CHAPTER 3

Materials & Methods
3.1. Plant material

For present investigation seeds of different varieties of rice were collected. The certified and disease free seeds of *O. sativa* L. (var. Pusa Basmati-1 cv. Indica) were procured from Indian Agricultural Research Institute (IARI), New Delhi, India. For a part of experiment, seeds of rice variety IR-64 were obtained from Punjab Agriculture University (PAU), Ludhiana, Punjab, India and Luna Suvarna (CR Dhan 403) and Luna Sankhi (CR Dhan 405) were collected from Central Rice Research Institute (CRRI), Cuttack, India.

*Salient features of different varieties*

- Pusa Basmati-1: Pusa Basmati-1 is a salt sensitive, dwarf, photoinsensitive, input responsive high yielding variety and enjoys distinctive status in global rice economy due to its special taste, aroma and flavour. The average yield was recorded to be 2.77 t ha\(^{-1}\) (George *et al.*, 2005).

- IR-64: IR-64 a salt-sensitive, semi-dwarf, high yielding rice variety with premium grain quality. It is the most widely grown indica rice cultivar in Southeast Asia. The average yield was recorded to be 6.40 t ha\(^{-1}\) (Makarim and Ikhwani, 2010).

- Luna suvarna: Luna Suvarna (CR Dhan 403) was developed at Central Rice Research Institute, Cuttack, India from a cross between Mahsuri/ Ormundakan. It was found promising through All India Coordinated Rice Improvement Programme, participatory varietal selection (PVS) and on-farm trials. The average yield was recorded to be 4.59 t ha\(^{-1}\). This variety can be grown in coastal belts with salinity stress tolerance in the range of 6-8 dSm\(^{-1}\).

- Luna Sankhi: Luna Sankhi (CR Dhan 405) was a breeding line of IR72046-B-R-3-3-3-1, cross between IR31142-14-1-1-3-2 / IR71350 from International Rice Research Institute. It was found promising through All India Coordinated Rice Improvement Programme, participatory varietal selection (PVS) and on-farm trials. The average yield was recorded to be 4.5 t/ha. This variety can be grown in coastal belts with salinity stress tolerance in the range of 6-8 dSm\(^{-1}\). 

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3.2. Chemicals, glassware/plasticware and washing procedure

The chemicals used during the study were procured from Sigma-Aldrich, Bio-Rad, New England Biolabs, Hi-Media, SRL, Merck, S.D. Fine or Qualigens brand (AR grade) unless otherwise specified. All glassware and plasticware were obtained from Borosil works Ltd., Mumbai and Tarson Ltd. India, respectively unless otherwise specified. In the present experiments, 28-Homobrassinolide (HBL; 22R, 23R, 24S)-2a, 3a, 22,23-tetrahydroxy-24-ethyl-ß-homo-7-oxo-5a-cholesterane-6-one] and 24-Epibrassinolide (EBL; 22R, 23R, 24R)-2a, 3a, 22,23-tetrahydroxy-24-methyl-ß-homo-7-oxo-5a-cholesterane-6-one] were procured from Sigma-Aldrich, USA. Two pesticides; Chlorpyrifos (CPF; O,O-diethyl-0-(3,5,6-trichlor-2-pyridyl) phosphorothioate; 20% active ingredient,) and Imidacloprid (IMI; 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine; Imidacloprid 20% active ingredient) were procured from Bayer Cropscience Ltd., India. Sodium Chloride (NaCl) (AR Grade, Hi Media Laboratories Pvt Ltd., India) was used as a source of salinity stress.

Laboratory glasswares were cleaned with neutral liquid detergent (Labolene) using bottle brushes. Subsequently, these were rinsed thoroughly with tap water and then rinsed twice with distilled water. Glassware washing was also sometimes performed by immersing them in chromic acid solution followed by vigorous tap water rinse as well as a final rinse with distilled water. Cleaned glassware was dried in oven at 150°C before use.

3.3. Seed sterilisation, treatments and inoculation

Healthy rice seeds were dehusked and surface sterilized with 70% ethanol (v/v) for 1 min, followed by 30 min in 0.4% sodium hypochlorite containing a drop of tween-20 with shaking at 180 rpm. Seeds were then washed 8-10 times with sterile distilled water and dried on autoclaved Whatman paper (3 mm) for 5 min. After surface sterilisation, seeds were soaked for 8h in distilled water (control) and in different concentrations of HBL/EBL (10^{-11}, 10^{-9} and 10^{-7} M). The stock solution (10^{-3} M) of HBL/EBL was prepared by dissolving BRs in ethanol (HPLC grade) and was stored at -20°C. The working concentrations of HBL/EBL (10^{-11} M, 10^{-9} and 10^{-7} M) were prepared by serially diluting the stock solution of BRs (10^{-7} M) with double distilled
water. Different concentrations for treatment of pesticides and salt were chosen based on their IC$_{50}$ values (the concentration of pesticide or salt at which growth of the rice seedlings is inhibited to 50%). It was determined on the basis of rate of germination and seedling growth under a range of concentrations of pesticides or salt (Liu et al., 2013). The IC$_{50}$ value for CPF, IMI, and salt was determined to be 0.04%, 0.015%, and 100 mM NaCl, respectively (Fig.3.1). In the present experiment, for treatments, three concentrations (IC$_{50}$ value, one concentration below and one above IC$_{50}$ value) were selected for each stress. After sterilization, seeds were sown in autoclaved sand moistened with different concentrations of IMI (0.01%, 0.015% and 0.02%), CPF (0.02%, 0.04% and 0.06%) and NaCl (75 mM, 100 mM and 125 mM). The above mentioned concentrations of pesticides and salt were applied alone as well as in binary combinations with BRs to rice seeds. In each box plastic box (dimensions 26 cm x 17.5 cm) 100 seeds were inoculated in and kept under controlled conditions; 25°C (day/night), 70-80% RH (day/night) and 14 h photoperiod for 12 days. The boxes were closed with the lid and hence the moisture content was maintained throughout the 12 days period of growth.

Fig. 3.1. Determination of IC$_{50}$ value for CPF, IMI and NaCl for rice var. Pusa Basmati-1.
Chapter 3: Materials and Methods

3.4. Harvesting of samples

Seedlings were harvested after 12 days (Fig. 3.2). They were removed from the boxes and were dipped in water to remove adhering sand particles. Representative lot of 15 seedlings were chosen for study of morphological parameters while remaining seedlings were flash frozen in liquid nitrogen and then stored at -80°C for further analysis.

![Fig. 3.2. 12-day old rice seedlings treated with HBL and growing in increasing concentration of CPF.](image)

3.5. Study of morphological parameters

For morphological parameter analysis, root and shoot length were measured using meter scale and observations for fresh weight of seedlings were made. Root number for each of the seedlings was recorded. The seedlings were then placed in an oven at 70°C till a constant weight is achieved and then observations for dry weight were recorded. Experiment was repeated thrice with three biological replicates.
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3.6. Study of biochemical parameters

Study of various biochemical parameters like chlorophyll contents (Chl a and Chl b and total Chl), total soluble proteins, free proline, malondialdehyde (MDA) content was performed on 12 day old seedlings. The activity of various antioxidant enzymes was also analyzed.

3.6.1. Chlorophyll content

Chlorophyll content was estimated following Arnon (1949) method with some modifications. 100 mg of fresh leaves from each of the sample were homogenized in liquid nitrogen. 1.5 ml of 80% acetone was added to it and the reaction was incubated in dark for 1 h at 25°C. The mixture was centrifuged at 14000g for 3 min and absorbance was recorded at 645 and 663 nm against 80% acetone which served as blank. The chlorophyll content was determined as follows

\[
\text{Total Chl (mg g FW}^{-1}) = 20.2 (A_{645}) + 8.02 (A_{663}) \times (\text{volume/1000}) \times \text{weight of tissue}
\]

\[
\text{Chl a (mg g FW}^{-1}) = 12.7 (A_{663}) - 2.29 (A_{645}) \times (\text{volume/1000}) \times \text{weight of tissue}
\]

\[
\text{Chl b (mg g FW}^{-1}) = 22.9 (A_{645}) - 4.68 (A_{645}) \times (\text{volume/1000}) \times \text{weight of tissue}
\]

3.6.2. Protein content

12-day old seedlings were harvested, frozen in liquid nitrogen and stored at -80°C. They were homogenized in ice chilled 50 mM phosphate buffer (pH-7.8) containing 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethane sulfonylfluoride (PMSF), 0.5% (v/v) triton X-100 and 10% (w/v) PVP-40. The mixture was centrifuged at 12000g for 20 min at 4°C, supernatant was collected and used for enzymes assay and protein content estimation. The extraction procedure was carried out at 0-4°C. Protein concentrations of samples were determined by bradford assay (Bradford, 1976) using protein estimation kit (Bradford macro method, Genei, Bangalore, India) and bovine serum albumin (BSA) as a standard. Standard curve was plotted between different known concentrations of BSA and their respective absorbance. The amount of protein in the samples was calculated from the standard linear equation and was expressed as mg g \^{-1}FW.
3.6.3. Proline content

Proline content in the seedlings was determined by following the method of Bates et al. (1973). Sample (0.5 g) homogenized in liquid nitrogen was extracted with 10 ml of 3% sulphosalicylic acid and centrifuged at 12000g for 15 min. In the extract, an equal volume of glacial acetic acid and acid ninhydrin solutions were added. Mixture was heated at boiling water bath for 1h and reaction was terminated on ice. Followed by addition of 4 ml of toluene and the absorbance of the toluene layer, when separated from the aqueous layer, was measured spectrophotometrically at 520 nm. Amount of proline was calculated from the standard curve and expressed as μmoles g⁻¹FW.

3.6.4. Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) assay, which determines MDA as an end-product of lipid peroxidation, was used to measure MDA content (Hodges et al., 1999). Seedlings (1g) were homogenized in 3 ml of 0.1% TCA kept at 4°C and then 3 ml of solution containing 0.5% Thiobarbituric acid (TBA) and 20% Trichloroacetic acid (TCA) was added. The mixture was incubated at 95°C for 30 min and then placed in ice to stop the reaction. The samples were centrifuged at 10000g for 15 min and the absorbance of the supernatant was measured at 532 nm, with a reading at 600 nm subtracted from it to account for nonspecific absorption. The MDA-TBA complex was quantified using the extinction coefficient as 155 mM⁻¹ cm⁻¹.

3.7. Histological determination of reactive oxygen species (ROS)

Superoxide radicals (O₂⁻) were visually detected in rice leaves according to method suggested by Wu et al. (2010). Plants were excised at the base of stems with a razor blade and supplied with 10 mM Na-citrate buffer (pH 6.0) containing 6 mM NBT for 8 h under light at 25°C. As the solution entered through the cut stem, pale yellow NBT reacted with superoxide radicals and formed a dark blue insoluble formazan compound. Superoxide radicals are thought to be the major oxidant species responsible for reducing NBT to formazan. The leaves were then decolorized by immersing them in boiling ethanol (95%) for 10 min to remove the green background of leaves except for the dark blue insoluble formazan deposits produced by the reaction of NBT with O₂⁻. After cooling, leaves were observed using stereomicroscope and photographed.
3.8. Antioxidant enzyme assay

3.8.1. Preparation of plant extracts for antioxidant enzyme analysis

Plant extract for enzymes assay was prepared in the same manner as explained above for the protein extraction. It was used for the estimation of enzyme activity for all the enzymes except monodehydroascorbate reductase (MDHAR). For MDHAR, 1 g seedlings were homogenized in 3 ml of 50 mM Tris-HCl buffer (pH-7.6) containing 2.5 mM ascorbic acid. The homogenates were centrifuged at 12000g for 20 min at 4°C. The supernatant was used for analysis of specific activity of MDHAR.

3.8.2. Superoxide dismutase, SOD (EC 1.15.1.1)

SOD activity was determined by measuring the ability of enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), as described by Beauchamp and Fridovich (1971). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 2 μM riboflavin, 75 μM NBT, 13 mM DL methionine, 100 μM EDTA and enzyme extract (50 μl). The reaction was initiated by illuminating the reaction mixture for 20 min at 25°C and absorbance was read at 560 nm. Identical tubes which were not illuminated served as blank. The positive control contained 50 μl of 50 mM phosphate buffer (pH 7.8) in place of the enzyme extract. One unit of activity was determined as amount of enzyme required to inhibit the photoreduction of NBT to blue formazan by 50% and was expressed as SOD unit activity mg protein⁻¹.

3.8.3. Ascorbate peroxidase, APX (EC 1.11.1.11)

Ascorbate peroxidase activity was determined by following the rate of oxidation of ascorbate (ε of ascorbate = 2.8 mM⁻¹ cm⁻¹) leading to decrease in absorbance at 290
nm which is observed spectrophotometrically at 25°C (Nakano and Asada, 1981). The reaction mixture (1 ml) comprised of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.5 mM ascorbate and 0.1 mM H$_2$O$_2$. The reaction was started by addition of 10 μl of the enzyme extract in a quartz cuvette. One unit of enzyme activity was calculated as the amount of enzymes required to oxidise 1 μmole of ascorbate min$^{-1}$mg protein$^{-1}$.

3.8.4. Catalase, CAT (EC 1.11.1.6)

Catalase activity was measured at 25°C by following the method of Aebi (1984). The reaction mixture contained 20 μl of enzyme extract, 10 mM H$_2$O$_2$ in 50 mM phosphate buffer (pH-7). CAT activity was estimated by following the decrease in absorbance of H$_2$O$_2$ at 240 nm and was expressed as μmole of H$_2$O$_2$ decomposed min$^{-1}$ mg protein$^{-1}$ using the extinction coefficient 39.4 mM$^{-1}$cm$^{-1}$.

3.8.5. Glutathione reductase, GR (EC 1.6.4.2)

Activity of glutathione reductase at 25°C was assayed as per the method of Jahnke et al. (1991) by following the decrease in absorbance at 340 nm due to the oxidation of NADPH. The 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 1 mM EDTA, 1mM oxidised glutathione (GSSG) and 25 μl of enzyme sample with 0.1 mM NADPH (ε of NADPH= 6.22 mM$^{-1}$cm$^{-1}$) which was added in the last to initiate the reaction. Enzyme activity was expressed as μmol of NADPH oxidized min$^{-1}$mg protein$^{-1}$.

3.8.6. Guaiacol peroxidase, GPX (EC 1.11.1.7)

Activity of Guaiacol peroxidase was determined spectrophotometrically at 25°C by following the increase in absorbance due to oxidation of guaiacol (Fernañá Ndez-Garcíaã et al., 2004). The reaction mixture comprised of 50 mM phosphate buffer (pH-7), 9 mM guaiacol, 10 mM H$_2$O$_2$ and 33 μl of enzyme extract in a total reaction volume of 1 ml. Enzyme activity was expressed as the amount of enzyme required to produce 1 μmol of guaiacol dehydrogenation product (GDHP) min$^{-1}$mg protein$^{-1}$ (ε= 26.6 mM$^{-1}$cm$^{-1}$).
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3.8.7. Dehydroascorbate reductase, DHAR (EC1.8.5.1)

Activity of DHAR was determined at 25°C according to the method of Nakano and Asada (1981) by measuring the increase in absorbance at 265 nm (ε=14mM⁻¹cm⁻¹) due to formation of ascorbate from dehydroascorbate using the reducing power provided by GSH. Reaction mixture (1 ml) contained 50 mM phosphate buffer (pH=7), 0.1 mM EDTA, 0.5 mM dehydroascorbate, 2.5 mM GSH and 25 µl enzyme extract in a total reaction volume of 1 ml. One unit of enzyme activity was calculated as the amount of enzymes required to produce 1 µmol of ascorbate min⁻¹mg protein⁻¹.

3.8.8. Monodehydroascorbate reductase, MDHAR (EC 1.6.5.4)

Enzyme activity was determined at 25°C according to Hossain and Asada (1985) by measuring the decrease in absorbance due to consumption of NADPH at 340nm (ε= 6.2 mM⁻¹cm⁻¹). The reaction mixture contained 50 mM Tris-HCl Buffer (pH-7.6), 0.15 units ascorbate oxidase enzymes, 2.5 mM ascorbic acid and 0.2 mM NADPH/NADH making a total volume of 1 ml. One unit of enzyme activity is described as the amount of enzyme required to oxidise 1 µmol of NADPH min⁻¹mg protein⁻¹.

3.9. Statistical analysis

All data obtained were subjected to two-way analysis of variance (ANOVA) for studying the interaction of stress (CPF, IMI and NaCl) with EBL/HBL and expressed as mean±SE of three independent replicates. The Fisher’s LSD test was applied for multiple comparisons using Sigmasstat version 3.5 and significance of difference between stress and BRs treatment was set at p≤0.05.

3.10. Expression analysis of genes

Semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (semi-qRT-PCR) was performed to study the expression profile of selected genes in response to various treatments. For the expression analysis of genes, the details of RNA isolation and cDNA preparation and amplification are described below.

3.10.1. RNA Isolation

Before RNA isolation, for inactivation of contaminating RNAases, all glasswares and plasticware were dipped in autoclaved DEPC-treated water for
overnight at 37°C, followed by autoclaving at 15 psi for 30 min. Gel running apparatus was cleaned with 0.5% SDS (Sodium dodecyl sulfate) solution, washed with DEPC-treated autoclaved water, treated with 3% H₂O₂ for 1 h and finally rinsed thoroughly with autoclaved DEPC treated water. All solutions are prepared in autoclaved DEPC-treated water. The total RNA was isolated from whole seedlings as well as from different parts of the plants using Trizol® reagents (Invitrogen, USA) as described below.

**Protocol for RNA isolation**

**Homogenization**
1. 100 mg of rice seedlings were homogenized in liquid nitrogen.
2. 1 ml of Trizol reagent was added to it followed by vortex for 30 sec.
3. The reaction was incubated in slow agitation at room temperature (25°C) for 10min.

**Phase separation**
4. The mixture was centrifuged at 4°C for 10 min at 12000g.
5. Supernatant was collected and 200 µl of chloroform was added to it.
6. Tubes were vortexed vigorously for 15 sec followed by incubation for 7 min at room temperature.
7. The mixture was then centrifuged at 4°C for 15 min at 12000g.

**RNA Precipitation**
8. After centrifugation, aqueous phase containing RNA was transferred to a new tube.
9. 0.5 vol of isopropanol and 0.5 vol of sodium citrate + NaCl solution were added to the aqueous phase containing RNA.
10. Reaction mixture was gently vortexed and incubated at 4°C for 15min.
11. The mixture was then centrifuged at 4°C for 10 min at 12000g.

**RNA Wash**
12. The supernatant was discarded and pellet was washed with 1 ml of chilled 70% ethanol, centrifuged for 5 min at 8000g at 4°C.
13. The supernatant was discarded and the pellet was dried in the laminar air flow for 10 min.
14. Pellet was dissolved in 20 µl of RNAase free H₂O was added and incubated for 15 min on ice.
15. Residual DNA was removed with DNase I treatment and subsequent purification. RNA quality and quantity were assessed by RNA agarose gel electrophoresis and spectrophotometric detection at 260nm, respectively.

3.10.2. RNA quantification and electrophoresis analysis

Total RNA was quantified spectrophotometrically according to Sambrook et al. (1989). Absorbance ratios \( \text{OD}_{260/280} \) and \( \text{OD}_{260/230} \) were used to evaluate protein and phenolic compounds residual contamination, respectively. The quality of the extracted RNA was determined by running the samples on 1.2 % (w/v) agarose gel prepared in 1X TAE buffer (Appendix). Agarose was heated in microwave oven for 2 min to dissolve agarose and cooled to 60°C. 0.5 µg ethidium bromide per ml of agarose solution was added to it. Agarose is then poured onto gel tray and allowed to cool for 20-30 min. Once the gel is solidified, it is placed carefully in an electrophoresis tank filled with 1X TAE. 2 µg of RNA was mixed with 6× loading RNAase free loading dye (Thermo scientific, R0611) and the volume was adjusted to 12 µl by adding RNAase-free ultra pure water (Life technology, AM9932). The sample was then loaded on to the wells of the gel. The gel was run at 50 V (constant) till the dye front moved about 2/3rd from the loading point. The gel was viewed under trans-illuminator and photographed on Gel Documentation Unit (Alpha Innotech Corporations, USA.).

3.10.3. Removal of genomic DNA contamination from RNA

To remove the genomic DNA contamination, RNA was treated with DNase I (RNase-free), procured from New England BioLabs® (NEB; Cat No. M0303S0). The reaction was set up as per the manufactures instruction.

**DNase reaction**

1. 10 µg RNA was resuspended in 1X DNase I reaction buffer.
2. 2 units of DNase I was added and it was mixed thoroughly.
3. The final volume was made to 100 µl.
4. It incubated at 37°C for 10 min.
5. 1 µl of 0.5 M EDTA was added (to a final concentration of 5 mM).
6. Heat inactivate at 75°C for 10 min.

After heat inactivation, the sample was kept in ice for 5 min and then diluted in the ratio of 1:1 with Phenol-Chloroform-Isoamylalcohol (PCI) (25:24:1). The tubes were thoroughly mixed and centrifuged for 5 min at 14000g at RT (25°C). The upper aqueous phase was collected and precipitated with 0.5 volumes 7.5 M ammonium acetate and 2 volumes 100 % ethanol for 1 h at -20°C. After 20 min centrifugation at 14000g at 4°C, the resulting pellet was washed with 70 % (v/v) ethanol, air-dried and resuspended in 20µl autoclaved DEPC treated water. The RNA was then used for synthesis of cDNA.

3.10.4. cDNA Preparation – Reverse Transcription

cDNA preparation was done using SuperScript III First-strand synthesis kit (Life Technologies, India).

1. Volume corresponding to 3-5 µg of total RNA was taken in a 0.2 ml centrifuge tube.
2. 1µl of dithiothreitol (DTT) was added to it and the mixture was incubated for 5 min at 65°C, then placed on ice for 5 min.
3. It was followed by addition of
   ● 0.5µl of RNase inhibitors
   ● 2µl of dNTPs
   ● 4µl of buffer (5X)
   ● 1µl of enzyme RT.
   ● 4µl of RNase free water was added
4. The mixture was spinned at 4°C for 30 sec.
5. The reaction mixture was incubated at 42°C for 1 hr and the reaction was terminated by incubating it at 70°C for 5 min. Chilled the tube on ice.
6. The cDNA was stored at -20°C for further use.
3.10.5. Semiquantitative RT-PCR amplification

The cDNA synthesised from mRNA of different samples were used as a template for PCR (Polymerase Chain Reaction). The PCR was carried out in PTC100 thermocycler (MJ Research Inc., USA). The gene specific primers presented in table 3.1 were designed using the PrimerQuest software of Integrated DNA Technologies (http://www.idtdna.com/Primerquest/Home/Index) under default parameters. The primers were further validated for unique amplicon using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All the primers were procured from Genei, Bangalore. Mother stocks of primers were prepared with concentration of 100 µM and they were further diluted to obtain a final working concentration of 10 µM.

A PCR reaction mixture (50 µl) included:
1. 5µl Taq Polymerase Buffer E (10X)
2. 1µl dNTPs
3. 1µl Forward Primer
4. 1µl Reverse Primer
5. 0.5µl TaqPolymerase
6. 1µl template
7. 40.5 µl autoclaved distilled water

The PCR program was as follows:

a. Step I: Initial Denaturation at 94°C for 4 min
b. Step II: Denaturation at 94°C for 2 min
c. Step III: Annealing at X°C for 1 min (for X refer Table 3.1)
d. Step IV: Extension at 72°C for 2 min
e. Step V: Repeat Step –II to Step IV 35 times (35 cycles)
f. Step VI: Final Extension at 72°C for 7 min
g. Step VII: Hold at 4°C for 8 min
### Table 3.1. Base-Sequence of Specific Primers and respective amplified product size (bp)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the Gene</th>
<th>Accession Number</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Size of amplified product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cu/Zn SOD</td>
<td>L19435.1</td>
<td>F: 5’-CCTCAAGCCTGGTCTCCAT-3’&lt;br&gt;R:5’-CAGGCTTGAAGTCGATGAT-3’</td>
<td>55°C</td>
<td>351</td>
</tr>
<tr>
<td>2.</td>
<td>Fe-SOD</td>
<td>AY770495.1</td>
<td>F: 5’-CTTGATGCCGGAACCTTA-3’&lt;br&gt;R : 5’-GCCAGACCCCAAAGTGATA-3’</td>
<td>52°C</td>
<td>348</td>
</tr>
<tr>
<td>3.</td>
<td>Mn-SOD</td>
<td>L19436.1</td>
<td>F: 5’-GCCATGAGGAGGTTTGG-3’&lt;br&gt;R: 5’-CAAGCAGTCGCATTTCGTA-3’</td>
<td>53°C</td>
<td>314</td>
</tr>
<tr>
<td>4.</td>
<td>CAT</td>
<td>D26484.1</td>
<td>F: 5’-GTTCGGTTCACCAGTGTG-3’&lt;br&gt;R: 5’-CCCTCATTGCGCTGAGTT-3’</td>
<td>52°C</td>
<td>311</td>
</tr>
<tr>
<td>5.</td>
<td>APX</td>
<td>D45423.1</td>
<td>F: 5’-CCAAGGTTCTGACCACCTA-3’&lt;br&gt;R: 5’-CAGTTCGAGGCTGTGGATG-3’</td>
<td>55°C</td>
<td>332</td>
</tr>
<tr>
<td>6.</td>
<td>GR</td>
<td>AB009592.1</td>
<td>F: 5’-AACAGCCGATGCCATAAAG-3’&lt;br&gt;R:5’-CAACCACCAGTTTCATGACG-3’</td>
<td>52°C</td>
<td>470</td>
</tr>
<tr>
<td>7.</td>
<td>OsBRI1</td>
<td>NM_001050612.1</td>
<td>F: 5’-CTTTCGCGCCTTTCCCTTG-3’&lt;br&gt;R: 5’-ACTCGCCTCCTATTTCGTA-3’</td>
<td>55°C</td>
<td>311</td>
</tr>
<tr>
<td>8.</td>
<td>OsDWARF</td>
<td>AB084385.1</td>
<td>F: 5’-TGGGCTGCTGAGGAAAAACTA-3’&lt;br&gt;R:5’-CTTCAACTCGGGGGAACCT-3’</td>
<td>55°C</td>
<td>323</td>
</tr>
<tr>
<td>9.</td>
<td>SalT</td>
<td>Z25811.1</td>
<td>F: 5’-GAGGGTCAGTCAGGACATC-3’&lt;br&gt;R:5’-GTCTTCAGTGGGAAATGTGA-3’</td>
<td>55°C</td>
<td>438</td>
</tr>
<tr>
<td>10.</td>
<td>EF1-α</td>
<td>D63580.1</td>
<td>F: 5’-GTACAAGATCGTGTTATT-3’&lt;br&gt;R: 5’-GGGTACTCAGAAGGCTCT-3’</td>
<td>50°C</td>
<td>560</td>
</tr>
</tbody>
</table>

3.10.6. Agarose gel electrophoresis of the amplified DNA

Amplified products were resolved on 1.5% (w/v) agarose gel prepared in TAE buffer containing 0.5 µg/ml ethidium bromide. The DNA sample was mixed with 6X loading dye (Thermo scientific, R0611) to make a final concentration of 1X. It was mixed properly then loaded on the agarose gel. 100 bp DNA ladder was loaded as a molecular weight marker (Genei, Bangalore). The gel was run at 100 V for 1 h. Gel was...
viewed in trans-illuminator and later photographed on gel documentation system (Alpha Innotech Corporations, USA).

3.11. Growth conditions and treatment of *Arabidospis* plants for phosphorylation site analysis

Seeds of *Arabidopsis thaliana* (ecotype Col-0 and Ws-2) and two transgenic lines expressing BRI1-Flag and BAK1–green fluorescent protein (GFP) were sterilized in absolute ethanol for 10 min followed by a 20 min treatment with a 30% commercial bleach solution containing 0.1% Triton X-100 as a surfactant. The seeds were thoroughly rinsed at least five times in a large volume of sterile distilled water and kept at 4°C for 2 days for vernalisation. After 2 days, seeds were then resuspended in sterilized 0.2% agarose (w/v) and inoculated in Petri plates containing 1/2 strength Murashige and Skoog salt and vitamins solid medium (Phyto Technology Laboratories), with 3% sucrose, pH 5.7 (referred to as ½ MS media). The petriplates were kept in growth chamber for three weeks at 22°C (Fig. 3.3). After three weeks, seedlings were ready for shifting to a controlled environment room in the North Carolina State University Phytotron for further growth. Seedlings were transferred to flats laid with

![Fig. 3.3. Germination of seeds of *A. thaliana* (ecotype Columbia-0, Ws-2) and two transgenic lines (expressing BRI1-Flag and BAK1–GFP) in MS media.](image-url)
‘Farfard 4P’ potting media containing 1:1:1 ratio of perlite, vermiculite and sphagnum moss. Plants were grown in the growth chamber at a constant temperature of 22°C at 100 µEm$^{-2}$ S$^{-1}$ constant light on a 14 h light /10 h dark cycle. Plants were watered regularly. After a month, peduncles emerged to about 4 cm and plants were supported with a collar. After about three months, mature seeds were harvested and stored at 4°C till further use (Fig. 3.4).

For producing plants for further biochemical and proteomic studies, 100 mg seeds each of *Arabidopsis thaliana* ecotype Ws-2, BRI1-Flag and BAK1–GFP were sterilized as mentioned above. Seeds were then resuspended in sterilised 0.2% agarose and inoculated in 1 L flask containing 100 ml of Gamborg’s B-5 basal medium (Phyto Technology Laboratories, USA), pH 5.7. Flasks were kept under continuous light at 80 rpm rotation for 11 days.
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At the end of 11 days of growth, flasks were divided into two groups and the old media was drained and was replaced with fresh media with or without 150 mM NaCl. The flasks were allowed to rotate at 80 rpm for 4 h. For each group of plants treated with or without salt, half of them were treated with 100 nM brassinolide (BL) and the other half by solvent control for 90 min (Fig. 3.5). After 90 min, plants were blotted dry, weighed, frozen in liquid nitrogen and stored at -80°C prior to membrane protein isolation. Three sets of biological replicates were prepared for each treatment.

3.11.1. Extraction of membrane protein

Total membrane protein was isolated from both the transgenic (BRI1-Flag and BAK1-GFP) as well as control lines according to the method suggested by Wang et al., (2005). Tissue (100 g) was homogenized in a blender in 200 ml of cold extraction buffer [20 mM Tris-HCl (pH 8.8), 150 mM NaCl, 1 mM EDTA (pH 8.0), 20% glycerol] containing 1% (v/v) polyvinylpolypyrrolidone (PVPP), 1 mM PMSF, 20 mM NaF, 50 nM microcystin and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN). The mixture was centrifuged at 6000g for 15 min at 4°C and supernatant was filtered through a mira cloth (Calbiochem® EMD Millipore). The filtrate was further ultra-centrifuged at 100,000g for 2 h at 4°C. After centrifugation, the microsomal fraction containing total membrane protein was pelleted down while the supernatant contained the cytosolic fraction. The pellet was resuspended in 2 ml of solubilization buffer [10 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1.0 mM EDTA, 500 nM microcystin, 10% glycerol, 1.0% Triton X-100, 1.0 mM PMSF, 20 mM NaF, and protease inhibitor cocktail (Roche)] and sonicated for 2 min. The sample was then centrifuged again at 21,000×g for 10 min and the total membrane protein in the clear supernatant was collected in a fresh tube. The protein in microsomal fraction was quantified by Bradford assay kit (www.bio-rad.com) using bovine serum albumin as the standard. Before immunoprecipitation of membrane protein, the protein concentration was adjusted to 1.0 mg/ml while concentration of triton X-100 was reduced to 0.2%.
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3.11.2. Western blot analysis

Proteins were separated by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using precast gels (Precise Protein Gels, 4-20%, Thermo Scientific) at a constant rate of 100 volt. Proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane by iBlot® dry Blotting System (Life technologies, USA). After protein transfer, the membrane was blocked with 5% non-fat dry milk in 50 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween-20 (TBS-T) for 1 h. The membrane was then incubated in an anti-Flag M2 primary antibody (Sigma-Aldrich) at 1:5000 dilution, anti-GFP antibody (Molecular Probes) at 1:2000, or antiphosphothreonine antibody (Cell Signaling Biotechnology, Beverly, MA) at 1:1000 for 1 h. It was followed by washing of the PVDF membrane with TBST buffer for 15 min with intermittent changing of the buffer after every 5 min. The membrane was then incubated in horseradish peroxidase–linked secondary antibodies at 1:2000 dilutions for 1 h. It was followed by 5 washes of the PVDF membrane with TBST for 5 min each. The secondary antibody was visualized by the ECL chemiluminescence detection system (ECL Plus Kit; GE healthcare, Piscataway, NJ). To ensure equal sample loading, after protein transfer, the membrane was incubated with Ponceau S stain (Sigma-aldrich, USA) and photographed. It was then rinsed with water for several times for complete removal of Ponceau S stain and was then blocked with milk. The experiment was done in duplicate to ensure consistent results.
3.11.3. Immunoprecipitation

BRI1-Flag and BAK1-GFP were immunoprecipitated from the solubilised total membrane protein fraction according to the method suggested by Wang et al. (2005) and further refined by Mitra et al. (2012). For BRI1-flag immunoprecipitation, microsomal protein diluted to 1.0 mg/ml was incubated with prewashed anti-Flag M2 agarose beads (Sigma-Aldrich) at 4°C overnight on a shaker. Beads were collected by centrifugation at 1,000g for 5 min while the supernatant was discarded. Beads were then washed extensively with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and centrifuged at 1,000g for 5 min. BRI1-flag was eluted with 100 µl of 2X SDS sample loading buffer (3% SDS, 94 mM Tris-HCl, pH 6.8, 15% glycerol, and 7.5% β-mercaptoethanol). After boiling for 5 min, 5 µl of supernatant was separated by SDS-
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PAGE using gradient precast gels (Precise Protein Gels, 4-20%, Thermo Scientific). Similarly, BAK1-GFP was immunoprecipitated from solubilized total membrane protein by first incubating the protein in anti-GFP mouse antibody (Molecular Probes, USA) for 10 h followed by another overnight incubation with protein A beads (Pierce, Rockford, IL). Immunoprecipitated proteins were detected by immunoblot analysis on PVDF membranes using anti-Flag and anti-GFP antibodies. For analysis of the samples on by mass spectrometry, the samples were loaded in 4-12% Bis-Tris NU-PAGE gel (Invitrogen) and stained with SYPRO Ruby.

3.11.4. Separation of membrane proteins using SDS-PAGE and in-gel trypsin digestion

Immunoprecipitated protein sample (20 µl) from treated as well as control samples was separated by one dimensional (1-D) SDS PAGE using pre-cast NuPAGE Novex 4-12% Bis-Tris gel, 1.0 mm, and NuPAGE MES (2-(N-morpholino)ethanesulfonic acid) buffer (Invitrogen, Carlsbad, CA) at a constant rate of 150 V. The gel was stained with SYPRO Ruby (Molecular Probes) and the bands corresponding to the molecular weight of BRI1-FLAG (~135 kDa) and BAK1-GFP (~98 kDa) were visualized. The bands corresponding to the protein of interest were excised in parallel from the lanes containing samples from transgenic and control lines. The excised gel bands were further cut into 1-5 mm small pieces and washed thrice for 15 min each with 500 µl of acetonitrile/100 mM NH₄HCO₃, pH 8.0 (1:1) to completely remove the stain. Gel slices were then dehydrated in 75% acetonitrile for 30 min and dried in a vacuum centrifuge. In-gel reduction, alkylation and trypsin digestion was performed on the excised bands according to a published protocol (Rowley et al., 2000). Protein in the gel slices was reduced with 15 mM dithiothreitol (DTT) for 30 min at 37°C, alkylated with 55 mM iodoacetamide for 30 min at room temperature. The gel pieces were then washed at least three times with neat acetonitrile and and 50 mM NH₄HCO₃ (pH 8.0). The slices were then incubated with 100 µl of 50 mM NH₄HCO₃, pH 8.0, containing 1 ng/µl trypsin overnight at 37°C. After tryptic digestion, peptides were extracted from the gel pieces with 200 µl of 2% acetonitrile /1% formic acid (v/v). The tubes were spun down at 6000g for 10 min. Supernatant was collected carrying tryptic peptides and was
dried via vacuum centrifugation (Savant, Thermo Fisher Scientific) and stored at -80°C until further analysis.

3.11.5. Phosphopeptide enrichment by immobilized metal ion chromatography (IMAC)

Tryptic peptides were enriched for phosphopeptides by immobilized metal affinity chromatography (IMAC) using Phos-select Iron affinity gel (Sigma, St. Louis, MO). The dried peptides were resuspended in 50 µl of buffer containing 250 mM glacial acetic acid and 30% (v/v) acetonitrile. 10 µl of Phos-select resin was equilibrated in the same buffer and was added to the resuspended peptide. The sample and beads were then incubated at room temperature for 1h on a shaker. Following the incubation, sample was spun down at 3000g for 1 min and beads were collected. Beads were then washed with resuspension buffer and the enriched phosphopeptides were eluted with two 50 µl aliquots of 100 mM NH₄HCO₃, pH 8.5. The aliquots were pooled, lyophilized and stored at -80°C.

3.11.6. Phosphopeptide identification

The dried peptides were resuspended in 20 µl of 0.1% formic acid of which 10 µl was used for MS analysis. Samples were analysed in the Mass Spectrometry Laboratory, North Carolina State University, Raleigh, NC, USA using one of two instruments, a Q-TOF Premier quadrupole time-of-flight system (Waters Corporation) or the Orbitrap Elite (Thermo Scientific). All samples were analyzed in triplicate (3 technical replicates per sample). For Q-TOF analyses, peptides were separated using a Symmetry C18 trapping column (180 µm id x 2 cm, 5 µm particles) coupled to a 25 cm BEH column (75 µm id, 1.7 µm particles). A nanoACQUITY ultra-high pressure liquid chromatography was used to deliver the 7-40% B gradient at a flow rate of 300 nl/min (A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile) over 30 min. The Q-TOF was operated in either LC/MSE data-independent analysis mode or LC/MS/MS data dependent mode as previously described (Wang et al., 2008). For Orbitrap analyses, peptides were separated using an easy-nLC 1000 system (www.thermoscientific.com) coupled on-line to the Orbitrap Elite mass spectrometer (www.thermoscientific.com). Peptides were subjected to a reversed-phase separation
using a 75 µm i.d. x 25 cm column packed in-house with 3 µm 200 Å Magic C18AQ stationary phase (www.michrom.com) coupled to an Acclaim PepMap 100 µm i.d. x 2 cm trapping column (www.thermoscientific.com). The mobile phases consisted of (A) 0.1% formic acid in 2% acetonitrile and (B) 0.1% formic acid in acetonitrile. Ten µg of total peptide digest was injected onto the reversed-phase column for each analysis and a linear gradient of 1% B/min from 5% B to 40% B was used for separation at a flow rate of 300 nl/min. A top 10 data-dependent acquisition method was used to acquire LC/MS/MS data. All the steps for analyzing in vivo phosphorylation sites have been summarized in Fig. 3.4.

3.11.7. LC/MS data analysis for phosphopeptide identification

Raw data files acquired on the Q-TOF were processed using Protein Lynx Global Server 2.4 software (Waters). For the data-dependent analyses, the raw data files were processed to generate plk files which were subsequently searched against the TAIR10 database (www.arabidopsis.org) using Mascot 2.4 search algorithm (Matrix Science, London, UK) running on an in-house server. Data was searched against the TAIR10 protein database (www.arabidopsis.org) to which the rabbit phosphorylase B protein sequence was appended. Trypsin was set as the primary digest reagent and no more than one missed cleavage site was allowed and search tolerances of 50 ppm and 0.05 Da for precursor and product ions respectively, were used. Cys-carbamidomethyl modification was fixed (+57.0215 Da) while dynamic mass modifications of phosphorylation of Ser, Thr and Tyr residues (+79.9663 Da) and oxidation of Met (+15.9949 Da) were used for database searches. For LC/MS\textsuperscript{E} raw files, data processing and database searching were accomplished completely within PLGS2.4. LC/MS\textsuperscript{E} data were searched against the modified TAIR10 database as described above, using automatic settings for mass accuracy. For Orbitrap analyses, raw LC/MS/MS data files were processed and searched using Proteome Discoverer 1.4. Searching was accomplished using Mascot with precursor and product mass accuracies of 10 ppm and 0.8 Da, respectively, with the same number of missed cleavage sites and variable modifications described above for Q-TOF analysis. All putative phosphopeptide assignments were manually curated.
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3.12. Plant materials, growth conditions and mode of treatment for total proteomics study

For total proteomics studies, 20 mg of *A. thaliana* ecotype Col-0 seeds were surface sterilized and grown in 100 ml of Gamborg's media for 11 days (as mentioned in section 3.11). On the 11th day, flasks were divided into two groups and the old media was drained and replaced with fresh media with or without 100 nM BL for 90 min. The flasks were allowed to rotate at 80 rpm for 90 min. The old media was again drained and replaced with the fresh media with or without 150 mM NaCl for 4 h. After 4 h, plants were blotted dry, weighed, frozen in liquid nitrogen and stored at -80°C prior to membrane protein isolation. Three sets of biological replicates were prepared for each treatment.

3.12.1. Extraction of membrane and cytosolic protein

Sample (10 g) treated each with or without 150 mM of NaCl and/or treated with or without $10^{-7}$ M of BL was further processed for membrane and cytosolic protein extraction by following the method given in section 3.11.1. The protein in the microsomal as well as cytosolic fraction was quantified by Bradford assay kit (www.bio-rad.com) using bovine serum albumin as the standard.

3.12.2. Precipitation and in-solution digestion of microsomal proteins

To each of the samples, from both the cytosolic and membrane fraction, 10 volumes of chilled acetone was added and kept at 4°C overnight for protein precipitation. The samples were centrifuged at 15,000g at 4°C for 20 min. The pellet containing the protein was washed with 80% acetone thrice and then air dried. Protein was then resolubilised in 8 M urea. Protein concentration was measured using Bradford assay kit (www.bio-rad.com).

Prior to in-solution digestion, samples were diluted with 50 mM NH$_4$HCO$_3$, pH 8.0 to reduce the urea concentration to 2 M. In solution reduction, alkylation and digestion was performed according to a published protocol (Rowley *et al.*, 2000). Protein in the solution was reduced with 15 mM dithiothrietol (DTT) for 30 min at 37°C, alkylated with 55 mM iodoacetamide for 30 min at room temperature. Digestion was carried out with Lyc-C dissolved in 50 mM NH$_4$HCO$_3$, pH 8.0. For 50 µg protein
concentration, 1 µg of Lyc-C was added and the solution was incubated overnight at 37°C. The solution containing the peptides was dried via vacuum centrifugation (Savant, Thermo Scientific, USA) and stored at -80°C until further analysis.

3.12.3. LC/MS/MS analysis

The dried peptides were resuspended in 200 µl of 0.1% formic acid of which 10 µl was used for LC/MS/MS analysis. Peptides were spiked with a predigested rabbit phosphorylase B internal standard (Waters, Milford, MA) at a level of 50 fmol per 10 µl injection. All samples were analyzed in triplicate (3 technical replicates per sample) by an easy-nLC 1000 system (www.thermoscientific.com) coupled on-line to an Orbitrap Elite mass spectrometer (www.thermoscientific.com). Peptides were subjected to a reversed-phase separation using a 75 µm i.d. x 25 cm column packed in-house with 3 µm 200 Å Magic C18AQ stationary phase (www.michrom.com) coupled to an Acclaim PepMap 100 µm i.d. x 2 cm trapping column (www.thermoscientific.com). The mobile phases consisted of (A) 0.1% formic acid in 2% acetonitrile and (B) 0.1% formic acid in acetonitrile. 10 µg of total peptide digest was injected onto the reversed-phase column for each analysis and a linear gradient of 1% B/min from 5% B to 40% B was used for separation at a flow rate of 300 nl/min. For LC/MS/MS analysis, the mass spectrometer was operated in the data-dependent mode where the top ten most intense ions were selected for CID.

3.12.4. Data processing for differential protein expression analysis

Raw Orbitrap LC/MS/MS data files were imported into the Progenesis (Nonlinear Dynamics) differential expression analysis software package for determination of protein abundance differences between treatments using a label-free approach. Mascot peptide assignments were imported into Progenesis for annotation of peptide level features, and protein level abundance changes summarized within Progenesis.

3.13. Two-dimensional gel electrophoresis analysis for rice seedlings

Rice seedlings treated with salt and EBL were analyzed by 2-DE. Four samples were chosen for the present study i.e. control, 10^{-7} M EBL, 100 mM NaCl and 10^{-7} M EBL+100 mM NaCl. Phenol based method was used for protein extraction.
3.13.1. Phenol based extraction of proteins

Rice seedlings (1 g) were crushed in liquid nitrogen and homogenized in 3 ml of extraction buffer containing 500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl and pH was set 8.0 with HCl. Just before use, 2% β-mercaptoethanol and 1 mM PMF were added to the extraction buffer. The mixture was vortexed and incubated by agitating for 10 min on ice. After incubation, 3 ml of tris-buffered phenol was added to it and the mixture was vortexed. It was incubated on a shaker for 10 min on ice and was followed by centrifugation for 10 min at 10000g at 4°C. A clear phase separation was seen after centrifugation. The upper phenolic phase was carefully collected in a separate tube without touching the lower phase. Again, 3 ml of the extraction buffer was added to the tris-buffered phenol and the extraction process was repeated as mentioned above. The mixture was again centrifuged, and the upper phenolic phase was collected in a new tube. 5 vol of 0.1 M ammonium acetate in cold methanol was added to the phenolic phase. The tube was shaken gently and the mixture was incubated overnight at –20°C. Protein was recovered by centrifugation at 12000g at 4°C for 10 min. The pellet so obtained was washed three times with cold 0.1 M ammonium acetate in cold methanol and then finally with cold acetone. The pellet was air-dried and stored at -80°C for 2-DE.

3.13.2. Protein solubilisation and quantification

The protein pellet was thoroughly resuspended in IEF (rehydration) buffer comprising of 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 15mg/ml Destreak reagent (Amersham Biosciences) and 0.2% Ampholytes. Protein was quantified using the Bradford protein assay as mentioned before.

3.13.3. Two-dimensional Electrophoresis (2-DE).

Protein (150 µg) dissolved in rehydration buffer was used to passively rehydrate IPG strips (7 cm, 3–10, 5-8, Readystrip, Cat.No. 163-200,163-2004, Bio-Rad) overnight. Isoelectric focusing (IEF) was carried out in Protean IEF Cell (Bio-Rad) at 20°C. The strips were focused at 250V for 40 min, 4000 V for 2 h with linear voltage amplification, and finally to 10,000 V h with rapid amplification. After isoelectric
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focusing, the strips were incubated with equilibration buffer (6M urea, 0.05M Tris-HCl pH 8.8, 20% glycerol, 2% SDS) containing 2% DTT for 15min for reduction. For alkylation of the proteins, the strip was further incubated with 2.5% iodoacetamide dissolved in equilibration buffer for 15 min. The second dimensional electrophoresis was performed using 12% polyacrylamide gel. After mounting the strip on the gel, it was sealed with 0.5% agarose containing 0.1% bromophenol blue (tracking dye). The protein molecular weight marker was loaded along with the strip in a small well made in agarose. Electrophoresis was performed at constant volt (100 V) for 2 h in tris glycine-SDS running buffer until the dye (bromophenol blue) reached the front end of the plate.

3.13.4. Gel staining, imaging and analysis

2-DE for 4 samples was performed in triplicates. The gels were stained with silver staining plus kit (Bio-Rad, USA) and stored in 5% acetic acid for further analysis. Gel imaging was done using Molecular Imager Gel Doc XR System (Bio-Rad, USA) and the images were analyzed using Imagev Master 2D Platinum version 7.0 software (Amersham, UK).


The behaviour of salT gene was studied by analysing its expression in various tissues and under various stress conditions. Rice seedlings var. Pusa Basmati-1, IR-64, Luna sankhi and Luna suvarna were grown in sand as mention in section (3.3) for 12 days in distilled water. On 12th day, the seedlings were harvested. Some seedlings of var. Pusa Basmati-1 were segregated into root, shoot and leaf from the seedling and the tissue was stored at -80°C for RNA isolation. Rests of the seedlings of Pusa Basmati-1 were transferred into test tubes containing solutions of 200 mM NaCl and 0.04% CPF. Heat and cold stress was inflicted on seedlings by keeping seedlings at 42°C and 4°C while drought stress was induced by keeping the seedlings for air-drying. After 8 h seedlings were processed further for expression study of salT gene.
3.14.1. In silico analysis of \textit{salT} gene

Structure organization of \textit{salT} gene of \textit{O. sativa} was predicted using gene structure display server (http://gsds.cbi.pku.edu.cn/). The full length nucleotide sequence obtained was translated using translate tool (http://www.expasy.ch/tools/dna.html) and the properties of deduced amino acid sequence were estimated using ProtParam (http://www.expasy.ch/tools/protparam.html) (Gasteiger \textit{et al.}, 2005) programs. Secondary structure was determined by SOPMA program (http://npsa-pbil.ibcp.fr) (Geourjon and Deleage, 1995). The promoter sequence was analyzed using PlantCARE, a database of plant \textit{cis}-acting regulatory elements (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). 1.5 kb region upstream of the start codon was analyzed for promoter sequence.

3.14.2. Constructing recombinant plasmid containing \textit{salT} gene

3.14.2.1. cDNA amplification with the designed primers

Total RNA was isolated from rice seedlings with Trizol® reagent and the cDNA was synthesized following the protocol described in section 3.10.1 to 3.10.3. Two sets of primers, one pair with stop codon, \textit{salT}+SP1 (\texttt{5′-ctgagctcTCAAGGGTGAGGTAGATGC-3′}) and \textit{salT}+SP2 (\texttt{5′-atgggatccATGACGCTGGTGAAGATTGG-3′}) and the other pair without stop codon \textit{salT}-SP1 (\texttt{5′-acgagctcAGGGTGGACTAGGTAGGCCAA-3′}) and \textit{salT}-SP2 (\texttt{5′-atgggatccATGACGCTGGTGAAGATTGG-3′}) were designed to amplify the \textit{salT} gene (NCBI accession no. Z25811). During the design of primers, specific restriction sites (BamHI and SacI) were introduced into the tails of the primers for cloning into different expression vectors. Choice of restriction sites were purely based on the enzymes that do not cut the gene (\textit{salT}) and were selected using NEB cutter software (http://tools.neb.com/NEBcutter2/). Designed primers were procured from IDT (Integrated DNA Technologies, http://eu.idtdna.com/site). Mother stocks of primers were prepared with concentration of 100 µM and they were further diluted to obtain a final working concentration of 10 µM. To amplify the gene, PCR was performed as follows:
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A PCR reaction mixture (50 µl) included:

1. 5µl Taq polymerase buffer E (10X)
2. 1µl dNTPs
3. 1µl Forward primer
4. 1µl Reverse primer
5. 0.5µl Taq polymerase
6. 1µl template
7. 40.5 µl autoclaved distilled water

The PCR program was as follows:

a. Step I: Initial denaturation at 94°C for 4 min
b. Step II: Denaturation at 94°C for 1 min
c. Step III: Annealing at 55°C for 1 min
d. Step IV: Extension at 72°C for 2 min
e. Step V: Repeat step -II to Step IV 35 times (35 cycles)
f. Step VI: Final extension at 72°C for 7 min
g. Step VII: Hold at 4°C for 8 min

The bands corresponding to the salT gene was extracted from gel using gel extraction kit (SIGMA, USA; Cat No. NA1111). The gel purified amplification products with stop codon and without codon were cloned separately into pGEM-T-Easy vector.

3.14.2.2 Cloning in pGEM-T easy vector

For cloning the amplified salT gene, ligation is done using the PCR product and the cloning vector pGEM-T. The T-overhangs at the insertion site in pGEM-T vector provides a compatible overhang for PCR products generated by certain thermostable polymerases. It also contains ampicillin as a bacterial selection gene. For cloning in pGEM-T vector, the ligation reaction was set having the final concentration of 1X Rapid ligation buffer, pGEM-T vector (50ng), purified PCR product (150-200 ng), and 1 µl DNA ligase (3 Weiss units/µl) making the final volume 10µl using autoclaved
distilled water. The reaction mixture was incubated overnight at 4°C. The ligated product was transformed to *E. coli* competent cell.

### 3.14.2.3. Competent cell preparation and bacterial transformation

**E. coli competent cell preparation protocol**

*E. coli* host cells were streaked on LB agar plate containing ampicillin (50 µg/ml) and incubated at 37°C to obtain single colony. 5 ml of LB media was inoculated with a single colony and incubated at 37°C overnight in incubator shaker and later the following protocol was followed for preparation of competent cell.

1. Inoculate 100 ml LB medium with 1ml of saturated overnight culture.
2. Shake at 37°C until $\text{OD}_{600}=0.4$ (usually 2-3 h).
3. Place in an ice bath for 10 min.
4. Transfer the culture into two pre-chilled 50 ml centrifuge tubes.
5. Centrifuge at 3000g for 10 min at 4°C.
6. Remove the medium, resuspend the cell pellet with 1.6 ml ice cold 100 mM CaCl$_2$ by swirling on ice gently.
7. Incubate on ice for 30 min.
8. Centrifuge at 3000g for 10 min at 4°C.
9. Remove the medium, resuspend the cell pellet with 1.6 ml ice-cold 100 mM CaCl$_2$ by swirling on ice gently.
10. Incubate on ice for 20 min.
11. Combine cells to one tube and add 0.5 ml ice-cold 80% glycerol and swirl to mix.
12. Freeze 100 µl aliquots in liquid nitrogen.
13. Store in -80°C.

**E. coli transformation protocol**

1. Thaw DH5α cells on ice (~ 5 min).
2. Ligation reaction (2-5 µl) containing usually 20 ng- 100 ng was added to the cells.

3. The tubes were gently flicked and incubated on ice for 30-45 min.

4. Cells are incubated for 60 seconds at 42°C.

5. Place the cells back on ice for 5 min.

6. 900 µl of LB was added and incubated at 37°C for 2 h with moderate shaking (~150 rpm).

7. Cells (100-200 µl) were plated on LB plates containing appropriate antibiotic, IPTG and X-gal.

8. Incubate overnight at 37°C. White colonies were selected and restreaked on LB plates with appropriate antibiotic.

3.14.2.4. Cloning of salT gene into expression vectors

Plasmids isolated from transformed colonies using plasmid isolation kit (Sigma-Aldrich, USA, Cat No. PLN350) following manufacturer's instructions. For cloning into different expression vectors, double digestion of the isolated pure plasmid of different expression vectors were carried out using BamHI and SacI restriction enzymes to generate sticky ends compatible for ligation with the double digested PCR product (salT). The pGEM-T- Easy vector harboring a salT cDNA was also digested with BamHI and SacI to yield ~ 450 bp fragment. The digested reaction mixtures were loaded on separate 1% agarose gels in TAE buffer and gel electrophoresis was carried out. After electrophoresis, each gel was visualized in a UV-transilluminator and the DNA fragments of expected size (~450 bp for salT) were excised from the agarose gel using a clean, sharp scalpel. PCR product gel extraction was carried out using gel extraction kit (SIGMA, USA; Cat No. NA1111). A ligation reaction was set up with insert (50 µg) and vector (150 µg) in 20µl reaction volume using 1 U of T4 DNA ligase (NEB, USA). The ligation reaction was incubated for 18 h at 16°C. The recombinant vector containing salT gene was introduced into E. coli and integrity was confirmed using colony PCR as well as restriction digestions (appendix). The salT gene was ligated to modified pCAMBIA 2301 vector between a CaMV 35S promoter and an rbcS
(ribulose-1,5-bisphosphate carboxylase small-subunit gene) terminator (Fig 3.7 a). Similarly, full-length cDNAs of salT (without stop codon) were cloned into a modified pCAMBIA1300 vector containing the CaMV 35S promoter and rbcS terminator (Fig 3.7 b). Three tandem repeats encoding the FLAG epitope were fused in-frame to the C-terminal end of salT cDNA. The resulting constructs, p35Spro: salT-rbcS and p35Spro: salT-FLAG was introduced into A. tumefaciens strain EHA105. To facilitate subcellular localization studies, further the ORF (open reading frame) of salT (without stop codon) was also cloned into the pBI221-GFP vector to create a CaMV 35S: salT-GFP fusion construct (Fig.3.7 c).

In order to express salT in E. coli, the entire coding region of the salT cDNA was cloned into pET 28a Vector (http://richsingiser.com/4402/pET28.pdf). The recombinant vector containing salT gene (pET28a-salT) was introduced into E. coli strain DH5α and integrity was confirmed using colony PCR as well as restriction digestions. The recombinant plasmid was later purified from E. coli strain DH5α and

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**Fig. 3.7.** salT gene of *O. sativa* ligated to a) modified pCAMBIA 2301 b) modified pCAMBIA1300 containing 3X FLAG c) a pBI221-GFP vector.
mobilized into BL21 competent cells. The transformed bacteria were grown at 37°C with shaking (200 rpm) in 20 ml LB broth containing 50 μg/ml kanamycin until the optical density at 600 nm (OD$_{600}$) of the culture reached 1.0. Then, the 10 ml of the culture was inoculated into 1000 ml LB broth containing 50 μg/ml kanamycin and incubated at 37°C with shaking (250 rpm) until OD$_{600}$ reached 0.5. The culture was then incubated at 20°C with shaking (200 rpm) for 30 min followed by the addition of 1 mM IPTG (isopropylthiogalactopyranoside). After the incubation for 2 h, the bacteria were harvested by centrifugation at 7000g for 10 min. The bacteria pellet was resuspended in 40 ml of buffer (50 mM sodium phosphate buffer, pH 8.0, containing 0.5 mM NaCl), and sonicated on ice (240× 2 s pulses with 4 s intervals). The homogenate was then centrifuged at 14000g for 10 min at 4°C, and the protein present in the supernatant was purified using the Ni-NTA affinity chromatography kit (Thermo scientific, USA) following manufacturers instructions. Purified SALT protein was observed on 10 % sodium SDS-PAGE gel.

3.15. In vitro regeneration of rice

3.15.1. Preparation of stock solutions for culture medium

The constituents of culture medium were divided into four different stock solutions: (I) Major salts, (II) Iron salts, (III) Organic nutrients except sucrose, (IV) Minor salts. The recipe for stock solutions is given in appendix. MS basal medium (Murashige and Skoog, 1962) stocks were also prepared as mentioned in appendix

3.15.2. Preparation of PGRs stocks

The phytohormone 2,4-D used for callus induction was prepared by dissolving the required amount of PGR in a few drops of 70% ethanol and then raising the final volume with distilled water so as give a final concentration of 5 mM. Other phytohormones like BAP, NAA and ABA were prepared by dissolving the PGR powder initially in 1 N NaOH and raising the final volume with distilled H$_2$O to reach the stock concentration to 5 mM. Stock solutions were stored at 4°C.
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3.15.3. Preparation of culture medium

To prepare 1L of medium, 50 ml of stock solution I and 10 ml each of the remaining three stock solutions (II, III and IV) were added to the flask. Additional compounds like myo-inositol (100 mg/l), L-proline (500 mg/l), L-glutamine (500 mg/l), and casein enzyme hydrolysate (300 mg/l) were also added along with stocks to the medium. Different media used for callus induction, maturation and regeneration (Appendix) differ from one another in the type of PGR added to it. For callus induction media only 2,4-D was used, for the maturation media PGRs like ABA, BAP and NAA were used and finally for the regeneration medium, only BAP and NAA were used. 30 g of sucrose was used as a carbon source. The final volume was raised to 1 L by adding the required amount of double-distilled water. After thorough mixing, the pH of the medium was adjusted to 5.8 using 0.1 N NaOH and/or 0.1 N HCl. To solidify the media, 2.8 g of clarigel was added to the solution. MS medium with 3% sucrose was prepared in tubes for rooting and shoot development. The medium was autoclaved for 20 min at 1.1 Kg/cm² pressure and 121°C temperature. After autoclaving, the media was cooled to around 60°C. For embryogenic calus induction, maturation and regeneration, sterilized media were aseptically poured into the petri plates in the laminar air-flow.

3.15.4. Surface sterilization and inoculation of seeds

Rice seeds were dehusked and washed with double distilled water containing 1-2 drops of tween-20 detergent. Seeds were then rinsed with 0.4% (v/v) solution of sodium hypochlorite containing a drop of tween-20 for 25 min followed by two washes with double distilled water. Seeds were subsequently treated with 0.01% (w/v) mercuric chloride solution containing a drop of tween-20 for 3-4 min and thereafter, rinsed many times with sterile distilled water to remove all the traces of sterilizing agents. 6-8 mature sterilized seeds were then inoculated into medium contained in Petri plates.

3.15.5. Embryogenic callus induction and regeneration

Seeds were inoculated in rice embryogenic callus induction medium (R-ECIM; Appendix) containing 2,4-D for 3 weeks in dark under controlled temperature (25±2°C) of culture room. The cultures were regularly screened for contamination and necrosis on the daily-basis. After 3 weeks, the scutellar embryogenic calli obtained from each single
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Explant were again subcultured to fresh R-ECIM medium. Later on, non-embryogenic calli (compact, non-friable calli that develop root like structures) were discarded and only embryogenic calli (organized, nodular) were selected and allowed to proliferate in the fresh R-ECIM medium for another 10 days in dark to attain a convenient size. After 10 days, the embryogenic calli were transferred to rice somatic embryo maturation medium (R-SEMM; Appendix). The cultures were kept at 25±2°C in dark for 2 weeks before they are transferred to regeneration medium. If any contamination is observed, the healthy calli were transferred to the fresh maturation media. After 2 weeks, the fully matured calli were transferred to the regeneration medium supplemented with PGRs (R-SERM; Appendix). These cultures were kept under light (2000 lux) of 40 W white cool fluorescence tubes. The temperature was maintained at 25±2°C with 14 h photoperiod. After regeneration, for proper development of roots and shoots, the shoots were transferred to, basal MS medium supplemented with 3% sucrose which was prepared from desired concentrations of stocks as mentioned in appendix.

3.16. Histological study

The histological analysis of the somatic embryos was carried out using the following protocol.

i) Killing, fixing and dehydration of material: The material was fixed in FAA (Formalin : Acetic Acid : Ethanol 50% :: 5 : 5 : 90 v/v) for 1 week. The material was preserved in 70% ethanol until use after which the tissue was dehydrated in the following TBA (t-butyl alcohol) series:

<table>
<thead>
<tr>
<th>Rect. alcohol (ml)</th>
<th>TBA (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 30</td>
<td>20</td>
</tr>
<tr>
<td>b) 50</td>
<td>20</td>
</tr>
<tr>
<td>c) 50</td>
<td>35</td>
</tr>
<tr>
<td>d) 45</td>
<td>55</td>
</tr>
<tr>
<td>e) 25 (Ethanol)</td>
<td>75</td>
</tr>
<tr>
<td>f) -</td>
<td>100</td>
</tr>
</tbody>
</table>
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Water was added to each grade (a-c) to make total volume up to 100 ml. The material was kept in each grade for 3-4 h except for 'c' where it was kept overnight.

ii) *Waxing:* The material was kept in an oven at 60°C and paraffin wax flakes were added after every 15-20 min. The whole process was carried out for a minimum of 4 h. Finally, it was kept in an oven overnight.

iii) *Block making and section cutting:* Blocks were made and sections (12 µm) thick were cut with the help of a rotary microtome.

iv) *Mounting and stretching:* A drop of egg albumen was applied on a clean slide after which 2-3 drops of distilled water were put on the slide and sections placed on it. These were stretched on a hot plate at 60°C and kept overnight at room temperature.

v) *Dewaxing:* Slides were put in pure xylol for 1-2 h and passed through each of the following grades for 2-3 min unless otherwise specified:

<table>
<thead>
<tr>
<th>Xylol (ml)</th>
<th>Ethanol (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 75</td>
<td>25</td>
</tr>
<tr>
<td>b) 50</td>
<td>50</td>
</tr>
<tr>
<td>c) 25</td>
<td>75</td>
</tr>
<tr>
<td>d) Rectified alcohol</td>
<td></td>
</tr>
<tr>
<td>e) 25 water</td>
<td>75 ethanol</td>
</tr>
<tr>
<td>f) 50 water</td>
<td>50 ethanol</td>
</tr>
<tr>
<td>g) 75 water</td>
<td>25 ethanol</td>
</tr>
<tr>
<td>h) Safranin (6-24 h)</td>
<td></td>
</tr>
<tr>
<td>i) 75 water</td>
<td>25 ethanol</td>
</tr>
<tr>
<td>j) 50 water</td>
<td>50 ethanol</td>
</tr>
<tr>
<td>k) 25 water</td>
<td>75 ethanol</td>
</tr>
<tr>
<td>l) Rectified alcohol</td>
<td></td>
</tr>
<tr>
<td>m) Ethanol</td>
<td></td>
</tr>
</tbody>
</table>
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n) Ethanol
o) Clove oil 25% in ethanol
p) Clove oil 50% in ethanol
q) Fast green (prepared in 50% clove oil)
r) Clove oil 50% in Xylol
s) Xylol (30 min)
t) Xylol (30 min)

vi) Mounted the slides in DPX

3.17. Agrobacterium strains and construct used for transformation

The transgene used in this study is the salT gene from rice (GenBank accession no LOC_Os01g24710.1). This was cloned in pCAMBIA2301 with Bam H1 and Sac1 restriction sites and the gene construct (Fig. 3.7) was finally transformed to EHA105 strain of A. tumefaciens. This vector has nptII (neomycin phosphotransferase) gene as the selectable marker, uidA (for GUS) as the reporter gene and a rbcS terminator. The salT gene was under the control of Cauliflower mosaic virus (CaMV 35S) promoter.

3.17.1. Preparation of Agrobacterium culture

Primary culture of Agrobacterium was prepared by inoculating single colony from a freshly streaked plate, in 5 ml of autoclaved liquid YEP medium (Appendix) supplemented with 10 mg/l rifampicin and 50 mg/l kanamycin. The culture was incubated for 16-18 h on a rotatory incubator shaker (ORBITEK, India) at 200 rpm in dark at 28°C. Secondary culture was prepared in a 500 ml baffled flask containing 100 ml YEP medium (supplemented with same antibiotics as used for primary culture) by adding 0.4% of the primary culture and grown under similar conditions. Once the OD\textsubscript{600} (optical density at 600 nm) reached ~1.0, Agrobacterium cells were pelleted by centrifugation at 8000g for 15 min at 4°C. The cells were resuspended in R-ECIM resuspension medium containing 100 μM acetosyringone to adjust the OD\textsubscript{600} of the bacterial suspension to 0.6.
3.17.2. Co-cultivation and selection of transformed calli

The 10 day subcultured embryogenic calli in R-ECIM medium were collected and Agro-infected by immersing them in the *Agrobacterium* (EHA105) culture for 15 min with intermittent gentle shaking at 40-50 rpm. The Agroinfected calli were blotted dry on sterile Whatman No. 3 filter paper for 5 min. Calli were then transferred to the co-cultivation medium (R2C; Appendix.) and incubated at 27±1°C in the dark for around 48 h. After the initial growth of *Agrobacterium* observed around most of the calli, the calli were rinsed 8-10 times with 250 mg/l cefotaxime in sterile distilled water, blotted dry on sterile Whatman No. 3 filter paper and transferred onto first selection medium- R₂S (200 µg/l cefotaxime, vancomycin 50 µg/l and 50 µg/l kanamycin) and incubated for 7 days at 27±1°C in dark. After the first selection, brown or black calli were removed and only creamish healthy calli were shifted to the fresh R₂CS Medium (Appendix) media for second selection and maintained at 27±1°C in dark. After second selection for about 20 days, microcalli could be observed which were finally transferred to fresh maturation media for third selection and allowed to proliferate for 5-7 days at 27±1°C in dark.

3.17.3. Regeneration of transformed calli

After third selection, black or brown microcalli were discarded and the putative transformed mature somatic embryos (with new growth) were transferred to regeneration medium (R-SERM; Appendix). These microcalli were incubated at 27±1°C in light for regeneration.