Chapter V

Validation of identified differentially expressed transcripts in field drought stressed population
Environmental stresses can negatively impact agricultural crop yield and quality. As an adaptive strategy, plant genomes encode genes that produce proteins which functions in stress response and tolerance. Plants can perceive abiotic stresses and elicit appropriate responses with altered metabolism, growth and development. The regulatory circuits include stress sensors, signaling pathways comprising a network of protein-protein interactions, transcription factors and promoters, and finally the output proteins or metabolites. Despite substantial research on response to abiotic stresses by plants, there are still knowledge gaps regarding the molecular mechanisms that regulate the diverse functions of environmental stress-associated plant genes and proteins.

5.1 Introduction

Tea (*Camellia sinensis*, L), being a rain-fed crop is greatly influenced by weather. Drought stress results in many morphological, bio-chemical, physiological and molecular changes in the plant. Genetic improvement of adaptation to drought in tea is usually addressed through the conventional approach by selecting for yield and its stability over locations and years. But such selection programs are slow in attaining progress due to several reasons. In annual plants, such as maize (*Zea mays* L., Chapman and Edmeades, 1999); wheat (*Triticum, aestivum* L., Richards *et al.*, 2000) and sorghum (*Sorghum bicolor* L., Tuinstra *et al.*, 1998), yield improvement in water limited conditions is achieved by identifying secondary traits contributing to drought resistance and selecting for those traits in breeding programs.

Different studies have been reported related to physiological and biochemical response of tea plant in response to drought. Damayanti *et al.* (2010) showed that rate of photosynthesis, stomatal conductance, transpiration rate, relative water content and total soluble sugar content are the parameters which could be successfully incorporated into a drought screening procedure. Cheruiyot *et al.* (2007) reported that decline in soil water content (SWC) reduces both growth and polyphenol content in tea. Drought tolerant clones maintain a high polyphenol content at low SWC and thus polyphenols can be used as a biochemical indicator for selection of drought tolerant tea cultivars. One of the
physiological traits that may affect drought tolerance is the decline in whole plant water use during a soil water deficit event. As soil water deficit develops, plants undergo a transition from the water saturated phase, in which whole plant water use is not dependent on soil water content, to a second phase where water use is directly related to the availability of soil water (Sinclair and Ludlow, 1986). This transition is associated with a reduction in the average stomatal conductance (Earl, 2003) which is a key variable influencing leaf gas and water vapour exchange. Soil moisture stress affects the stomatal conductance and transpiration of tea leaves (Fordham 1971; Callander and Woodhead 1981; Gee et al., 1982; Saikia and Dey 1984; Squire 1990). Wijeratne et al. (1998) reported that the drought tolerant tea clone tides over dry periods by conservation of water through efficient stomatal control and effective osmotic adjustment, which enable it to absorb soil water at low water potential. Thus physiological functions of the leaf canopy play a decisive role in the water economy of plants which determines their growth and survival during water deficit condition.

Plant adaptive strategies to stress are coordinated and fine-tuned by adjusting growth, development, cellular and molecular activities. Soon after the perception and recognition of external changes, different signaling pathways are activated in order to convert a physical stress into a biochemical response, each of them promoting the expression of a set of stress-responsive genes; the full activation of signal cascades induced by a given stress event promotes acclimation and leads to stress tolerance. Tea plants can grow in to a 10-15 m tall tree (C. assamica) or a 5-8 m tall shrub (C. sinensis) if they are allowed to grow uninterrupted. However, in commercial plantations, tea is maintained as a bush of about 1 m tall, having a flat-topped foliage canopy with a depth of about 0.6 m (Costa et al., 2007). Thus, in normal field condition tea plant is always under stress. There is a probability that a gene which has been reported as stress induced in other plants may show constitutive expression in tea or vice-versa.

Genes induced during drought stress conditions are thought to function not only in protecting cells from water deficit by the production of important metabolic proteins, but also in the regulation of genes for signal transduction...
in the drought stress response (Shinozaki et al., 2003). Thus the gene products are classified into three major groups- (i) those that encode products that directly protect plant cells against stresses such as heat stress proteins or chaperones, LEA proteins, osmoprotectants, antifreeze proteins, detoxification enzymes and free radical scavengers (Bray et al., 2000); (ii) those that are involved in signaling cascades and in transcriptional control, such as mitogen-activated protein kinases (MAPKs), calcium dependent protein kinases (CDPKs) (Ludwig et al., 2004); SOS kinases (Zhu, 2001), phospholipases (Frank et al., 2000) and transcription factors (Shinozaki and Yamaguchi Shinozaki, 2000); (iii) those that are involved in water and ion uptake and transport such as aquaporins and ion transporters (Blumwald, 2000). Stress inducible genes have been used to improve stress tolerance of plants by gene transfer. It is important to analyze the functions of stress inducible genes not only to understand the molecular mechanisms of stress tolerance and responses of plants, but also to improve stress tolerance by gene manipulation (Seki et al., 2003) and/or molecular breeding.

Many agriculturally important traits such as productivity and quality, tolerance to environmental stresses, and some forms of disease resistance are controlled by polygenes and are “multifactorial” that, greatly depend on genetic × environmental (G × E) interactions. These complex traits are referred to as quantitative trait loci (QTLs), and it is challenging to identify QTLs based on only traditional phenotypic evaluation (Ross-Ibarra et al., 2007; Collard et al., 2005). Drought tolerance is also a polygenic trait which affects morphological, physiological, biochemical and molecular processes of plants (Zhu, 2002). Plant cellular water-deficit stress may occur under conditions of reduced soil water content. Under these conditions, changes in gene expression take place, with simultaneous up as well as down regulation. The changes in gene expression may be regulated directly by the stress conditions or may result from secondary stresses and/or injury responses (Hanson and Hitz, 1982). These are induced by a complex series of signal transduction events that have not been clearly delineated. Signals may result in changes of gene expression due to an injury response or it may responsible for inducing genes that may have adaptive functions. Gene expression
patterns are influenced by the severity, extent and rate of application of stress (Bray et al., 2000). Gene expression pattern may be altered at the initial step – increasing the transcription rate of specific gene – or at subsequent step that controls specific mRNA levels or the translation of specific mRNA. Together a complex pattern of gene expression is established that is the result of specific stress conditions.

Hanson and Hitz (1982) hypothesized that when stress is imposed rapidly a greater number of responses will be injury induced than under a slower long term application of water-deficit stress. Induction of gene expression does not necessarily imply that a gene will play an adaptive role. Depending upon the condition to which the plant was subjected, the gene expression pattern may change. Also, the drought under controlled condition (green house) may not be similar to what actually happens in the field. Field drought is a slow mechanism and the plants go through an adaptive process in contrast to the drastic changes of rapid dehydration in case of controlled experiments. For the present study, Chapter IV documents a set of drought responsive genes under controlled condition using SSH. But there is a strong probability that identified genes may not show same pattern in field drought condition. Thus, it becomes very important to check the expression of genes in field drought condition before any conclusion can be drawn. The expression of few genes may simply be a response to injury and doesn’t play any role against drought stress.

As explained earlier, drought tolerance is a combined effort of many loci resulting adaptation at the physiological and molecular level. Also, there exist genotypic differences towards adaptation leading to tolerant and/or susceptible characteristics. The aim the present experiment was to identify genes and physiological functions that are central to the establishment of drought tolerance in tea. This study was done to get an overview of correlation and/or association between transcript abundance (of selected drought induced genes) and physiological parameters in a parent progeny combination during field drought condition.

5.2 Materials and methods
5.2.1 Plant materials and experimental site

Experiments were conducted at Tocklai Experimental Station, Jorhat, Assam of the Tea Research Association and its out-stations in North-East India during 1970 to 1990 to screen out germplasm based on drought tolerance/susceptibility (Singh and Handique, 1993). TV series clonal cultivars from TV1 to TV26 and garden series were evaluated for drought tolerance. From the earlier studies done at Tocklai it was found that TV19 can be considered as drought tolerant and TV1 as drought susceptible. Fifty progenies from a mapping population (TS-463) generated from a cross between TV19 and TV1 was selected for the present study. This experiment was conducted at the Experimental Garden of Tocklai Experimental station, TRA, Jorhat. This area normally experiences seasonal dry spells during the months of September to March. The soil characteristics and weather condition of the experimental site are summarized in Table 5.1.

Table 5.1: Soil properties and weather conditions of field drought experimental site (July to September 2007).

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Elevation (meters above sea level)</td>
<td>96.5 amsl</td>
</tr>
<tr>
<td>2</td>
<td>Latitude and longitude</td>
<td>26°47’N ; 94°12’E</td>
</tr>
<tr>
<td>3</td>
<td>Soil type</td>
<td>Sandy-Loam</td>
</tr>
<tr>
<td>4</td>
<td>Soil pH</td>
<td>4.8 – 5.1</td>
</tr>
<tr>
<td>5</td>
<td>Average maximum temperature</td>
<td>31.6°C</td>
</tr>
<tr>
<td>6</td>
<td>Average minimum temperature</td>
<td>25.16°C</td>
</tr>
<tr>
<td>8</td>
<td>Organic matter content</td>
<td>0.8 – 1.2 % w/w</td>
</tr>
<tr>
<td>9</td>
<td>Bulk density</td>
<td>1.3 – 1.4 g/cc</td>
</tr>
<tr>
<td>10</td>
<td>Single super phosphate</td>
<td>500 g /cubic metre of soil</td>
</tr>
</tbody>
</table>

5.2.2 Drought Experiment

Fifty progenies (three years old) of TS-463 mapping population (raised from cuttings) were planted (June, 2006) in two replica plots (plot A and plot B) in a random complete block design at the Tocklai Experimental Garden (Figure 5.1). In June 2007, the rain-out shelter was constructed equipped with a
plastic roof and plastic barriers 4 feet below ground to prevent uncontrolled water in-flow and with plastic nets to exclude insects (Figure 5.2). The plants were regularly irrigated under the shelter until July 2007. Thereafter, irrigation was stopped to induce gradual water stress until the symptoms of wilting become evident in some plants. Physiological data and samples were collected for each day after ten days of drought stress. The first five plants showing severe wilting were considered as most drought susceptible compared to others in the plots. Samples were collected for these wilted progenies in plot A for expression analysis and labeled as DS/TS-463/W progenies (‘DS’ refers to drought susceptible and ‘W’ refers to wilting stage). The samples collected ten days before wilting day was considered as ‘before wilting stage’ of the plant and labeled as DS/TS-463/BW (‘BW’ refers to before wilting stage). Thereafter, wilted progenies were revived in plot A, while drought was continued in plot B to find out the most tolerant progenies. The five progenies showing wilting symptoms at the last in plot B were considered as most tolerant. Samples collected from these progenies were labeled as DT/TS-463/W progenies (‘DT’ refers to drought tolerant and ‘W’ refers to wilting stage). For before wilting stage of tolerant progenies same rule was followed i.e., samples collected ten days before wilting day. The before wilting samples of tolerant progenies were labeled as DT/TS-463/BW.

The drought tolerant progenies from plot B was revived before it enter into the permanent wilting stage. The samples for recovery stage were collected 15 days after irrigation and labeled as DS/TS-463/R for susceptible progenies and DT/TS-463/R for tolerant progenies. Samples in the present study refer to 3rd and 4th leaf of the plant. The lay-out of this experiment is shown in figure 5.3.

5.2.3 Physiological measurements

5.2.3.1 Gas exchange parameters

A portable photosynthesis system (PP system: CIRAS-I) was used to determine the stomatal conductance (Gs) and transpiration rate according to the procedure described in the user manual.
Figure 5.1: TS-463 parents and progenies in replica plot A and B. (I) Progenies and parents in two replica plot under rain-out shelters. (II) After 45 days of induced drought. Parents and progenies with brown background represent the most susceptible group showing wilting phenotypes. (III) Plants revived after irrigation (Plot A). In Plot B, plants with brown background represents permanently wilted condition while green background represents the tolerant progenies.
Figure 5.2: Experimental plants under rain-out Shelter.

Figure 5.3: Lay-out of induced drought stressed experiment of TS-463 parents and progenies.
Chapter V
Validation of identified differentially expressed transcripts in field drought stressed population.

5.2.3.2 Water use efficiency (WUE) (µ mol/m mol)

The water use efficiency was calculated using the data obtained from portable photosynthesis system (PP system: CIRAS-I) as described by Wibbe and Blanke (1995).

5.2.3.3 Relative water content (RWC) (%)

Leaf relative water content (RWC) was measured as described in Barrs and Weatherley (1968). Leaves were excised, weighed (FW), floated on distilled water for at least 8 h for full hydration of the plant tissue, blotted dry to remove surface water, and weighed again (TW). Dry weight (DW) was subsequently determined after oven-drying for 2 days and RWC was calculated using the equation:

$$RWC \% = \left[ \frac{(W-DW)}{(TW-DW)} \right] \times 100.$$  

Where, W: sample fresh weight; TW: sample turgid weight; DW: sample dry weight.

5.2.4 Real Time Quantitative PCR analysis

5.2.4.1 Total RNA extraction

Total RNA was isolated from leaf samples using RNAqueous Kit (Ambion, USA, Cat. No. AM1912) and the concentration were determined spectrophotometrically (BioPhotometer, Eppendorf, Germany). Following extraction the isolated total RNA was electrophoresed in 1% agarose gel to check for RNA quality, integrity and concentration.

5.2.4.2 mRNA Isolation

Poly A+ RNA was purified from total RNA using the PolyATtract® mRNA isolation system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The concentration of eluted mRNA was checked using a spectrophotometer (BioPhotometer, Eppendorf, Germany).
5.2.4.3 Reverse transcription

Moloney Murine Leukaemia virus (MMLV) reverse transcriptase with RNase H activity was used to generate cDNA strands following manufacturers protocol. 1 µg of total RNA or 10 ng of mRNA was taken as a starting material. The RNA was then mixed with oligo-dT primers and incubated at 65°C for 10 minutes (to inactivate the secondary structure of the single stranded RNA). This was followed by the addition of other components namely first strand synthesis buffer, reverse transcriptase, dNTPs and RNAse inhibitor. The resulting mix was incubated at 50°C for an hour. The activity of the reverse transcriptase was stopped by incubating the reaction mix at 85°C for 5 minutes. The first strand of cDNA so synthesized was stored at -20 °C till further use.

5.2.4.4 Internal standards (House-keeping genes)

Sequences for the house keeping genes were obtained from the public databases viz. the DNA Data Bank of Japan (DDBJ) and NCBI. The four genes considered in the study were 18S rRNA, 26S rRNA, Camellia bis-phosphate carboxylase small sub-unit and Camellia tubulin. The corresponding GenBank accession number and primer pair sequence for the gene are described in Appendix I. Primers for genes considered for study was designed using primer3 and DNASTAR primer designing soft wares.

5.2.4.5 Real time PCR assays

Real time PCR was done using LightCycler 480 SYBR Green I Master Kit (Roche Cat No. 04 707 516 001). PCR primers used were 0.4 µM concentration each (both forward and reverse primers) in the reaction. The PCR parameters programmed for PCR run with the LightCycler 480 SYBR Green Master using a LightCycler 480 Multiwell Plate 96 and the temperature targets as given in Appendix II and III.

The PCR Mix was prepared in a 1.5 ml reaction tube on ice. In short, 4.7 µl of supplied PCR grade water was mixed with 0.4 µl of forward and reverse primer and 4 µl of ‘LightCycler 480 SYBR green I Master Mix’ to get a final volume of
9.5 µl. Finally, 0.5 µl of template was added. The multi-well plate was sealed with LightCycler 96 multi-well sealing foil. Melting curve was run immediately after the last cycle to exclude any influence of primer-dimers pairs. Each reaction was performed in triplets to increase the reproducibility.

5.2.4.6 Real time PCR data acquisition and analysis

The data generated in a real time PCR machine is the crossing point values or the CP values which is the optimum number of PCR cycles required to reach a threshold transcript number i.e. around $10^{11}$ transcripts. This is the point where enough transcripts have accumulated and the fluorescence emitted at the said cycle number is just optimum for the camera to capture. So the expression of about 4 housekeeping genes in all the experimental samples was studied to derive with a normalization factor, which was then used as a function to obtain the normalized expression ratios for all other genes of interest. GeNorm was used to estimate the normalization factor (NFn) using n multiple reference genes, by calculating the geometric mean of the expression levels of the n best reference genes (Cavallari et al., 2009). The optimisation of the number of reference genes starts with the inclusion of the two genes with the lowest M value, and continues by sequentially adding genes with increasing values of M.

5.2.4.7 Hierarchical clustering

Hierarchical clustering was done as hit map generated online at www.genepattern.com. The data sheets were first transformed into a GCT file as an input file format for analysis in the gene pattern software.

5.2.5 Statistical analysis

For physiological parameters like WUE, leaf moisture content and rate of transpiration, and for quantification of gene expression normalized values by RT-PCR, the results are presented as mean values for three individual samples with standard errors. Statistical analyses were performed using principal component analysis (PCA) functions of the XLStat-Pro 7.5 (Addinsoft, New York, USA).
York, USA) software. A Pearson (n) correlation matrix was used to perform PCA analysis.

5.3 Results

5.3.1 Physiological responses and grading of drought stressed progenies

To get a better insight of drought stress response in tea, TS-463 progenies and parents (TV1 and TV19) were subjected to drought in field condition using a rain-out shelter. Although, all the plants were grown and exposed to drought stress from the same day, few plants showed symptoms of wilting very early than the other plants. As shown in Figure 5.3, few progenies showed wilting (drooping of leaves and loss of leaf turgor) after 45 days of water deficit stress. These plants were grouped as DS/TS-463/W. The samples collected ten days before wilting day is the before wilting stage. Thus susceptible progenies were at before wilting stage on 35\textsuperscript{th} day of water deficit stress. On the other hand the drought tolerant progenies showed symptoms of wilting on 75\textsuperscript{th} day of water deficit stress and thus the samples collected on 65\textsuperscript{th} day was considered as before wilting stage (DT/TS-463/BW). In the experiment, the samples that were collected after 15 days of irrigation were considered for recovery stage analysis.

During drought experiment, it was found that the drought tolerant group had shown varying degrees of drought effect like dullness in leaves, little loss of turgidity earlier than drought susceptible group but these tolerant plants continued to stay in a similar condition for a prolonged period (75 days of water deficit stress ‘DWS’) till wilting. On the other hand the susceptible plants showed symptoms of drought effect later than tolerant group but went sharply to wilting stage (45 DWS). Also, the susceptible progenies showed flowering near to wilting stage. The whole experiment of water deficit stress continued for 91 days. It should be noted that the days stated above are the average day of five progenies. TS-463-63; TS-463-74; TS-463-66; TS-463-62 and TS-463-70 was found to be positively heterotic (tolerant) whereas TS-463-23; TS-463-42; TS-463-43; TS-463-41; TS-463-71 was found to be negatively heterotic (susceptible).
Validation of identified differentially expressed transcripts in field drought stressed population.

Water deficit stress has exerted a negative effect on relative water content in both parents and progenies. As shown in Figure 5.4, there is a sharp decrease in RWC from before wilting stage to wilting stage in both the parents and progenies. The DT/TS-463 progenies can maintain high RWC compared to DS/TS-463 progenies at a given soil moisture content. Transpiration rate also decreases with the application of water deficit stress. The rate of transpiration of TS-463 parents and progenies at three stages of water deficit stress is shown in Figure 5.5. With the application of water deficit stress there is a sharp decrease in transpiration rate from before wilting stage to wilting stage. But transpiration rate is higher in drought tolerant progenies compared to drought susceptible progenies. WUE of TS-463 parents and progenies at three stages of water deficit stress is shown in Figure 5.6. As evident from the figure, drought stress has a negative impact on WUE i.e., with the increase in stress, there is a gradual decrease in water use efficiency. As DT/TS-463 progenies shows higher transpiration rate, they showed a lower WUE compared to DS/TS-463 progenies at before wilting stage. WUE throughout the water deficit stress experiment in DS/TS-463 as well as DT/TS-463 progenies is shown in Figure 5.6. The DS/TS-463 progenies initially maintained high WUE but sharply decrease after 35th DWS and reach approximately zero on 45th DWS. On the other hand, DT/TS-463 progenies although showed initial lower WUE, are able to maintain it upto 70th DWS. Thus DT/TS-463 progenies have certain adaptive features which enable them to maintain WUE at low soil moisture content up to a prolonged period.

5.3.2 Responses of drought stress at gene expression level

The expression of 21 genes (identified as ‘drought induced’ and given in Appendix VII) were studied at three stages of the field drought i.e., BW, W and R. As shown in Figure 5.7, wilting stage (W) of parents and progenies were identified as the day when the WUE becomes approximately zero. DS/TS-463 progeny showed wilting on 45th DWS whereas DT/TS-463 progenies showed wilting on 75th DWS.
Validation of identified differentially expressed transcripts in field drought stressed population.

Figure 5.4: Relative water content (%) of TS-463 parents and progeny at three stages of water deficit stress.

Figure 5.5: Rate of transpiration of TS-463 parents and progenies at three stages of water deficit stress.

Figure 5.6: WUE of TS-463 parents and progenies at three stages of water deficit stress.
The hierarchial clustering formed four distinct groups, based on stages of water deficit stress (drought stressed parents and progenies) (Figure 5.8). Cluster I includes P2/TV19/W, P1/TV1/W and DS/TS-463/W. Cluster II includes P1/TV1/BW, P2/TV19/BW, DS/TS-463/BW and DT/TS-463/BW. P1/TV1/R, P2/TV19/R, DS/TS-463/R and DT/TS-463/R were grouped together to form cluster III. The fourth cluster includes only the DT/TS-463/W progenies. Therefore, the clustering grouped samples on the basis of stages of water deficit except for cluster IV. The clustering of genes formed three clusters. Cluster I include genes which showed significantly higher expression in DT/TS-463/W. Cluster III includes genes showing higher expression in DS/TS-463/BW and DT/TS-463/BW. Cluster II genes didn’t showed any conclusive pattern of expression. Genes included in cluster I are amine oxidase, hydrogen peroxide induced protein, diaminopimelate decarboxylase, lipase, ethylene responsive transcription factor, ACC oxidase and hexose transporter. Genes included in cluster III are dehydrin, cinnamoyl CoA reductase, ABA ripening protein, ascorbate peroxidase, galactinol synthase, glutathione peroxidase, LEA and glutathione S-transferase.

The relative expression of selected genes at three stages of water deficit stress in P1 (TV1), P2 (TV19), DS/TS-463 progenies and DT/TS-463 progenies is shown in Figure 5.9, 5.10, 5.11 and 5.12 respectively. The expression of genes shows four patterns- (i) genes showing gradual higher expression from before wilting to recovery, (ii) genes which show initial higher expression at BW, expression decreases at wilting stage but expression again increases at recovery, (iii) genes which initially showed lower expression at before wilting, expression increases at wilting stage but again decreases at recovery, (iv) pattern four includes those genes which shows gradual decrease in expression from before wilting to recovery. The list of genes showing the four patterns of expressions in TV1, TV19, DS/TS-463 and DT/TS-463 is shown in Table 5.2. Thus it was found that most of the genes of TV19 and DT/TS-463 falls under pattern III and IV. On the other hand in TV1 and DS/TS-463 shows pattern I and II (Figure 5.13).
Three stages of susceptible progenies in Plot A.

Three stages of tolerant progenies in Plot B.

Figure 5.7: Different stages of water deficit stress where expression analysis of the selected genes was done. SB = TS-463 susceptible progenies at before wilting stage; SW = TS-463 susceptible progenies at wilting stage; S = TS-463 susceptible progenies at recovery stage; TB = TS-463 tolerant progenies at before wilting stage; TW = TS-463 tolerant progenies at wilting stage; TR = TS-463 tolerant progenies at recovery stage.
Figure 5.8: Hit map for expression patterns generated from selected genes at three stages of water deficit stress.
Validation of identified differentially expressed transcripts in field drought stressed population.

Figure 5.9: Relative expression of selected drought induced genes in P1 (TV1) at three stages of water deficit stress.

Figure 5.10: Relative expression of selected drought induced genes in P2 (TV19) at three stages of water deficit stress.

Figure 5.11: Average relative expression of selected genes in drought susceptible TS-463 progenies at three stages of water deficit.
Validation of identified differentially expressed transcripts in field drought stressed population.

Genes which showed pattern III expression in DT/TS-463 includes diaminopimelate decarboxylase, lipase, hexose transporter, ethylene responsive transcription factor, Drm3, amine oxidase, proline rich protein, hydrogen peroxide induced protein and ACC oxidase. These genes showed higher expression at wilting stage of drought stress. Genes which showed pattern IV type of expression includes dehydrin, ABA ripening protein, glutathione peroxidase, cinnamoyl CoA reductase, calmodulin binding protein. These genes showed higher expression at before wilting stage of drought stress.

The comparative expression of genes at three stages of water deficit stress between parents and progenies shows significant differences at before wilting stage (Figure 5.14) and wilting stage (Figure 5.15) of drought stress. Most of the genes selected have exhibited higher expression in DT/TS-463 progenies. But at recovery stage (Figure 5.16), no significant differences were observed. The probable reason may be that the genes selected for the present study were from SSH libraries constructed from before wilting and wilting stage samples (Chapter IV).
Validation of identified differentially expressed transcripts in field drought stressed population.

Figure 5.13: Expression pattern of selected drought induced genes in TS-463 parents and progenies.

Figure 5.14: Relative expression of selected genes in parents and progenies of TS-463 at before wilting stage of water deficit stress.
Table 5.2: Expression pattern of drought induced genes in TS-463 parents and progeny.

<table>
<thead>
<tr>
<th>Gene Expression</th>
<th>TV1</th>
<th>TV19</th>
<th>DS/TS-463</th>
<th>DT/TS-463</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PATTERN I</strong></td>
<td>Amine oxidase, Aquaporin, Proline rich protein, Diaminopimelate decarboxylase, Zinc finger protein, Lipase, ACC oxidase, Calmodulin related protein, Ethylene responsive TF, DNA J,</td>
<td>Zinc finger protein, Proline rich protein, Diaminopimelate decarboxylase, Zinc finger protein, Lipase, ACC oxidase, Calmodulin related protein, Ethylene responsive TF, DNA J,</td>
<td>Amine oxidase, Proline rich protein, Diaminopimelate decarboxylase, Zinc finger protein, Lipase, ACC oxidase, Calmodulin related protein, Ethylene responsive TF, DNA J,</td>
<td>Zinc finger protein, Proline rich protein, Diaminopimelate decarboxylase, Zinc finger protein, Lipase, ACC oxidase, Calmodulin related protein, Ethylene responsive TF, DNA J,</td>
</tr>
<tr>
<td><strong>PATTERN II</strong></td>
<td>Dehydrin, ABA ripening protein, Ascorbate peroxidase, glutathione peroxidase, Ethylene responsive TF, DNA J,</td>
<td>ABA ripening protein, Ascorbate peroxidase, glutathione peroxidase, Ethylene responsive TF, DNA J,</td>
<td>Dehydrin, ABA ripening protein, Ascorbate peroxidase, glutathione peroxidase, Ethylene responsive TF, DNA J,</td>
<td>Ascorbate peroxidase, DNA J, Galactinol synthase</td>
</tr>
<tr>
<td><strong>PATTERN III</strong></td>
<td>Hydrogen peroxide induced protein, Hexose transporter, Drm 3</td>
<td>Hydrogen peroxide induced protein, Hexose transporter, Drm 3</td>
<td>Hydrogen peroxide induced protein, Hexose transporter, Drm 3</td>
<td>Hydrogen peroxide induced protein, Hexose transporter, Drm 3</td>
</tr>
<tr>
<td><strong>PATTERN IV</strong></td>
<td>LEA, Galactinol synthase, Cinnamoyl CoA reductase</td>
<td>LEA, Galactinol synthase, Cinnamoyl CoA reductase</td>
<td>LEA, Galactinol synthase, Cinnamoyl CoA reductase</td>
<td>LEA, Galactinol synthase, Cinnamoyl CoA reductase</td>
</tr>
</tbody>
</table>


5.3.3 Co-relation between transcript abundance and physiological parameters:

A principal component analysis (PCA) was carried out to find any correlative relationships that may exist between transcript abundance (of selected drought induced genes) and physiological parameters like WUE, leaf moisture content and rate of transpiration in the 3rd and 4th leaves of the parent-progeny combination at three stages of drought stress (BW, W and R) considered for this study. PCA analysis was performed for the different variables (physiological parameters and normalized gene expression values) using all the genotypes as
a reference. The PCA type that was used during the computations is the Pearson's correlation matrix, which corresponds to the classical correlation coefficient. Axes F1 and F2 were used to display plots as the percentage of variability represented by these two factors which was high (69.5%). The correlation matrix thus obtained is given in Appendix VIII. From the correlation matrix itself it is clear that the genes selected for the present study is correlated to one or more physiological parameters at significant level. Water use efficiency is negatively correlated to leaf moisture content and rate of transpiration. Genes which were found to be significantly correlated with WUE includes amine oxidase, dehydrin, hydrogen peroxide induced protein, diaminopimelate decarboxylase, lipase, hexose transporter, ethylene responsive transcription factor, drm3 and ACC oxidase. Leaf moisture content and rate of transpiration are positively correlated. Leaf moisture content was found to be negatively correlated with genes amine oxidase, aquaporin, proline rich protein, hydrogen peroxide induced protein, diaminopimelate decarboxylase, zinc finger protein, lipase, ethylene responsive transcription factor and ACC oxidase. On the other hand, LEA, ABA ripening protein, ascorbate peroxidase, glutathione peroxidase, galactinol synthase and cinnamoyl coA reductases were positively correlated with leaf moisture content.

The Appendix IX (a) shows the eigenvalues which reflect the quality of projection from the higher dimensional initial table to a lower number of dimensions. The first eigenvalue equals to 10.039 and represents 41.829 % of the total variability (Figure 5.17). The second eigenvalue equals to 6.640 with % variance of 27.667 and cumulative variance of 69.49%. Each eigenvalue corresponds to a factor and each factor to a one dimension. A factor is a linear combination of initial variables, and all the factors are un-correlated (r=0). The eigenvalues and the corresponding factors are sorted by descending order of how much of initial variability they represent (converted to %). Therefore, if the data generated in the present study are represented with axis F1 and F2, the outcome is 69.49 % of total variability of data. So, axis F1 and F2 were selected for representation of the data. The correlation circle (Figure 5.18) thus
developed shows the correlation between the genotypes and the variables. It shows that the horizontal axis (F1) is linked with transpiration rate, amine oxidase, diaminopimelate decarboxylase, LEA, ABA ripening protein, Ascorbate peroxidase, lipase, ethylene responsive transcription factors, ACC oxidase, galactinol synthase and glutathione S-transferase. On the other hand vertical axis (F2) is linked with dehydrin and cinnamoyl CoA reductases. This trend can also be confirmed with squared cosine table (Appendix IX b). The observation graph (Figure 5.19) shows all the genotypes and their interrelationships based on the variables considered and represented between axis F1 and F2. It also shows DT1/TS-463/BW to DT5/TS-463/BW and DT1/TS-463/W to DT5/TS-463/W as a separate group. This can be also derived from squared cosines of the observation table given in Appendix IX c. Also the drought tolerant progenies are separated from the drought susceptible progenies. Thus, between the germplasm there is a high variability of the studied traits as they are separated on the basis of time-points during drought and this variability is genetically controlled.

Figure 5.17: Eigenvalues between various axis.
The Biplot (Figure 5.20) is representing genes and physiological parameters and their relationship with the genotypes. This also shows the distribution of clusters of variables in order to determine if they could be differentiated by specific relationships existing between these variables. For example, most of the genes and physiological parameters are linked with DT/TS-463 progenies at BW and W. DT/TS-463/BW is more related to transpiration rate and leaf moisture content, whereas DT/TS-463/W is more related to WUE.

Figure 5.18: Correlation circle using axis F1 and F2 showing the correlation between the variables (physiological parameters like WUE, leaf moisture content and transpiration rate, and gene expression normalized values) [TR- Transpiration rate, LMC – Leaf moisture content, LEA – Late embryogenesis abundant protein, GS- Galactinol synthase, GT- Glutathione transferase, AP- Ascorbate peroxidase, ARP- ABA ripening protein, GP- Glutathione peroxidase, CCR- Cinnamoyl CoA reductases, DH- Dehydrin, CB- Calmodulin binding protein, HT- Hexose transporter, HPP- Hydrogen peroxide induced protein, ACCO- ACC oxidase, WUE- Water use efficiency, AO- Amine oxidase, ERTF- Ethylene responsive transcription factor, DAD- Diaminopimelate decarboxylase, LP- Lipase, PRP- Proline rich protein, AQ- Aquaporin, ZFP- Zinc finger protein.
Chapter V

Validation of identified differentially expressed transcripts in field drought stressed population.

Figure 5.19: Correlation between TS-463 parents and progenies considered at three stages of drought stress.

Figure 5.20: Biplot showing the correlation between the genes and physiological parameters with TS-463 parents and progenies.
5.4 Discussion

Adaptation to drought is undoubtedly one of the complex processes, involving numerous changes including attenuated growth, the activation/increased expression or induction of genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways. However, no consensus has been reached in defining the key processes determining tolerance and the secondary follow-up processes.

Drought avoidance is characterized by anatomical and morphological changes that enable plants to maintain high water potentials by reducing transpiration and increasing water uptake, but may negatively affect productivity. Drought tolerance, on the other hand ensures that cellular and molecular structures are not damaged even under severe desiccation (Vartania 1996). In the present study drought stress symptoms were first visible in drought tolerant progenies but they survived longer. By contrast susceptible progenies showed symptoms lately but wilted earlier. It is inferred that the tolerant progenies are able to sense water stress much earlier and the morphological effects seen is the part of their adaptation so that they can prepare for the forthcoming stress. The flowering of susceptible progenies shows that the plant is trying to avoid stress by completing its life cycle. The tea industry needs plants which can sustain drought condition without loss of minimal yield (two and a bud). In the present experiment it was interesting to find few progenies which didn’t showed avoidance character; rather they continued to grow (tolerant).

RWC represents a useful indicator of the state of water balance of a plant, essentially because it represents the absolute amount of water, which the plant requires to reach artificial full saturation (González and González-Vilar, 2001). In the present experiment, RWC decreases with increasing stress showing a negative relation. Similar type of results has also been reported in other plants (Mekliche et al., 1992). The DT/TS-463 progenies can maintain a high RWC
compared to drought susceptible progenies. David (2002) reported that low relative water content strongly reduced photosynthesis, stomatal conductance and evaporation activities in olive trees. The higher transpiration rate of drought tolerant progenies indicates that the plant is able to maintain turgor at low soil moisture content. DaMatta et al. (2003) concluded that osmotic adjustment under drought conditions was the main factor that contributes to maintain higher transpiration efficiency. The DT/TS-463 progenies are able to maintain WUE upto 70\textsuperscript{th} DWS whereas DS/TS-463 progenies show nil WUE on 45\textsuperscript{th} DWS. Similar type of results has also been reported in other plants (Donovan and Ehleringer, 1994; Dudley, 1996; Heschel et al., 2002).

The hierarchical clustering formed four cluster based on stages of water deficit stress. The parents and progenies at before wilting stage and recovery stage formed cluster II and cluster III respectively. At wilting stage the parents and susceptible progenies formed cluster I, but the tolerant progenies at wilting stage was grouped separately as cluster IV. Thus the tolerant progenies seem to adapt better at wilting stage. The clustering of genes showed interesting results. Three clusters were formed. Cluster I genes showed more expression in DT/TS-463 progenies at wilting stage and cluster III includes genes showing higher expression at before wilting stage of DT/TS-463 and DS/TS-463 progenies. Based on these observations it is inferred that cluster I genes are late responsive genes and cluster III genes are early responsive genes.

The selected drought induced genes showed four types of expression pattern at three stages of water deficit stress. Most of the genes in TV19 and DT/TS-463 showed III and IV expression pattern. Pattern IV includes genes (dehydrin, ABA ripening protein, glutathione peroxidase, cinnamoyl CoA reductase, calmodulin binding protein) which showed higher expression at before wilting stage. The expression of these genes may be related with the ability of DT/TS-463 to maintain turgor at low soil water content along with WUE. All of these genes may be considered as early responsive genes. As explained earlier DT/TS-463 showed effect of drought stress earlier than DS/TS-463. This morpho-
physiological effect seen could be related now to higher expression of calmodulin related protein and ABA ripening protein at before wilting stage of water deficit stress. Drought stress stimulates ABA biosynthesis and accumulation by activating genes involved in ABA biosynthetic pathway, which itself could be mediated by calcium dependent phosphorylation cascade. ABA could also up-regulate expression of ABA biosynthetic genes via calcium signaling pathways (Xiong et al 2002, Zhu, 2002). ABA stimulates osmotic adjustment (Ober and Sharp, 1994), induces the synthesis of protective proteins such as LEA (Bray, 1993) and it has also been shown to induce the expression of various stress induced genes (Shinozaki and Yamaguchi-Shinozaki, 2007). Various transcription factors such as DREB2A/2B, AREB1, RD22BP1 and MYC/MYB are known to regulate ABA responsive gene expression through interacting with their corresponding cis-acting elements (Tuteja, 2007). Upon exposure to abiotic stress, ROS forms in the plant tissues. These are compounds such as superoxide anions, hydrogen peroxide, and hydroxyl radicals that damage cells by oxidizing vital biomolecules (Carraggio and Tuberosa, 2004). Antioxidants act on different cell compartments, scavenging ROS free radicals to detoxify the plant. Enhance ROS scavenging which results in improve detoxification system seems to provide tolerance to DT/TS-463 progenies with the higher expression of genes such as glutathione peroxidase and glutathione S-transferase. Another important function which could be related is that chaperones stabilizes membranes and proteins by keeping them properly folded and repairing them (Coraggio and Tuberosa, 2004). This prevents the enzymes and proteins from malfunctioning, which is often the case under drought stress. LEAs are thought to benefit plants both by targeting free radicals and acting as chaperones (Shinozaki and Yamaguchi-Shinozaki, 2007). HVA, a LEA gene in barley, has been successfully manipulated to increase salt and drought tolerance. Plants carrying an up-regulated form of the gene that were exposed to stress took longer to show symptoms of damage, had greater yield and recovered more quickly than controls (Corragio and Tuberosa 2004). Another group with chaperones like
function is dehydrin. They are the group of heat stable plant proteins believed to play a protective role during cellular dehydration (Close, 1996; Champell and Close, 1997) and in stabilizing cells under stress (Close, 1996; Suprunova et al., 2004). Another gene showing higher expression at before wilting stage of DT/TS-463 is aquaporin. Aquaporins are considered to be the main channels for the transport of water, as well as small neutral solutes and CO₂, through the plant cell membrane (Tyerman et al., 2002; Uehlein et al., 2003).

Pattern III includes diaminopimelate decarboxylase, lipase, hexose transporter, ethylene responsive transcription factor, Drm3, amine oxidase, proline rich protein, hydrogen peroxide induced protein and ACC oxidase, all of which showed higher expression at wilting stage. It seems that these genes play a role towards enhanced tolerance in DT/TS-463 progenies by maintaining cell structure and functions. Proline rich proteins are thought to provide stability to other proteins in osmotic stress (Ingram and Bartels, 1996).

Plant hormone ethylene has been reported in regulating leaf senescence. It has been reported that defects in ethylene synthesis or perception delay leaf senescence in tomato and Arabidopsis (Abeles et al., 1992; Davies and Grierson, 1989; Grbic and Bleecker, 1995; John et al., 1995; Picton et al., 1993). ACC oxidase catalyzes the last step of ethylene biosynthesis in plants. The increased expression of ACC oxidase in DT/TS-463 at wilting stage shows that the plant now tries to shed older leaves to reduce its metabolism and energy consumption. At wilting stage, plant promotes increased ethylene production by increasing ACC synthesis and ethylene biosynthesis (Apelbaum and Yang, 1981; McKeon et al., 1982; McMichael et al., 1972). Another gene supporting it is lipase which is also found to be up-regulated at wilting stage of water deficit stress. Lipase has been reported to be up-regulated during leaf senescence (Kong et al., 2006) and transgenic Arabidopsis plants in which levels of the senescence-induced lipase protein has been reduced show delayed leaf senescence.
The original aim of the present study was to determine through a statistical clustering analysis, if physiological traits related to water stress and its components were correlated with molecular traits viz., gene expression and transcript accumulation in the third and fourth leaves of tea, which are considered as metabolically most active. It was observed that physiological parameter like WUE formed a close group with genes such as calmodulin binding protein, *drm3*, hexose transporter, hydrogen peroxide induced protein, *ACC oxidase*, *lipase*, ethylene responsive transcription factor and *diaminopimelate decarboxylase*. This means that for tolerance during wilting stage, the above mentioned genes and WUE are the markers. Such a strategy may be a way of determining the predictive value of a physiological trait or a group of physiological traits of a given organ or tissue in the amelioration of water stress. The result obtained means that there is a complex interaction between the expression of these traits that depends both on the genetic background and on the genotype examined. Therefore, clustering various physiological and molecular traits in a given organ can provide information on the capacity of a specific progeny to use or remobilize precursors both at the physiological and molecular levels. It will be interesting to determine if the genetic variability observed, for the most interesting set of marker traits evaluated in a given progeny, can be further used in a breeding programme to produce commercial hybrids, tolerant to water stress.

5.5 Conclusion

The present study is an effort towards understanding the expression pattern of selected drought induced genes during field drought condition taking a parent progeny combination. Study was focused on the expression of drought induced genes at three stages of water deficit stress *viz.*, before wilting, wilting and recovery. The drought stress showed negative impact on all the considered physiological parameters. Drought tolerant group showed initial lower WUE but it was able to maintain it up to prolonged period compared to susceptible group.
The expression analysis revealed a set of genes with higher expression at before wilting stage which includes dehydrin, ABA ripening protein, glutathione peroxidase, cinnamoyl CoA reductase, calmodulin binding protein. The enhanced expression of these genes was related to increased tolerance of DT/TS-463 at before wilting. Statistical analysis showed co-relation between genes and physiological parameters with the tolerant TS-463 progenies and thus these genes can be a potential marker for identification of drought tolerant genotype for MAS in tea. The present work not only provides a clue for better understanding of the mechanisms by which tea plants deal with drought stress, but also to aid the tea industry on its search for drought tolerant tea plants. The selected five tolerant progenies can withstand drought stress and thus can be used for future breeding purposes.