fluorescence with both the dyes indicating that higher amount of Na$^+$ accumulated inside these cells and increased cell death (Fig. 30A). However, in transgenic lines the accumulation of Na$^+$ was significantly reduced (showed less fluorescence) and they also showed deposition of only a few dead cells in the root tip region (Fig. 30B, C).
Figure 30: Confocal microscopy images showing reduced accumulation of sodium salt in root tips of *PgRab7* transgenic lines compared to WT plants stained with CoroNa Green and propidium iodide (PI) fluorescent dye after 150 mM salt stress for 48 h. (A) Root tips of WT plants stained with CoroNa Green and PI showing accumulation of more sodium inside the root after 150 mM salt stress for 48 h. (B), (C) Staining of root tips of P36 and P40.1 transgenic lines stained least with CoroNa Green and PI staining showed accumulation of negligible sodium ions inside the root.
**Results**

**DAB Staining of Leaf Tissue of *PgRab7* Transgenic Lines Under Control and Different Stress as Compared with WT Plant**

DAB staining of leaf and root tissues was carried out to see the accumulation of superoxide and hydrogen peroxide during NaCl and methyl viologen (MV) stress. Equal sized green leaf tissues were taken from 24 h of 150 mM NaCl and 12 h MV stressed seedlings along with control and stained with DAB solution for 12 h. The chlorophyll was bleached out by washing overnight with warm 96% ethanol and the images were taken at constant light intensity and at the same focal length. DAB staining developed brown colour indicating the formation of superoxide and hydrogen peroxide, hence an indication of oxidative stress.

![DAB staining images](image)

**Figure 31:** *PgRab7* transgenic plant experienced less oxidative stress compared to WT plants under different stress condition confirmed by DAB staining of leaf and root tissues. (A) DAB staining of leaf tissue of WT and *PgRab7* transgenic plants grown under control condition, 150 mM NaCl stress (24 h), methylviologen (1 µM) stress for 12 h. (B) DAB staining root of WT and *PgRab7* transgenic plants grown under control condition, 150 mM NaCl stress (24 h), methylviologen (1 µM) stress for 12 h. (C) Histogram showing mean average DAB staining image of leaves in control and under stress condition.
In control conditions, leaf tissue of transgenic lines P36 and P40.1 showed 13.28% and 12.61% less brown colour than the WT plant respectively. These results suggest that plants do get a basal level of oxidative stress in unstressed conditions (Fig. 31A).

At 24 h of NaCl stress, transgenic lines P36 and P40.1 showed 14.74% and 20.22% less brown colour with respect to WT plant respectively. In case of 12 h of MV stress, transgenic lines P36 and P40.1 showed 20.56% and 23.60% less brown colour with respect to WT plant respectively (Fig. 31C). These data suggest that during 24 h salt stress or 12 h MV stress transgenic lines P36 and P40.1 experience less oxidative stress as compared to WT plant. Similar results were obtained with root tissues as well as leaf tissues (Fig. 31B).

**Seed Production in PgRab7 Transgenic Lines Under NaCl Stress**

Total seed weight per plant data from transgenic lines after completion of life cycle in 200 mM NaCl stress showed that WT seeds did not germinate in 200 mM NaCl stress shows 100% yield penalty in salt stress as compared to their water control. Transgenic lines such as P24, P27, P36, P40.1 and P40.2 showed 67%, 52.4%, 51.89%, 50.98%, 51.15% yield penalty as compared to their respective water controls (Fig. 32A).

Total seed weight per plant data from WT and two month old transgenic lines provided with 200 mM NaCl stress showed that WT plants did not set seed in 200 mM NaCl stress, showed 100% yield penalty in salt stress as compared to their water control. Transgenic lines such as P24, P27, P36, P40.1 and P40.2 showed 53%, 41%, 33.62%, 33.3%, 35.8% yield penalty as compared to their respective water controls (Fig. 32B).

**Figure 32:** (A) Comparison of seed weight of WT and transgenic lines grown on water and 200 mM NaCl stress throughout their life cycle. (B) Comparison of seed weight of WT plants and transgenic lines grown on water and 200 mM NaCl stress, the stress was given to two month old plants.
**Response of *PgRab7* Transgenic Lines to Drought Stress**

To check if *PgRab7* overexpressing transgenic lines also show tolerance to drought stress, watering was completely withdrawn for 12 days in two months old WT plants and transgenic lines and then re-watered till seed maturation stage (Fig. 33A, B). The total seed weight per plant data were compared between water control and 12 days drought stress of WT plants and transgenic lines (Fig. 36).

![Figure 33](image)

**Figure 33:** *PgRab7* transgenic lines shows better resistance to 12 days drought stress. (A) WT plants and transgenic lines are growing in water and 12 days drought stress condition. (B) WT plants and transgenic lines are growing in water and 15 days continuous watering after 12 days drought stress condition.

**Comparison of Total Chlorophyll in WT Plants and *PgRab7* Transgenic Lines in Drought Stress**

Total chlorophyll content was measured in WT and different transgenic lines in water control and under drought stress condition. As shown in Fig. 34, the *PgRab7* transgenic lines had more chlorophyll than WT plants. In control condition, transgenic lines such as P24, P27, P36, P40.1 and P40.2 have 30.7%, 12.27%, 12.47%, 15.45%, 41.95% respectively more chlorophyll than WT plants. During drought stress, there was reduction of 53.21% in WT plants; P24, P27 showed reduction of 0.37%, 1.8%
chlorophyll where as P36, P40.1, P40.2 recoded 28.89%, 33.52% and 2.39% more chlorophyll respectively (Fig. 34).

**Figure 34:** *PgRab7* transgenic lines shows high quantity of total chlorophyll in drought stress condition as compared to WT plants.

**Measurement of Different Photosynthesis Parameters of *PgRab7* Transgenic Lines by IRGA During Drought Stress**

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 (Fig. 35A-F).

From the data it was observed that in control condition, transgenic lines P24, P27, P36, P40.1, P40.2 showed 0.32% to 13.4% higher photosynthesis, 12.4% to 26.16% higher ETR, 13.31% to 24.18% higher PSII, 1.31% to 3.04% higher Fv/Fm, 3.55% to 8.92% higher photochemical quenching and 7.33% to 12.25% lower non-photochemical quenching than the WT plants (Fig. 35A-F).

During drought stress transgenic lines P24, P27, P36, P40.1, P40.2 showed 123.57% to 184.1% higher photosynthesis, 53.8% to 84.58% higher ETR, 23.79% to 45.37% higher PSII, 3.76% to 7% higher Fv/Fm, 21.41% to 30.56% higher photochemical quenching and 7.77% to 23.23% lower non-photochemical quenching than the WT plants (Fig. 35A-F). From the measurement of different parameters it follows that in *PgRab7* transgenic lines photosynthetic efficiency is much higher in both control and drought stress as compared to WT plants.
Results

Figure 35: Measurement of different photosynthesis parameters of transgenic plants over-expressing *PgRab7* and WT in control and drought stress condition by IRGA (A) Rate of photosynthesis. (B) Electron transport rate. (C) Photosystem II. (D) Fv/Fm. (E) Photochemical quenching (qP). (F) Non-photochemical quenching (qN).

*PgRab7* Transgenic Lines Show Better Yield in Drought Stress

WT plants showed 82.5% yield penalty in terms of seed weight per plant in drought stress as compared to plants which were irrigated throughout. Transgenic lines P24 showed 3.4% yield penalty whereas P27, P36, P40.1, P40.2 showed 2.9%, 30.6%, 3.4%, 2.7% higher yield as compared to their respective water controls (Fig. 36).

Figure 36: Comparison of seed weight of WT plants and transgenic lines grown on water control and 12 days of drought stress.
Effect of Sortin1 and Brefeldin in Morphology of WT and Transgenic Plants

The WT plants and transgenic lines P24, P27, P36, P40.1, P40.2 were germinated hydroponically in Yasoda medium for 14 days and then treated with Sortin1 (227 µM) and Brefeldin (20 µg/ml) for 48 h in darkness. After 48 h it was observed that both WT plants and all five transgenic lines started dying in both Sortin 1 and Brefeldin (Fig. 37A, B).

Figure 37: A comparative survival test of two week old transgenic lines overexpressing PgRab7 and WT under control, (A) Sortin1 (227 µM) and (B) Brefeldin (20 µg/ml) for 48 h.

Transcriptome of PB1 WT and PgRab7 Transgenic Lines in Response to Salinity and Drought Stress

To study the transcriptome level changes in both the WT and PgRab7 transgenic lines under different experimental condition, microarray experiment were 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 stress condition, were chosen for microarray data analysis. The microarray data were analyzed 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.
The number of genes up- and down-regulated in different combinations are summarized in Table 7. The details of three comparisons are presented in Annexure-I.

**Table 7**: Differentially expressed genes in various comparisons at FDR of 0.05

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<th>S.No</th>
<th>Comparison</th>
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<th>Fold change</th>
<th>Up-regulated gene</th>
<th>Down-regulated gene</th>
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<td>WT_NaCl vs. WT_CONT</td>
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<tr>
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<td>903</td>
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<td>2.56</td>
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<td>209</td>
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<td>3.1</td>
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<td>1053</td>
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<td>2.9</td>
<td>394</td>
<td>308</td>
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</table>

The Venn diagram representation of the microarray results suggest that 55 genes are up-regulated between T_DROU vs. T_CONT and WT_DROU vs. WT_CONT. Between WT_DROU vs. WT_CONT and T_CONT vs. WT_CONT 603 genes are up-regulated. Between T_CONT vs. WT_CONT and T_DROU vs. WT_DROU 53 genes are up-regulated. Between WT_DROU vs. WT_CONT and T_CONT vs. WT_CONT and T_DROU vs. WT_DROU 11 genes are up-regulated. Between T_DROU vs. T_CONT and WT_DROU vs. WT_DROU vs. WT_DROU 5 genes are up-regulated. Between T_DROU vs. T_CONT and WT_DROU vs. WT_DROU vs. WT_DROU vs. WT_DROU 2 genes are up-regulated. No gene was up-regulated between T_DROU vs. T_CONT and T_CONT vs. WT_CONT and between T_DROU vs. T_CONT and WT_DROU vs. WT_DROU vs. WT_DROU and T_CONT vs. WT_CONT (Fig. 38A).

Between T_DROU vs. T_CONT and WT_DROU vs. WT_CONT 10 genes are down-regulated. Between WT_DROU vs. WT_CONT and T_CONT vs. WT_CONT 792 genes are down-regulated. Between T_CONT vs. WT_CONT and T_DROU vs. WT_DROU 217 genes are down-regulated. Between T_DROU vs. T_CONT and WT_DROU vs. WT_CONT and T_CONT vs. WT_CONT 3 genes are down-regulated. Between WT_DROU vs. WT_CONT and T_CONT vs. WT_CONT and
Results

T_DROU vs. WT_DROU 20 genes are down-regulated. Between T_DROU vs. T_CONT and WT_DROU vs. WT_CONT and T_DROU vs. WT_DROU 4 genes are down-regulated. No gene was down-regulated between T_DROU vs. T_CONT and T_CONT vs. WT_CONT and between T_DROU vs. T_CONT and WT_DROU vs. WT_CONT and T_DROU vs. WT_DROU (Fig. 38B).

Similarly, 537 genes are up-regulated between WT_NaCl vs. WT_CONT and T_CONT vs. WT_CONT. Between T_CONT vs. WT_CONT and T_NaCl vs. WT_NaCl 64 genes are up-regulated. Between WT_NaCl vs. WT_CONT and T_CONT vs. WT_CONT and T_NaCl vs. WT_NaCl 32 genes are up-regulated. No genes were up-regulated between WT_NaCl vs. WT_CONT and T_NaCl vs. WT_NaCl, between T_NaCl vs. WT_NaCl and T_NaCl vs. T_CONT, between T_CONT vs. WT_CONT and T_NaCl vs. WT_NaCl and T_NaCl vs. T_CONT, between WT_NaCl vs. WT_CONT and T_CONT vs. WT_CONT and T_NaCl vs. T_NaCl vs.

Figure 38: Venn diagrams showing the classification of genes inducible by drought, and salinity stresses on the basis of microarray. (A) Intersection of genes that were up-regulated by WT control, transgenic control and WT drought stress, transgenic drought stress. (B) Intersection of genes that were down-regulated by WT control, transgenic control and WT drought stress, transgenic drought stress. (C) Intersection of genes that were up-regulated by WT control, transgenic control and WT salinity stress, transgenic salinity stress. (D) Intersection of genes that were down-regulated by WT control, transgenic control and WT salinity stress, transgenic salinity stress.
Results

Between WT NaCl vs. WT CONT and T CONT vs. WT CONT and T NaCl vs. WT NaCl and T NaCl vs. T CONT (Fig. 38C).

Between WT NaCl vs. WT CONT and T CONT vs. WT CONT 476 genes are down-regulated. Between T CONT vs. WT CONT and T NaCl vs. WT NaCl 321 genes are down-regulated. Only 1 gene is down-regulated between T NaCl vs. WT NaCl and T NaCl vs. T CONT. Between WT NaCl vs. WT CONT and T CONT vs. WT CONT and T NaCl vs. WT NaCl 89 genes are down-regulated. No genes were down-regulated between WT NaCl vs. WT CONT and T NaCl vs. WT NaCl, between T CONT vs. WT CONT and T NaCl vs. WT NaCl and T NaCl vs. T CONT, between WT NaCl vs. WT CONT and T CONT vs. WT CONT and T NaCl vs. WT NaCl and T NaCl vs. T CONT and between WT NaCl vs. WT CONT and T CONT vs. WT CONT and T NaCl vs. WT NaCl (Fig. 38D).

Data as shown in the Venn diagrams of microarray results, suggest that 4 genes are up-regulated in T NaCl vs. T CONT. Between T DROU vs. T CONT 361 genes are up-regulated. Between T DROU vs. T NaCl 431 genes are up-regulated. Whereas 2 genes are up-regulated in T NaCl vs. T CONT and T DROU vs. T CONT. Between T DROU vs. T CONT and T DROU vs. T NaCl 664 genes are up-regulated. Also 1 gene is up-regulated between T NaCl vs. T CONT, T DROU vs. T CONT and T DROU vs. T NaCl. No gene was up-regulated in T NaCl vs. T CONT and T DROU vs. T NaCl (Fig. 39A).

Between T NaCl vs. T CONT, 10 genes are down-regulated. Between T DROU vs. T CONT 49 genes are down-regulated. Between T DROU vs. T NaCl 185 genes are down-regulated. Whereas 2 genes are down-regulated in T NaCl vs. T CONT and T DROU vs. T CONT. Between T DROU vs. T CONT and T DROU vs. T NaCl 357 genes are down-regulated. No gene was down-regulated between T NaCl vs. T CONT and T DROU vs. T NaCl and between T NaCl vs. T CONT, T DROU vs. T CONT and T DROU vs. T NaCl (Fig. 39B).

Similarly, 251 genes are up-regulated between WT NaCl vs. WT CONT. Between WT DROU vs. WT CONT 564 genes are up-regulated. Between WT DROU vs. WT NaCl 129 genes are up-regulated. Between WT NaCl vs. WT CONT and
WT_DROU vs. WT_CONT 580 genes are up-regulated. Between WT_DROU vs. WT_CONT and WT_DROU vs. WT_NaCl 300 genes are up-regulated. Between WT_NaCl vs. WT_CONT and WT_DROU vs. WT_CONT and WT_DROU vs. WT_NaCl 121 genes are up-regulated. No gene was up-regulated in WT_DROU vs. WT_NaCl and WT_NaCl vs. WT_CONT (Fig. 39C).

Between WT_NaCl vs. WT_CONT 180 genes are down-regulated. Between WT_DROU vs. WT_CONT 746 genes are down-regulated. Between WT_DROU vs. WT_NaCl 161 genes are down-regulated. Between WT_NaCl vs. WT_CONT and WT_DROU vs. WT_CONT 600 genes are down-regulated. Between WT_DROU vs. WT_CONT and WT_DROU vs. WT_NaCl 133 genes are down-regulated. Between WT_NaCl vs. WT_CONT and WT_DROU vs. WT_CONT and WT_DROU vs. WT_NaCl 51 genes are down-regulated. No gene was down-regulated between WT_DROU vs. WT_NaCl and WT_NaCl vs. WT_CONT (Fig. 39D).

**Figure 39:** Venn diagrams showing the classification of genes inducible by drought, and salinity stresses on the basis of microarray. (A) Intersection of genes that were up regulated by transgenic control, transgenic salinity and transgenic drought stress. (B) Intersection of genes that were down-regulated by transgenic control, transgenic salinity and transgenic drought stress. (C) Intersection of genes that were down-regulated by WT control, WT salinity and WT drought stress. (D) Intersection of genes that were down-regulated by WT control, WT salinity and WT drought stress.
The scatter plots (Fig. 40A-I) also suggested that the genes are more disturbed in WT plants facing salinity and drought stress in comparison to transgenic lines. So in scatter plot, the genes are scattered more in both above and below the axis. The spots above and below the red lines indicate up- and down-regulated probe sets at the specified fold change. In Fig. 40A, C, G, the signal intensity is scattered more above and below the axis. This indicates more genes are up- and down-regulated. In Fig. 40B, D the signal intensity is scattered more above the axis. This indicates that more genes are up- than down-regulated. In Fig. 40E, H, I, the signal intensity is scattered more below the axis, indicating more genes to be down-regulated than up-regulated. In Fig. 40F, the signal intensity is scattered very less above and below the axis. This indicates very less number of genes are either up- or down-regulated.

Figure 40: Scatter plots showing the differential expression of genes with P-value < 0.05, FDR = 0.05 and fold change >2 (blue dots) in transgenic plant overexpressing *PgRab7* as compared to WT plant under control, salinity and drought stress condition (A) Transgenic control Vs WT control. (B) WT salinity stress Vs WT control. (C) WT drought stress Vs WT control. (D) WT drought stress Vs WT salinity stress. (E) Transgenic salinity stress Vs WT salinity stress. (F) Transgenic salinity stress Vs Transgenic control. (G) Transgenic drought stress Vs WT drought stress. (H) Transgenic drought stress Vs Transgenic control (I) Transgenic drought stress Vs Transgenic salinity stress.
Results

*Agrobacterium Mediated Transformation of Rice (Oryza sativa cv IR64) with PgRab7 for Development of Transgenic Plants*

To further test the abiotic stress tolerance of *PgRab7* in IR64 background and later use the transgenic lines for commercial purpose, IR64 transgenic plants overexpressing *PgRab7* were generated. For *Agrobacterium* transformation, the embryogenic calli were developed from mature dehusked seeds in MS callus induction medium containing 2, 4-D (2.5 mg/l) and BAP (0.2 mg/l) in the presence of casein hydrolysate and L-proline, kept in darkness for three to four weeks. The callus induction frequency was found to be very high i.e. 95%. Embryogenic calli were subcultured into 2-3 small pieces and kept on the same callus induction medium for another period of 4-5 days in darkness. The calli were infected with *Agrobacterium* and selected using hygromycin antibiotic (50 mg/l). After first selection, most of the cream coloured calli were transformed to the second selection where the brownish or black coloured calli were discarded. After the second selection, minute outgrowth were seen coming from the selected calli and these microcalli were then transferred to the first regeneration medium and kept in darkness. During this phase, somatic embryos were seen developing from the globular round calli. During the second phase of regeneration, the microcalli growing on first regeneration medium under darkness were shifted directly to light for 5 days without any change of medium. In the second phase, new shoots came out from the somatic embryos within two weeks.

![Figure 41: Agrobacterium-mediated transformation of pCAMBIA-PgRab7 construct in IR64 calli. (A-I) various steps of transformation and regeneration of IR64 rice. (A) IR64 seeds on callus induction medium. (B) Callus induction. (C) Callus subculture (D) Calli co-cultivated with Agrobacterium. (E) Calli in selection medium. (F) Microcalli regenerated. (G) Regenerated explants are on rooting medium. (H) Putative transgenic line in vermiculite. (I) Putative transgenic line in soil.](image-url)
The regenerated shoots were transferred to rooting medium having hygromycin (40 mg/l). The average transformation efficiency of IR64 was found to be approximately 5% using LBA4404 Agrobacterium strain. When roots developed properly under hygromycin selection, the putative transgenic lines were shifted to vermiculite and subsequently to soil pot having garden soil for hardening (Fig. 41A-I).

**Confirmation of Putative Transgenic Lines by PCR Analysis**

To confirm the gene integration, putative transgenic lines (T₀ plants) were confirmed by PCR (Fig. 42A). For PCR confirmation, three pairs of primers were used. The first pair is PgRab7 gene specific. The forward primer was designed from the junction of 35SCaMV promoter and 5' end PgRab7 gene and reverse from the 3' end of PgRab7 gene. This primer set gave ~ 700 bp amplification product (Fig. 42B). For the second pair, forward primer was designed from the 5' end of 35SCaMV promoter and the reverse primer was designed from 3' end of PgRab7 gene. This primer set gave ~ 1.1kb amplification product (Fig. 42C). The third pair consisted of hptII forward and reverse primers. This primer set yielded 1 kb amplification product (Fig. 42D). All the transgenic lines showed single product of expected size in all the three cases and no such band was observed in the WT plants. The PCR positive plants were subsequently transferred to vermiculite pots for hardening and finally transferred to soils in the earthen pot.

**Figure 42:** Confirmation of putative IR 64 transgenic plants by PCR. PCR confirmation of PgRab7 putative transgenic plants by using (A) Rab7 forward and reverse primers. (B) 35S forward and Rab7 reverse primers. (C) hptII forward and reverse primers. (D) PCR confirmed IR64 transgenic plants.
Leaf Disc Senescence Assay of *PgRab7* Transgenic Rice Under Different Concentration of NaCl Stress

A comparative leaf disc senescence study was carried out between WT plant and *PgRab7* transgenic line. Equal sized (1.2 cm) segments of leaves were floated on water and 100, 200, 400 mM NaCl solutions and kept in light under green house condition for 72 h. It was observed that under control conditions, leaf discs of *PgRab7* transgenic line were equally green like WT plant. Under salt stress, leaf discs of WT plant completely bleached and turned yellow while in case of transgenic line, the leaf discs remained green (Fig. 43A).

![Leaf Disc Senescence Assay](image)

**Figure 43:** Determination of salt tolerance of transgenic plants overexpressing *PgRab7* by leaf disc senescence assay. (A) Leaf discs of WT and transgenic plants IR1 of *PgRab7* were subjected to 100, 200, 400 mM NaCl treatments as indicated. As control, leaves were treated in water. (B) Total Chlorophyll estimation of the leaf discs 72 h following treatment.

In control condition, the total chlorophyll content in the WT plant was estimated to be 0.8 mg/g of tissue where as in transgenic line IR1 the value was 0.97 mg/g of tissue. In 100 mM NaCl stress, the total chlorophyll content in the WT plant reduced to 0.35 mg/g of tissue where as in transgenic line IR1 the value was 0.9 mg/g of tissue. In 200
mM NaCl stress, the total chlorophyll content in the WT plant reduced to 0.093 mg/g of tissue where as in transgenic plant IR1 the value was 0.81 mg/g of tissue. In 400 mM NaCl stress, the total chlorophyll content in the WT plant reduced to 0.073 mg/g of tissue where as in transgenic line IR1 the value was 0.48 mg/g of tissue. A histogram of the above data shows that the transgenic lines retained more chlorophyll as compared to WT plant under stress condition (Fig. 43B). This indicated that overexpression of *PgRab7* gene provides salt stress tolerance to the transgenic rice plants.