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In-silico identification of phytohormone pathway genes in *Camellia sinensis* and expression analysis under combined water and herbivore stress

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ABSTRACT

Tea (*Camellia sinensis*) is a popular beverage worldwide. Abiotic and biotic stresses due to recent climate change have significant effect on yield of tea. Plant hormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) plays an important role in regulating plant defense responses to different kind of stresses. In this study homologous phytohormone genes of ABA, JA, SA and ET pathway in tea plant were identified from the public domain transcriptomic database and the expression of the rate-limiting genes of phytohormone pathway were analyzed in tea plants subjected to combined water and herbivore stress to understand the interaction among the stress-induced phytohormone pathways genes. Vegetatively propagated TV1 clones of tea plant were subjected to three level of water stress treatments: 1) well watered control 2) mild water stress 3) severe water stress for three months and then infested with *Hyposidra talaca* (looper caterpillar). The constitutive expression (without infestation) of the rate-limiting genes of ABA and ET pathway were positively regulated by water stress whereas JA and SA pathway genes were negatively regulated. On looper caterpillar infestation (induced expression) water stressed plants showed significant decrease in expression of the rate-limiting phytohormone genes except *ACC* synthase. Our study showed that on herbivore infestation well watered plants have higher capacity to induce the phytohormone genes and water

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stress played a major role in regulation of gene expression than herbivore infestation. The presence of an initial water stress not only affected the tea plant constitutive defense but also significantly altered the phytohormone defense gene expression towards subsequent herbivore stress. The water stressed tea plant with weak induced expression of defense associated phytohormone genes may be at a higher risk for incidence of pest and pathogen attack compared to well watered plants.

KEY WORDS: *CAMELLIA SINENSIS*, EXPRESSION ANALYSIS, HERBIVORE INFESTATION, *HYPOSIDRA TALACA*, *IN SILICO* IDENTIFICATION; PHYTOHORMONE PATHWAY

INTRODUCTION

Tea, *Camellia sinensis* is a major economic crop worldwide and its young leaves are used for preparing beverage. Huge losses in tea leaf yield is incurred due to the present climate change scenario. As the climatic change event is expected to increase the incidence of water shortage and outburst of insect population, the monoculture cultivation of tea may face severe crop loss in recent future. Drought is one of the major abiotic stress that influences the quality and productivity of crops by growth inhibition, increase in organic solutes concentration and changes in the endogenous phytohormones content (Wijeratne et al., 2007; Bhagat et al., 2010, Aimar et al. 2011, Chen and Chen, 2012 and Anderegg et al. 2015).

Biotic stressors such as insects and pathogens, also contribute significantly towards the enormous damage to the crops (Hammond-Kosack and Jones, 2000). Plants have both inherent and adopted mechanisms to cope with the environmental stresses by producing certain proteins and secondary metabolites that are toxic or have repellant effect on the biotic agents (Rani and Jyothsna, 2010; War et al., 2011a; War et al., 2011b; War et al., 2012). Abiotic and biotic stresses in plants trigger the activation of a number of phytohormone pathway genes which simultaneously activate other interconnected defense network to help plants sustain the stress period (Fraire-Velázquez et al., 2011). The primary phytohormones abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), ethylene (ET) are involved as messengers triggering the specific defense pathways against environmental stress and may act individually or in combinations depending upon the stress perceived, (Atkinson and Urwin, 2012, Verma et al., 2016; Wani et al., 2016).

ABA is produced in response to water-deficit stress (Osakabe et al., 2013). Enhanced accumulation of ABA in *Arabidopsis thaliana* seedlings has been reported under drought conditions (Huang et al., 2008). Exogenous application of ABA delay wilting and is reported to induce drought tolerance in plants (Lu et al., 2009). ABA functions both synergistically and antagonistically with JA, SA and ET signaling pathways which play a dominant role during biotic stress (Chen and Yu, 2014). Under the combination of abiotic and biotic stresses, ABA mostly acts as an antagonist to JA/SA/ET making the plant susceptible to disease and pathogen attack (Rejeb et al., 2014). However, a positive interaction has also been observed, whereby an increase in ABA level under abiotic stress results in stomatal closure which prevents the entry of biotic agents and protects the plants from both biotic and abiotic stresses (Melotto et al., 2006). SA, an endogenous growth regulator, induces systemic acquired resistance (SAR) in plants against different pathogens, particularly microbes and serves as a signal molecule by producing pathogenesis related (PR) proteins (Gao et al., 2015; Verma et al., 2016).

SA is also involved in plant response to different abiotic stresses such as drought, temperature variations, heavy metals and osmotic stress (Rivas-San Vicente and Plasencia, 2011). JA, a key regulator of plant response to pathogens and insects, is involved in both direct and indirect defenses of plants to herbivory (Creelman and Mullet, 1995). JA also participates in plant's response to drought and salinity (Riemann et al., 2015). ET, the gaseous phytohormone for defense, helps in both direct and indirect response of plants to abiotic and biotic stresses. The effects of ET can be transitory or long lived as its biosynthesis shows a diurnal rhythm and controls its own biosynthesis (Eyidogan et al., 2012; Gamalero and Glick, 2012; Verma et al., 2016).

Depending on the type of stress perceived by the plant, different signaling pathways are activated which synergistically or antagonistically influence the type of response generated. The interactions among the different signal transduction pathways are considered as crosstalk between the pathways which helps the plants to sustain the stress period (Rejeb et al., 2014). In the event of climatic change where both the abiotic and biotic stress will co-occur, it is largely unknown how the phytohormone based plant defense network would behave as majority of the studies so far considered single stress factor either abiotic or biotic at a time. It also remain unpredictable how the presence of an initial stress affects the plant defense network on perception of a subsequent stress. As the frequency and extent of drought as well as insect infestation are projected to increase due climate change it is essential to understand the response of plants to combined stress conditions. Knowledge of the molecular mechanisms underlying these effects is very limited. The

molecular study of the stress-related hormonal genes and their interactions would help to understand the synchronization of plant constitutive and induced defense responses to insect infestation in plants under abiotic stress. Tea plant faces water stress event round the year and also plethora of insect infestation. Looper caterpillar infestation stand out to be the most destructive insect infestation in terms of crop loss. The water stress may change the overall metabolism of the tea plant and alter its defense interaction with the biotic agents. It will be vital to know the interaction of the phytohormone defense gene network in perception of combined water and herbivore stress.

In this study, a comparative genomics approach was undertaken to mine the phytohormone pathway genes of *C.sinensis* and the expression pattern of the rate limiting genes of the four phytohormone pathway (ABA, JA, SA, ET) was analyzed in a clone (TV1) of tea plant which was subjected to different regime of water stress (abiotic stress) treatment with subsequent insect infestation (looper caterpillar). This study will help to better understand the stress-induced phytohormones defense interaction at transcriptional level in tea plant under combined water and herbivore stress.

MATERIALS AND METHODS

IN SILICO IDENTIFICATION OF PHYTOHORMONE PATHWAY GENES IN *C. SINENSIS*

The genes or transcripts involved with ABA, JA, SA and ET phytohormone pathway in *C. sinensis* were mined from different databases using *Arabidopsis* sequences as reference. The *Arabidopsis* full-length coding sequences (CDS) were collected from the TAIR database (https://www.arabidopsis.org/) and subsequently the sequences were subjected to BLASTN with *C. sinensis* Expressed Sequence Tags (EST), Transcriptome Shotgun Assembly (TSA) and Non-Redundant (NR) nucleotide databases of NCBI. The sequences showing significant similarity with an E-value ≤ $1e^{-15}$ were selected and assembled using CAP3 program to remove redundancy and get consensus sequences. Each of those sequences were screened for the presence of open reading frame (ORF) using NCBI ORF Finder (Wheeler et al., 2003) and sequences with the longest ORF having both start and a stop codon were sorted out. The sequences were further subjected to BLASTX with NCBI NR (Non-Redundant) protein database to confirm their annotation. Based on the BLAST annotation and alignment results, the sequences found similar to *Arabidopsis* reference sequences and nearby plant species sequences were retained and others were filtered out. The best representative sequences were subjected to blast and functional classification following the Gene Ontology (GO) scheme using BLAST2GO suite (Conesa and Götz, 2008). The transcripts were classified into the major GO categories, namely, cellular component, molecular function and biological process. Further the rate-limiting gene sequences of the phytohormone pathways were used in expression study.

MOTIF AND DOMAIN IDENTIFICATION

MEME and MAST programs (Bailey and Elkan, 1994; Bailey and Gribskov, 1998) were used for the identification of the motif cluster present in the phytohormone pathway gene sequences. MEME program performs motif discovery on DNA, RNA or protein datasets. Whereas, MAST program searches sequences for matches to a set of motifs and sorts the sequences by the best combined match to all motifs. The sequences from *C. sinensis* and *Arabidopsis* were analyzed together for the easy identification of common motifs between them. The motif discovery mode was set to normal. The maximum number of motif was set to 20. Domain search was performed using CD-search tool available at the conserved domain database (CDD) of National Center (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

SELECTION OF GENE FOR qRT PCR AND PRIMER DESIGN

The rate limiting gene/enzyme of each phytohormone pathway was searched from literature references and the expression of the corresponding genes was analyzed across different treatments. For the ABA pathway, the reaction catalyzed by 9-cis-epoxycarotenoid dioxygenase (*NCED*) i.e- the oxidative cleavage of neoxanthin is considered as the rate-limiting step (Tan et al., 1997; Qin and Zeevaart, 1999) and chosen for our study. In the ET biosynthesis pathway, the conversion of S-AdoMet to 1-aminoacyclopropane 1-carboxylate (*ACC*) by *ACC* synthase is taken as the rate-limiting step (Wang et al., 2002). Allene oxide synthase (*AOS*), the first enzyme in the branch pathway leading to the formation of JA act as rate-limiting step in JA biosynthesis (Harms et al., 1995; Sivasankar et al., 2000). In the SA pathway, isochorismate synthase (*ICS*) acts as the rate-limiting enzyme (Serino et al., 1995; Gaille et al., 2003).Thus *NCED* gene for ABA, *ACC* synthase gene for ET, *AOS* gene for JA and *ICS* gene for SA pathway was chosen for gene expression analysis. The primers for the corresponding genes were designed using primer3 (http://frodo.wi.mit.edu/primer3/) software. The primers were designed as such that the product length was within 100-200 base pair. List of the primers used in the study is provided in Supplementary Table 1.

EXPERIMENTAL SET UP AND SAMPLE COLLECTION

Two years old vegetatively propagated TV1 clones of tea plant were collected from the nursery of Tocklai Tea Research Institute, TRA, Jorhat. The plants from nursery were replanted in black poly-sleeves (18 cm diameter and 23cm height) with field soil (sandy loam, pH 4.8-5.1, bulk density 1.3-1.4 Mg m^{-3}, single super phophate 0.5 kg. m^{-3} of soil) and allowed to acclimatize for 30 days in natural environmental condition with sufficient irrigation. After the acclimatization period the plants were transferred to a polyhouse and were allowed to acclimatize within the polyhouse for 10 days before starting the stress experiments. The plants were covered with nets for protecting it from external pest infestation. Thereafter the plants were subjected to three level of water stress treatment: 1) well-watered control; 2) mild water stress; 3) severe water stress. The plants in each drought stress level received same amount of water and watered simultaneously. The well-watered control plants received water every 3rd day such that the soil remained constantly moistened. Mild drought stressed plants were watered once the soil water content drops to 7-8% and received 40-50 % of water supplied to the well-watered control plants. The severe water stressed plants were watered when the soil water content reaches 4-5 % and received around 15–20% the amount of water supplied to well-watered control plants. At the end of the three months the plants were rehydrated with small amount of water overnight in all the treatment and then subjected to *Hyposidra talaca* (looper caterpillar) infestation.

Rehydration of plants was done to ensure that the gene expression of plants reflect the effect of pulsed water stressed treatment which is often faced by tea plant in natural environment rather than continuous drought. The leaves were collected before insect infestation i.e - at 0 hours (0 TPI), and after insect infestation i.e - 24, 48 hours (24 TPI, 48 TPI) in all the treatments. The leaves collected at 0 TPI were taken as undamaged control. As only the non-damaged control tissue was measured at the initial time point (time = 0 hrs), the main effect of time represents time post herbivory infestation or time post infestation and is designated at TPI. For each treatment and for each time point there were three biological replicates. Once the plants were used to collect sample they were discarded and not used further in the experiment. For all the treatment the third leaf of tea plant from top was collected and immediately stored at -80^{0}C to prevent any enzymatic activity.

RNA ISOLATION AND cDNA PREPARATION

Total RNA was extracted from 100 mg of each sample according to the protocol of Zaman et al., 2016. RNA integrity was determined using a 1% agarose gel and concentration was quantified using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). After verifying the integrity of the RNA, equal concentration of RNA from each sample was used for first strand cDNA preparation using QuantiTect Rev. Transcription Kit (Cat No./ID: 205311, QIAGEN, Germany).

QUANTITATIVE REAL-TIME PCR ANALYSIS

Four important genes known to be involved in the rate-limiting step of the phytohormone biosynthesis pathway (ABA, JA, ET, SA) in tea were selected based on the comparative *in-silico* identification of homologous genes. The expression of the selected four genes was studied in tea plant subjected to three level of water stress treatments with subsequent insect infestation. Quantitative Real-Time PCR was performed in a Roche Light Cycler 480 real time machine (Roche, Germany) using QuantiTect SYBR Green PCR Kit (Cat No./ID: 204145, QIAGEN, Germany). The reverse transcribed first strand cDNA of each sample was used as template in the assay and amplified by gene-specific primers. The PCR was performed in 10 µl reaction volume and prepared according to the protocol mentioned in the kit manual. In short, 3.5 µl of supplied PCR grade water was mixed with 0.5 µl of forward and reverse primer and 5 µl of 'QuantiTect SYBR Green I Master Mix' to get a final volume of 9.5 µl. Finally, 0.5 µl of template was added. The relative expression levels of all the genes were calculated using the ddCt method. The raw Ct values were normalized against Ribulose-1, 5-bisphosphate carboxylase/oxygenase housekeeping gene. The log 2 fold change values for all the samples was calculated relative to well-watered control sample at 0 TPI.

STATISTICAL ANALYSIS

The transcript relative expression values were log 2 fold transformed and analyzed with a 3 × 3 (T x TPI) mixed model analysis of variance (ANOVA) followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing. "T" stands for water stress treatment and "TPI" stands for time post infestation. All the expression data are expressed as mean ± standard deviation (SD). Each expression value is the mean of three biological replicates. Data were analyzed using IBM SPSS Statistics, Version 20.0.

RESULTS AND DISCUSSION

IDENTIFICATION AND ANALYSIS OF PHYTOHORMONE PATHWAY GENES

Gene mining of the four phytohormone pathways (ABA, JA, SA and ET) resulted in a large number of *C. sinen-*

sis homologues for each gene (Table1). CAP3 clustering removed the redundancy of the sequences, still a significant number of homologues were retained after clustering in many instances. In the ABA pathway few genes like ABA 8'-hydroxylase, 9-cis-epoxycarotenoid dioxigenase (*NCED*) etc. were represented by more than one homologue of *C. sinensis* having significant similarity and they possessed most of the common motifs present in corresponding *Arabidopsis* reference sequences (Table 1, Fig.1 (A, B)). Homologue gene mining of JA pathway resulted in the identification of nine genes and each gene best representative homologue is listed in Table1. For the SA pathway a total of four genes were mined but only three gene homologues were retained. The *C. sinensis* homologues obtained for salicylic acid carboxyl methyltransferase gene had an E-value >1e^{-15} and hence it was not considered for further study. Two full-length sequences of ET pathway genes namely 1-aminocyclopropane-1-carboxylate synthase (*ACCS*) and 1-aminocyclopropane-1-carboxylate oxidase (*ACCO*) were obtained based on keyword search in the NCBI NR nucleotide database.

Further the result of Blast2GO program with the details of sequence similarity, GO classification, enzyme list, InterPro scan domain etc. for the four pathway genes are provided in Supplementary Table 2. MEME/MAST search, for the putative functional and common motif occurrence showed that most of the motifs are conserved among the *C. sinensis* homologues and its *Arabidopsis* counterpart except in few cases, where it was seen that few motifs were missing in *C. sinensis* homologues. This may be due to the presence of partial transcript sequences of *C. sinensis*. Few representative genes displaying the occurrence of common motifs between *Arabidopsis* and *C. sinensis* homologues are shown in Fig.1 (A, B), Supplementary Fig. 1 (A-D) & Supplementary Fig. 2 (A-D). The result of the functional domain identification using CD-search tool showed the presence of conserved domains between *Arabidopsis* and *Camellia sinensis* homologues (Supplementary Table 3). The presence of conserved common motif and domain in sequential pattern between the phytohormone pathway genes of *C. sinensis* and *Arabidopsis* clearly confirms their identity and support the results of our comparative genomics approach.

Gene expression of the rate-limiting genes 9-cis-epoxycarotenoid dioxygenase (*NCED*), Allene oxide synthase (*AOS*), 1-aminocyclopropane-1-carboxylate synthase (*ACC* synthase) and Isochorismate synthase (*ICS*) of ABA, JA, ET and SA biosynthesis pathway respectively was studied at different time point in the three water stress treatment with subsequent insect infestation. The relative gene expression values discussed here are expressed in terms of log 2 Fold Change and the well watered treatment at 0 TPI is taken as control for calculation of relative gene expression fold change for other time points and treatments.

Considering the expression of rate limiting genes at 0 TPI (without insect infestation) the expression of *NCED* gene of ABA pathway in case of mild and severe water stress treatment was higher than well watered plants. Mild water stress plant showed a log 2 FC value of 1.31 while severe stress plant showed a value of 1.62 (Fig.2 (A)). In mild water stress plants the *AOS* gene of JA pathway had almost similar expression value with control and the difference was not statistically significant. Whereas in severe water stress plants there was significant down regulation of *AOS* expression with a log 2 FC value of -2.49 compared to control plants ($P< 0.05$, Fig.2 (B)). *ICS* gene of SA biosynthesis pathway showed higher transcript accumulation in mild water stress plant with a value of 2.84 whereas in severe stressed plants it was down-regulated with a value of -4.04 compared to control ($P< 0.05$, Fig.2 (C)). In case of ET pathway, with the increase in water stress intensity the expression of *ACC* synthase gene also increased proportionally with a value of 1.88 and 2.59 in mild and severe water stress treatment respectively (Fig.2 (D)). The transcript expression at 0 TPI represent the constitutive expression of tea plant and it mainly reflect the effect of the water stress treatment on the expression of the phytohormone pathway genes. The expression at 24 TPI and 48 TPI mainly represent the induced expression of the tea plant after insect infestation under different levels of water stress treatment.

At 24 TPI, the expression of *NCED* gene in well-watered plants increased significantly (log 2 FC: 2.13) compared to control at 0 TPI. However the expression at 48 TPI was somewhat less (log 2 FC: 1.42) compared to 24 TPI. In the mild stress plants there was up-regulation at 24 TPI followed by down-regulation at 48 TPI with a value of -0.04. The severe stress plant showed a decline in expression pattern with the increase of time (1.62 at 0 TPI, 1.38 at 24 TPI and 0.04 at 48 TPI). *AOS* gene of JA pathway at 24 TPI showed significant induction in expression (log 2 FC 3.05, $P<0.05$) of well-watered plants. As the time elapsed the gene expression declined at 48 TPI (Fig.2 (B)). However the expression was still significantly higher compared to 0 TPI. Mild and severe stressed plant followed the same trend with increase of transcript accumulation at 24 TPI and then a decline at 48 TPI. The expression of *ICS* gene in case of control and severe stressed plants increased at 24 TPI and decreased at 48 TPI whereas in mild stressed plant the expression declined both at 24 TPI and 48 TPI (Fig.2 (C)). Expression of *ACC* Synthase of ET pathway increased at 24 TPI and then declined at 48 TPI in all the water stress conditions (Fig.2 (D)). Two way ANOVA showed that for the

Table 1. Results of in-silico mining of *Camellia sinensis* phytohormone genes..

Arabidopsis reference sequence accession numbers	Sequence Name	Associated Pathway	Total No. of Homologs (EST + NR + TSA) before CAP3	Total sequences after CAP3 assembly (Contig + singleton)	Camellia sinensis homologous sequences retained after processing	CAP3 (Contig, singleton) Number	Accession No.s of Contigs & singletons	Blast Annotation	BlastX similarity percentage and best hit organism
AT4G19230, AT2G29090, AT5G45340, AT3G19270	ABA 8'-hydroxylase (ABA8ox)	Abscisic acid pathway	22	5	ABA 8'-hydroxylase (ABA8ox) [Singleton1, Singleton2]	Singleton1	HP733304.1	ABA 8'-hydroxylase	86% Citrus sinensis
						Singleton2	HP764411.1	PREDICTED: abscisic acid 8'-hydroxylase 4	83% Vitis vinifera
AT1G52400	ABA glucosidase		12	6	ABA glucosidase [Contig1]	Contig1	KA279844.1, KA279587.1, HP733896.1, GH710784.1, GH710770.1, FE942881.1	beta-glucosidase-like protein	99% Camellia sinensis
AT2G27150	abscisic aldehyde oxidase (ABAO)		6	1	abscisic aldehyde oxidase (ABAO) [Contig1]	Contig1	KA288987.1, HP767578.1, KA286688.1, KA282538.1, HP727479.1, HP770168.1	PREDICTED: aldehyde oxidase 4-like	87% Vitis vinifera
AT1G16540	molybdenum cofactor sulfurase		3	2	molybdenum cofactor sulfurase [Contig1]	Contig1	HP742704.1, JK475554.1	PREDICTED: molybdenum cofactor sulfurase-like	70% Vitis vinifera
AT4G18350, AT3G14440, AT1G30100, AT3G24220, AT1G78390	9-cis-epoxycarotenoid dioxigenase (NCED)		18	4	NCED [Singleton1, Contig2]	Singleton1	HP727751.1	9-cis-epoxycarotenoid dioxygenase 1	88% Diospyros kaki
						Contig2	HP765237.1, BJ999395.1	putative 9-cis epoxycarotenoid dioxygenase	88% Daucus carota subsp. sativus
AT1G67080	neoxanthin synthase (NSY)		2	1	NSY[Contig1]	Contig1	KA283257.1, HP702818.1	neoxanthin synthase	73% Citrus sinensis
AT1G52340	xanthoxin dehydrogenase (XD)		7	6	XD[Contig1]	Contig1	HP701207.1, JK341963.1	short chain alcohol dehydrogenase	79% Citrus sinensis
AT5G67030	zeaxanthin epoxidase (ZEP)		4	1	ZEP[Contig1]	Contig1	HP760589.1, HP739212.1, HP727722.1	zeaxanthin epoxidase 1	82% Vitis vinifera

AT3G45140	13-lipoxygenase/LOX2	Jasmonic acid pathway	20	5	LOX2[Contig1]	Contig1	KA280187.1, DY523322.1, HP756288.1, FE942952.1	lipoxygenase	99% Camellia sinensis
AT4G16760, AT5G65110, AT1G06290, AT3G51840, AT2G35690	acyl-CoA oxidase		38	13	ACX[Contig4, Contig5]	Contig4	FE943071.1, KA288172.1, KA280456.1, HP709220.1, HP724420.1 , HP740917.1, HS399721.1, KA280456.1	Peroxisomal acyl-coenzyme A oxidase 1-like	70% Nicotiana tomentosiformis
						Contig5	HP754805.1, KA298085.1, KA300729.1	acyl-coenzyme A oxidase 2	85% Brassica oleracea var. oleracea
AT3G25760, AT3G25770, AT3G25780	allene oxide cyclase		46	5	AOC[Singleton1, Singleton2]	Singleton1	HP769131.1	allene oxide cyclase	74% Camellia sinensis
						Singleton2	KA282377.1	allene oxide cyclase	99% Camellia sinensis
AT5G42650	allene oxide synthase (CYP74A1)		4	3	AOS[Contig1]	Contig1	KA283779.1, HP717071.1	cytochrome P450 allene oxide synthase	77% Populus trichocarpa
AT5G07010	hydroxyjasmonic acid sulfotransferase		5	4	hydroxyjasmonic acid sulfotransferase (AtST2a)[Contig1]	Contig1	KA297677.1, HP755751.1	PREDICTED: flavonol sulfotransferase-like	74% Vitis vinifera
AT1G19640	jasmonic acid carboxyl methyltransferase		1	NA	NA	KA286401.1	KA286401.1	Jasmonate O-methyltransferase, putative	67% Ricinus communis
AT2G46370	jasmonic acid-amino acid synthase		6	4	JAR[Contig1]	Contig1	HP705426.1, KA286035.1	JAR1-like protein	80% Nicotiana attenuata
Keyword Search	OPC-8:0-CoA ligase		NA	NA	NA	NA	FS957551.1	OPC-8:0-CoA ligase	NA
AT1G20510	OPC-8:0-CoA ligase		6	4	OPCL1[Contig1]	Contig1	HP750023.1 ,KA293135.1, KA286488.1	4-coumarate--CoA ligase-like 5	76% Sesamum indicum
AT2G06050	OPDA reductase		9	2	OPR3[Contig1]	Contig1	HP743074.1, KA280376.1, HS399901.1, HP725651.1, HP746582.1, HS398253.1, HS398153.1, KA302045.1	PREDICTED: 12-oxophytodienoate reductase 3	82% Vitis vinifera

AT1G18870, AT1G74710	isochorismate synthase	Salicylic acid pathway	5	2	ICS[Contig1]	Contig1	HP734410.1, KA297692.1,	isochorismate synthase, putative	69% Ricinus communis
AT2G23620, AT2G23600, AT2G23560, AT4G37150	methyl salicylate esterase		13	6	Methyl_salicylate_ esterase [Singleton1]	Singleton1	KA281093.1	PREDICTED: polyneuridine-aldehyde esterase	63% Vitis vinifera
AT2G43840, AT2G43820	salicylic acid glucosyltransferase		14	8	salicylic acid glucosyltransferase [Singleton1, Contig2]	Singleton1	KA286158.1	PREDICTED: UDP-glycosyltransferase 74F2	71% Vitis vinifera
						Contig2	HP768085.1, KA296966.1	PREDICTED: UDP-glycosyltransferase 74E1-like	80% Vitis vinifera
Keyword Search	1-aminocyclopropane-1-carboxylate synthase	Ethylene pathway	NA	NA	NA	NA	EF205149.1	1-aminocyclopropane-1-carboxylate synthase	NA
Keyword Search	1-aminocyclopropane-1-carboxylate oxidase		NA	NA	NA	NA	DQ904328.1	ACC oxidase	NA

NCED gene expression there was significant main effect for time post infestation and interaction effect between water stress treatment and time post infestation whereas for *AOS*, *ICS* and *ACC* synthase gene expression both the main effect (water stress treatment, time post infestation) and there interaction effect was significant (Fig 2 A- D). The homogeneity and specificity of the single PCR product was determined by the melting curve and melting peak of the four analyzed genes and are provide in Supplementary Fig. 3 (A-D), Supplementary Fig. 4 (A-D) For supplementary data please see:https://drive.google.com/drive/folders/1qVqzAqW1IEgkKnh6RA75zkdVIUxx0xH?usp=sharing.

It can be seen that as the water stress (0 TPI) increased the transcript abundance of *NCED* gene also increased (Fig.2 (A)). Water stress is known to increase the expression of *NCED* gene followed by accumulation of ABA (Shinozaki and Yamaguchi-Shinozaki, 2007; Wang et al., 2009). The function of ABA in the control of stomata closure and the responses to abiotic stress is well-established (Mittler and Blumwald, 2015). ABA integrates various stress signals and is known to controls stress responses during water deficit stress (Raghavendra et al., 2010; Ye et al., 2012). After insect infestation the expression of *NCED* gene in well watered plant showed higher induction than mild and severe stressed plants (24 TPI) and then there was a drop in transcript level at 48 TPI for all treatment with well watered plant retaining the highest transcript abundance.

When the expression folds were analyzed for statistical significance main effect of water stress treatment (T) on *NCED* gene expression was not statistically significant ($P > 0.05$). However the main effect of time post infestation (TPI) and interaction effect (TxTPI) was significant ($P<0.05$). Herbivore infestation and the interaction of water stress treatment and time post infestation significantly regulated the expression differences. The strong induction of *NCED* expression in well-watered and mild stressed plants along with the increase in *AOS* gene expression of JA pathway at 24 TPI (Fig. 2(A) , Fig.2(B)) strongly suggest the synergistic role of *ABA* and JA signaling pathway genes in plant herbivore defense. On the other hand severe stressed plant produced a smaller induction for both *NCED* and AOS gene. It is very likely that water stress severity negatively affected the induced response of the *ABA* and JA biosynthesis pathway genes on herbivore attack. *ABA* have been reported to interact with JA signaling and enables *N. attenuata* plants to mount a full defense response against chewing herbivores (Dinh and Baldwin, 2013).

The expression of *AOS*, JA pathway rate-limiting gene, was significantly down-regulated in severe water stressed plants with a value of -2.49 compared to well watered control plants (0 TPI, $P<0.05$, Fig. 2 (B)). Irrespective of water treatment on herbivore infestation *AOS* gene expression increased at 24 TPI for all

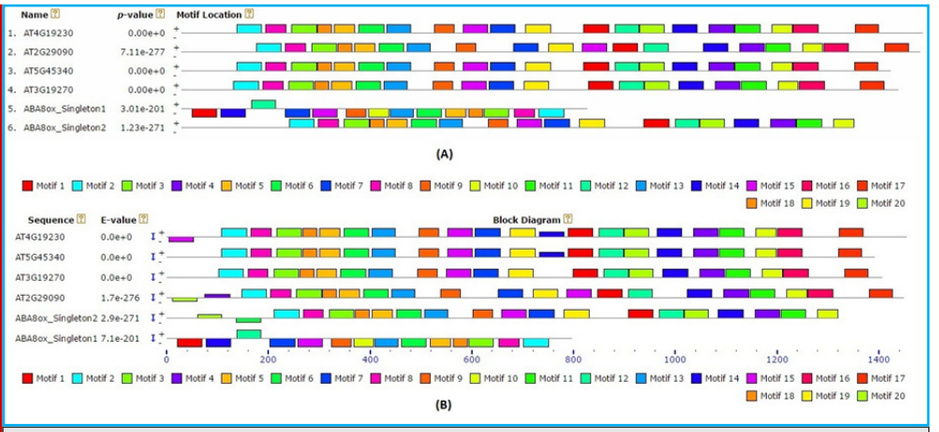

FIGURE 1. (A) Schematic representation of occurrence and distribution of motifs in the ABA 8'-hydroxylase sequences of *Arabidopsis* and *Camellia sinensis* - MEME program output (B) Schematic representation of motifs and sorting of *Arabidopsis* and *Camellia sinensis* ABA 8'-hydroxylase sequences by the best combined match to all motifs - MAST program output.

the plants with control showing maximum transcript abundance. It is noteworthy to mention that severe stressed plant had maximum induction value when the induced expression fold change of individual treatment at 24 TPI is calculated relative to its own expression at 0 TPI. Wound induced elevated level of *AOS* gene expression was found to correlate with the increase in endogenous JA content (Wilmowicz et al., 2016). JA is mainly involved in biotic stress response and its role biotic defense is well established. The detailed molecular mechanisms of the role of jasmonates for drought stress signaling are still unclear (Riemann et al., 2015). Plant

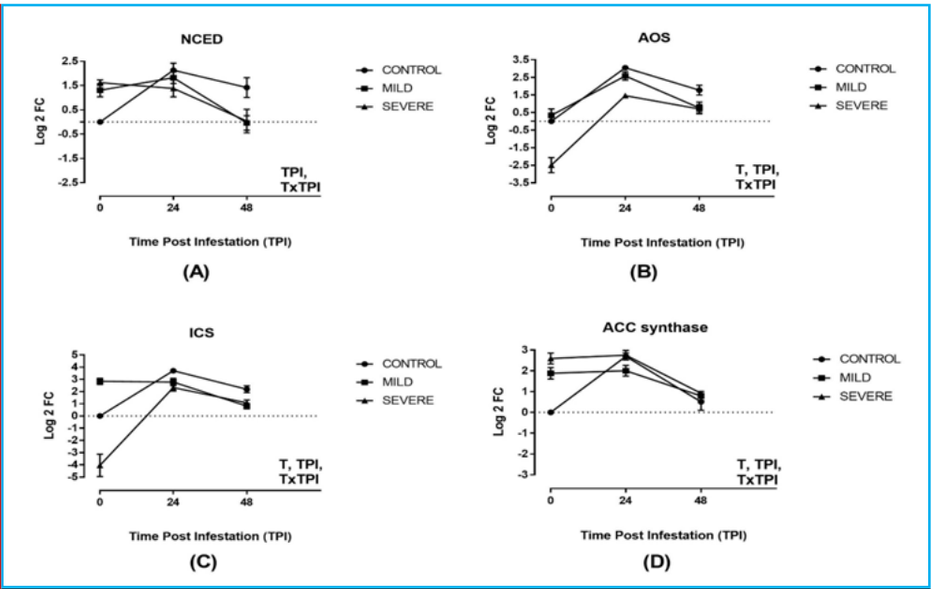

FIGURE 2. Expression of 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene of ABA pathway (B) Expression of Allene oxide synthase (*AOS*) gene of JA pathway (C) Expression of Isochorismate synthase (*ICS*) gene of SA pathway (D) Expression of 1-aminocyclopropane-1-carboxylate synthase (*ACC synthase*) gene of ET pathway. Significant main effect of water stress treatment and time post infestation are indicated by T and TPI respectively, while significant interaction are indicated by TxTPI ($P<0.05$). All the expression data are calculated relative to well watered control sample at 0 TPI and are expressed as mean ± standard deviation (SD). Each expression value is the mean of three biological replicates.

responses to combined abiotic stresses and biotic stress are largely controlled by different signaling pathways that may interact and inhibit one another (Suzuki et al., 2014). In our study the expression of *AOS* gene of JA biosynthesis pathway is clearly suppressed in severe water stressed plants (0 TPI, 24 TPI). High transcript accumulation of *AOS* gene in controls and mild water stressed plants compared to severe stressed plants indicates that water availability play an important role in stronger constitutive and induced JA pathway defense. Plants under increasing drought stress may be at danger of herbivore infestation with lower activation of JA pathway defense. In some plants, it has been reported that a specific abiotic stress enhanced the resistance of plants to biotic stress (Rouhier and Jacquot, 2008). However, in most cases, prolonged exposure of plants to abiotic stresses, such as drought resulted in the weakening of plant defenses (Mittler and Blumwald, 2010). Plants under combinations of abiotic and biotic stresses may prioritize responses to address the potentially more damaging abiotic stress (Atkinson et al., 2013).

Increase in *ICS* gene expression at 0 TPI in mild water stress points towards the role of SA pathway in moderate water stress condition. SA significance has been increasingly recognized in enhanced plant abiotic stress-tolerance via SA-mediated control of major plant-metabolic processes (Khan et al., 2015). Studies extensively found and reviewed the role of SA pathway in the improvement of plant abiotic stresses tolerance such as drought (Horváth et al., 2007; Pal et al., 2013; Fayez and Bazaid, 2014; Miura and Tada, 2014). In induced response (24 TPI) well watered plant maintained higher *ICS* gene expression followed by mild and severe water stressed plant. It is known that upon insect attack usually two signaling pathways Salicylic acid (SA) and Jasmonic acid (JA), mediate plant responses. Severe water stress negatively affected the *ICS* gene expression at constitutive and induced level. The lower expression of SA biosynthesis pathway gene in water stress tea plant may increase their susceptibility to herbivores and necrotrophic pathogens. A significant amount of literature mentioned that the induction of the SA signaling pathway suppresses JA signaling (Niki et al., 1998; Preston et al., 1999; Koornneef et al., 2008a, 2008b).

However, in our study, we have seen a completely different picture where SA and JA associated gene expression increased co-currently in control and mild stressed plants. Whereas in case of severe stressed plant both the genes were significantly down-regulated. Thus JA and SA pathway associated gene expression may not always act antagonistically and may act in synchrony according to the type and severity of stress it is undergoing. In tea cultivation, water stress severity is an important factor which needs to be taken care to avoid disastrous pest and pathogen attack due to weakening of SA and JA associated plant defense.

ACC synthase expression showed maximum peak in severe stressed plants at 0 TPI. The increased constitutive expression in water stressed plants may be associated with ET signaling role in osmotic stress adjustment. ET signaling is known to act as an important controller of the hormone-regulated defense pathways in biotic stress (Broekgaarden et al., 2015) as well as helping plants to adjust to drought stress (abiotic stress) by increasing the compatible solutes accumulation (Cui et al., 2015). Pairwise comparison showed that induced expression value was not significant between control and severe stressed plant ($P > 0.05$, Fig.2 (D)). However it was significant between control and mild stressed plant ($P < 0.05$, Fig.2 (D)). When the fold change at 24 TPI in all treatment is calculated relative to its own expression at 0 TPI the increase in well-watered fold induction in maximum. Thus the increase of *ACC* synthase expression in response to herbivore infestation points towards ET signaling role in biotic stress signaling and well-watered plant have higher capacity of transcript induction on insect infestation. Ethylene transcripts mediated role in plant stress and pathogen responses have been already reported in literature (Abeles et al., 1992; O'Donnell et al., 1996). In rice plant, *ACC* oxidase and *ACC* synthase transcript up regulation in response to feeding by the brown planthopper, *Nilaparvata lugens* (Stal) have been documented (Zhang et al., 2004).

Overall from our study it has been seen that water stress positively affected ABA and ET pathway genes with increase in constitutive expression. However JA and SA pathway genes known to be involved with herbivore and pathogen defense signaling were negatively affected in severe stressed plants. In case of induced expression (24 TPI), severe water stressed plants showed significant decrease in expression of the rate-limiting phytohormone genes compared to well water plants except *ACC* synthase gene. The crosstalk among the phytohormone pathway transcriptional defense in combination with other intrinsic defense mechanism will ultimately govern the of tea plant response.

The presence of abundant antagonistic or synergistic interactions among the pathways provide the plant with an extensive regulatory potential for the activation of specific defenses (Vos et al., 2013). When combination of water and herbivory stress was applied, it seems to be most likely that plant transcriptional defense machinery was largely controlled by water stress severity and herbivory stress regulation of phytohormone genes was overpowered by water stress gene regulation. Thus our results clearly shows that well-watered plants on insect infestation will have higher capacity to induce the expression of phytohormone genes than water stressed plants.

CONCLUSION

It can be concluded that the presence of an initial water stress not only affected the tea plant constitutive defense but also significantly altered the phytohormone defense gene expression towards subsequent herbivore stress. On prolonged drought events tea plant with weak induced defense may face higher incidence of pest and pathogen attack.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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