CHAPTER III
MATERIALS AND METHODS

The present investigation entitled “Study of differential gene expression in rice under drought stress” was carried out in the department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, Assam during my Ph.D. programme.

The techniques employed and materials used therein are described in this chapter.

3.1 Plant materials

The experiment was conducted with rice germplasm, which were collected from the Regional Agricultural Research Station, Gosaigaon, Assam Agricultural University and Instructional cum Research (ICR) farm, Assam Agricultural University, Jorhat. At first comparisons of some physiological and biochemical parameters were done between a drought tolerant local rice variety viz., Banglami (Kalita, 1996) and a high-yielding rice variety viz., Luit. As Banglami is a drought tolerant variety and all the studied parameters also are in support of it, so subsequently Banglami was taken for whole transcriptome analysis to study the differential gene expression under drought stress and well-watered condition.

3.2 Experimental site

The experimental site is situated at 26°47' N latitude, 94°12’ E longitude having an elevation of 86.6 m above mean sea level.

3.3 Weather and climate

The experimental area, Jorhat falls within the upper Brahmaputra valley Agro-Climatic zone of Assam. The zone is characterized by a subtropical climate with hot humid summer and relatively dry and cold winter.

3.4 Meteorological conditions

The meteorological data during the period of investigation (the growing season of the crop under study including the period of water stressed condition) with monthly averages were recorded at the Meteorological Observatory of Assam Agricultural
University, Jorhat. The total rainfall during the crop season (March, 2012 to August, 2012) was 1431.2 mm. The rainfall was lowest in the month of March (36.7 mm) and highest in the month of July (401.3 mm). The average rainfall during the crop season was (238.5 mm). The mean temperature during the crop season was 30.2°C (maximum) and 22.1°C (minimum) (Fig. 3.1, Appendix-II). Some important meteorological parameters during the crop growing season are as mentioned in Fig. 3.1.

3.5 Experimental site and treatment details

The crop was grown in pots, which were kept in the Net house of the Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat. Proper care was taken to control the water inputs from rainfall so that even a single drop of rainwater could not fall on the experimental site. Two varieties of rice viz., Banglami and Luit were studied at physiological and biochemical levels to compare them under well-watered control and drought stressed conditions. For differential gene expression study, the Banglami Variety, which is reported as drought tolerant (Kalita, 1996) was taken. For estimation of physiological and biochemical parameters ten replications were used for each treatments.

1. Treatment Details for Physiological and Biochemical Studies

   T₁ = Well watered control
   T₂ = Drought stressed for 15 Days

2. Varietal Details for Physiological and Biochemical Studies

   V₁ = Banglami
   V₂ = Luit

3. Treatment Details for differential gene expression study of Banglami Variety

   A = Well watered control
   B = Drought stressed for 15 Days

3.6 Rain shelter

As the experiment was conducted in the net house having a PVC roof, so 100 percent protection from rainwater was there.
FIG. 3.1. GRAPHS SHOWING DATA OF SOME METEOROLOGICAL PARAMETERS DURING THE CROP SEASON, 2012
3.7 Seed selection

Seeds of both the variety were put in two different beakers containing plain water and stirred well. Sunken seeds were selected rejecting the floating ones.

3.8 Seed treatment

Seeds of both the variety were put in two different containers and captan @ 2.5 g/kg was added to it. The fungicide was mixed thoroughly with seeds by agitating them for five minutes.

3.9 Growth conditions

Fine and sterilized field soil was used to germinate the rice seeds of two different varieties in earthen pots (22 cm height and 24 cm diameter). The pots were filled with a mixture of soil and organic matter (50:50) (Pieters and Souki, 2005). The pots were also fertilized with recommended doses of fertilizers (N: P2O5: K2O @ 40:20: 20 kg/ha).

3.10 Method of planting

Seeds were planted at a depth of 2-3 cm of the soil. After planting, seeds were covered by fine soils.

3.11 Moisture stress treatment

Water deficit stress was imposed by suspension of irrigation, to half of the plants of both the varieties and other half was kept irrigated. The moisture stress at seedling stage was imposed by withdrawing the water for 15 days between 30 and 45 days after sowing as described by Kalita (1996). Swain et al. (2014) also imposed moisture stress to 30 days old seedlings of rice for characterization of some Indian native land race rice accessions for drought tolerance at seedling stage.

Soil moisture content was measured using gravimetric method (Dastane, 1972). Soil samples were augured out from an average depth of 15-20 cm and collected in aluminium containers. The container along with the soil sample was weighed. Then the soil was dried in an oven at 105°C. Drying was allowed to continue until a constant weight was attained. After attaining the constant weight, the weight of the container plus dry soil was taken. The weight of the empty container was also recorded. The difference between the fresh weight and dry weight of soil determined the moisture content in the soil, which is expressed as percentage.
\[ W = \frac{W_1 - W_2}{W_2 - C} \times 100 \]

Where, \( W \) = Soil moisture content expressed in percent

\( W_1 \) = Weight of moist soil + container

\( W_2 \) = Weight of dry soil + container and

\( C \) = Weight of container

The average moisture content of soil ranged from 36-37 per cent under irrigated condition and it was maintained between 8-9 percent under moisture stress conditions during crop growth period (Table 3.1, Fig. 3.2). Under moisture stress condition the soil moisture content decreased to a level in which the normal growth and development of crop plants was hampered. In our study there was no any significant difference between the soil moisture content under water stress condition for both the varieties (Table 3.1).

**Table 3.1. Soil moisture content (%) under control and drought stress condition**

<table>
<thead>
<tr>
<th></th>
<th>Banglami (V₁)</th>
<th>Luit (V₂)</th>
<th>t-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_1 )</td>
<td>36.33</td>
<td>36.51</td>
<td>1.91(^{\text{NS}})</td>
</tr>
<tr>
<td>( T_2 )</td>
<td>8.82</td>
<td>8.93</td>
<td>1.78(^{\text{NS}})</td>
</tr>
</tbody>
</table>

\( t \)-Value 189.63** 151.27**

**: Significant at 1% level of significance

NS: Non-significant

![FIG. 3.2. AVERAGE SOIL MOISTURE CONTENT](image)
The photographs of Banglami and Luit variety of rice grown under well watered control condition and drought stressed condition are shown in Plate 3.1 and Plate 3.2. The photographs of root of both the variety grown under well watered control condition and drought stressed condition are also shown in Plate 3.3 and Plate 3.4.

3.12 Aftercare and intercare operation

The experimental site and pots were kept free from weeds by periodic hand weeding. Proper care was also taken so that the plants are free from pest and diseases.

3.13 Plant sampling

Five observational plants were tagged at random per pot for study of some physiological and biochemical parameters. For every case only the physiologically active leaves were used for studies and four leaves from different plants were sampled each time.

3.14 Methods used for evaluation of physiological and biochemical parameters

Sampling

Leaves were detached from five different plants from each treatment before 12:00 noon when solar PPFD ~ 500µmol m\(^{-2}\)s\(^{-1}\). The estimation of physiological and biochemical parameters were carried out using ten replications for each observation. For enzymatic study samples were quickly immersed in the bucket and 4°C temperature was maintained throughout the estimation.

3.14.1 Relative leaf water content (RLWC)

Twenty discs were taken from the leaves of each treatment and weighed. Leaf discs were submerged in water in a petridish for four hours. They were blotted and their saturated weight was measured. Discs were dried at 80°C till constant weight. Relative water content was calculated by following equation (Weatherly and Barrs, 1962) and was expressed as percentage.

\[
\text{Relative leaf water content} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100
\]

3.14.2 Chlorophyll content

Leaf chlorophyll was estimated by non-maceration method using Dimethyl Sulphoxide (DMSO) (Hiscox and Israelstam, 1979) and light absorption at 663 nm and 645 nm was recorded in spectrophotometer. The amount of chlorophyll content was calculated using absorption coefficients.
Plate 3.1. Seedlings of Banglami variety of rice: (a) Grown under well watered control condition and (b) Grown under drought stressed condition

Plate 3.2. Seedlings of Luit variety of rice: (a) Grown under well watered control condition and (b) Grown under drought stressed condition
Plate 3.3. Root of Banglami variety of rice at seedling stage: (a) Grown under well watered control condition and (b) Grown under drought stressed condition

Plate 3.4. Root of Luit variety of rice at seedling stage: (a) Grown under well watered control condition and (b) Grown under drought stressed condition
The fresh leaf material (0.5 g) in test tube containing 5 ml of DMSO was kept in an oven at 65°C for about 4 hours. Chlorophyll was extracted in a test tube and the volume was made 10 ml by using DMSO. The optical density (OD) of the extract was read at 663 nm and 645 nm using spectrophotometer. The chlorophyll content was determined by using the following formula and expressed as mg g⁻¹ leaf fresh weight.

\[
\text{Chlorophyll a} = \frac{V}{1000 \times W} \times [12.7 (A_{663}) - 2.69 (A_{645})]
\]

\[
\text{Chlorophyll b} = \frac{V}{1000 \times W} \times [22.9 (A_{645}) - 4.68 (A_{663})]
\]

\[
\text{Total chlorophyll} = \frac{V}{1000 \times W} \times [20.2 (A_{645}) + 8.02 (A_{663})]
\]

Where,

- \( A \) = Absorbance at specific wavelength
- \( V \) = Final volume of chlorophyll extract in 80% acetone
- \( W \) = fresh weight of the tissue extracted

3.14.3 Proline content

Free proline content in the leaves was determined following the method of Bates et al. (1973).

Reagents

(i) Sulphosalicylic acid (3%): Three gram of sulphosalicylic acid was dissolved in 100 ml of distilled water.

(ii) Orthophosphoric acid (6N): Required volume of orthophosphoric acid (38.1 ml) was taken and volume was made to 100 ml, using distilled water to get 6N phosphoric acid.

(iii) Acid ninhydrin: Ninhydrin (1.25 gram) was dissolved in a blend of 30 ml of glacial acetic acid and 20 ml of 6N orthophosphoric acid.

Method

Leaf sample (0.5 gram) was homogenized in 5 ml of sulphosalicylic acid (3%) using mortar and pestle. 2 ml of extract was taken in test tube and to it 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent were added. The reaction mixture was boiled in water bath at 100°C for 30 minutes, brick red colour was developed. After cooling the
reaction mixtures, 6 ml of toluene was added and then transferred to a separating funnel. After thorough mixing, the chromophore containing toluene was separated and absorbance was read at 520 nm in spectrophotometer against toluene blank. Concentration of proline was estimated by referring to standard curve of proline.

3.14.4 Nitrate reductase activity

In vivo nitrate reductase (NR, EC 1.6.6.1) activity was estimated spectrophotometrically at 540 nm (Thimmaiah, 1999).

Reagents

(i) Phosphate buffer (0.1 M, 7.5 pH)
(ii) Potassium nitrate, KNO$_3$ (200 mM)
(iii) 0.5% n-propanol
(iv) 1N HCl
(v) Sulphanilamide (1% in 1N HCl)
(vi) 0.02% naphthylethylene diamine hydrochloride (NEDD)

Method

0.3 gram fresh leaf sample was cut into small pieces and put in a test tube filled with incubation medium (0.1 M phosphate buffer at 7.5 pH and 200 mM KNO$_3$ and 0.5% n-propanol) and kept in dark for 2 hours at 30°C. Thereafter, to 1 ml aliquot, 1 ml sulphanilamide and 1 ml 0.02% NEDD was added and shaken thoroughly. After keeping for 25 minutes for colour development absorbance was read at 540 nm. Nitrate reductase activity was calculated employing standard curve of nitrate and expressed in µmol NO$_2$ g$^{-1}$ FW hr$^{-1}$ on a fresh weight basis.

3.14.5 Antioxidative enzymes

(a) Superoxide dismutase (SOD; EC 1.15.1.1)

The superoxide dismutase (SOD; EC 1.15.1.1) activity was determined spectrophotometrically by measuring the inhibition of blue diformazone formation in the presence of riboflavin/ nitro-blue tetrazolium (NBT) and light (Beauchamp and Fridovich, 1971).
Reagents

(i) Methionine solution (200 mM)
(ii) Riboflavin solution (75 µM)
(iii) Nitro-blue tetrazolium (NBT) solution (1.125 mM)
(iv) Ethylene diamine tetra acetic acid (EDTA) disodium solution (1.5 mM)
(v) Phosphate buffer (50 mM; pH 7.0)
(vi) Phosphate buffer (50 mM; pH 7.8)

Enzyme extraction

Leaf sample weighing 500 mg was homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.5 mM EDTA in a pre-chilled pestle and mortar. The homogenate was centrifuged at 4°C for 30 minutes at 30,000 x g. The supernatant was transferred to another tube and was used as enzyme extract.

Assay

The assay solution consisted of 0.2 ml methionine (200 mM), 0.2 ml NBT (1.125 mM), 0.2 ml EDTA (1.5 mM), 40 µL enzyme extract, 2.19 ml phosphate buffer at pH 7.8 and 0.2 ml Riboflavin (75 µM). Riboflavin was added as the last component. Tubes were shaken and placed 30 cm below a light source, consisting of two 15 W fluorescent tubes. The reaction was started by switching on the light and was allowed to run for 10 minutes. Light was switched off after 10 minutes to stop the reaction. The tubes were covered with black cloth immediately after switching off the light. A non-irradiated reaction mixture containing enzyme extract, which did not develop colour, was used as control. Blanks were lacking of enzyme in reaction mixture and developed maximum colour. The absorbance of the reaction mixture was read at 560 nm. The volume of the enzyme extract producing 50 percent inhibition of the reaction was read from the resultant graph. Activity is expressed at unit mg⁻¹ protein. One unit of SOD activity is defined as the enzyme which causes 50 percent inhibition of the initial rate of reaction in the absence of enzyme (Beauchamp and Fridovich, 1971).

(b) Catalase (CAT; EC 1.11.1.6)

Catalase activity in leaves was measured following the method of Teranishi et al. (1974) with minor modification. Reaction mixture without enzyme developed maximum colour with titanium reagent. Catalase activity based colour
intensity was measured spectrophotometrically at 415 nm. The enzyme activity was assayed by estimating the residual H$_2$O$_2$ in the reaction using H$_2$O$_2$ standard.

**Reagents**

(i) 0.1 M potassium phosphate buffer (pH 7.5)

(ii) Titanium reagent

(iii) H$_2$O$_2$ solution (2 mM)

**Enzyme extraction**

Fresh leaf sample of 500 mg was macerated in 0.1 M potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 12,000 x g for 20 minutes at 0°C. The supernatant was collected and used as enzyme extract.

**Assay**

The reaction mixture comprised of 100 µL of enzyme extract, 1 ml of 2 mM H$_2$O$_2$ and 1.9 ml of 0.1 M potassium phosphate buffer in 10 ml test tube. The reaction mixture was incubated at 30°C for five minutes. The reaction was stopped by adding 4 ml of titanium reagent and resultant mixture was centrifuged at 3,000 x g for 10 minutes to get clear supernatant. The absorbance was recorded at 415 nm in spectrophotometer. The residual H$_2$O$_2$ content in sample were computed with the help of standard curve. Catalase activity was expressed as µmol H$_2$O$_2$ min$^{-1}$mg$^{-1}$ protein.

**Ascorbate peroxidase (APOD; EC 1.11.1.11)**

Ascorbate peroxidase activity was measured by a modified spectrophotometric procedure based on the rate of decrease in absorption of ascorbate at 290 nm during ascorbate oxidation (Dalton *et al.*, 1986).

**Reagents**

(i) 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.0)

(ii) 0.5 mM EDTA

(iii) 250 µM ascorbate

(iv) 1.5 mM H$_2$O$_2$
Enzyme extraction

Leaf sample weighing 500 mg was homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.5 mM EDTA in a pre-chilled pestle and mortar. The homogenate was centrifuged at 30,000 x g in 4°C for 30 min. The supernatant was transferred to another tube and used as enzyme extract.

Assay

The assay was performed in a 1 ml cuvette containing 250 µM ascorbate, 50 mM KH$_2$PO$_4$/ K$_2$HPO$_4$ (pH 7.0) and 0.5 mM EDTA, 1.5 mM H$_2$O$_2$ and 100 µL enzyme extract. The rate of decrease in absorption of ascorbate at 290 nm during ascorbate oxidation recorded spectrophotometrically and enzyme activity was expressed as n mol min$^{-1}$ g$^{-1}$ FW.

(d) Peroxidase (POD; EC 1.11.1.7)

The peroxidise activity was measured spectrophotometrically based on the changes in the absorption at 430 nm (Thimmaiah, 1999).

Reagents

(i) 0.1 M Potassium phosphate buffer (ph 6.0)
(ii) 0.01 Mo-Dianisidine in methanol
(iii) 0.02 M Hydrogen peroxide
(iv) 2 N H$_2$SO$_4$

Enzyme extraction

Fresh leaf sample of 1 gram was homogenized in 10 ml of ice-cooled 0.1 M phosphate buffer (pH 6.0). The homogenate was centrifuged at 16,000 x g at 4°C for 20 minutes. The supernatant was used as enzyme extract.

Assay

The assay mixture comprised of 0.2 ml enzyme extract, 1 ml phosphate buffer, 1 ml o-Dianisidine, 0.5 ml H$_2$O$_2$ and 2.4 ml distilled water. The blank was lacking of H$_2$O$_2$. After 5 minutes of enzyme extract addition, the reaction was stopped by adding 1 ml of 2 N H$_2$SO$_4$. The absorbance was measured spectrophotometrically at 430 nm. The specific activity of enzyme was expressed as µmol H$_2$O$_2$ reduced mg$^{-1}$ protein min$^{-1}$.
(e) **Glutathione reductase (GR; EC 1.6.4.2)**

Glutathione reductase activity was measured by a spectrophotometric procedure based on the rate of increase in absorbance at 412 nm in presence of substrate, oxidized glutathione (GSSG) following the method of Smith *et al.* (1988).

**Reagents**

(i) 0.1 M potassium phosphate buffer (pH 7.5)

(ii) 0.2 M potassium phosphate buffer (pH 7.5)

(iii) 0.5 mM EDTA

(iv) 1 mM EDTA

(v) 3 M DTNB

(vi) 2 mM NADPH

(vii) 20 mM GSSG (oxidized glutathione)

**Enzyme extraction**

Leaf sample weighing 500 mg was macerated in 10 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.05 mM EDTA and filtered through cloth. The filtrate was centrifuged for 15 minutes at 15,000 x g. Supernatant was collected and used as enzyme extract.

**Assay**

The assay mixture comprised of 0.5 ml of 3 M DTNB (5,5-dithio bis-2 nitro benzoic acid) prepared in phosphate buffer (pH 7.5), 0.1 ml of 0.2 M potassium phosphate buffer (pH 7.5 containing 1 mM EDTA), 0.2 ml of 2 mM NADPH and 0.2 ml of enzyme extract and final volume was made to 2.9 ml with distilled water. The increase in absorbance at 412 nm was measured immediately after adding GSSG over a period of 10 minutes with an interval of 1 minute. Glutathione reductase activity was expressed as \( \Delta OD_{412nm} \text{ min}^{-1} \text{mg}^{-1} \text{ protein} \)

(f) **Enzyme protein estimation**

The enzyme protein content was determined according to the method of Lowry *et al.* (1951) using BSA as standard.
3.14.6 Photosynthesis

The rate of photosynthesis was measured on leaves using portable Infrared Gas Analyzer (LI-COR Biosciences, USA). Infrared gas Analysis of CO₂ is the method of determining CO₂ concentration for the measurement of photosynthesis and respiratory rates of plants.

Operation

The target leaf was inserted in the leaf chamber facing the sun. The vapour pressure was monitored on the display board after inserting the leaf, and flow rate was adjusted till a constant rate was obtained. The incident photon flux density was monitored from the quantum sensor mounted on the leaf chambers. Assimilation by the enclosed leaf area caused a depletion of CO₂ concentration inside the chamber and this depletion was measured for three consecutive times of 10 seconds each. Based on the CO₂ depletion and other factors such as temperature, pressure and volume of the enclosure, the rate of photosynthesis was computed by the in-built microcomputer of the IRGA, which was expressed in µmol CO₂ m⁻² s⁻¹.

3.14.7 Transpiration

The rate of transpiration was measured on leaves using a portable Infrared Gas Analyzer (LI-COR Biosciences, USA) during recording of the photosynthesis data.

3.14.8 Stomatal conductance

The stomatal conductance was measured following the method of Centritto et al. (1999) using a portable Infrared Gas Analyzer (LI-COR Biosciences, USA). The stomatal conductance was expressed as mmol m⁻² s⁻¹.

3.15 Statistical analysis

Statistical analysis of data was performed by following the method of Student’s t- test described by Gomez and Gomez (1984) and Panse and Sukhatme (1989). Analysis was performed using software “SPSS for MS Windows” (Version 7.0). The significance was tested at 1% and 5% level of significance.

3.16 Collection of the leaf sample for RNA isolation

For isolation of RNA for differential gene expression study, only Banglami variety of rice which is reported as drought tolerant (Kalita, 1996) was selected and plants grown under control (soil moisture content: 36.33% and relative leaf water content: 88.79%) and drought stress (soil moisture content: 8.82% and relative leaf water content: 71.54%) conditions were tagged. Physiologically active tender leaves from the respective
plants grown under different conditions were collected separately. Immediately after collection, the leaf samples were stored at low temperature until use.

3.17 Isolation of RNA

3.17.1 General precautions for handling RNA

Care was taken to avoid contamination with RNase enzymes which cause destruction of RNA. All glassware were baked overnight in a 180°C oven. Plastic ware were incubated overnight with 0.1% DEPC and then autoclaved for 30 min. General lab equipments, pipettors and working surfaces were cleaned with RnaseZAP (Ambion, USA) which is effective in eliminating RNase contamination. Gloves were frequently changed.

3.17.2 Isolation of total RNA

Total RNA was isolated from leaf samples using Trizol Reagent (Invitrogen) by following the procedures as follows:

1. Homogenization: The leaf sample was ground in liquid nitrogen and then 5 ml of Trizol Reagent was added to the grinded sample and subsequently vortexing was done.

2. Phase Separation: After vortexing, the sample was incubated for 5 minutes at room temperature. After 5 minutes of incubation, 1 ml of chloroform was added and a vigorous shaking was done by hand for 15 sec and then again incubated at room temperature for 2-3 minutes. Then the samples were centrifuged at 12000 x g for 15 minutes at 4ºC. (Three layers were observed: the upper is aqueous phase; the middle is inter phase; and the lower is chloroform phase).

3. RNA precipitation: The aqueous phase was transferred to a fresh tube. Then the RNA was precipitated by mixing with 2.5 ml of isopropyl alcohol (2-propanol). After that the sample was incubated at room temperature for 10 minutes and subsequently centrifuged at 12000 x g for 10 minutes at 4ºC.

4. RNA wash: Supernatant was removed and subsequently the pellet was washed with 5 ml of 75% ethanol. Then the sample was mixed by vortexing and centrifuged at 6500 x g for 5 minutes at 4ºC.

5. Re-dissolving the RNA: The RNA pellet was air dried (for 20 minutes). After that RNA pellet was dissolved in 200µL of RNAase-free water by passing the solution a
few times through a pipette tip, and subsequently incubated for 10 minutes at 55-60°C.

6. Then the isolated RNA was stored at -70°C.

3.17.3 Quantification

The RNA thus extracted was subjected to spectrophotometric analysis. Quantification of RNA was done by monitoring the absorbance at 260 nm. Spectrophotometric reading was also recorded at 280 nm to know the quality of isolated RNA and thus $A_{260}/A_{280}$ ratio was calculated.

The concentration of RNA sample was calculated by using the formula:

$$\text{Concentration of RNA in } \mu \text{g/ml} = A_{260} \times \text{dilution factor} \times 40$$

(1 OD$_{260}$ Unit = 40µg/ml of ssRNA)

The total yield of RNA was calculated by using the formula:

$$\text{Total Yield} = \text{Concentration} \times \text{Volume of sample in milliliters}$$

3.17.4 Denaturing agarose gel electrophoresis of total RNA

The quality of total RNA was checked on 1% denaturing Agarose gel (loaded 5µl) for the presence of 28S and 18S bands.

3.17.4.1 Preparation of the gel

For preparation of 1% formaldehyde agarose gel of size 10 x 8 x 0.5 cm 0.5 g of agarose was heated in 36 ml of RNase-free water (DEPC treated autoclaved water) for dissolving the agarose and then it was cooled to 60°C. After that 5ml of 10X MOPS running buffer and 9 ml of 37% formaldehyde (12.3M) were added. Then the gel was poured using a comb that forms wells large enough to accommodate enough samples. After solidification the gel was assembled in the tank, and enough 1X formaldehyde agarose gel running buffer was added to cover the gel by a few millimeters, then comb was removed. Prior to running, the gel was equilibrated in the running buffer for 30 minutes.

(Composition of 10X MOPS running buffer: 0.4M MOPS, pH 7.0, 0.1M sodium acetate, 0.01 M EDTA)
3.17.4.2 Preparation of 1X Formaldehyde agarose gel running buffer

50 ml of 10X MOPS, 10 ml of 37% (12.3M) formaldehyde and 440 ml of RNase-free water were mixed for preparation of 1X Formaldehyde agarose gel running buffer.

3.17.4.3 Preparation of RNA sample

10μl of RNA, 10μl of formamide, 2μl of ethidium bromide, 3μl of 10X MOPS and 4μl of formaldehyde were mixed and the solution was incubated at 80ºC for 20 minutes and then it was chilled in ice water for 30 minutes. After that the solution was centrifuged for 5 seconds for collecting all the fluids at bottom of the centrifuge tube and then 3μl of 10X formaldehyde gel loading buffer was added to the solution. Immediately after addition of the loading buffer, the centrifuge tubes containing the solution were transferred to ice bucket till loading.

3.17.4.4 Loading of the gel

The prepared RNA sample was loaded into the well of the gel.

3.17.4.5 Electrophoresis of the gel

The electrophoresis was done at 5-6V/cm and it was done until the bromophenol blue (the faster migrating dye) has migrated to 2/3 length of the gel. Gel of same size running at 100 V for 30 minutes also gave good result.

3.17.4.6 Visualization of the gel

The gel was visualized in a Gel documentation system (Bio Rad, USA) using UV light. Photograph was taken for record.

3.18 Illumina NextSeq 2 x 150 PE library preparation

The pair-end cDNA sequencing libraries were prepared using Illumina TruSeq RNA Library Preparation Kit as per protocol. Library preparation was started with mRNA enrichment and fragmentation. The mRNA were converted into first-strand cDNA, followed by second-strand generation, end repair, A-tailing, adapter ligation, and finally ended by index PCR amplification of adaptor-ligated library. Library quantification and qualification was performed using DNA High Sensitivity Assay Kit.

The detail protocols starting from mRNA enrichment and fragmentation to validation of library are described below:
3.18.1 mRNA enrichment and fragmentation

This process enriches the polyA containing mRNA molecules using oligo-dT attached magnetic beads using two rounds of purification. During the second elution of the polyA RNA, the RNA is also fragmented and primed for cDNA synthesis.

Preparation

- The buffers (bead binding buffer, bead washing buffer, elution buffer and resuspension buffer) and Elute, Prime, Fragment Mix were removed from -25°C to -15°C storage and thawed at room temperature.
- The RNA purification beads tube was removed from 2°C to 8°C storage and then it was allowed to bring to room temperature.
- The thermal cycler was pre-programmed with the following programs:
  - The pre-heat lid option was chosen and it was adjusted at 100°C
  - 65°C for 5 minutes, 4°C hold — save as mRNA Denaturation
  - 80°C for 2 minutes, 25°C hold — save as mRNA Elution 1
  - 94°C for 1 minutes, 4°C hold — save as Elution 2 -Frag -Prime

Procedure

- The total RNA of each samples were diluted with nuclease-free ultra pure water to a final volume of 50 μl in two separate 0.2 ml PCR tubes.
- The RNA purification beads tubes, which were kept at room temperature, were vigorously vortexed to resuspend the oligo-dT beads.
- 50 μl of RNA purification beads was added to each of the PCR tube to bind the polyA RNA to the oligo-dT beads. The entire volume of each tube was gently pipetted up and down for 6 times to mix thoroughly.
- The tubes were placed on pre-programmed thermal cycler. Then the lid was closed and mRNA Denaturation was selected (65°C for 5 minutes, 4°C hold) to denature the RNA and facilitate binding of the polyA RNA to the beads.
- The tubes were removed from the thermal cycler when it reaches 4°C.
- The tubes were placed on the bench and then incubated at room temperature for 5 minutes to allow the RNA to bind to the beads.
Then the tubes were placed on the magnetic stand at room temperature for 5 minutes to separate the polyA RNA bound beads from the solution.

All of the supernatant from the tubes were removed and discarded.

The tubes were removed from the magnetic stand.

The beads were washed by adding 200 μl bead washing buffer in each tube to remove unbound RNA. The entire volume of each tube was gently pipetted up and down for 6 times to mix thoroughly.

Again the tubes were placed on the magnetic stand at room temperature for 5 minutes.

The thawed elution buffer was centrifuged at 600 x g for 5 seconds.

Then all of the supernatant from each tube were removed and discarded. The supernatant contains most of the ribosomal and other non-messenger RNA.

The tubes were removed from the magnetic stand.

Then 50 μl of elution buffer was added to each tube. The entire volume of each tube was gently pipette up and down for 6 times to mix thoroughly.

The tubes were placed on the pre-programmed thermal cycler. The lid was closed and then mRNA Elution 1 was selected (80°C for 2 minutes, 25°C hold) to elute the mRNA from the beads. Both the mRNA and any contaminant rRNA that have bound to the beads nonspecifically were released.

The tubes were removed from the thermal cycler when it reaches 25°C.

Then 50 μl of bead binding buffer was added to each tube. This allows mRNA to specifically rebind the beads, while reducing the amount of rRNA that nonspecifically binds. The entire volume of each tube was gently pipette up and down 6 times to mix thoroughly.

The tubes were incubated at room temperature for 5 minutes.

Then the tubes were placed on the magnetic stand at room temperature for 5 minutes.

All of the supernatant from each tube were removed and discarded. Then the tubes were removed from the magnetic stand.

The beads were washed by adding 200 μl bead washing buffer in each tube.
The entire volume was gently pipette up and down for 6 times to mix thoroughly.

Then the tubes were again placed on the magnetic stand at room temperature for 5 minutes.

All of the supernatant from each tube was removed and discarded. The supernatant contains residual rRNA and other contaminants that were released in the first elution and did not rebind the beads.

The tubes were removed from the magnetic stand and 19.5 μl of Elute, Prime, Fragment Mix was added to each tube. The entire volume of each tube was gently pipette up and down 6 times to mix thoroughly. (The Elute, Prime, Fragment Mix contains random hexamers for RT priming and serves as the first strand cDNA synthesis reaction buffer).

The tubes were placed on the pre-programmed thermal cycler. Then the lid was closed and Elution 2 - Frag - Prime was selected (94°C for 8 minutes, 4°C hold) to elute, fragment, and prime the RNA.

The tubes were removed from the thermal cycler when it reaches 4°C and was centrifuged briefly.

Then just after that immediately proceeded to synthesize first strand cDNA.

3.18.2 First strand cDNA synthesis

This process reverse transcribes the cleaved RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase and random primers.

Procedure

The tubes containing RNA fragments primed with random hexamers were placed on the magnetic stand at room temperature for 5 minutes. The tubes were not removed from the magnetic stand.

17 μl of the supernatant (fragmented and primed mRNA) from each tube was transferred to the corresponding new 0.3 ml PCR tube.

The thawed tube of first strand master mix was centrifuged at 600 × g for 5 seconds.
8 μl (Superscript II 1 μl to 9 μl first strand mix) was added to each tube. Then the entire volume of each tube was gently pipette up and down for 6 times to mix thoroughly.

The tubes were placed on the pre-programmed thermal cycler. The lid was closed, and then the 1st Strand program was selected and started:

- 25°C for 10 minutes
- 42°C for 50 minutes
- 70°C for 15 minutes
- Hold at 4°C

### 3.18.3 Second strand cDNA synthesis

This process removes the RNA template and synthesizes a replacement strand to generate ds cDNA. AMPure XP beads (Beckman Coulter) are used to separate the ds cDNA from the second strand reaction mix.

- The thawed second strand master mix was centrifuged at 600 × g for 5 seconds.
- Then 25 μl of thawed second strand master mix was added to each tube. The entire volume of each tube was gently pipette up and down for 6 times to mix thoroughly.
- The tubes were placed on the pre-heated thermal cycler. Then the lid was closed and was incubated at 16°C for 1 hour. Subsequently the tubes were allowed to stand for bring it to the room temperature.

### Purifications

- The AMPure XP beads (Beckman Coulter) were vortexed until they were well dispersed.
- 90 μl well-mixed AMPure XP beads (Beckman Coulter) were added to each tube containing 50 μl ds cDNA. The entire volume of each tube was gently pipette up and down 10 times to mix thoroughly.
- The tubes were incubated at room temperature for 15 minutes.
- Then the tubes were placed on the magnetic stand at room temperature for 5 minutes to make sure that all of the beads were bound to the side of the wells.
135 μl of the supernatant from each tube was removed and discarded.

The tubes were kept on the magnetic stand while performing the following 80 percent ethanol wash steps.

With tubes on the magnetic stand, 200 μl of freshly prepared 80 percent ethanol was added to each tube without disturbing the beads.

Incubated at room temperature for 30 seconds, and then all of the supernatant was removed and discarded from each well.

For a total of two 80 percent ethanol washes, one more time was repeated

Then the tubes were allowed to stand at room temperature for 15 minutes to dry, and then the tubes were removed from the magnetic stand.

The thawed, room temperature resuspension buffer was centrifuged at 600 × g for 5 seconds.

52.5 μl of resuspension buffer was added to each tube. The entire volume of each of the tube was gently pipette up and down 10 times to mix thoroughly.

Incubated at room temperature for 2 minutes. The tubes were placed on the magnetic stand at room temperature for 5 minutes.

50 μl of supernatant (ds cDNA) from each tube was transferred to two new 0.2 ml PCR tubes.

3.18.4 End-Repair

10 μl of RSB (resuspension buffer) and 40 μl of properly thawed end repair mix were added to each PCR tube having 50 μl of ds cDNA and then it was gently mixed by pipetting (pipetting was done at least for 10 times).

Then the mixtures were placed on pre-heated thermal cycler and incubated at 30°C for 30 min. Then proceeded for purification.

Purification/ Clean Up

The whole content of each of the tube were transferred to two separate fresh 1.5 ml eppendorff tubes. 160 μl of well mixed AMPure XP (Beckman Coulter) beads were added to 100 μl of end repaired ds cDNA of each of the sample, then it was gently mixed by pipetting and incubated for 15 min at room temperature.
The tubes were placed on magnetic stand at RT for 5 min. Then the clear supernatant was discarded (approx. 250 μl for each sample).

200 μl of 80 percent ethanol was added to each tube without disturbing the beads, then the mixture was hold for 30 seconds on magnetic stand and subsequently the clear supernatant was discarded.

The wash step repeated for one more time and then the tubes were allowed to stand for 15 min at room temperature over the magnetic stand for complete drying of residual ethanol.

The tubes were removed from the magnetic stand, 17.5 μl of RSB was added to each tube, thorough mixing was done and then incubated for 2 min at room temperature and was placed on the magnetic stand at room temperature for 5 min.

15 μl of clear supernatant from each tube was transferred to two new PCR tube.

3.18.5 3’ End adenylation and adapter ligation

2.5 μl of RSB and 12.5 μl of properly thawed A-Tailing Mix were added to 15 μl of end repaired cDNA of each sample; it was gently mixed with pipette and was placed on pre-heated thermal cycler and incubated at 37°C for 30 min. After incubation immediately proceeded for adapter ligation.

RNA adapter index was chosen according to the number of libraries to be pooled in a single run and it was noted in work-sheet (index compatibility chart was referred).

2.5 μl of RSB, 2.5 μl of properly thawed ligation mix and 2.5 μl of appropriate RNA adapter index were added and then it was gently mixed by slow pipetting. This was also followed for both the sample.

Placed on thermal cycler and incubated for 20 min at 30°C. After incubation 5 μl of stop ligation buffer was added to each tube, mixed thoroughly and proceeded for clean up.
Purification/ Clean Up

- The whole content of both tube were transferred to two separate fresh 1.5 ml eppendorff tube. Then 42 µl of well mixed AMPure XP beads (Beckman Coulter) were added to 42.5 µl of adapter ligated cDNA of each sample, then it was mixed gently by pipetting and incubated for 15 min at room temperature.

- The tubes were placed on magnetic stand at room temperature for 5 min and then clear supernatant was discarded (approx. 80 µl for each sample).

- 200 µl 80 per cent ethanol was added to each tube without disturbing the beads, holded for 30 seconds on magnetic stand and then the clear supernatant was discarded.

- The wash step was repeated for one more time and then the tubes were allowed to stand for 15 minutes at room temperature over the magnetic stand for complete drying of residual ethanol.

- The tubes were removed from the magnetic stand, 52.5 µl of RSB was added to each tube, mixed thoroughly, incubated for 2 minutes at room temperature and was placed on the magnetic stand at room temperature for 5 minutes.

- 50 µl of clear supernatant from each sample was transfer to new PCR tubes and then proceeded for 2nd purification.

- 50 µl of well mixed AMPure XP beads (Beckman Coulter) were added to 50 µl of purified adapter ligated cDNA of both samples, then it was gently mixed by pipetting and incubated for 15 minutes at room temperature.

- The tubes were placed on magnetic stand at room temperature for 5 minutes. Clear supernatant was discarded (approx. 80 µl for each sample).

- 200 µl 80 percent ethanol was added to each tube without disturbing the beads, holded for 30 seconds on magnetic stand and the clear supernatant was discarded.

- The wash step was repeated for one more time and then the tubes were allowed to stand for 15 minutes at room temperature over the magnetic stand for complete drying of residual ethanol.
The tubes were removed from the magnetic stand, 22.5 μl of RSB was added to each tube, thoroughly mixed and then incubated for 2 minutes at room temperature and was placed on the magnetic stand at room temperature for 5 minutes.

20 μl of clear supernatant from each sample was transferred to new PCR tubes.

### 3.18.6 Library enrichment

The PCR master mix and PCR primer cocktail were removed from -20°C storage. Then thawed at room temperature and spinned for a short period after which they were placed on ice.

Then 5 μl of properly thawed PCR primer cocktail was added and subsequently 25 μl of thawed PCR master mix were added to 20 μl of adapter ligated library of each sample from above to a total volume of 50 μl.

Thorough mixing was done with slow repeated pipetting followed by a short spinning.

Then the reaction mixture containing tubes were placed on a pre-programmed thermal cycler with the following program.

The pre-heat lid option was chosen and was set to 100°C

- 98°C for 30 seconds
- 15 cycles of: 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

### PCR Clean Up

The whole content of each tubes were transferred to separate fresh 1.5 ml eppendorff tubes. 50 μl of well mixed AMPure XP beads (Beckman Coulter) were added to 50 μl of PCR product. The entire volume of each tubes were gently pipetted up and down for at least 10 times to mix thoroughly and then incubated for 15 minutes at room temperature.

The tubes were placed on magnetic stand at room temperature for 5 minutes. Then clear supernatant from each tube (approx. 90 μl from each tube) was discarded.
200 µl of 80 percent ethanol was added to each tube without disturbing the beads, held for 30 seconds on magnetic stand and the clear supernatant was discarded from each tube.

The wash step was repeated for one more time and then the tubes were allowed to stand over the magnetic stand for 15 minutes at room temperature for complete drying of residual ethanol.

The tubes were removed from the magnetic stand, 50 µl of RSB was added to each tube and thorough mixing was done, then tubes were incubated for 2 minutes at room temperature and subsequently the tubes were placed on the magnetic stand for 5 minutes at room temperature.

25 µl of RSB was added to each tube, then thorough mixing was done and incubated for 2 minutes at room temperature and then the tubes were placed on the magnetic stand for 5 minutes at room temperature.

23 µl of clear supernatant from each tube was transferred to two new PCR tubes.

3.18.7 Library validation

1 µl of resuspended construct from each sample was loaded on an Agilent Technologies 2100 Bioanalyzer using DNA High Sensitivity kit as per protocol provided by manufacturer.

The size and purity of the samples were checked.

3.19 Cluster generation and sequencing run

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyzer profile, 1.8 pM of Library was loaded into the reagent cartridge 300 cycles PE (Illumina). Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions. Cluster generation was carried out by hybridization of template molecules onto the oligonucleotide-coated surface of the flow cell. Immobilized template copies were amplified by bridge amplification to generate clonal clusters. This process of cluster generation was performed on NextSeq using reagent Kit 300 cycles PE (Illumina). The kit reagents were used in binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the
reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

3.20 Data Generation on NextSeq

The raw data of two plant samples were generated on NextSeq. At first, the libraries have been sequenced using 2 X 150 PE chemistry. Then the generated raw reads were filtered to obtain high quality reads. The raw reads were filtered using Trimmomatic (v 0.30) (Bolger et al., 2014) with quality value QV > 20 and other contaminants such as adapters were trimmed. Parameters considered for filtration are as follows:

- Adapter trimming was performed
- SLIDINGWINDOW: Perform a sliding window trimming of 10 bp, cutting once the average quality within the window falls below a threshold of 20
- LEADING: Cut bases off the start of a read, if below a threshold quality of 20
- TRAILING: Cut bases off the end of a read, if below a threshold quality of 20
- MINLENGTH: Drop the read if it is below 100 bp length

3.21 Mapping reads to the reference genome and annotated transcripts

The Oryza sativa subsp. indica genome and gene information (GTF file) were downloaded from Ensemble Plant database (http://plants.ensembl.org/Oryza_indica/Info/Annotation). Bowtie2 (v2.1.0) (Langmead et al., 2009) was used to index the reference genome and to map sequencing reads to the indexed genome. After trimming low quality bases (Q<20) from 5' and 3’ ends of the reads, high quality reads of two plant samples (both are FastQ files) were mapped on to the Oryza sativa subsp. indica reference genome using TopHat (version-2.0.9) (Kim et al., 2013) and then mapped reads were assembled by using cufflinks (v 1.3.0) (Trapnell et al., 2012) with default parameter to generate transcript annotation. For alignment, TopHat was run with the default parameters except for a maximum intron length of 5000 bp.

3.22 Analysis of differential gene expression

The expression analysis or the expression profiling of the transcripts/genes were carried out with cufflinks pipeline using reference annotation-based transcript (RABT) assembly (Roberts et al., 2011), which is based on GTF annotation file. The assembly file (GTF file) generated for both well watered control...
(Sample-A) and drought stressed sample (Sample-B) were compared to the reference annotation and then resulted GTF file was taken as input for analysis of differential gene expression by cuffdiff (Trapnell et al., 2013). The expressions of the transcripts/genes were quantified in terms of FPKM (Fragment per kilobase per million mapped reads).

\[
\text{FPKM} = \frac{(\text{No. of reads mapped} \times 10^9)}{\text{(Length of gene} \times \text{Total no. of reads})}
\]

The FPKM values for each transcript/gene were calculated for the combination of control and drought samples with Cufflinks pipeline. These FPKM values were further used to calculate the log fold change \([\log_2 (\text{FPKM}_{\text{Drought}}/\text{FPKM}_{\text{Control}})]\). Moreover, an uncorrected p-value of the test statistic for each transcript/gene was also computed. The analysis was carried out for commonly expressed transcripts/genes reported between control and drought samples, respectively. These transcripts/genes were further divided on the basis of their statistical significance [which can be either “yes” or “no”, depending on whether p value is less than 0.05 and the FDR (False discovery rate) 0.05 after Benjamin-Hochberg correction for multiple-testing] for their significant expression. These transcripts/genes were further categorized as up-regulated and down-regulated in drought stressed sample as compared to well watered control sample. Gene that exhibited an FDR<=0.05 and estimated absolute log2 fold change were determined to be significantly expressed genes (Log2 fold change value > 0 for Up regulation and Log2 fold change value < 0 Down regulation).

The differentially expressed transcripts/genes were represented graphically by Heat map and Volcano plot. For representation by Heat map, an average linkage hierarchical cluster analysis was performed on top 100 differentially expressed transcripts/genes using Multiple experiment viewer (MEV v4.8.1) (Saeed et al., 2003) and for representation by Volcano plot, Cummerbund (R package) (Goff et al., 2014) was used.

### 3.23 Downstream analysis

#### 3.23.1 Gene Ontology enrichment analysis

In order to determine the biological function of differentially expressed genes between well watered control and drought stressed samples, Gene Ontology (GO) based enrichment tests were performed. Gene Ontology enrichment analysis was conducted using AgriGO tool (Du et al., 2010) with a background reference of Rice TIGR gene model which has total annotation GO number 30241.
3.23.2 Pathway analysis

KAAS (KEGG Automatic Annotation Server) (Moriya et al., 2007) was used to functionally annotate differentially expressed transcripts/genes by BLAST comparisons against KEGG GENES database (Kanehisa et al., 2014). KEGG is the major public pathway-related database, providing classification that is valuable for research on genetically and biologically complex behaviours. It contains systematic analysis of inner-cell metabolic pathways and functions of gene products, which aid in studying the complex biological behaviours of genes. The BBH (Bi-directional best hit) option was used to assign KO terms (representative gene data set Oryza sativa). The KEGG database (http://www.genome.jp/kegg/ko.html) (Kanehisa et al., 2014) was used for pathway mapping.

3.23.3 Mapping of gene to drought responsive QTLs of rice genome

In order to map the differentially expressed genes/transcripts to drought responsive QTLs of rice genome, the QTL information with transcription profiles was derived from Gramene (http://www.gramene.org/qtl/) (Monaco et al., 2014). Then, at the whole genome level the mapping of the differentially expressed genes/transcripts and QTL by their individual positions on the rice genome was done and represented by using Circos (Krzywinski et al., 2009).