3. MATERIALS AND METHODS
3. Materials and Methods

3.1 Materials

3.1.1 Chemicals, reagents, and consumables

| Chemicals for Bacterial Media Preparations | Hi-Media Inc Ltd India |
| Plasmid Miniprep purification modules | Qiagen Inc GMBH, Machery Nagel GMBH, Novagen USA. |
| Dynamic ET Terminator Mix for Automated sequencing | Amersham Pharmacia Biotech, Sweden |
| Sequencing Capillaries, Matrix, Loading buffer for automated capillary sequencing | Amersham Pharmacia USA. |
| Trizol | Life technologies USA |
| RNA ZAP, and RNA Latter | Ambion, USA |
| SuperScript™ Indirect cDNA Labeling System for DNA Microarrays | Life Technologies, USA |
| PCR product purification modules | Machery Nagel, GMBH. |
| cDNA post labelling dye kit for microarrays Alexa Fluor 488, 555, 594, 647 dyes, ARES DNA labeling Kits, TOTO-dyes. | Molecular probes, USA |
| Super amine coated Microarray substrates | Telechem, USA |
| Microarray CMP3 stealth pins | Telechem, USA |
| First strand cDNA purification GFX columns | Life Technologies, USA |
| Lucidea universal score card | Amersham, USA |
| Lambda DNA and RNA | Takara, Japan |
| Hybri-slips | Sigma, Schleicher & Schuell GMBH |
| Yeast-t-RNA, Poly DA | Sigma, USA |
| Succinic anhydride, Methyl pyrildenone, 200 pint absolute alcohol | Aldrich, USA |
| Anchored Primer | Life Technologies, USA |
| Sequencing Primers | MWG Biotech GMBH |
| Tri-sodium citrate, SDS, Sod.acetate, Agarose, Sephadex G25, G50, Iso-propanol, glycogen, BSA, Low Temperature microarray Hybridization buffer, NaoH, Sod. Borate. | Sigma-Aldrich. USA |
| DNA modifying and restriction enzymes | Gibco-BRL USA, MBI fermentas, Lithuania, New England Biolabs, USA, Bangalore Genie Ltd, Bangalore |
| 0.2 micron filters | Nalgene, GMBH |
| All plastic ware for microarray | Nalgene, Nunc, USA, eppendorf GMBH, Tarsons, India. |
| Tris, EDTA, Agarose, DEPC, and all molecular biology grade chemicals | USB chemicals, USA. |
3.1.2 Plasmid vectors

The cloning vector used to clone normalized cDNA library is PT7T3 D Pac from Amersham Pharmacia. The super promoter cassette, pE1806, was from Dr. Stanton Gelvin, Purdue University, USA. Cloning vector pBluescript SK+ was from Stratagene, La Jolla, USA.

3.1.3 Description of rice lines

Nagina 22 is an early maturing indica rice cultivar with deep roots and also both drought and heat tolerant. It is used as a parent in crosses for developing drought tolerant cultivars. The IR64, is a high yielding cultivar but susceptible to drought. It is one of the most popular rice cultivar in India.

3.1.4 Normalized cDNA library source

Normalized cDNA library (Reddy et al., 2000) constructed from the drought stressed N22 seedlings is the primary source for generating ESTs in this study. The vectors and procedures of normalization were described (Reddy et al., 2002).

3.1.5 Sequence repositories

The sequence repositories used in the study are given below:

1. EST sequences from N22 libraries, both earlier and present study.
2. Rice genome sequence of the O. sativa sub sp japonica cv Nipponbare generated by the International Rice Genome Sequencing Project (IRGSP)
3. Draft sequences of the O. sativa sub sp. indica cultivar generated by the Beijing Genomics Institute (BGI) available in the GenBank.
5. The rice full-length cDNA consortium sequences of Nipponbare cultivar.
7. The nucleotide, protein and EST databases at NCBI were utilized for homology search using BLAST program.

8. Protein motifs were searched through protein data bases at InterPro (http://www.ebi.ac.uk/InterProScan/) (R. Apweiler 2001).

3.1.6 Software used for annotation

Standard sequence processing tools Phred (Ewing and Green, 1998), Phrap, and Cross_match (Smith & Waterman 1981; Gotoh, 1982) were used with CodonCode InterPhace. Homology search in the NCBI database was carried out using network client software with the DNATools interface http://www.crc.dk/dnatools. CAP3 (Huang and Madan, 1999) assembly algorithms were used to assemble the individual ESTs into clusters of sequences deriving from the same transcript as tentative consensus sequences (TCs) and singletons representing unique transcripts. Transcript mapping and localization of ESTs onto rice genome was done using cMAP software (Fang et al 2003). The unigene set derived from CAP3 assembly were searched for Protein motifs using InterProScan v3.3 (http://www.ebi.ac.uk/InterProScan/). All computations were performed on a 32-node Linux Cluster running Red Hat 9.0.

Genscan, GeneMarkHMM, Glimmer R, FGENESH, Rice Genome Automated Annotation System (Rice GAAS) were used for accurate gene prediction. Sim4, LALNVIEW V2.0 were used for identifying gene structure. TIGR tm4, Avadis (Strand genomics, India) suite was used for microarray data analysis, TIGR spot finder and Quantarray (Genomic solutions, USA) for quantification of intensities.
3.2 Methods

3.2.1 Bacterial transformation

*E. coli* strain DH5α was grown at 37°C either on solid (1.5% agar) or in liquid LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract). Liquid cultures were grown initially in 2 ml of LB medium in a test tube, and later in 1-liter flasks for plasmid isolation. Competent cells of *E. coli* were prepared as follows. One ml of DH 5α cells from an overnight grown culture was inoculated in 100 ml of LB medium without antibiotic. The cells were grown till they reached an A₆₀₀ of 0.4-0.6. Cells were then harvested into pre-cooled 50 ml falcon tubes by centrifugation at 3000 rpm for 10 min at 4°C. All the operations were performed under sterile conditions at 4°C. After the centrifugation, the cells were resuspended in 15 ml 0.1 M CaCl₂ and incubated on ice for 10 min. This suspension was centrifuged at 3000 rpm for 10 min. The resultant pellet was resuspended in 4 ml of 0.1 M CaCl₂ (in 10% glycerol) for every 100 ml of original culture, dispensed into 200 µl aliquots, frozen and stored at –70°C for future use.

Transformation of the competent cells was done as follows: Frozen *E. coli* cells were thawed on ice to which 1 ng of plasmid DNA or 100 ng of ligation mix were added. The suspension was carefully mixed with pipette tip and incubated on ice for 30 min. A heat shock of 42°C for 45 sec was applied followed by incubation on ice for another 2 min. 800 µl of LB medium was added and the bacterial suspension was incubated at 37°C with shaking for 1 h. Aliquots of the suspension were spread evenly on LB supplemented with an appropriate antibiotic. The plates were incubated at 37°C overnight. Single colonies were picked up following day and inoculated for plasmid mini preparation.

3.2.2 Expressed Sequence Tags generation

3.2.2.1 Template preparation

The Normalized cDNA library was transformed in chemically competent *E.coli* DH5α by heat shock at 42°C and kept for expression for 30 min and then plated at low density on Luria-bertani medium containing 20µg/ml ampicillin, and were incubated at 37°C overnight. Individual colonies were selected randomly and inoculated in 5ml Luria-Bertani broth containing 20µg/ml ampicillin and incubated at 37°C overnight in an
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orbital shaker at 250 RPM. The overnight grown culture was used in plasmid DNA preparation for nucleotide sequencing. Plasmid DNA was prepared using a DNA purification column (Qiagen Plasmid Isolation Kit). These plasmid preparations represent clones of amplified normalized cDNA library. The quality and concentration of the template plasmid DNA was controlled on 1% agarose (USB biochemicals) gels and was used directly for sequencing.

3.2.2.2 Sequencing

3.2.2.2.1 Preparing of sequencing reaction

For EST generation, 3’ end single pass sequencing of 7740 cDNA clones using M13 (-40) Reverse primer 5’ CGCCGAGGTTTTCCCAGTCACGAC 3’, and M13 (-20) Reverse primer 5’ GTAAAACGACGGCCAGTG 3’, were performed on automated high throughput 48 capillary genetic analysis system, MegaBACE 500 (Amersham Pharmacia). DYEnamic ET terminator chemistry (Amersham Pharmacia) was used for sequencing reaction set-up, post-sequencing reaction clean-up and loading samples on MegaBACE was adjusted to suite our conditions and the rest were followed according to the manufacturer’s instructions. We have standardized the optimal template concentration to be used, which ranged from 300 ng to 400 ng for a quarter strength reaction for a vector size in between 3kb to 6kb. The primer is diluted in water or in buffer containing no more than 0.1 mM EDTA. And for each reaction 2.5 mM of sequencing grade primer is used (PAGE purified or HPLC purified). For each template to be sequenced, we use a 1/4th strength Dyenamic ET terminator reagent premix (Amersham Pharmacia), and 1/2 strength dilution buffer, and 2.5 mM primer and 400 ng of plasmid DNA or 15 to 60 ng of PCR product are added depending on the size of the template, and set for cycle PCR with the following cycling parameters: 95°C for 20 seconds, 50°C for 15 seconds and 60°C for 1 minute for 30 cycles and stopped the thermal cycler with rapid thermal ramp to 4°C and hold. In our studies, 30 cycles are found to be optimum for achieving low noise and good read length.
3.2.2.2 Post reaction clean-up

The post reaction clean-up is either done by ethanol precipitation or by passing the samples through sephadex G 25 (Amersham Pharmacia). Ethanol precipitation method worked effectively both in terms of cost and quality. Millipore filter membrane in a 96 well format was used to load appropriate bed volume of sephadex G 25 (Amersham Pharmacia) and was allowed for 2 days to swell in milliQ water. Before the samples were loaded, the excess water was drained down by centrifuging at 1000 RPM for 2 min in order to minimize the sample loss. The bed was pre-wetted with 10µl of sterile milliQ water and the samples were loaded on top and spun at 3100g for 5 min to retain the samples. Samples were directly injected into the MegaBace capillary sequencer at 3 Kva for 40 sec. Ethanol precipitation was carried out by adding 1µl of 7.5M Ammonium acetate and 2.5 volumes (27.5µl) of 100% ethanol for a final concentration of 75%. The samples were incubated at -20°C for 30 min. The samples were centrifuged at either room temperature or 4°C in a micro centrifuge for 15 min at ~ 12000 rpm. The 96-well plates are centrifuged for 30 min at 2500 x g or more. If plates are centrifuged at a lower relative centrifugal force, the time of centrifugation was increased. We routinely use 3100 x g for 30 min. The supernatant was removed by aspiration or by a brief inverted spin. The pellet was washed in 100-200 µl of 70% ethanol to remove any leftover salt that can have a deleterious effect during injection in capillary electrophoresis. The supernatant was removed by aspiration or by a brief inverted spin. Further, the pellet was vacuum-dried or air-dried care was taken that the samples are not over-dried. Each pellet was resuspend in 10 µl of loading solution (70% formamide) and vortexed vigorously for 10-20 sec to ensure complete resuspension. The plates were briefly centrifuged to collect the sample at the bottom of the tube/well and to remove any bubbles.

3.2.2.3 Injection and run parameters

For the greatest reproducibility, we use a voltage of 2 KV for injection (2 to 3 KV is standard). Injection time can be varied widely during optimization with injections as short as 5 sec or as long as 400 sec being equally successful. In the present study an injection time of 50KV for 50 Sec was found to be optimal and therefore used routinely. Using our standard run conditions of 200 min at 6 KV, an average read-length of greater than 800 bases with 98% accuracy were reproducibly obtained.
3.2.2.3 Sequence processing and analysis

The raw data was exported from MegaBace in SCF format, and the raw sequences were base called using Phred. The low quality regions present at the beginning and end of each sequence were trimmed using a Phred 20 cutoff value. Vector screening was performed using the cross_match program with Codoncode InterPhace software. Sequences were edited for the removal of oligo dT tracks and other contaminants. A batch file of ESTs having greater than 100 bp length of sequence reads were submitted to the NCBI db EST division of GenBank. After the rice genome sequence was largely completed (IRGSP, 2005), all ESTs from this project were compared to the genomic sequence. All sequences that did not exhibit excellent nucleotide homology with the Nipponbare genomic sequence were removed from GenBank, with the assumption that they were most likely to be derived from microbial contaminants.

3.2.3 Annotation

Homology searches were performed against non-redundant (nr) nucleotide and protein sequence databases using BLASTN 2.2.2 and BLASTX 2.2.2 versions of the BLAST programs (Altschul et al., 1997) through BLAST 2.0 network client software with the DNAtools interface (http://www.crc.dk/dnatools). The BLASTN program was used to identify rice EST hits and rice BAC/PAC clones in the non-redundant (nr) nucleotide sequence database, High Throughput Genomic Sequences (HTGS) division of GenBank and the Beijing WGS (whole genome shotgun contigs) draft sequence of the indica rice genome (Yu et al., 2002) in the NCBI database.

3.2.4 EST clustering and transcript mapping

Phrap and CAP3 (Huang and Madan, 1999) assembly algorithms were used to assemble the individual ESTs into clusters of sequences derived from the same transcript as tentative consensus sequences (TCs) and singletons representing unique transcripts. Assembled N22 unigenes were aligned to the TIGR japonica rice assembly (ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_1.0) using BLASTN (Altschul et al., 1990) genes were annotated if they met the following stringency criteria: E-value $\leq 1 \times 10^{-10}$; percent identity $\geq 95$%; and alignment length $\geq 50$bp. Functional annotations were then associated to mapped unigenes. These
positions were displayed using CMAP software (Fang et al., 2003). Also the putative functions of the stress responsive genes were also displayed along with the unigenes.

3.2.5 Data mining, compilation, and identification of putative stress responsive ESTs

The ESTs associated with stress response were identified from multiple sources based on the compiled list of stress regulated genes documented or presumed to be relevant to abiotic stress tolerance in more than one plant species (http://stress-genomics.org/stress.fls/expression/expression.html). Further, it is based on the microarray expression profiles of possible candidate gene sequences; 650 from Arabidopsis (Seki et al., 2001, 2002; Kreps et al., 2002), 150 from barley (Ozturk et al., 2002) and 100 from rice (Matsumura et al., 1999; Kawasaki et al., 2001; Rabbani et al., 2003). These were compared to the EST data set using TBLASTX with E-value >1e-20. All the stress responsive gene sequences were retrieved from the above studies and a local database was constructed and utilized for BLAST analysis.

3.2.6 Isolation of drought inducible transcriptional factors DREB1B and CRT/DRE binding factor from Nagina 22

Through in-silico analysis of the ESTs generated the sequence showing homology to the Os-DREB 1b Accession Number AF300972 was sequenced from both end to get the full length 862 nucleotides. The 1128 bp sequence of CRT/DRE binding factor was amplified from genomic DNA of N22 using the forward primer carrying SalI restriction site (5’ ACG CGT CGA CCC ATC ATC ACC GAG ATC GAC TCG AC – 3’) and the reverse primer with NotI restriction site (5’- ATA AGA ATG CGG CCG CTC ATT GTT CGC TCA CTG GGA G – 3’). PCR amplification was carried out in a MJ Research thermal cycler. The amplified product of 1.2 kb was gel eluted and cloned in TA vector pTZ57R using InsT/Aclone PCR product cloning kit (MBI Fermentas). The cloned product, 1128 nucleotides in length, was sequence confirmed on MegaBace 500 using M13 forward and reverses primers. The CRT/DRE fragment was gel eluted by restriction digestion with SalI and NotI and was cloned directionally in pBluescript.

3.2.6.1 Construction of plant expression vector for DREB1B

The DREB1B fragment was released from pT7T3Pac vector by digesting the vector with HindIII and EcoRI present in the 3’ UTR of the gene and was cloned
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directionally in pBluescript at *Hind*III and *Eco*RI sites. Further the 840 nucleotide DREB1B cDNA was excised from pBluescript KS+ by digesting with *Xho*I and *Xba*I. The fragment was gel excised and cloned in the same sites in pE 1806 under the super promoter sequences from mannopine synthase (mas) and the octopine synthase (ocs) genes. The restriction map of the recombinant binary vector, pE1806 and their identities were confirmed by restriction analysis using suitable restriction endonucleases.

3.2.6.2 Construction of the bacterial expression vector for DREB1B

The 862 bp sequence of DREB 1b was gel eluted from the pT7-T3 vector using *Not*I and *Xho*I and cloned directionally in frame at *Not*I and *Xho*I sites of T7 RNA polymerase expression vector pET 28a. The restriction map of the recombinant plasmid and their identities were confirmed by restriction analysis using suitable restriction endonucleases.

3.2.6.3 Construction of the bacterial expression vector for CRT/DRE binding factor

The 947 bp open reading frame from the Os CRT/DRE cDNA cloned in pBlue Script was PCR amplified using the forward primer carrying *Nde*I site (5’- GGG AAT TCC ATA TGG AGA AGA ACA CCG CCG C –3’) and the reverse primer with *Not*I restriction site (5’- ATA AGA ATG CGG CCG CTC ATT GTT CGC TCA CTG GGA G – 3’) PCR amplification was carried out in a MJ Research Thermal Cycler. The amplified product of 1.0 kb was gel eluted and cloned in TA vector pTZ57R using InstAclone PCR product cloning kit (MBI Fermentas). The cloned product was confirmed by sequencing and a 947 bp length fragment was obtained (Fig-3). The CRT/DRE fragment was gel eluted by restriction digestion with *Nde*I and *Not*I and was cloned directionally in frame at *Nde*I and *Not*I sites of T7 RNA polymerase expression vector, pET 28a. The restriction map of the recombinant plasmid and their identities were confirmed by restriction analysis using suitable restriction endonucleases.

3.2.6.4 Restriction digestion and ligation of fragments

Restriction digestion of the plasmid DNA and the amplicons was carried out in a total volume of 20 µl containing 0.5 µg of plasmid DNA, 2 µl of appropriate 10 X restriction enzyme buffer, 1 µl BSA (1 µg/µl, if necessary), 15 (or 16) µl of double distilled water and 1 Unit of restriction enzyme. The reaction mixture was incubated at
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37º C (or at an appropriate temperature according to the restriction enzyme used) for 1 hour and the digestion pattern was analyzed on 1% agarose gel. The following principle was used to calculate the concentration of fragment and plasmid DNA needed (3:1 ratio of fragment to vector) for ligation reaction. Fragment size/vector size X 100 X 3 where “100” denotes the amount (ng) of vector DNA and “3” denotes the number of times of fragment DNA (ng) required for ligation. The ligation reaction was carried out in a total reaction volume of 20 µl containing 100 ng of restriction digested vector DNA, appropriate amount of fragment DNA, 2 µl 10 X ligase buffer, 1 µl of T4 DNA ligase (5 U/µl) and sterile double distilled water to make up the volume. The reaction was incubated at 16º C for 2 h or overnight where necessary. After completion of the reaction, an aliquot of 10 µl was used for transformation.

3.2.7 Simulated field drought stress

3.2.7.1 Experimental design

In order to conduct a field experiment for gene expression profiling, we have constructed a rain out shelter with specifications described below. A plot of 13 feet width and 16 feet length was first excavated and filled with soil up to 5 feet depth. A sand bed of 1 ft was laid across the length in a slanting manner such that the end of the plot has just 1 inch sand, so that seepage of water will be towards sand end. Also, all the sides were filled with sand so as to leach the water out of the experimental plot. This has allowed us to maintain drought conditions and avoid any accidental seepage. Eight plots of the size 3ft x 4ft were partitioned and constructed with concrete walls with water proof agents to avoid seepage of water across plots. The inner walls were of 5ft and the outer walls facing the exterior soil apart from the black clayey soil were of 3ft long. The plots are raised enough to avoid accidental overflow from the control plots to the experimental.

All plots were filled (5ft depth) with black clayey soil in which the rice crop is usually cultivated. To maintain fertility, recommended amount of farm yard manure was added. Rainout shelter was constructed to cover the plots during rainy days to keep the plants protected from rain during experiment. The rainout shelter was provided with free moving rail tracks to move forward and backward. Proper care was taken to avoid any
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Drought stress induction was monitored through high-precision digital irrigation controllers procured from Netafim Inc (Israel). Irrigation was given taking Plot A1 /AR1 as 100% field capacity, which is a control plot in this experiment, and irrigation was set up in the other plots as shown in the table below, with moisture probes/Timer controllers using digital controllers.

<table>
<thead>
<tr>
<th>No.</th>
<th>Plot</th>
<th>Genotype</th>
<th>Irrigation Levels</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1</td>
<td>N22</td>
<td>100%</td>
<td>NR</td>
</tr>
<tr>
<td>2</td>
<td>A2</td>
<td>N22</td>
<td>70%</td>
<td>NR</td>
</tr>
<tr>
<td>3</td>
<td>A3</td>
<td>N22</td>
<td>40%</td>
<td>NR</td>
</tr>
<tr>
<td>4</td>
<td>A4</td>
<td>N22</td>
<td>10%</td>
<td>NR</td>
</tr>
<tr>
<td>5</td>
<td>A1</td>
<td>IR 64</td>
<td>100%</td>
<td>NR</td>
</tr>
<tr>
<td>5</td>
<td>AR1</td>
<td>N22</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>AR2</td>
<td>N22</td>
<td>70%</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>AR3</td>
<td>N22</td>
<td>40%</td>
<td>87 days</td>
</tr>
<tr>
<td>8</td>
<td>AR4</td>
<td>N22</td>
<td>10%</td>
<td>80 days</td>
</tr>
</tbody>
</table>

NR- Not subjected to Recovery
AR1 –Control Recovery

Table 1: Irrigation plan to for induction of different degrees of drought stress

3.2.7.2 Rice seedling culture, stress treatments, and transplantation

Rice seeds were imbibed in water, surface sterilized with 5% sodium hypochlorite (v/v) for five minutes, thoroughly washed with sterile water and germinated in water upon filter papers in dark. Two-day-old germinated seeds were transferred to growth chambers and were supplemented with Hogland’s medium. The average temperature during seedling culture was 28 ± 1°C and a photoperiod of 16 hours light and 8 hours dark was maintained. Seedling trays were examined twice daily to maintain constant moisture content. Fifteen day old seedlings were transplanted into field for drought stress induction. The transplanted seedlings were grown in 100% field capacity with 2cm standing water until day 30; from day 31 field drought stress was initiated at 100%, 60%, 40% and 10% field capacities in A1, A2, A3, and A4. It took nearly 8 days for the onset of the actual drought for the experimental plots to reach to their designated field capacities from 100% FC. Prior to this actual experiment a trail run was performed to
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check whether the electronic digital irrigation controllers are functioning properly all through the experiment. Samples were collected to check differential gene expression in various drought stress regimes based on the developmental stages of the plant. The genotype under study, N22, is a short duration crop and completes within 95 to 100 days. The vegetative stage ends on 50 to 55 days, pre-bloom stage is between 55 to 60 days, milking stage is between 60 to 70 days and seed maturing stage is between 70 to 100 days. Samples were collected as mentioned in table 2.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Developmental Type</th>
<th>Tissue Type</th>
<th>Day</th>
<th>Sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vegetative</td>
<td>Leaf</td>
<td>45</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>I</td>
<td>Vegetative</td>
<td>Leaf</td>
<td>52</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>I</td>
<td>Vegetative</td>
<td>Leaf</td>
<td>55</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>II</td>
<td>Pre-bloom stage</td>
<td>Leaf</td>
<td>59</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>II</td>
<td>Pre-bloom stage</td>
<td>Panicle</td>
<td>59</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>III</td>
<td>Milking stage</td>
<td>Leaf</td>
<td>63</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>III</td>
<td>Milking stage</td>
<td>Panicle</td>
<td>63</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>III</td>
<td>Milking stage</td>
<td>Leaf</td>
<td>67</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>III</td>
<td>Milking stage</td>
<td>Panicle</td>
<td>67</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Leaf</td>
<td>71</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Panicle</td>
<td>71</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Leaf</td>
<td>75</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Panicle</td>
<td>75</td>
<td>A1 A2 A3 A4</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Leaf</td>
<td>78</td>
<td>- - - - -</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Panicle</td>
<td>78</td>
<td>- - - - -</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Leaf</td>
<td>81</td>
<td>- - - - -</td>
</tr>
<tr>
<td>IV</td>
<td>Seed setting</td>
<td>Panicle</td>
<td>81</td>
<td>- - - - -</td>
</tr>
</tbody>
</table>

Table 2: Sample collection of field drought stressed samples

Table 2 legend: A1- 100%FC, A2- 60%FC, A3- 10%, AR2- 60% FC Recovery, AR3- 40% FC Recovery, AR4- 10% FC Recovery, REC-Recovery.
3.2.7.3 Seedling stress experiments: lab experiments

N22 seedlings were grown as mentioned above and fifteen-day-old seedlings were treated with either 20% poly ethylene glycol (PEG 8000) or 150 mM sodium chloride or 100 µM ABA or dehydration, solutions prepared in Hogland’s solution. Control plants received only Hogland’s solution. The treatments were given three hours after beginning of light period. Samples were collected at 1, 2, 3, 6, 12, 24, and 48 hours after treatment. The collected samples were frozen in liquid nitrogen and stored in -70 °C for RNA isolation and microarray expressional analysis.

3.2.8 cDNA template amplification for array fabrication

The amplified clones of cDNA normalized library from which ESTs have been generated were used for amplification of cDNA inserts from the plasmid templates. 5664, plasmid templates have been diluted to an average 5ng/µl. The cDNA inserts were amplified using 15 ng of the plasmid template with M13 (-40) forward primer 5’ CGCCGAGGTTTTCCACGTCGAC 3’, and M13 (-20) Reverse primer 5’ GTAAAACGACGGCCAGTG 3’, in an 100µl reaction volume with 10 mM each of forward and reverse primer, 5 mM dNTPs, 5U Taq DNA polymerase and the PCR conditions were, an initial denaturation at 95°C for 5 min followed by 94°C, 1 min; 55°C, 1 min; and 72°C, 4 min for 35 cycles. The amplified products were screened on 1% agarose gel electrophoresis. The PCR products were scored for low conc., absence, and nonspecific amplifications and were documented accordingly. These were purified using silica membrane based bind, wash and elute columns procured from Machery Nagel using vacuum manifold in 96 well plate formats, according to the Manufacturer’s instructions. The PCR products were eluted in MilliQ water and were reformatted into 384 well griddler compatible plates (Nunc 384 well square well plates) such that a 96 well plate clones are printed in series in 2 subarrays. 7µl of the PCR product was aliquoted in each well of 384 well plate to which 7µl of DMSO was added to make a final concentration of 50% DMSO (v/v). Likewise 1152 pearl millet cDNA clones amplified from differential cDNA λ phage libraries using PBSK+ forward and reverse primers were purified using GFX columns (Amersham Pharmacia) were also resuspended to a final concentration of 50% DMSO. In all 6816 cDNA clones of rice and pearl millet, 24
artificial genes designed from yeast genomic sequence information procured from Amersham Biosciences USA, and λ DNA procured from Takara Inc Japan, which were aliquoted in 48 different wells, were used for arraying on superamine coated glass microarray slides.

3.2.9 Array fabrication

3.2.9.1 Array design

cDNA microarray chip consisting of 15552 features with 6144 ESTs from drought stress induced cDNA library of *Oryza sativa* cv Nagina22, 1152 ESTs from drought stressed *Pennisetum glaucum*, 24 yeast artificial genes, λ DNA controls were printed in duplicate on superamine coated glass substrate using Omnigrid (GeneMachines). The machine was calibrated for 384 Nunc square well plates, CMP3 print head and stealth printing pins and 75mm x 25 mm microarray glass slides. We have used 16 pins altogether in 4 x 4 format, which gave us an option to print 4 subarrays along Y direction and 4 arrays in X direction. The origin offset values set were 3000 microns in X direction and 2000 microns in Y direction. We have used 225 µ spot to spot spacing in a subarray. The number of spots printed in a subarray is 18 clones in X direction and 18 clones in Y direction, the number of clones printed in one subarray is 18 x 18 = 324 stamps, in which 162 clones have been printed onto each subarray. The maximum number of arrays which would be printed were 4 in which we limited to 3 arrays, with a spacing of 500 microns each, in which a maximum number of 7776 clones in duplicate could be printed. The above format allowed us to print 7680 samples in duplicate including blanks.

For printing, the sample intake was for every 100 slides, the dip time being 1000 milliseconds, with each dip the sample is taken in by capillary action and is sufficient enough to print 200 stamps. The sample volume which is delivered in each stamping is around 0.5 to 0.7 nl. After each printing the pins were sonicated for 2000 milliseconds, followed by washing in sterile water for 3000 milliseconds, and dried under vacuum for 2000 milliseconds which is repeated thrice, the final cycle has an extended drying time of 3000 milliseconds. After each round the 384 well plate and the pins were also sonicated.
thrice for 3000 milliseconds. In order to avoid the possibility of sample sticking to the walls of the pins, during each dip, which can cause excess sample being deposited on slides resulting in run over of samples, the pins were initially blotted on a poly-L-Lysine coated blot pad 10 times with 150 micron spot to spot spacing. The time taken by the robotic griddcr to print 7680 samples in duplicate is 36 hours. The cDNAs were printed at a relative humidity between 45 to 50%, which is very critical in printing good and reproducible microarrays. Also we found that before printing, calibration of wash station, dry station, sonicator, and slides across the platen of the slide holder is of utmost importance for uniform spot morphology in all the printed slides.

3.2.9.2 Array printing

Microarraying was by robotic systems built by Gene Machines, California (Genomic Solutions). We have used aminosilane coated slides from Telechem, SuperAmine, aminosilane coated glass microscope slides have been found to be the most consistent, when printed in 50% DMSO as a printing solution at 45% relative humidity and 72°F (22°C). We have used quill pens (Arrayit ChipMaker3 microspotting pins from Telechem International Inc.) which were very durable and could reproduce high-quality spots with good precision. All arrays were printed with the same set of ChipMaker 3 pins for more than six months. Following printing, the slides were rehydrated on a steam coming from a 500ml conical flask containing MilliQ water, which was kept boiling on a hot plate. Care was taken such that all slides were uniformly rehydrated; the slides were passed over the steam with the array side down 2 to 3 times until a thin layer of mist was formed on the printed side. Extreme care was taken not to rehydrate such that the spots run into each other. Following rehydration, the slides were immediately snap dried for 5 sec on a hot glass plate. The slides were allowed to dry and spotted DNA was fixed to slide by UV cross-linking at 320 mJ using a Stratalinker (Stratagene, Cat# 400071) and by baking at 80º C for 2 hrs. Printed slides were stored in a light-tight box in a bench-top dessicator, with desiccant and stored at 4ºC until they were used for hybridization.

3.2.10 Extraction of total cellular RNA

Total RNA was isolated from leaf and panicle samples collected at different time intervals after stress treatment using Trizol (Chirgwin et al., 1979; Chomczynski and
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Sacchi, 1987) from Life Technologies with minor modifications. The harvested tissues were stored immediately in RNA later (Ambion) and stored at 4 °C for 24 hours after which the RNA latter was removed and the samples were quick frozen in liquid nitrogen and stored at −70°C. Five grams of freshly harvested or frozen tissues were ground in liquid nitrogen to a fine powder and transferred to 50ml tubes with 15ml of Trizol. The samples were suspended in Trizol and incubated at room temperature for 5 minutes. Cellular debris was removed by centrifugation at 2700g for 15min at 4°C. The supernatant was transferred to a fresh RNAase free 50 ml oakridge tube. To it 3 ml of chloroform (0.2 ml/1 ml Trizol) was added and shaken vigorously for 1 minute, and incubated at room temperature for 5 min. The samples were centrifuged at 2700 g for 15min at 4°C to remove proteins and high molecular weight DNA. The top supernatant layer containing RNA was carefully removed without disturbing the protein layer and was transferred to a fresh RNAase free Oakridge tube. To this, an equal volume of isopropanol was added to precipitate the RNA and incubated at RT for 15 min. The sample was centrifuged at 21,000g for 15 minutes, the supernatant discarded, and the pellet resuspended in 70% ethanol. The RNA pellet was resuspended in diethylpyrocarbonate (DEPC) treated water. The quality of the total RNA was determined through 1.2% denatured agarose formaldehyde gel and concentration estimated spectrophotometrically. For labeling at “University of Georgia” the samples were resuspended in 1/10th volume DEPC treated sodium acetate and 2.5 volumes ethanol. Prior to labeling the precipitated RNA was spun at 12000 RPM for 10 min and washed with 70% ethanol. The RNA was dried in a Savant rotary vapor trap and resuspended in RNAase free milliQ water for labeling.

3.2.11 RNA labeling

3.2.11.1 Labeling procedure

Probes for microarray analysis were prepared from RNA templates by first strand synthesis of cDNA containing amino-allyl-labeled nucleotide dCTP and amino-hexyl modified dATP for both control and stress samples, followed by a covalent coupling to the NHS-ester of the appropriate Cyanine fluor. This method has proven highly efficient for labeling RNAs from all kinds of tissues from rice for expression analysis. Among the reverse transcriptases used, including AMV and MMLV, we found the Superscript III RT
(Life Technologies; Cat# L-1014-02) generates probes with significantly greater activity. Coupling of modified cDNA with fluorescent probes was done by photostable Alexafluor fluorescent dyes (we have used Alexafluor 488, and 555 for control labeling and Alexafluor 594, and 647 NHS ester dyes for coupling stressed samples). The photosensitivity of dyes was minimized by avoiding exposure to light during labeling, hybridization, washing, and scanning processes. Upon receipt, these NHS ester dyes were stored in light-proof tubes and stored at -20°C until needed. All reactions were carried out in foil-wrapped tubes and all hybridizations and washes in foil-wrapped containers.

3.2.11.2 First strand amino modified cDNA synthesis recipe step 1

Prior to labeling, the quality of total RNA for both control and stressed experimental sample, were quantitated and suspended in 40 µg of total RNA in not more than 10 µl DEPC treated water. Anchored oligo DT primer was used; this oligo (20mer oligo DT) primed with equal concentrations of A, C, G, or T, allowed proper priming of the primer to the 5' end of the cDNA. This helped us in avoiding reverse transcription of long stretches of dT in cDNA. Five microgram of anchored primer was mixed with 40 µg of total RNA and the reaction was incubated for 5 min at 70°C, following which the reaction was snap cooled for 1 min on ice, to which the following components were added, 5X first strand buffer - 6.0 µl, 10 mM dNTP mix (contains modified aminoallyl dUTP and aminohexyl dATP - 1.5µl, RNAse out 20units/µl - 1.5µl Super script RT III 400 units/µl - 2.0µl). DEPC treated water was added to make-up a final volume of 30.0 µl. The above reaction mixture was incubated for 2 hours at 42°C in a thermocycler.

3.2.11.3 Alkaline hydrolysis and neutralization step 2

To the reaction from step 1, 15 µl 1N NaoH was added to denature the RNA through hydrolysis from RNA-DNA hybrid and was incubated for 10 min at 70°C for complete RNA denaturation. To this 15µl of 1N HCl was added to neutralize the pH and mixed gently. To the above reaction, 20µl sodium acetate, pH 5.2, was added and mixed gently. Once the cDNA was generated with amino-modified nucleotides, it was purified to remove unincorporated dNTPs and hydrolyzed RNA, using bind wash elute SNAP columns (Life Technologies) as per manufacturers instructions following which, the
cDNA was ethanol precipitated by adding 1/10th volume sodium acetate and 2.5 volumes of ethanol.

### 3.2.11.4 First strand cDNA dye coupling step 3

The aminoallyl and aminohexyl modified cDNA precipitated in step 2 was further centrifuged at 12000 RPM for 10 min to pellet it down and the pellet was washed with 70% ethanol and dried down in Speedvac (Savant). The pellet was dissolved in 6 µl sterile water. For coupling of the dye, the dye vials (Alexa fluor dyes) were allowed to come down to room temperature in order to avoid condensation of water inside the vial. The dyes were dissolved in 4 µl of DMSO. For coupling, 10 µl of 2X coupling buffer (sodium bicarbonate) was added to modified cDNA to bring the final concentration to 1X, to the above reaction 4µl of the dissolved dye was added and allowed to couple overnight in dark at room temperature. Further, the cDNA was purified by bind wash elute SNAP columns (Life Technologies) as per manufacturers instructions to remove any unreacted free dye molecules.

### 3.2.11.5 Quantification and assessing labeling efficiency of the cDNA

The relative efficiency of a labeling reaction was evaluated by calculating the approximate ratio of bases to dye molecules. This ratio has been determined by measuring the absorbance of the amino modified cDNA at 260 nm and the absorbance of the dye at its absorbance maximum (max) ($\lambda_{\text{max}}$ for Alexa 488, 555, 594, and 647 are 492nm, 555nm, 588nm and 650 nm), and by using the Beer-Lambert law: $A = \varepsilon \times \text{path length (cm)} \times \text{concentration (M)}$, where $\varepsilon$, is the extinction coefficient in cm$^{-1}$M$^{-1}$. The absorbance measurements were used to determine the concentration of nucleic acid in the sample. Most fluorescent dyes absorb light at 260 nm as well as at their $\lambda_{\text{max}}$. To obtain an accurate absorbance measurement for the nucleic acid, it was therefore necessary to account for the dye absorbance using a correction factor (CF260). Hence using the CF$^{260}$ values in the following equation: $A_{\text{base}} = A_{260} - (A_{\text{dye}} \times \text{CF260})$ exact absorbance $A_{\text{base}}$ was calculated.

### 3.2.11.6 Measurement of base:dye ratio

The ratio of bases to dye molecules was calculated using the following equation:
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Base:Dye = \( \frac{A_{\text{base}} \times \varepsilon_{\text{dye}}}{A_{\text{dye}} \times \varepsilon_{\text{base}}} \) where \( A_{\text{base}} \) is the dye corrected absorbance calculated previously, \( \varepsilon_{\text{dye}} \) is the extinction coefficient for the fluorescent dye and \( \varepsilon_{\text{base}} \) is the average extinction coefficient for a base in single-stranded DNA (ssDNA). The values of \( \varepsilon_{\text{dye}} \) for Alexa 488, 555, 594, and 647 are 62,000, 150,000, 80,400 and 239,000, whereas the values for \( \varepsilon_{\text{base}} \) is 8919.

3.2.11.7 Measuring the concentration of nucleic acid

The absorbance values, \( A_{260} \) and \( A_{\text{dye}} \), and the Beer-Lambert law were used to determine the concentration of cDNA. In order to obtain an accurate measurement for a dye-labeled nucleic acid, a dye-corrected absorbance value \( A_{\text{base}} \) was used, in the following formula [N.A.] (mg/mL) = \( \frac{A_{\text{base}} \times \text{MW}_{\text{base}}}{\varepsilon_{\text{base}} \times \text{path length}} \), whereas \( A_{\text{base}} \) is the dye corrected absorbance calculated previously, \( \text{MW}_{\text{base}} \) is average molecular weight of base which is 330 g/mol, \( \varepsilon_{\text{base}} \) is 8919, and path length is 1 cm. Using the above formulas, the amino modified cDNA concentrations and efficiency of dye incorporation was calculated for all the samples for hybridization to probes on the arrays with equal concentrations of cDNA, which helped in employing efficient normalization strategies.

3.2.12 Hybridization

The dye labeled cDNA once quantified, equal amounts of cDNA from tester and reference samples were pooled and was precipitate by adding 1/10\(^{th}\) of sodium acetate and 2.5 volumes of ethanol, the cDNA populations were spun at 8000 RPM for 10 min, to pellet down the cDNA. The pellet was washed with 70% ethanol, dried and the pellet was dissolved in 50\(\mu\)l of low temperature hybridization buffer from Sigma at room temperature.

3.2.12.1 Preparation of slides for pre-hybridization/hybridization

Our protocols employed yielded reproducible and high-quality hybridizations while maximizing the measured fluorescence from the array. Aminosilane coated slides bind DNA with high efficiency. Prior to hybridization, the free amine groups on the slide were blocked or inactivated to avoid nonspecific binding of labeled cDNA to the slide. The slides were blocked in sodium borate and succinic anhydride blocking reagent.

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Microarray slides stored at 4 °C were allowed to come to room temperature. The required number of slides were dipped in the above solution and kept for shaking on an orbital shaker for 15 min. Slides were placed in opposite direction such that the non printed side touches each other. This was scaled up if more than 2 slides were processed using a Lipshaw slide holder and a jar. After 15 min, the slides were washed in 0.1% SDS for 2 min in a clean glass jar (Lipshaw dish staining assembly), the SDS solution was removed and a brief 10sec wash was performed with milliQ water. Slides were transferred to a fresh dish jar containing 2XSSC, washed twice with 2X SSC for 2 min each.

3.2.12.2 Denaturing and fixing
Denaturation was performed by dipping the slides in boiling hot water at 95 °C and incubating them for 2 min. The denatured DNA was fixed by transferring the slides in to ice cold 200ml 95% ethanol (Aldrich). Slides were retained in ice cold ethanol for 2 to 3 min before being transferred to a 50ml falcon tube with a tissue paper at the bottom. The slides were spun without cap to a 1000 RPM for 5 min at RT. After spinning the slides were immediately used for hybridization.

3.2.12.3 Target hybridization
The labeled cDNA dissolved in low temperature hybridization buffer from Sigma. Two blocking agents, yeast tRNA (Sigma) at a concentration of 20 µg per hybridization, and 2 µg poly dA (Sigma) per hybridization were used to block non-specific hybridization. The pre-hybridized slides were heated to bring them to 45 °C for hybridization. After denaturation the labeled probe was applied to a pre-hybridized microarray slide and covered with 22mm x 60mm plastic hybri-slips (Sigma, Schleicher, and Schuell Gmbh). Once the coverslip is properly placed without any air bubble trapped between the slide and the coverslip, the slides were inverted (array side down) and kept inside a 50ml falcon tube, which were used as a makeshift hybridization chamber. A small piece of tissue paper was soaked with 1ml of 3x SSC and was kept inside the bottom of falcon tube to maintain humidity during hybridization and to avoid drying of the target. The Falcon tube was placed in hybridization bottle (Amersham Pharmacia) and dipped in hot water bath set at 50 °C. Weight rings were used between the bottles to
avoid floating of the hybridization bottles. Hybridization was allowed for 16 hours in dark at 50 Deg C.

The slides from the water bath were taken out after 16 hours of hybridization and dipped in wash buffer 1 (1x SSC, 0.03% SDS). They were shaken gently until the coverslip come off by itself. Once the coverslips come off, the slides were transferred to wash buffer 2 (0.2x SSC) and washed for 2 min by gently shaking and then transferred to wash buffer 3 (0.05x SSC). After gentle agitation for 5 min, the slides were then transferred to a 50ml Falcon tube and with its lid open were spun at 1000 RPM for 5 min to dry out completely for scanning.

3.2.13 Data collection, normalization, and analysis

Scanning of the hybridized slide was done using ScanArray 3000. This scanner uses 4 Helium-Neon lasers operating at 488 nm, 543 nm, 594 nm and 633 nm to excite Alexa 488, 555, 594 and 647, respectively. Hybridized slides were scanned first in the high wave length channel (594 nm and 647 nm), and then the lower wavelength (488 nm and 555 nm). Data from each fluorescence channel was collected and stored as a separate 16-bit TIFF image. To quantify the right intensities slides were scanned as soon as possible after washing. The slides were scanned at appropriate PMT gain and 100% laser power, that is, low to high at 30 microns scan resolution, then appropriate PMT settings for both the dye wavelength was standardized. Scanning was done at PMTs where we could observe 20 to 30 white spots, such that the overall intensities are good. This was determined by observing the RI plot of intensities. The slides were scanned at 10micron. During quantification we used the highest threshold limits for spot intensity calculation and ANOVA performed for these low PMT and High PMT technical replicates.

Scanned images generated from microarray experiment were processed with TIGR Spotfinder and Quantarray, to identify and quantify the spots on the array. The two images were loaded on to the two different channels of Spotfinder and Quantarray. Grids were generated taking printing style into consideration. Overlay of the two channel intensities revealed the hybridization was good and more or less similar intensities were observed in adjacent in-slide replicas. A total of 48 blocks with 324 features in each were generated with 12 rows and 4 columns in each covering all the 15552 features including controls. A sum of 15552 cells were generated with 5 - 23 pixel spot size adjusted as to
cover all the features of the superimposed slide. In house custom perl scripts were written to facilitate the annotation to the features on the array. Annotation including gene function, chromosome BAC/PAC alignment, and map position of the ESTs, unigenes cluster, functional classification, and redundancy were provided to facilitate further analysis with ease. Otsu threshold algorithm has been adopted to estimate the spot size since it does not require close estimate of spot size and requires just one value reasonably close. All the grids were processed and spots were quantified in the cells eliminating local background. The spots with poor quality in either of the slide were flagged off and were eliminated during the normalization. If the intensity values approached to a maximum \((2^{16-1})\) per pixel for a 16 bit scanner comparison, those are no longer meaningful as the array elements become saturated and all these were excluded. Quantified spots from each analysis were exported for further analysis.

3.2.14 Data normalization

Total intensity normalization was first applied to all the data points assuming the initial RNA taken for the hybridization is equal in both the samples. It has been observed based on pre and post normalized RI plot (log ratio intensity vs log intensity product) the factor calculation for scaling up the data was not appropriate. Keeping the systemic errors like systematic dependence of \(\log_2\) values on the intensity, the LOWESS (LOcally WEighted linear regreSSion) normalization technique was applied to all the data points and compared with the total intensity of normalized plots. LOWESS normalization was performed which considered all the genes on the array, considered house keeping genes and internal control in calculating normalization factor.

3.2.15 Clustering of co-regulated genes

3.2.15.1 Analysis across the stress regimes

The genes were clustered with Hierarchical classification after normalizing expression data and unsupervised classification was followed. In all the data points from day 45 to day 81 of the development stages A2, A3, A4, vectors were taken for the (A2 denotes 60% FC, A3 denotes 40 % FC, A4 denote 15% FC) stress regime analysis of the expression data. Hierarchical clustering was carried out for either genes or experiments at an instance on whole slide data, as it is a memory intensive process. Average linkage
clustering technique was followed for hierarchical clustering. Significant gene clusters were identified by k-means clustering to ~10-20 groups based on hierarchical clustering results.

3.2.15.2 Analysis across the developmental stages:

The intensities captured at each developmental stage from 45-75 days within the stress regime were considered for time series analysis and carried out at all the stress regimes viz. A2 (60%FC), A3 (40%FC), A4 (15%FC). Panicle sample from day 59 to 75 were analyzed at each stress regime for expression patterns in panicle tissue in stress across the developmental stages.