Transcriptome-based identification of drought responsive genes in the tea plant

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ABSTRACT

Tea (*Camellia sinensis* L. (O) Kuntze) is one of the most widely consumed beverages worldwide. Tea growing areas in Kenya often experience drought periods which cause accumulated soil water deficit. These adversely affect tea production and hence necessitate a need to develop drought-adapted tea cultivars that can withstand the stress challenge. Development of such cultivars can be facilitated by better understanding of genetic mechanisms underlying tolerance of the tea plant to water deficit. Tea plants respond to water deficit through poorly understood molecular processes. The present study was therefore, designed with the objective of identifying genes putatively conferring tolerance in the tea plant. Drought tolerant (TRFCA SFS150) and susceptible (AHP S15/10) tea cultivars, both 18-month old, were each separately exposed to water stress or control conditions of 18% and 34% soil moisture content, respectively, for three months in a randomized complete block (RCB) design with three replicates. Fresh shoots ($n = 5$) were randomly selected and separately harvested from each treatment and replicate. Total RNA of the shoots were extracted, their mRNA reverse transcribed and sequenced on Roche 454 high-throughput pyrosequencing platform. Overall, 232,853 reads were generated. The reads were quality-filtered, trimmed and assembled into 460 long transcripts (contigs). Contigs were annotated using BLAST searches against similar proteins in the Arabidopsis proteome and blast2go against non-redundant database to determine gene ontologies. Drought-related genes including heat shock proteins (HSP70), superoxide dismutase (SOD), catalase (cat), peroxidase (PoX), calmoduline-like protein (Cam7) and galactinol synthase (*Gols4*) were induced in plants exposed to drought. Additionally, the expressions of HSP70 and SOD were higher in the drought tolerant relative to the susceptible cultivar under drought conditions. Loci with known functional links to physiological and biochemical features of drought response appear to mediate tolerance to drought in *C. sinensis.* The loci present potential molecular markers for drought tolerance that can be explored through functional genomics to better understand molecular mechanisms underlying drought tolerance in *C. sinensis.*

Key words: Cultivars, Drought, Genes, Tea Plant, Water Deficit.

INTRODUCTION

Tea leaf extracts are among the most widely consumed beverages in the world (Cabrera *et al.,* 2003). The popularity of this beverage is ascribed to its aroma, pleasant taste and medicinal benefits; the latter conferred by its potent antioxidant activity (Frei and Higdon, 2003; Lin *et al.*, 2003). In Kenya and several African and Asian countries, tea is an economically important crop contributing significantly to foreign exchange earnings and rural development (Wachira and Ronno, 2004). Tea plants grow as an evergreen bush that can attain a height of up to 15 m high in the wild. However, in cultivation, the crop is maintained at 0.6-1.0 m high to facilitate harvesting of the leaves (Vo, 2006). Cultivated tea is diploid ($2n = 2x = 30$ (Bezbaruah, 1971; Kondo, 1977) with a relatively large genome size of about 4.0 GB (Tanaka and Taniguchi, 2006).

In most countries where tea is cultivated, it is grown under rain-fed conditions. The crop flourishes in minimal but well distributed rainfall averaging 1150-1400 mm per year (Carr, 1972). Most of the tea growing areas in the world are prone to drought. In Kenya, stress associated with drought is responsible for a 14% to 20% reduction in yield (Ngetich *et al.*, 2001) and 6% to 19% mortality of the plants (Cheruiyot *et al.*, 2007). Like other plants, tea responds to drought at the physiological (Shakeel *et al.*, 2011; Maritim *et al.*, 2014), biochemical (Chaves *et al.*, 2003; Xu *et al.*, 2003; Maritim *et al.*, 2014) and molecular levels (Bartels and Sunkar, 2005; Shinozaki *et al.*, 2003). Since plant responses are controlled by the genome, most studies focus mainly on molecular response to stress (Mohammad and Lin, 2010). To better understand the mechanism involved in molecular response to water stress, genes responsible must be characterized. Studies have shown that several classes of genes including those responsible for regulation, signalling and cellular adaptation are induced in response to water deficit (Mohammad and Lin, 2010). The genetic basis of drought tolerance in *C. sinensis,* however, still remains poorly understood with limited amount of data available in the databases. With respect to drought, dr1, dr2 and dr3 drought responsive ESTs from leaf tissues have been reported using differential display technique (Sharma and Kumar, 2005). Another 572 EST from young roots of drought tolerant tea cultivars have also been discovered using suppression subtractive hybridization (SSH) technique (Das *et al.*, 2012). Muoki *et al.* (2012) also reported ESTs responsible for cell rescue, defence, cellular transport, metabolism, energy, protein synthesis, cell cycle and DNA processing, signal transduction, transcription and biogenesis of cellular components in drought stressed tea. In the drought tolerant cultivar, chaperones and defence related genes, traumatin-like proteins, chitinase and heat shock proteins, were over-expressed in leaf tissues as compared to the susceptible cultivar (Muoki *et al.*, 2012). As a result of this technique, sequencing of cDNA library clones, generation and analysis of ESTs of *C. sinensis* provide mRNA expression profile and a rapid, low cost and efficient way to identify functional genes. Other than the tea ESTs, 1 Genome Survey Sequence, two sequences reads archives, and 770 proteins have been deposited in genebank. A better understanding of the genetic basis of drought tolerance in tea is essential in enhancing the efficiency and effectiveness of tea breeding programmes. The present study was, therefore, designed to identify drought-responsive gene(s) in two tea cultivars widely grown in Kenya to facilitate future research and development of drought tolerant tea cultivars.

EXPERIMENTAL MATERIALS AND DESIGN

The experiment was carried out in a rain-out shelter at the Tea Research Foundation of Kenya (TRFK) located in Kericho County at Latitude 0° 22'S, Longitude 35° 21' E, and altitude of 2180 m a.m.s.l. The rain-out shelter was constructed as described by Cheruiyot *et al.* (2008). 18 month old, vegetatively propagated, and well-rooted drought tolerant (TRFCA SFS150) and drought susceptible (AHP S15/10) tea plantlets were separately planted in 1000-gauge black polythene tubes 0.3 m in diameter by 0.3 m deep. Drought sensitivities of the cultivars were previously confirmed by breeders, and commercially established on the basis of yield stability

during drought periods (Kamunya *et al.*, 2009). The potted plants were allowed to establish for two months before transferring them to the rain-out shelter. The potted plants were arranged according to treatments in a completely randomized design and replicated three times giving a total of 72 experimental units. All plantlets were regularly and uniformly watered to established field capacity levels (34% soil moisture content (SMC) as determined using a time domain reflectometer (TDR) soil moisture meter) for two weeks to acclimatise (Cheruiyot *et al.*, 2007) after which watering was progressively reduced on weekly basis to respective treatment levels. The three soil moisture content treatments subjected to the eight cultivars were 34% v/v (high soil moisture/field capacity), 26% v/v (moderate soil moisture) and 18% v/v (low soil moisture). These soil water regimes were based on calculations determined by Cheruiyot *et al.* (2008). Each experimental plot was maintained at the predetermined soil moisture content during the experimental period. The control (non-droughted), at 34% soil moisture content, was watered throughout the experiment. The soil moisture content was routinely checked using a TDR soil moisture meter (TRIME-FM-2-Eijkelkamp Agrisearch Equipment, The Netherlands) according to the manufacturer's instructions, and maintained at desired levels by watering. SMC (% v/v) measurement was done twice daily at 1000hrs and 1500hrs and maintained within ±2% of the treatment level.

The growing conditions in the rain-out shelter were also monitored by determining temperature and relative humidity using maximum and minimum thermometer and hygrometer, respectively.

SAMPLING AND EXTRACTION OF RNA FROM *CAMELLIA SINENSIS* LEAVES

The third and fourth leaves (n = 10) of fresh shoots were randomly selected and separately harvested from each treatment and replicate, and immediately snap frozen in liquid nitrogen. Total RNA was isolated from each of the liquid nitrogen frozen (100mg per replicate) and grounded leave samples using the ZR Plant RNA Miniprep Kit *(Zymo Research,* Irvine, CA, USA). Subsequently, mRNA was isolated from the total RNA using a mRNA Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. In all cases, the integrity of extracted RNA was validated by electrophoresis in 1.0% agarose (Sigma-Aldrich Chemie, Gmbh) RNA denaturing gel in 1.4% sodium phosphate with 1 μg/ml ethidium bromide staining for visualization. The concentration of total RNA and mRNA was also determined spectroscopically (Sambrook *et al.*, 1989) using a 2000-NanoDrop spectrophotometer (Thermo Fisher Scientific, DE, USA).

PREPARATION AND PYROSEQUENCING OF *CAMELLIA SINENSIS* cDNA LIBRARY

cDNA libraries were synthesized from the isolated mRNA using a cDNA Rapid Library Preparation kit for GS FLX Titanium Series (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The products were purified to remove fragments less than 50 bp long using Individual Sample Cleanup (ISC) sizing solution. The cDNA libraries were subsequently quantified and assessed for quality using a TBS 380 Fluorometer (Turner Biosystems, USA) and Agilent Bioanalyzer High Sensitivity DNA chip (Agilent Technologies, Germany), respectively. Additionally, clonal amplification of the product was done through emulsion PCR (emPCR) using the emPCR kit for GS FLX Titanium series (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The PCR program used comprised: 1 cycle at 94^0C for four minutes, 50 cycles at 94^0C for 30 seconds, 58^0C for 4.5 minutes, 68^0C for 30 seconds, followed by a 10^0C hold. Sequencing primer was annealed to the PCR products to form a library of clonally amplified DNA fragments for each treatment and replicate that were subsequently loaded onto a PicoTiterPlate™ (Roche Applied Science, Mannheim, Germany) and separately sequenced on a half-plate run on a 454 GS FLX Titanium

Series sequencer. The emergent data was processed using GS FLX gsRunBrowser version 2.5.3 (Roche Applied Science, Mannheim, Germany) to obtain 454 sequence fasta files (sff) with quality scores.

DATA PROCESSING AND ANALYSIS

The raw reads were processed by removing adaptor sequences, redundant reads and those containing more than 10% N (ambiguous bases in reads), and low-quality reads (containing more than 50% bases with Q-value < 20). The quality of the reads data was assessed based on the base-calling quality scores using FastQC software version 0.10.1, Babraham Bioinformatics, UK. The reads were subsequently *de novo* assembled using Newbler program version 1.03 (Roche Applied Science, Mannheim, Germany). All the assembled contigs longer than 100 bp were annotated by BLAST analysis (Altschul *et al.,* 1997) against similar proteins in the *Arabidopsis* proteome (https://www.arabidopsis.org/), and BLASTx routine with *E*-value threshold of10^{-5} in Blast2GO software (Conesa *et al.,* 2005) against NCBI non redundant (nr) (http://www.ncbi.nlm.nih.gov), and Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg), databases to determine category and metabolic pathway ontologies of the differentially induced genes between the treatments.

RESULTS

De novo transcripts from the *Camellia sinensis* leaf transcriptome

Overall, 232,385 reads were generated from the cDNA libraries from all treatments and replicates. The reads length ranged between 40 and 1143bp in length with an average of 369bp. FastQC analysis revealed that all four libraries had Phred-like quality scores greater than Q20 level (with an error probability of 0.01). All high-quality reads were deposited in the National Centre for Biotechnology Information (NCBI) Short Read Archive (SRA) database under the accession number SRX485271. The *pre-processed* sequences were assembled into 460 contigs of 100-2,466 bp length (mean 250.28 bp) with GC content of 43.91%.

Gene ontology and KEGG functional annotation of the tea transcript

The contigs were categorized into three broad categories (biological processes, cellular component and molecular functions) as established for the *Arabidopsis* proteome (Figure 1). In the biological process category, the "metabolic processes" related genes were dominant (20%), followed by "multicellular organismal development" (15%) and "cellular processes" (12%). It was also notable that 10% of the genes in this category represented genes related to "stimulus response". The "secretion" related genes (0.4%) were the least in this category. In the cellular component category, genes assigned to the intracellular region accounted for the largest group (78%) followed by cell part (2%) whereas genes of the extracellular region were the least (1%). In the molecular function category, the highest percentage was covered by binding related genes (43%), followed by the catalytic activity related genes (27%), nucleic acid binding (10%), and structural molecule activity related genes (10%). The signal transduction (2%) and transporter activity (2%) related genes were the least in this category of genes.

The most dominant biological pathways that were active in the leaf of *C. sinensis* from the present study are presented in Figure 2. The pathways with the most representation of the contigs were related to "oxidative phosphorylation" and photosynthetic processes 53% and 31%, respectively. "RNA synthesis" related contigs accounted for 8% while "nitrogen metabolism", "ribosome", "carbohydrate metabolism" and "energy metabolism" -accounted for the least number of contigs.

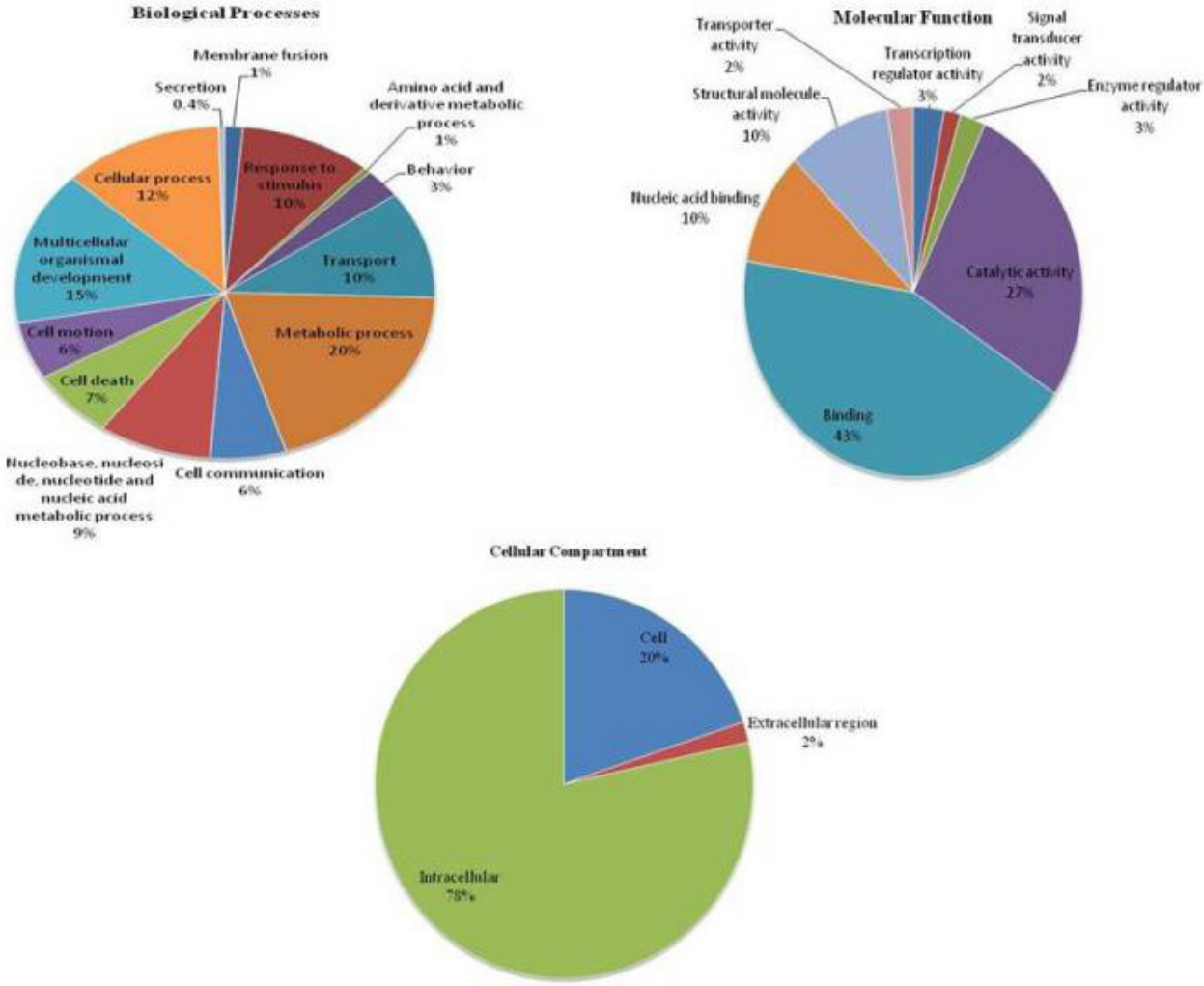


Figure 1: Gene ontology classification of *Camellia sinensis* contigs as summarized into biological processes, molecular functions and cellular components. The percentage shows the proportion of genes related to various activities within the three main functional categories of genes.

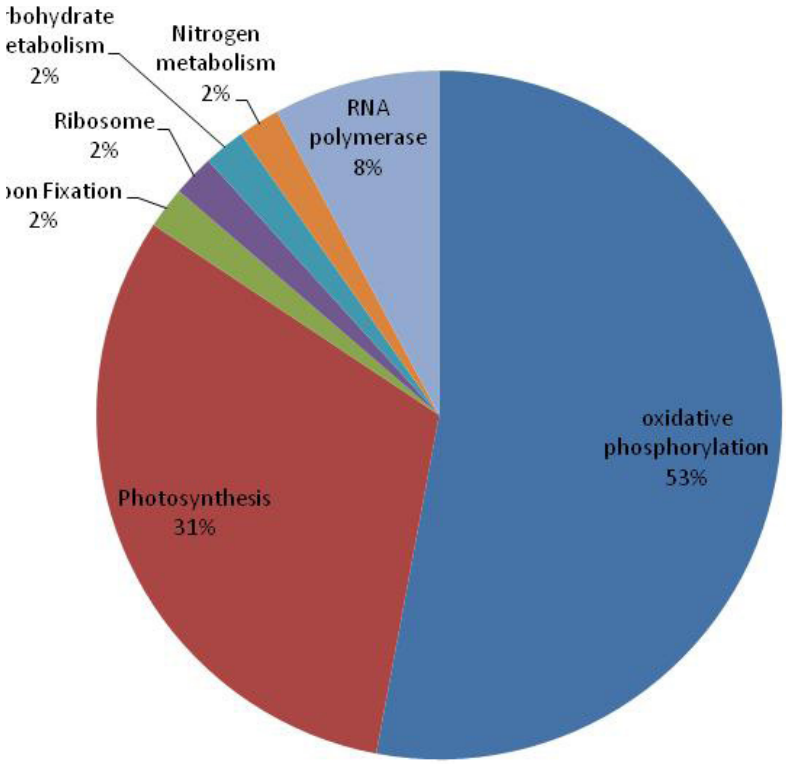
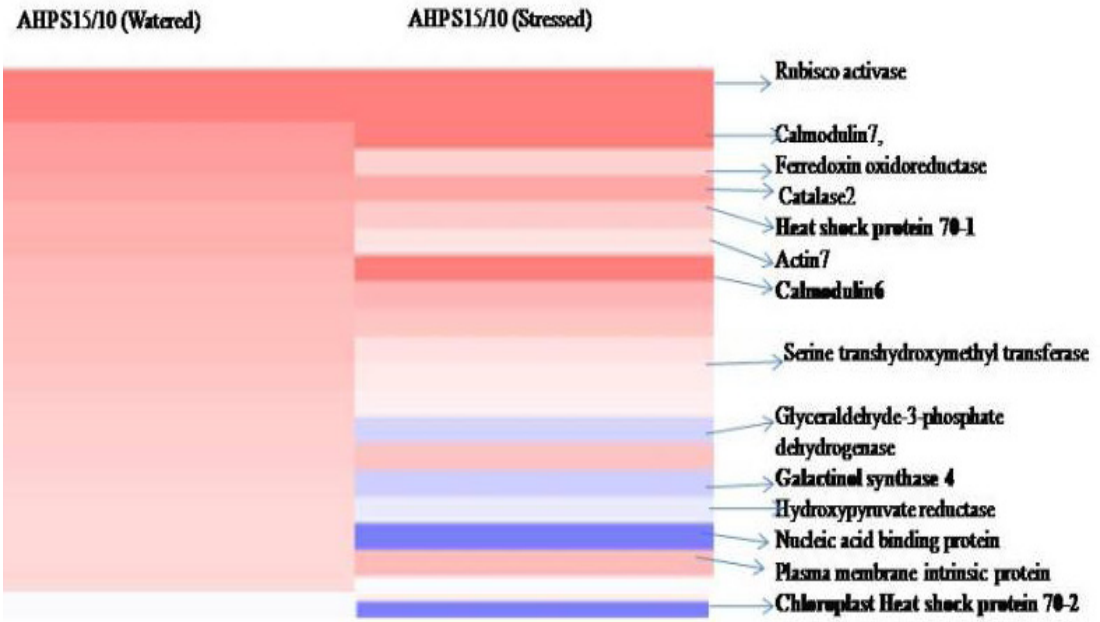


Figure 2: The biologically active pathways in the leaf transcriptome of tea.

Drought responsive genes in tea

The potential water stress responsive genes presented in a form of heat map are shown in Figure 3. The classification of the genes was based on sequence similarity to those in *Arabidopsis proteome*. The drought sensitive Cultivar AHP S15/10 showed that genes responsible for defence against drought were repressed at low soil moisture content (stressed). The stressed tea plants showed the repression of heat shock protein related genes (cpHsc70-1 and 2), and Galactinol synthase related gene (GolS4) as compared to the unstressed plant. However, the "calmodulin" gene (CAM6), signal inducer, was induced (Figure 3). Other genes that were minimally expressed under the stress conditions as compared to control conditions were the nucleic acid binding protein and glyceraldehydes-3-phosphate dehydrogenase.



Legend

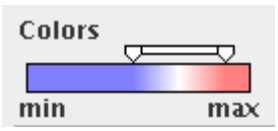


Figure 3: Heat map of expression pattern of genes in the susceptible cultivar (AHP S15/10) with response to water deficit.

However, the data generated from the well watered TRFCA SFS150 were very few to use in expression analysis. Comparative expression of potential genes in the stressed tolerant and susceptible cultivar showed various genes expressed and or repressed (Figure 4). The signalling genes, CAM7 and CAM6, were induced in the susceptible cultivar under water stress conditions. Other than calmodulin-like proteins, another signalling gene related to calcium dependent protein kinase "CDPK" was also upregulated in the water stressed plant. The transcript related to defence against effects of drought like heat shock protein was also expressed at higher levels in the tolerant cultivar TRFCA SFS150 as compared to the susceptible cultivar, AHP S15/10.

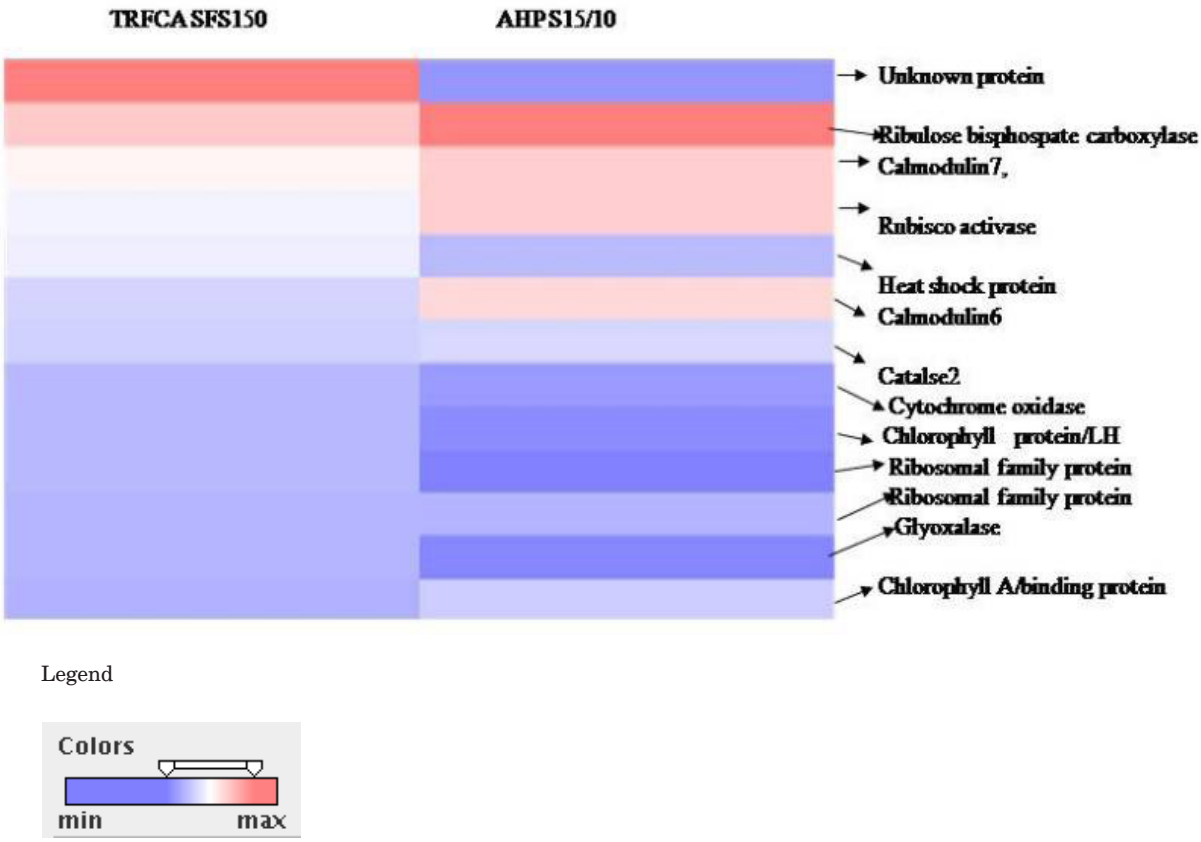


Figure 4: Heat map of expression pattern of genes in the tolerant cultivar TRFCA SFS150 and susceptible AHP S15/10 in response to water deficit.

Other notably expressed genes were those related to photosynthetic processes such as Rubisco activase and the Ribulose bisphosphate carboxylase. Individual analysis on transcripts from the four libraries showed that transcripts related to calatase *(*Cat2), Peroxidase family protein (PRXR1, and Superoxide dismutase, SOD2) were also expressed by the test tea cultivars. The tolerant cultivar, TRFCA SFS150, expressed all the three antioxidant molecules whereas the susceptible cultivar, AHP S15/10, expressed the catalase and peroxidases activity only. The level of expression differed with cultivar, for example the SOD2 was expressed in the tolerant cultivar, TRFCA SFS150, and not in the susceptible cultivar AHP S15/10.

DISCUSSION

In this study, gene ontology (GO) categories associated with contig annotations as derived from sequence homology to *A. thaliana* gene, showed that majority of contigs in the biological processes were associated with metabolic processes, cellular development and response to stimulus. This indicates that the diverse metabolic processes are active in the *C. sinensis* leaf, and a variety of metabolites are synthesized in the leaf. The dominance of contigs associated with cellular development and response to stimulus is an indication that whereas the plants under high SMC are actively growing, the stressed plants induce stimulus response related genes in order to mediate signalling as a result of exposure to water deficit and other drought related incidences like heat.

Majority of the contigs were assigned to metabolic pathways, which included categories such as carbohydrate metabolism, energy metabolism/oxidative phosphorylation and photosynthesis. A significant proportion of the contigs were related to photosynthetic processes. This can be attributed to the fact that the leaf is the main photosynthetic site in a tea plant. Carbohydrate metabolism and energy metabolism were also represented in the KEGG pathway indicating that many active metabolic processes occurred in tea leaves. The leaf acts as the main organ for complex carbohydrate synthesis and energy conversion in plants (Wu *et al.*, 2012).

During drought, the tea plants up regulated or down regulated several genes to mitigate against cellular damage. The genes varied from signalling to defence related genes. In this study, the signalling gene calmoduline-like protein was found to be up-regulated at various levels in the stressed tea plants. It is hypothesized that this is to allow the tea plants under stress to transduce calcium ion signals that activate major pathways by which extracellular signals such as growth factors, hormones and abiotic stress stimuli are converted into intracellular response (Munnik and Meijer, 2001). Calmodulin-like protein was expressed at a higher level in the susceptible cultivar, AHP S15/10, as compared to the tolerant cultivar TRFCA SFS150, under water stress conditions. The induction of this gene at various levels in the tea cultivars suggests that it plays a significant role in signal transduction during stress.

Transcripts showing homology to galactinol synthase (*Gols*) were also shown to be down regulated in the water stressed susceptible cultivar, AHP S15/10. This implies that the drought adaptation or tolerance of this cultivar to water stress conditions is limited; a trait that had been determined earlier through morphological studies. Galactinol l synthase functions as an osmo-protectant in plants (Nishizawa *et al.*, 2008). It has been shown to be induced in plants subjected to drought such as *Cucumis melo* (Volk *et al.*, 2003)*, Coffea arabica* L and *Arabidopsis thaliana* (Taji and Ohsumi, 2002). The induction of *Gols* in these species has been shown to confer some level of drought tolerance (Gupta *et al.*, 2012). The expression of galactinol synthase gene in the tolerant cultivars studied corroborated their role in defence.

Another category of transcripts that showed homology with heat shock proteins were induced in the test cultivars under water stress conditions. The heat shock proteins (HSP70-1) were induced in water stressed tea cultivars in this study. Heat shock proteins serve as intra-cellular chaperones for other proteins and are also involved in plant stress response (Gupta *et al.*, 2012). Heat Shock Proteins (HSPs) are involved in protection by controlling protein folding and protection of macromolecules and membranes from dehydration during drought (Das *et al.*, 2012). Genes encoding HSPs have been reported to be upregulated in tolerant Indian tea cultivars subjected to water stress (Muoki *et al.*, 2012). The results generated from this study, corroborated this observation; with the tolerant cultivar TRFCA SFS150, showing higher levels of HSP70-1 when compared with the less tolerant cultivar, AHP S15/10. This implies that the upregulation of heat shock proteins, HSp70-1, confers drought tolerance. Similar observations have been reported in *Pinus* (Heath *et al.*, 2002) and *Apple* (Wisniewki *et al.*, 2008). On the other hand, the inability of cultivar AHP S15/10 to withstand drought can be attributed to the low level of expression of heat shock proteins in the leaf tissues. Such an observation has been reported in *Populus euphratica* (Bogeat-Tribo *et al.*, 2007).

Transcripts showing homology with reactive oxygen scavengers such as peroxidase family protein (PRXR1), catalase (Cat2) and superoxide dismutase (SOD) were also induced in the assayed tea cultivars in this study. The catalase and peroxidase protein were expressed in the tolerant cultivar TRFCA SFS150 and the susceptible AHP S15/10 when exposed to water stress conditions. However, the SOD was only expressed in the tolerant cultivar, TRFCA SFS150, under the same condition. The accumulation of antioxidant molecules is attributed to their role in scavenging for reactive oxygen species which damage the photosynthetic machinery in plants (Das *et al.*,

2012). Accumulation of antioxidant molecules such as superoxide dismutase, acts as the first line of cellular defence against oxidative stress by catalyzing the dismutation of $O2^-$ to H_2O_2. The catalases and peroxidases, on the other hand, catalyse the removal (Chaves *et al.*, 2003) and conversion of H_2O_2 into water (Rossel *et al.*, 2006), respectively. The existence of a balance between *SOD* and other H_2O_2 scavenging enzymes is crucial in maintaining a steady level of oxidant molecules. Expression of SOD has been shown to confer tolerance and enhance shoot regeneration in transgenic pepper under water stress conditions (Chatzidimitriadou *et al.*, 2009). The upregulation of SOD in the tolerant cultivar TRFCA SFS150 in this study suggests that this antioxidant molecule plays a role in regulating response of tea to drought. The absence of the genes in the susceptible cultivar on the other hand is the probable contributor to its susceptibility to water deficit.

The identified genes which are all DNA-based are potential targets for developing markers associated with water deficit response in tea. Use of molecular markers in breeding and selection can help in identification of traits of interest at early stages of the breeding cycle and hence reduce the breeding period (Shalini *et al.*, 2007). The advantage of this approach is that molecular markers are not influenced by environmental factors and developmental stage of plant and therefore can be selected for at any stage of the plants phenology and in any environment. They can also be used to screen for resistance to a stress condition in the absence of the stress factor (Mphangwe *et al.,* 2013). DNA-based molecular markers have been exploited in breeding programmes of various crops, for example in rice, apples, eucalyptus and maize. Tea has, however, not benefited much from this biotechnological advancement. Initially, this approach was considered less applicable to tea because of the limited genetic information that was available in the public domain. Good progress has, nevertheless, been made in identifying molecular markers associated with various agronomic traits (Mphangwe *et al.*, 2013) including work on QTL associated with yield (Kamunya *et al.*, 2010) and for diversity studies in tea (Wachira and Waugh, 1995). However, the molecular markers that have been identified in tea are probably still too few considering the big tea genome and therefore necessitate more research work on molecular markers. Development of such markers will help in the identification of resistant tea cultivars at the early stages of breeding without subjecting the plant to water stress, thus facilitating marker assisted selection. Using conventional tea breeding approaches, an elite tea variety can take up to 23 years to be developed but with the use of molecular marker techniques, there is likelihood that this period can be reduced by about ten years.

REFERENCES

Altschul S.F.; Madden T.L.; Schaffer A.A.; Zhang J.; Zhang Z.; Miller W. and Lipman D.J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*. 25:3389-3402.

Bartels D. and Sunkar R. (2005). Drought and salt tolerance in plants. *Critical Reviews in Plant Science.* 24:23-58.

Bezbaruah H.P. (1971). Cytological investigation in the family Theacea - I. Chromosome numbers in some Camellia species and allied genera. *Carylogia* .24:421 – 426.

Bogeat-Tribo L.; Brosche M.; Renaut J.; Jouve L.; Thiec D.L., Fayyaz P.; Vinocur B.; Witters E.; Laukens K.; Teichmann T.; Altman A.; Hausman J.F.; Polle A.; Kangasjarvi J. and Dreyer E. (2007). Gradual soil depletion results in reversible changes of gene expression, protein profiles, ecophysiology and growth perfomance in *Popolus euphratica*, a popular growing in arid regions. *Plant Physiology* .143:876-892.

Cabrera C.; Gimenez R. and Lopez M.C. (2003). Determination of tea components with antioxidant activity. *Agricultural and Food Chemistry.* 51: 4427-4435.

Carr M.K.V. (1972). The climatics requirements of tea: A review. *Experimental Agriculture.* 8:1-14

Chatzidimitriadou K.; Nianiou-Obeedat I.; Madesis P.; Perl-Treves R. and Tsaftari A. (2009). Expression of SOD transgene in pepper confer stress tolerance and improve shoot regeneration. E*lectronic Journal of Biotechnology.* 12:1-9.

Chaves M.M., Maroco J.P. and Pereira J.S. (2003). Understanding plant response to drought-from genes to the whole plant. *Functional Plant Biology.* 30:239–264.

Cheruiyot E.K.; Mumera L.M.; Ngetich W.K.; Hassanali A. and Wachira F.N. (2008). Threshold soil water content for growth of tea (*Camellia sinensis* (L.) *O. Kuntze). Tea.* 29:30-38.

Cheruiyot E.K., Mumera L.M., Ng'etich W.K., Hassanali A. and Wachira F. (2007). Polyphenols as Potential Indicators for Drought Tolerance in Tea (Camellia sinensis (L.) *O. kuntze*). B*iosciences Biotechnology Biochemistry.* 71:2190-2197.

Conesa, Götz S.; Garcia-Gomez J.M.; Terol J. and Talon M.R. (2005). Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics.* 21:3674-3676.

Das A.; Das S. and Mondal T.K. (2012). Identification of differentially expressed gene profiles in young roots of tea (*Camellia sinensis* (L.) *O. Kuntze*) subjected to drought stress using suppression subtractive hybridization. *Plant Molecular Biology.* 30:1088-1101.

Frei B. and Higdon J.V. (2003). Antioxidant activity of tea polyphenols in vivo: Evidence from animal studies. *Journal of Nutrition.* 133:3275S–3284S.

Gupta S.; Bharalee R.; Bhorali P;, Agarwal N.; Ahmed P.; Saikia H.; Borchetia S.; Kalita M.S.; Handique A.K. and Das S. (2012). Identification of drought tolerant progenies in tea by gene expression analysis. *Functional Intergrative Genomics.* 12:543-63.

Heath L.S.; Ramakrishnam N.; Sederoff R.R.; Whetten R.W.; Chevone B.L.; Struble C.A.; Jouenne V.Y.; Chen D.; Zyl L. and Green R. (2002). Studying the functional genomics of the stress responses in Loblolly pine with the expresso microarray experiment management system. *Comprehensive Functional Genomics.* 3:226-243.

Kamunya S.M.; Wachira F.N.; Pathak R.S.; Korir R.; Sharma V.; Kumar R.; Bhardwaj P.; Chalo R.; Ahuja P.S. and Sharma R.K. (2010). Genomic mapping and testing for quantitative trait loci in tea (*Camellia sinensis* (L.) *O. Kuntze*). *Tree Genetics and Genomes.* 6:915-926.

Kamunya S.M.; Wachira F.N.; Pathak R.S.; Muoki R.C.; Wanyoko J.K.; Ronno W.K. and Sharma R.K. (2009). Quantitative genetic parameters in tea (*Camellia sinensis* (L.) *O. Kuntze*): I Combining abilities for yield, drought tolerance and quality traits. *African Journal Science.* 3:93-101.

Kondo K. (1977). Chromosome numbers in the genus *Camellia*. *Biotropica.* 9:86 – 94.

Lin Y.S.; Tsai Y.J.; Tsay J.S. and Lin J.K. (2003). Factors affecting the levels of tea polyphenols and caffeine in tea leave. *Journal of Agricultural Food Chemistry.* 51:1864-1873.

Mohammad I. A. and Lin T.P. (2010). Molecular analysis of dehydration in plants. *Plant Science.* 1:21-25.

Mphangwe N.I.; Vorster J.; Steyn M.; Nyirenda H.E.; Taylor N.J. and Apostolides Z. (2013). Screening of tea (*Camellia sinensis*) for traits associated molecular markers. *Applied Biochemical Biotechnology.* 171:437-449.

Munnik T. and Meijer H. (2001). Osmotic stress activate distinct lipid and MAPK signalling pathway in plants. *FEBS Lett.* 498.

Muoki R.C.; Paul A. and Kumar S. (2012). A shared response of thaumatin like protein, chitinase and late embryogenesis abundant protein3 to environmental stresses in tea (*Camellia sinensis* (L.) *O. Kuntze*). *Functional Intergrative Genomics.* 12 (3): 565-571.

Ngetich W.K.; Stephen W. and Othieno C.O. (2001). Responses of tea to environment in Kenya. III. Yield and yield distribution. *Experimental Agriculture.* 37:361-372.

Nishizawa A; Yabuta Y. and Shigeoka S. (2008). Galactinol and Raffinose Constitute a novel function to protect plants from oxidative damage. *Plant Physiology.* 147:1251-1263.

Rossel J.B.; Water P.B.; Hendrickson L.; Chow W.S.; Poole A.; Mullineaux P.M. and Pogson B.J. (2006). A mutation affecting ascorbate peroxidase 2 gene expression reveals a link between responses to high light and drought tolerance. *Plant Cell Environment.* 29:269-281.

Sambrook J., Fritsch E.F. and Maniatis T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press: New York, USA.

Shakeel A.A.; Xiao-yu X.; Long-chang W.; Muhammad F. S. and Chen M.; L.W. (2011). Morphological, physiological and biochemical responses of plants to drought stress. *African Journal of Agricultural Research.* 6:2026-2032.

Shalini K.V.; Manjunatha S.; Lebrun P.; Berger A.; Baudouin L.; Pirany N.; Ranganath R.M. and Prasad T.D. (2007). Identification of molecular markers associated with mite resistance in coconut (*Cocos nucifera* L.). *Genome.* 50:35-42.

Sharma P.; Kumar A. (2005). Differential display-mediated identification of three drought-responsive expressed sequence tags in tea (*Camellia sinensis* (L.) *O. Kuntze*). *Journal of Bioscience.* 30:231-235.

Shinozaki K.; Yamaguchi-Shinozaki K. and Seki M. (2003). Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology*. 6:410-417.

Taji T. and Ohsumi (2002). Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant Journal.* 29:417-426.

Tanaka J. and Taniguchi F. (2006) Estimation of the genome size of tea (*Camellia sinensis*), *Camellia* (C. *japonica*), and their interspecific hybrids by flow cytometry. *Journal of the Remote Sensing Society of Japan.* 101:1-7.

Vo T.D. (2006). An assessment on genetic diversity in Vietnam tea using morphology, ISSR and microsatellites. Dissertation.

Volk G.M.; Haritatos E.E. and Turgeon R. (2003) Galactinol synthase gene expression in melon. *American Society of Horticultural Science.* 128:8-15.

Wachira F. N. and Waugh R. (1995). Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. *Genome.* 38:201-210.

Wachira F.N. and Ronno W. (2004). Current research on tea in Kenya. In:. *Proceedings of 2004 International Conference on O-Cha (Tea) Science*). Shizouka, Japan 59-65.

Wisniewki M.; Bassett C.; Norelli J.; Macarisin D.; Artlip T.; Gasic K. and Korban S. (2008). Expressed sequence tag analysis of the response of apple (*Malus x domestica Royal Gala*) to low temperature and water deficit. *Physiology Plant.* 133:298-317.

Wu H.; Chen D.; Li Jiaxian; Yu B.; Qiao X.; Huang H.; He Y. (2012) De novo characterization of leaf transcriptome using 454 sequencing and development of EST-SSR markers in tea (*Camelia sinensis)*. *Plant Molecular Biology Report.* 31.

Xu Z.Z.; Zhou G.S.; Shimizu H. (2009). Effects of soil drought with nocturnal warming on leaf stomatal traits and mesophyll cell ultrastructure of a perennial grass. *Crop Science.* 49:1843–1851.