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RESEARCH ARTICLE

Biochemical and molecular studies on the resistance mechanisms in tea [*Camellia sinensis* (L.) O. Kuntze] against blister blight disease

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Abstract Tea (*Camellia sinensis*) plantations are exposed to biotic and abiotic stresses. Among the biotic factors, blister blight (BB), caused by *Exobasidium vexans*, affects the quality and quantity of the product and demands high fungicide application. A long term solution for disease resistance would require the knowledge of the basic molecular and biochemical changes occurring in plant as an attempt to resist the pathogen and limit the spread of the disease which can further help in developing resistant cultivars using biotechnological tools. Thus, gene expression studies using the cDNA based suppressive subtractive hybridization library, characterization of genes for pathogenesis related (PR) proteins [chitinase (*CsCHIT*), glucanase (*CsGLUC*), phenylalanine ammonia lyase (*CsPAL*)] and genes in flavonoid pathway were accessed in the BB resistant and susceptible cultivars, SA6 and TES34, respectively. Further, biochemical analysis of PR and antioxidant enzymes (POX, APX, SOD) involved in BB resistance have been carried out to investigate the potential molecular and biochemical changes. Various stages of pathogen development had varied impact on PR protein, flavonoid pathway and anti-oxidative enzymes and indicates the possible role of reactive oxygen species, lignins, flavonoids, anthocyanins and other synthesized compounds in acting as antimicrobial/antifungal agents in tea cultivars.

Keywords Blister blight · Flavonoid · Pathogenesis · Resistance · SSH · Tea

Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is a perennial crop, with an economic life span of over 60 years. Extensive cultivation, improved technology, nutrition and fertility management, introduction of high yielding clones and longer pruning cycle have helped in production increase but have also encouraged productivity limit due to biotic stresses caused by insects, mite pests and various other pathogens (Senthilkumar et al. 2012; Singh et al. 2015).

As the crop is being cultivated for its young succulent leaves, diseases infecting these leaves also affect the quality and quantity of harvest (Senthilkumar et al. 2012; Singh et al. 2015). Among the foliar diseases, blister blight (BB) is caused by the obligatory biotrophic fungal pathogen, *Exobasidium vexans* (Order: *Exobasidiales*).

Indiscriminate usage of fungicides to control BB may lead to resistance development and may exceed the acceptable maximum residue limits of residue (1–2 ppm for systemic fungicides).

Various attempts have been made to correlate resistance with the chemical composition of tea leaf like changes in saccharide, increased activity of polyphenol oxidase and peroxidase, and decrease in chlorophylls and carotenoids induced by BB infection (Rajalakshmi and Ramarethinam 2000). Study of the molecular changes that take place in

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the tea during the course of BB infection has not been attempted so far.

In order to understand the molecular basis of disease resistance in plants, it is necessary to find a method to assess and isolate the differentially expressed genes related to the infection. Study of the defence gene regulation in intact plant-pathogen interactions would aid in the identification of specific genes involved against biotic stresses. Suppression subtractive hybridization (SSH) followed by validation studies is a powerful technique that enables cDNA libraries representing differentially expressed transcripts in different tissues and different stages (Thirugnanasambantham et al. 2011; Senthilkumar et al. 2012).

Hence, the present study aimed to identify the differentially expressed genes during BB infection in the tolerant cultivar, SA-6, by SSH technique. Further, the studies on biochemical composition, free-radical scavenging enzymes, and PR proteins of the leaves were carried out to correlate the changes in gene expression data in different cultivars with varied degree of tolerance towards the disease and also during different stages of infection. Changes in the pattern of gene expression in the flavonoid pathway genes during different infection stages have been studied to correlate the expression levels and their possible role in defense.

Materials and methods

Plant material

The healthy and blister infected leaves from twelve cultivars, classified as highly resistant (SMP-1 and SA-6), resistant (UPASI 5 and UPASI 6), tolerant (UPASI 2 and UPASI 10), moderately susceptible (UPASI 3 and UPASI 8), susceptible (UPASI 9 and UPASI 12) and highly susceptible (UPASI 14 and TES 34) to BB infection in field conditions (Baby 2006), growing in the tea garden of the UPASI—Tea Research Institute were selected in the present study. Only healthy leaves were collected from the SMP 1 cultivar which did not show any blister infestation during the study. All the cultivars used in the study are 50 years of age with ten successive pruning cycle of 5 years interval.

Blister infected leaves corresponding to (1) initial stage with 0.5–1.0 mM sized lesion (S1), (2) blister formation stage (S2), (3) incipient stage of sporulation (S3) and (4) stage showing sporulating lesions (S4) and uninfected, healthy leaves (C) (Fig. 1) from a single bush were collected and immediately frozen in liquid nitrogen and transferred to − 80 °C for further use.

SA-6 was selected for SSH library. SA-6 and TES 34 were used to study the gene expression of three important

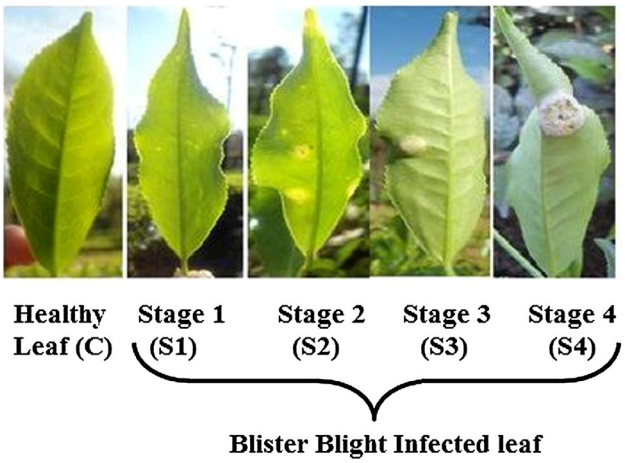

Fig. 1 Schematic representation of healthy and blister blight (BB) infected leaves showing the four stages of BB infection used in this study

PR protein encoding genes, chitinase (*CsCHIT*), β 1,3-glucanase (*CsGLUC*) and phenylalanine ammonia lyase (*CsPAL*) and the flavonoid biosynthetic pathway specific genes encoding cinnamate 4-hydroxylase (*C4H*), coumarate coA-ligase (*4CL*), chalcone isomerase (*CHI*), dihydroflavanol reductase (*DFR*), flavanone 3-hydroxylase (*F3H*), flavonoid 3,5-hydroxylase (*F3*,*5*,*H*), anthocyanin synthase (*ANS*) and anthocyanidin reductase (*ANR*), during the different stages of the infection in comparison to the uninfected, healthy conditions.

The bud, first, second, third and maintenance leaves of the healthy and infected shoots as well as the first leaf tissues during different stages of infection were used for quantitative analysis of anti-oxidative enzymes, PR proteins and gene expression pattern. These tissues were harvested between 9 and 10 a.m., transferred immediately in liquid nitrogen and stored at − 80 °C till further use.

RNA isolation and cDNA synthesis

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) followed by DNase I (Fermentas) treatment as per the manufacturer's protocol. Total RNA isolated from the leaves of SA6 cultivar corresponding to different stages of blister infection was pooled together and that of the healthy leaf was used as such for SSH analysis.

Suppression subtractive hybridization (SSH)

PCR-select cDNA subtraction was performed using PCR-Select^{TM} cDNA subtraction Kit (Clontech) according to the manufacturer's protocol. cDNAs from pooled RNA of SA6 cultivar was used as the 'tester' and from the healthy leaf tissue as 'driver' in forward subtraction and vice versa for

reverse subtraction. The subtracted cDNAs were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instruction. Primers of tea β-actin gene (GenBank accession no. CA148161) were designed (FP: 5′-GTATTGTTCTCGACTCTGGTGATGG-3′; RP: 5′-TCTCAGGTGGTGCAACGACC-3′) and used to amplify for 15, 20, 25, 30 and 35 cycles to test the subtraction efficiency of the library before cloning.

Cloning of SSH PCR products

The purified secondary SSH-PCR products were cloned into pTZ57R/T (Fermentas) and transformed into *E. coli* DH5α. To assess the size of inserts, colony PCR was performed, and analyzed on 1% agarose/EtBr gel. Each colony was inoculated in 150 µL of freezing broth/ampicillin (100 mg/L), grown overnight at 37 °C and finally stored at 4 °C for further use.

EST sequencing and analysis

Plasmid DNA was extracted from overnight grown individual colonies with miniprep-DNA Purification System (Himedia) and sequenced in MegaBACE 500 DNA Sequencer (Amersham Biosciences). ESTs were compared with non-redundant public databases, using the BLASTN and BLASTX algorithms of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ESTs with E value < 0.01 were deemed to have significant homology and were categorized according to their function. Homologies exceeding 50 nucleotides that showed more than 90% identity to sequences in the database were considered significant. The putative physiological function of these ESTs was classified according to Gene Ontology (http://www.geneontology.org).

Semi-quantitative RT-PCR

Single strand cDNAs were synthesized from total RNA (1 µg) using cDNA synthesis kit (Clontech). cDNA (50 ng) were subjected to PCR amplification using gene specific primers (Table 1) designed for validating the differentially expressed cDNAs obtained from SSH library and PR protein encoding genes, eight major flavonoid pathway specific genes encoding *C4H*, *4CL*, *CHI*, *DFR*, *F3H*, *F3*,*5*,*H*, *ANS*, and *ANR* during different stages of the infection in comparison to the uninfected, healthy conditions.

PCR conditions for each primer set was optimized and amplification was carried for 24 cycles with denaturation at 94 °C for 30 s, annealing at standardized temperature (Table 1) for 45 s, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min. PCR products were analyzed on 1% agarose/EtBr gel. Forward and reverse primers (Table 1) of *Camellia sinensis* 26S rRNA (*Cs26SrRNA*, GenBank accession no. AY283368) were used as internal control. The semi-quantitative PCR assay was performed and analyzed at least two times independently. The intensity of bands was quantified using ImageJ software (http://rsb.info.nih.gov/ij/) and normalized against *Cs26SrRNA* band intensity. An arbitrary measure of the expression (at transcript level) was calculated for each gene from two independent RT-PCR reactions.

Total protein extraction

1 g of tissue was ground in 0.01 M sodium acetate (pH 5.0, 1:3, w/v) and centrifuged at 15,000×g for 10 min at 4 °C, and the supernatant was used for biochemical and enzymatic activity. The protein content of the supernatant was also measured (Bradford, 1976).

Pathogenesis related (PR) protein analysis

Chitinase, β 1,3-glucanase and phenylalanine ammonia lyase (PAL) activities in the tissue were determined using colloidal chitin, laminarin and phenylalanine substrates as described by Sun et al. (2006), Pan et al. (1991) and Sadasivam and Manickam (1996), respectively (Supplementary file 1).

Anti-oxidative enzyme analysis

Peroxidise (POX), ascorbate peroxidise (APX), and superoxide dismutase (SOD) activities in the collected tissue samples were determined as purpurogallin formation from pyrogallol, oxidation of ascorbate, and NBT reduction, respectively by following the methods described by Palanisamy and Mandal (2014) (Supplementary file 1).

Data analysis

The enzyme quantification experiments were performed at 25 °C in triplicate, and the results are expressed as mean values with ± standard error.

Results

Isolation of differentially expressed transcripts during blister infection using SSH library

RNA extraction and library construction

PCR-select cDNA subtractions were performed using BB infected leaves as the tester and healthy leaves as the driver

Table 1 Primers used for the specific amplification of genes encoding PR proteins and in flavonoid biosynthetic pathway during different stages of blister blight infection

Gene	DNA sequence (5′–3′)	Annealing temperature	Optimum cycle
CsCHIT-FP	CACAATGATAGGAAGAGCCGAC	58	32
CsCHIT-RP	CAAGGGA ACGGGCTTGGCAGAATC		
CsGLUC-FP	CAGTCTAGAATGGCATTGCTTCTTCTTCTGC	60.5	28
CsGLUC-RP	CGAGGATCCTCATAAGAAAACAAGAGACCATGATAG		
CsPAL-FP	AGATAGATCCAACTGCTTCA	63	30
CsPAL-RP	TTGTTCCAATGCGATAAGAA		
CsC4H-FP	CGCGTGGTGGCTTGCAAACAACCCC	60	29
CsC4H-RP	TGGCCTGTCCCGGAGGAGGCAAGAG		
Cs4CL-FP	GATGATGAGGTTATTGTTGATCGAT	60	29
Cs4CL-RP	CCTGTTTCGCTATGAATTCTTTAACA		
CsCHI-FP	CGTGGAGGATATCACTGCCAAGGCT	65	28
CsCHI-RP	TGTGGGTATAGGCCCTGCATAGGAG		
CsDFR-FP	GCTGACACGAACTTGACAC	65	28
CsDFR-RP	CCACCCCACCATACAAAAAC		
CsF3H-FP	ATGGCGCCAACAACGCTTAC	63	30
CsF3H-RP	TCAAGCAAAAATCTCATCAGTG		
CsF3,5H-RP	TCAAGCGTGCTCACGAAGAA	58	32
CsF3,5H-RP	AAGCCACAGGAAACTAAAACAACA		
CsANS-FP	ATGACTACAGTGGCTGCCCCGAGAG	55	33
CsANS-RP	CTGAGCAAAAGTCCTCGGCGGGAA		
CsANR-FP	ATGGAAGCCCAACCGACAGCTC	63	27
CsANR-RP	TCAATTCTTCAAAATCCCCTTAGCCT		

and vice versa for the forward and reverse libraries respectively. The subtracted cDNA products thus obtained were cloned into pTZ57R/T cloning vector and then transferred into competent *E. coli* cells. From the forward library, 192 colonies and from the reverse library, 630 colonies were obtained.

EST sequencing and analysis

Colonies with insert size > 250 bp were selected by colony PCR. About 96 colonies from the forward library and 384 colonies from the reverse library were selected based on insert size (> 250 bp) and sequenced. Vector and adaptor sequences were removed from the sequences and the processed sequences were submitted to NCBI database (Genbank Accn no. JG463673 to JG463746, JG294142 to JG294233).

From the forward SSH library, transcripts with high similarity to early light inducible protein, DNA damage/repair toleration protein 112,2-oxoglutarate dehydrogenase, glyceraldehyde 3 phosphate dehydrogenase, NAD + transporter 1, ATP dependent zinc metalloprotease, α-1,3 glucosyltransferase and cyclin dependent kinase were obtained, which are involved mainly in flavonoid biosynthesis, catabolic activities, ion transport, protein folding, cell division and repair (Table S1). In addition, transcripts encoding stress responsive proteins like photosystem II 5kD protein, asparagine-linked glycosylation protein, thioredoxin M type 4, and heptahelical transmembrane protein 1 related to wounding stress, hydrogen peroxide stress, oxidative stress and hormone stress respectively, were obtained from the forward library. Gene sequences similar to few of the generalised cellular processes were also obtained along with three ESTs of hypothetical proteins and 24 ESTs with no significant match, considered to be novel sequences were obtained (Table S1).

The reverse SSH library did not yield much functional transcripts except for the long-chain specific acyl-coA dehydrogenase involved in lipid catabolic activity. Fourteen ESTs highly similar to hypothetical proteins were also obtained in the reverse library.

Gene ontology analysis

Functional annotation of the forward SSH sequences using Gene Ontology (GO) analysis categorized the sequences as 44.07% associated with hypothetical/unknown proteins,

11.86% each involved in carbohydrate metabolism and photosynthesis, 8.47% involved in protein metabolism, 5.08% involved in transport and 1.69% each for flavonoid metabolism, 2-oxoglutarate metabolism, methylglyoxal catabolism, photosystem repair, photorespiration, cell cycle, leaf morphogenesis and stress like oxidative, hormone, wounding and hydrogen peroxide stress (Fig. 2). The reverse SSH sequences resulted in match with proteins involved in lipid biosynthesis required for cell wall synthesis and reinforcement.

Expression pattern of PR protein encoding genes

To further validate the results of SSH library, expression pattern of genes coding for PR proteins, chitinase, β-1,3 glucanase and PAL was analysed using semi quantitative RT PCR under optimized conditions. The expression of *CsCHIT* in the cultivar SA6 was 1.32–13.81 fold higher during BB infection (Fig. 3a). The *CsCHIT* expression started increasing with the intensity (1.32 fold) of the infection from the first stage (SA6-S1) and reached the maximum in the fourth stage (SA6-S4; 13.81 fold). In contrast, the highly susceptible cultivar TES34 had decreased expression of *CsCHIT* in the first and second stages (TES34-S1 and TES34-S2) with a slight increase in the third and fourth stages (TES34-S3 and TES34-S4) of infection (Fig. 3b). The expression of *CsGLUC* in SA6 did not register a marked increase during infection (Fig. 3a). The first stage (SA6-S1) of the disease had a mild increase in the *CsGLUC* expression followed by a decrease in the second (SA6-S2) and an increase in the third (SA 6-S3) with a fall in the expression at fourth stage (SA6-S4) of infection. The cultivar TES34 had a higher expression of *CsGLUC* in the first stage (TES34-S1; 4.15 fold) with a gradual decrease in the second (TES34-S2) and third (TES34-S3) and then reached to maximum increase (5.91 fold) in the fourth stage (TES34-S4) of the disease. The expression range of *CsGLUC* was higher in the highly susceptible cultivar than the highly resistant cultivar (Fig. 3c).

The expression of *CsPAL* in SA6 decreased until the third stage (SA6-S3) and then reached the maximum in the fourth stage (SA6-S4). In TES 34, expression of *CsPAL* increased rapidly in the first (TES34-S1) and then gradually decreased until the fourth stage (TES34-S4) of infection.

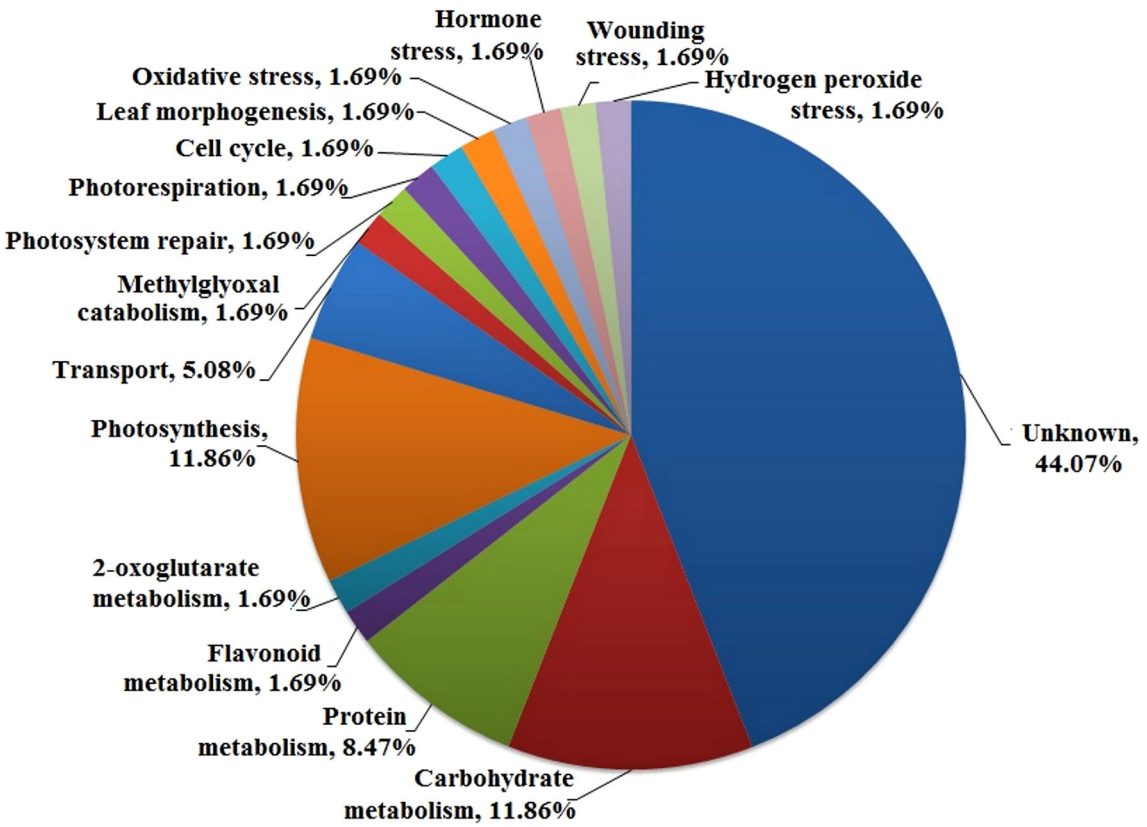

Fig. 2 Functional categorization of differentially expressed genes identified from SSH library in response to BB infection in tea. The relative frequencies of assigned Gene Ontology (GO) hits were presented as percentage of GO-annotated ESTs

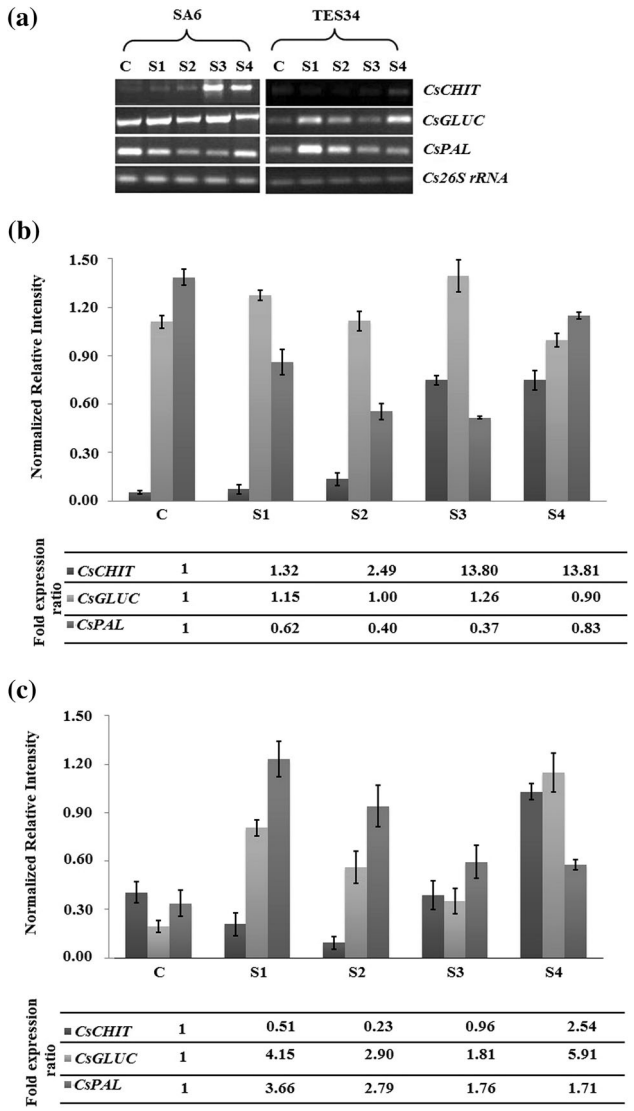

Fig. 3 **a** Semi-quantitative RT-PCR, **b**, **c** expression analysis of PR protein encoding genes, *CsCHIT* (Acc No. JN392472); *CsGLUC* (Acc No. AF399920); *CsPAL* (Acc No. D26596) during BB infection in BB resistant, SA6 and susceptible cultivar, TES 34 respectively. *Cs26S rRNA* (Acc No. AY283368) was used as internal control. Lane C to S4: cDNA (50 ng/µl) of the respective samples (control and infected stages). Each value represents the average of the expression ratios between the normalized relative intensity of analyzed gene in infected (Stage 1–4 of blister blight infected leaf) and control (healthy uninfected leaf) from two independent RT-PCR

Overall, *CsCHIT* expression was the highest in the resistant, SA6 than the susceptible cultivar, TES34 and vice versa for *CsGLUC* and *CsPAL.*

Role of PR proteins and anti-oxidative enzymes during the different infection stages, tissues and cultivars

To further support the expression analysis, PR related proteins and anti-oxidative enzymes were analyzed in different infection stages, and tissues collected from infected and uninfected tea bushes of SA6 and TES34. In addition, twelve tea cultivars grouped according to their varied degree of resistance to the BB infection were also analysed for the enzyme levels.

Chitinase

The levels of the enzyme, CsCHIT increased markedly during the first stage of the BB disease with further increase in the second and third stages until reaching a maximum expression in the fourth stage of the disease, in comparison to the uninfected leaves, in SA6. In TES34, there was an increase in the first stage, followed by a decrease in the third and then an increase in the fourth stage of the disease. However, the range of increased expression was several folds higher in the highly resistant cultivar SA6 than highly susceptible cultivar TES34 (Fig. 4a).

The expression of the enzyme in the tissues of the infected plant of SA6 were higher than that in the healthy plant in the first, second and third leaves whereas in the bud, the expression decreased in the infected plant when compared with healthy plant. In the cultivar TES34, the bud and first leaf showed increase in the enzyme levels and decrease in the second and third leaves in the infected plant in comparison to the healthy plant (Fig. 4b).

The incidence of BB disease increased the level of enzyme in all cultivars except the highly susceptible ones. In healthy leaves, the expression of chitinase was highest in the highly resistant cultivars and decreased with increasing susceptibility, with the least activity in UPASI14. The level of induction was highest in SA6 while the lowest induction was in UPASI12. The cultivars UPASI14 and TES34 showed a decrease in enzyme activity during disease incidence (Fig. 4c).

β 1,3-glucanase (CsGLUC)

The enzyme CsGLUC in both SA6 and TES34 followed same pattern of expression during different stages of infection. Expression increased initially during the first stage of the disease after which there was a decrease in its levels and then again an increase in the third stage followed by a decrease in the fourth stage of the disease. However there was a marked difference in the range of variation in the CsGLUC levels between the highly resistant and highly susceptible cultivars under study (Fig. 4a).

In the infected plant, the levels of CsGLUC increased in the bud, first and second leaves whereas in the third leaf, the enzyme levels decreased when compared to the healthy plant of SA6. In TES34, there was increase in the enzyme

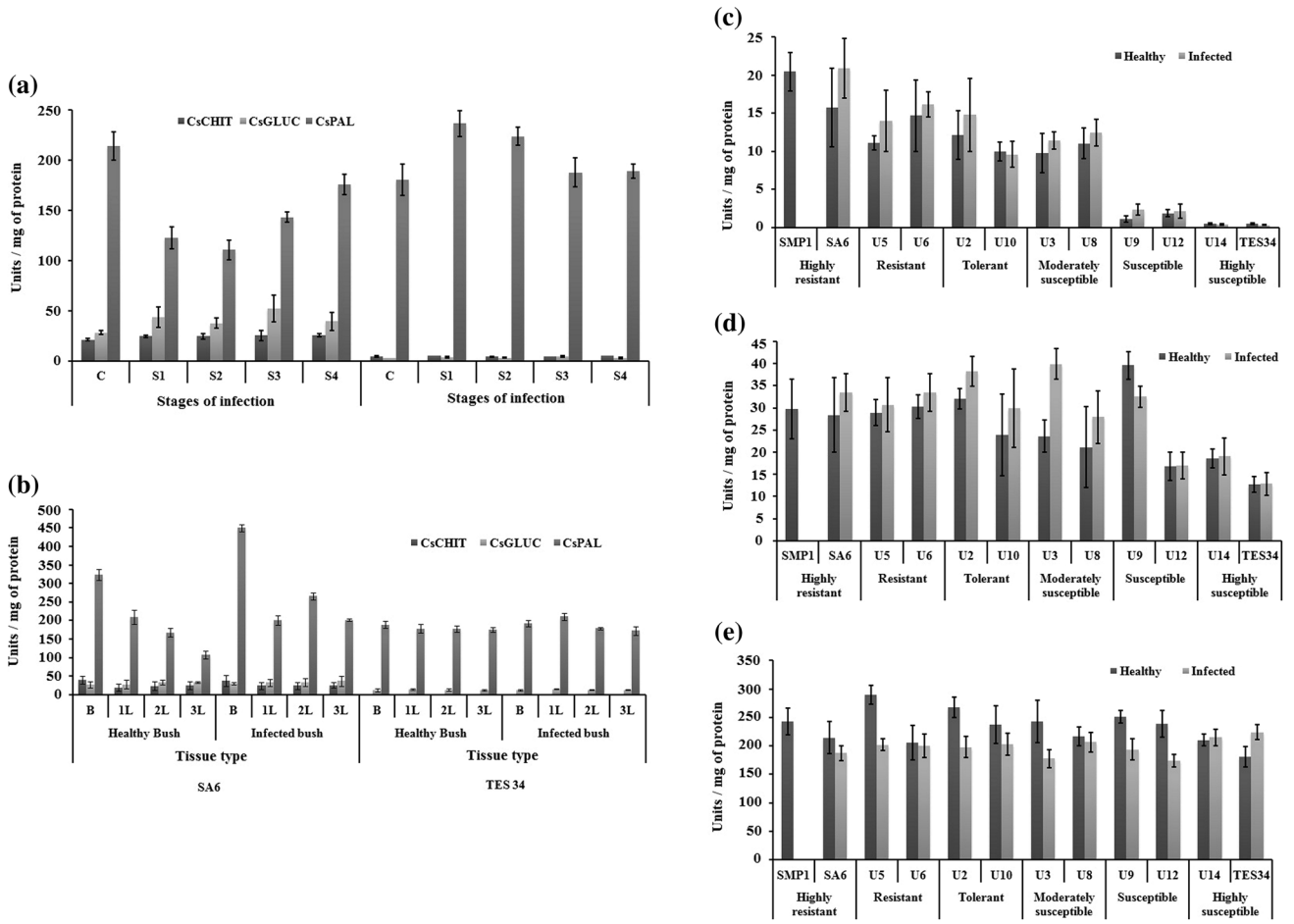

Fig. 4 Biochemical activity analysis of PR protein CsCHIT, CsGLUC and CsPAL in **a** different infection stages of SA 6 and TES 34, **b** different tissues of a healthy and infected bush of SA6 and TES34. The healthy and BB infected leaves of tea cultivars with varying degrees of resistance/susceptibility to BB infection were also analyzed for **c** CsCHIT, **d** CsGLUC, and **e** CsPAL activity. Data is presented as mean ± SE of three replicates and error bars in the chart represent ± SE

levels in all the tissues of the infected plant than the healthy plant (Fig. 4b).

The level of this enzyme was found to be higher in the resistant, highly resistant and tolerant cultivars whereas it began to decrease in the susceptible cultivars except in UPASI9, in the absence of disease symptoms. However the incidence of disease symptoms induced enzyme expression in almost all of the cultivars except UPASI9, which showed a decrease in activity. The induction was highest in UPASI3 followed by UPASI2 and UPASI8. The induction was the least in UPASI12, UPASI14 and was almost negligible in TES34 (Fig. 4c).

Phenylalanine ammonia lyase (CsPAL)

In SA6, levels of CsPAL decreased during the initial and second stage of infection after which there was slight increase in its levels in the third and fourth stages, but none had an increase as that in the healthy leaf. In TES34, CsPAL levels were higher in the infected stages than the uninfected leaf with an increase in the initial stage itself. Further stages saw a decrease in the CsPAL levels but none had decreased below that in the healthy leaf (Fig. 4a).

The levels of the enzyme in SA6 increased in the bud, second and third leaves whereas it decreased in the first leaf of the infected plant when compared to the healthy plant. In TES34, the levels increased in the infected plant in the bud, first and second leaves but decreased in the third leaf in comparison to the healthy plant (Fig. 4b).

Levels of CsPAL decreased in all of the cultivars except the highly susceptible ones during the BB infection. The decrease in the levels was least in UPASI 6 followed by UPASI 8 whereas it was more pronounced in other cultivars. UPASI 14 showed a little increase in the activity when compared to TES 34 which showed an increase of almost 44 U during infection (Fig. 4c).

Peroxidase (CsPOX)

In the highly resistant cultivar SA6, CsPOX showed a marked increase in its levels during the first stage of disease incidence when compared to the uninfected condition. In the second and third stage of the disease, the enzyme levels decreased gradually followed by a steep increase in the fourth stage under study. The highly susceptible cultivar TES34 did not show any change in the POX levels at the very first stage of the disease with respect to the uninfected condition. Gradual increase was seen in the second and third stages followed by the highest production in the fourth stage (Fig. 5a).

The enzyme CsPOX showed higher expression in the tissues of the infected plant than in the healthy plant of both the cultivars under study. However the susceptible cultivar, TES34, had higher expression in the infected plant tissues when compared to the enzyme levels in the infected plant tissues of the highly resistant cultivar, SA6. Exceptionally, the third leaf in the cultivar TES34 had decreased levels of POX during infection (Fig. 5b).

The constitutive level of the enzyme was found to be higher in the susceptible cultivars than the tolerant cultivars. The healthy leaves of UPASI10 possessed high level of CsPOX whereas UPASI14 showed least activity. During blister blight incidence in leaves, tea cultivars, irrespective of being resistant, tolerant or susceptible, registered an increase in CsPOX activity and highest induced expression was in UPASI10, a tolerant cultivar. In this study, the difference in enzyme levels of infected and healthy leaves increased with increased range of susceptibility. The highly resistant cultivar, SA6, showed an increase of 8.9 U of enzyme during disease incidence whereas the most susceptible cultivar, TES34, showed 11.2 U increase (Fig. 5c).

Ascorbate peroxidase (CsAPX)

The levels of CsAPX in SA6 initially increased during first stage of disease and then decreased in second stage of infection. There was a marked increase again in the third stage and the levels came down rapidly during the last stage of disease. In the case of TES34, the levels saw a

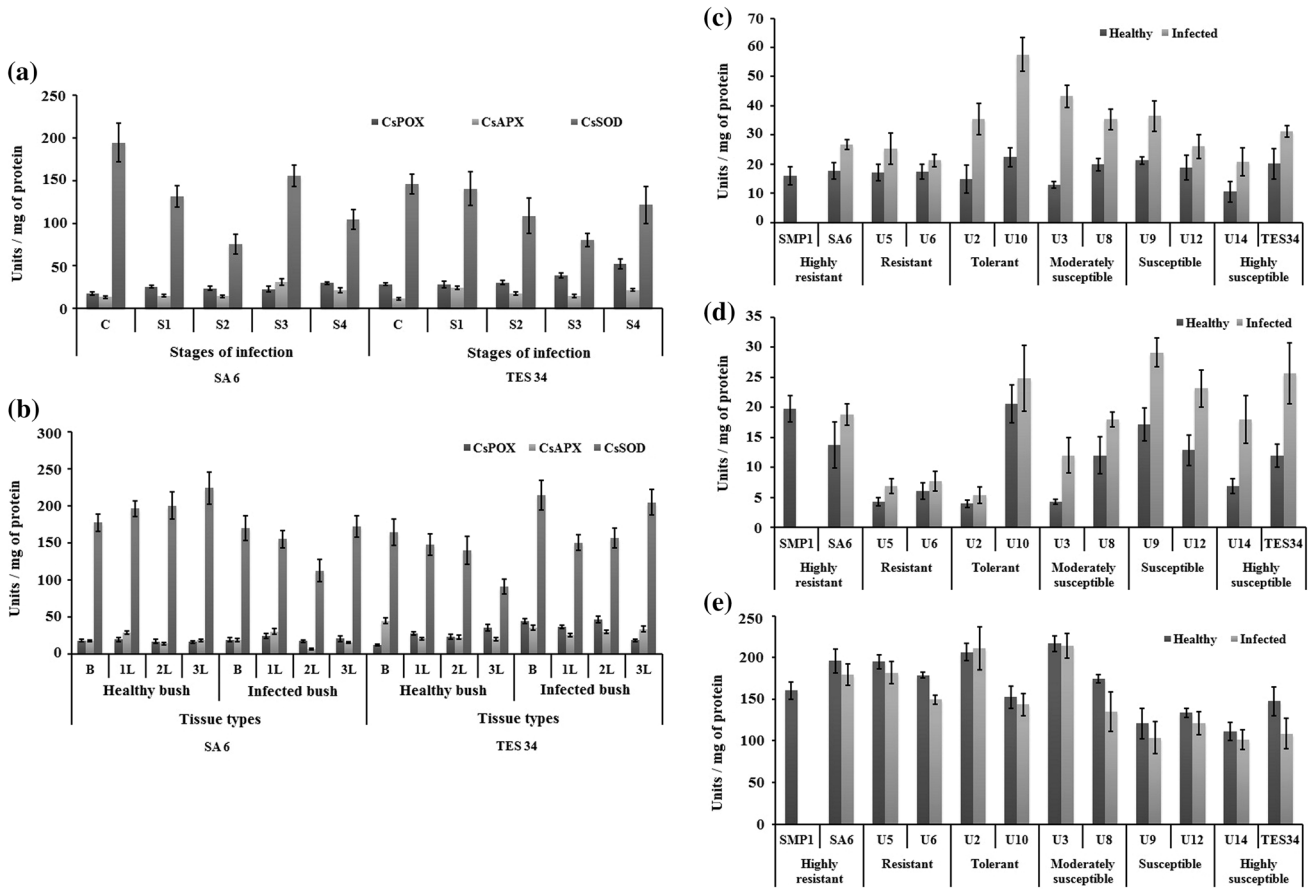

Fig. 5 Biochemical activity analysis of PR related anti-oxidant enzyme activity of CsPOX, CsAPX and CsSOD in **a** different infection stages, **b** different tissues of a healthy and infected bush of SA 6 and TES 34. The healthy and BB infected leaves of tea cultivars with varying degrees of resistance/susceptibility to BB infection were also analyzed for **c** CsPOX, **d** CsAPX, and **e** CsSOD activity. Data is presented as mean ± SE of three replicates and error bars in the chart represent ± SE

marked increase in the first stage and then the second and third stages showed a gradual decrease in its levels, whereas there was again an increase at the last stage (Fig. 5a).

CsAPX levels increased in the bud and first leaf of the infected plant than in the healthy plant of the cultivar SA6. However in the same cultivar, the levels decreased in second and third leaf of the infected plant than in the healthy plant. In the case of TES34, APX level decreased in bud and increased in first, second and third leaves of the infected plant than in the healthy plant (Fig. 5b).

The enzyme, in healthy conditions, did not follow a particular trend among the cultivars. The highest level was found in UPASI10 and lowest level in UPASI2. However, the level of the enzyme increased during disease incidence in both tolerant and susceptible cultivars. The induced expression started to increase with increasing susceptibility of the cultivars, the highest induction being in TES34 followed by UPASI14, whereas the lowest was in UPASI2. This enzyme increased by 5.14U in the cultivar SA6 and by 13.71U in TES 34 (Fig. 5c).

Superoxide dismutase (CsSOD)

In comparison to the healthy leaves, the levels of the enzyme CsSOD decreased enormously during the first two stages of the disease and showed a slight increase in the third and again decreased in the fourth stage in SA6 whereas in TES34, the levels decreased gradually till the third stage of the disease and then showed a higher expression in the fourth stage (Fig. 5a).

Levels of CsSOD decreased in the infected plant tissues when compared to the healthy tissues in the cultivar SA6. In contrast, the cultivar TES34 showed increased levels of the enzyme in the infected plant tissues than the healthy tissues (Fig. 5b).

The enzyme was found to be highly expressed in the resistant and tolerant cultivars than in susceptible cultivars in the absence of infection. Incidence of the disease decreased the levels of the enzyme in all the cultivars. The difference in enzyme levels in infected and healthy leaves was more pronounced in moderately susceptible cultivar, UPASI8, followed by highly susceptible cultivar, TES34, whereas it was the least in UPASI 3. The cultivar UPASI 2 had higher expression of the enzyme during disease infestation in contrast to the trend generally shown by other cultivars under study (Fig. 5c).

Effect of pathogen infection on flavonoid pathway specific genes

Expression of *CsC4H* increased considerably in the first (SA6-S1) and third (SA6-S3) infection stages and decreased in the second (SA6-S2) and fourth stage (SA6-S4) in SA6 whereas it increased till the second stage (TES34-S2) and then decreased in TES34 (Fig. 6a). Expression of *Cs4CL* in SA6 increased on the onset of infection and reached the maximum in the second stage (SA6-S2), after which it gradually decreased. In TES34, disease incidence decreased *Cs4CL* expression initially and then increased in the second stage (TES34-S2) and again decreased in the third (TES34-S3) and fourth stages (TES34-S4) (Fig. 6b).

The expression of *CsCHI* decreased in all of the infection stages except the third stage (SA6-S3) where there was a mild increase in SA6 whereas in TES34, the levels decreased in the first stage (TES34-S1) in comparison to the healthy leaf. The second stage (TES34-S2) had increased expression after which the induction reduced gradually (Fig. 6c). Expression of *CsDFR* was suppressed by pathogen invasion in the initial stage (SA6-S1) in SA6 and later induced in the second (SA6-S2) and third stage (SA6-S3) and then decreased. In TES34, the pathogen invasion suppressed the *CsDFR* expression in all the stages except the third stage (TES34-S3) when compared to the uninfected leaf (Fig. 6d).

The expression of *CsF3H* in SA6 increased mildly in the first (SA6-S1) and second stages (SA6-S2), after which there was a fall in its expression level in third stage (SA6-S3) and then again increased in the fourth (SA6-S4) while in TES34, the expression of *CsF3H* increased several folds in the first stage (TES34-S1) and then decreased tremendously in the second (TES34-S2) and then again increased in the third (TES34-S3) and fourth stages (TES 34-S4) of infection (Fig. 6e). The expression of *CsF3*,*5*,*H* in SA6 decreased in all the four stages of infection with respect to the uninfected leaf. Expression in the second (SA6-S2) and fourth stages (SA6-S4) was not higher than the expression in the healthy leaf. In TES34, its expression was suppressed in the first stage (TES34-S1) and later induced in the second (TES34-S2) and fourth stages (TES34-S4) (Fig. 6f).

In SA6, the expression of *CsANS* increased gradually during disease incidence reaching maximum in the second (SA6-S2) followed by fourth stages (SA6-S4) of infection. TES34 followed the same pattern as SA6 but the induction levels were higher than in SA6 (Fig. 6g). Expression of *CsANR* was suppressed in the first stage (SA6-S1) and then induced till the third stage (SA6-S3) and again decreased in SA6, whereas the level of expression remained suppressed in all of the infection stages in comparison to the uninfected leaf of TES34 (Fig. 6h).

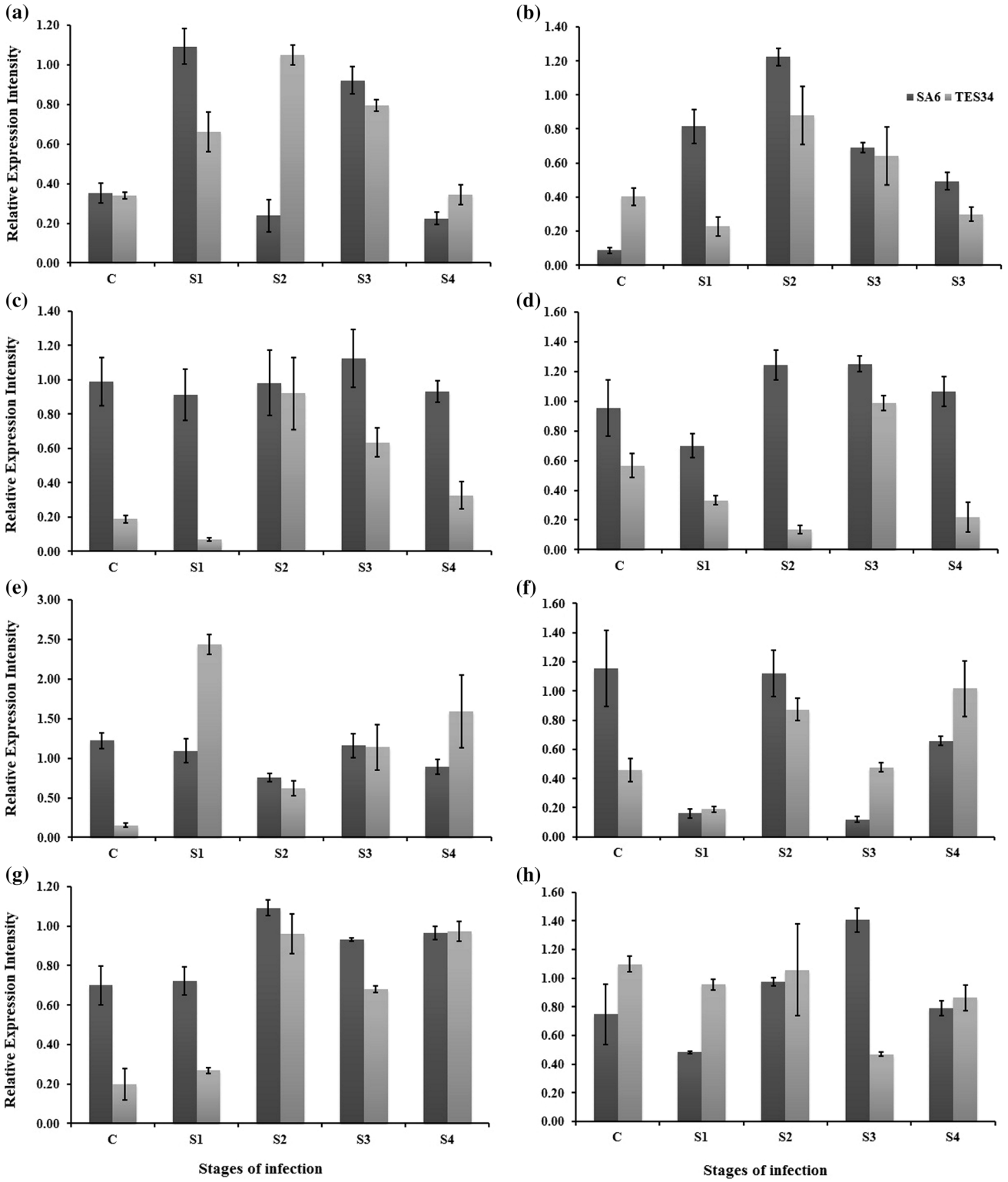

Fig. 6 Expression analysis of flavonoid pathway specific genes. **a** *CsC4H*, **b** *Cs4CL*, **c** *CsCHI*, **d** *CsDFR*, **e** *CsF3H*, **f** *CsF3′5′H*, **g** *CsANS* and **h** *CsANR* during BB infection in BB resistant cultivar, SA6 and susceptible cultivar, TES34. Each value represents the average of the expression ratios between the normalized relative intensity of analyzed gene in infected samples (Stage 1–4 of blister blight infected leaf) and the control (healthy uninfected leaf) from two independent RT-PCR

Discussion

Suppression subtractive hybridization (SSH) library

Most of the up-regulated genes identified in forward SSH were related to general metabolism (23.71%) and stress (6.76%). In forward SSH library, the EST encoding early light inducible protein (ELIP) (GenBank Accn no: JG463735) (Table S1) is involved in a number of cellular processes and structurally related to light harvesting complex (LHC) proteins. Several studies have demonstrated the role of ELIP in response to a variety of stress-related signals such as salt, drought, heat, abscisic acid, cold, desiccation, aluminium, high CO_2, and senescence (Provart et al. 2003).

Glycolysis pathway genes encoding fructose 1,6-biphosphatase (GenBank Accn no: JG463680) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank Accn no: JG463719) were up-regulated in forward SSH (Table S1). GAPDH is a NAD(P)(+)-binding central glycolytic protein with pivotal role in energy production and also involved in photosynthetic metabolism and responses to abiotic and biotic stresses. The osmotic potential of cell is increased by accumulation of sugar and also participate in specific signal transduction mechanisms in stressed plants. The stressed plants tissue require energy to exhibit tolerance to stress and the energy is derived from carbohydrate that is metabolizable (Radomiljac et al. 2013).

The obtained ESTs encoding 2-oxoglutarate dehydrogenase (OGDH; GenBank Accn no: JG463734) and Chloroplastic M-type thioredoxins (TRXM) (GenBank Accn no: JG463738) from the forward SSH library are the key regulators in TCA cycle and redox regulation in photosynthesis, respectively. Thioredoxin M documented to regulate NADP-malate dehydrogenase (NADP-MDH) which catalyses the conversion of malate into oxaloacetate, producing sufficient quantity of NAD(P)H, that can then be used to form H_2O_2.

In addition to central metabolic pathways, other biochemical pathways for generating energy are also up-regulated during the defence response. NADP-MDH enzyme is up-regulated in response to pathogen infection and has been speculated to be involved in energy for plant defence through the generation of pyruvate and NADPH (Widjaja et al. 2009). Similarly, OGDH up-regulation could speed up the energy metabolism and provide more energy for stress resistance. In addition, oxoglutarate dehydrogenase complex (OGDHC) is targeted by reactive oxygen species (ROS) and thus able to generate ROS, thereby making it significant in bioenergetics (Tretter and Adam-Vizi 2005).

NAD + transporter 1 (GenBank Accn no: JG463723) found to import NAD + into the mitochondria, play vital roles in signalling via the generation and scavenging of ROS and in systems controlling adaptation to environmental stresses. Further, the transported NAD and NADP nucleotides could be the fundamental common mediators of nearly all major biological activities, including mitochondrial function, energy metabolism, calcium homeostasis, antioxidation/generation of oxidative stress, gene expression, immunological functions, aging, and cell death (Ying 2008).

The ESTs encoding photosystem II 5 kDa protein (GenBank Accn no: JG463703) and cyclin dependent kinase (CDK) E (GenBank Accn no: JG463731) are found to be involved during different plant-pathogen interactions and key regulator of cell cycle, respectively.

Several of the other forward SSH sequences are those involved in the general metabolism and photosynthesis (Table S1) while the reverse SSH did not yield any of the functional gene sequences except for an acyl-coA dehydrogenase involved in lipid catabolism. The construction of SSH library did not provide any fruitful insight into the resistant mechanism of the tea cultivar against blister blight pathogen except for the identification of the above discussed genes found to be up-regulated during infection.

Expression analysis of PR protein encoding genes

Induction of *CsCHIT* transcript expression was found to be higher in the blister infected leaves of resistant cultivar, SA6 than susceptible cultivar, TES34 (Fig. 3b, c). The antifungal potential of chitinase in plant-pathogen interactions are well documented (Singh et al. 2015) and accordingly our findings supported the role of tea chitinase in defence against the fungal pathogen. In resistant cultivar (SA6), the expression level of *CsCHIT* was high and reached to maximum level of 13.81 fold in the fourth stage of infection whereas the susceptible cultivar (TES34) showed very low constitutive expression of *CsCHIT* at each stage of disease development (Fig. 3b, c).

The mRNA level of *CsGLUC* did not increase upon pathogen infection in SA6 as rapidly as in TES34 where the level increased in the initial infection stage and then decreased up to the third stage and again increased (Fig. 3b, c). Such low levels of the *CsGLUC* mRNA could lead to the prediction that the half-life of tea *CsGLUC* mRNA could be relatively short for their increased accumulation to be detected by the RT-PCR in SA6. This relatively short half-life prediction out of this study is supported by the response of *GLUC* of bean leaves (Vogeli-Lange et al. 1988) and peach (Thimmapuram et al. 2001). Further, this prediction is supported by the observed enzymatic analysis data, where the levels of glucanase increase with increasing incidence of disease in the resistant cultivar SA6.

The mRNA levels of *CsGLUC* induced as a result of pathogen invasion is translated immediately into the respective protein in the resistant cultivar (SA6) and accumulates in the leaf. In contrast, the cultivar TES34 showed detection of *CsGLUC* mRNA during the infection stages that was not successfully translated as in the case of the resistant cultivar, SA 6. This is reflected in the protein levels where there is no much accumulation of these proteins during infection in TES34. This could be due to the interference of the pathogen in the translation machinery of the susceptible cultivar that prevents the accumulation of the protein. Further investigations in this area are needed to support this prediction derived out of the results obtained from this study.

Transcripts encoding pathogenesis-related (PR) proteins, such as chitinases, glucanases have been or over-expressed in crop plants to defend against fungal pathogens directly (via degradation of the fungal structural barrier) or indirectly (via elicitor activity of fungal cell wall degradation products) (Balasubramanian et al. 2012).

Similarly, the accumulation of chitinase and β 1,3-glucanase in tea leaves during diseased conditions increased rapidly in all the cultivars irrespective of that being resistant or susceptible, though higher increase was seen in the resistant cultivars. The similar trend shown by these enzymes during different stages of infection in SA6 and TES34 which is in accordance to the higher production of these enzymes as documented in various plant-pathogen interactions (Recupero et al. 1997). Increased PR related enzyme levels in the tissues of the infected bush indicate effective signalling from other parts of the plant upon pathogen infection. These reports are in corroboration with that given for the tea cultivars in response to *Pestalotiopsis theae* infection (Senthilkumar et al. 2012).

In SA6, the *CsPAL* mRNA transcripts were observed to decrease upon infection in the order of first < second < third stage and then a mild increase in the fourth infection stage, still lesser than that in the healthy leaves. The transcript levels in TES34 showed a dramatic increase in the first stage of infection and then gradually decreased till the fourth stage but was always higher than healthy leaf. These observations are in agreement with the PAL enzyme level results of both the cultivars (Fig. 3b, c). Further, decreased PAL activity was observed during the increased activities of POX, APX, PPO, chitinase, and glucanase in tea leaves infested with tea mosquito bug (Chakraborty and Chakraborty 2005), and in wheat (Rybka et al. 1998).

Our observations with regard to PAL are in contrary to the studies on tea plant during diseases like grey blight (Senthilkumar et al. 2012), bird's eye spot (Gnanamangai et al. 2011) and sclerotial blight disease (Indramani and Bishwanath 2010) and in different plant-pathogen interactions where there was increase in both the transcript levels and protein in the resistant hosts and decrease in the susceptible hosts (Senthilkumar et al. 2012).

From these observations, the resistance against blister blight disease shown by the cultivar SA6 and other cultivars is not entirely PAL reliant but is dependent on the activation of other PR proteins like chitinase and glucanase. The increased expression of *PAL* in TES34 with decreased chitinase and glucanase activity indicate that the induction of *PAL* alone is not sufficient to resist the pathogen entry. Further work would be required to ascertain our assumption out of this observation.

Role of anti-oxidative enzymes during blister blight infection

The activity of POX and APX enzymes increased during disease incidence in all the cultivars, however the increase being more pronounced in the susceptible cultivars than the resistant ones. The role of the enzymes could more easily be predicted by looking at the status of the enzyme activity during different stages of infection in SA6 and TES34. There is increased activity in TES34 during all stages but the resistant cultivar behaves in a way to minimise the accumulation and thus activity of POX and APX.

All these findings suggest that increased POX and/or APX do not increase the resistance/tolerance of the cultivar against the fungal pathogen. The gray blight disease infection in tea is also shows a similar trend in POX and APX activity (Senthilkumar et al. 2012).

The levels of POX during subsequent stages of infection in resistant and susceptible cultivar suggest the possible involvement of reactive oxygen species (ROS) in the induction of resistance. ROS are known to act as potent microbial agents where micromolar concentrations of H_2O_2 inhibited spore germination of a number of fungal pathogens in vitro. An accumulated level of H_2O_2 is known to activate the MAPK cascade for activation of various defence pathways (Zwerger and Hirt 2001).

Defense pathways are activated in the distant uninfected cells as a consequence of ROS produced upon pathogen attack. The down-regulation of APX has been found associated with the expression of resistance, rather than susceptibility. Elevated levels of cellular H_2O_2 is observed in transgenic tobacco which has antisense gene for APX, suggesting supressive role of APX, and thus an enhanced cell death occurs in antisense tobacco on pathogen attack (Mittler et al. 1999).

The level of SOD in infected leaves of both resistant and susceptible cultivars is seen to decrease in contrary to most of the reports. This was however in accordance with the decreased SOD levels in both resistant and susceptible cultivars during the gray blight disease induction in tea (Senthilkumar et al. 2012).

Effect of pathogen infection on flavonoid pathway specific genes

Flavonoid compounds are involved in a wide range of functions in plants, including protection against abiotic and biotic stresses. They are one among the several factors contributing to plant resistance. Many of the flavonoid compounds can function as passive or inducible barriers against herbivores or microbial pathogens, and flavonoid content or composition can increase or change in response to pathogen (Miranda et al. 2007; Carlsen et al. 2008). Genes encoding almost the entire pathway from phenylalanine and malonyl-CoA to proanthocyanidin synthesis were induced in poplar as a response to *M. medusae* infection (Miranda et al. 2007). Similarly, up-regulation of genes involved in the phenylpropanoid pathway, mainly those leading to isoflavone and isoflavonoid compounds was shown in *M. truncatula* (Foster-Hartnett et al. 2007). However, the species also decides the role of flavonoids in plant defense (Carlsen et al. 2008).

The expression of genes involved in the pathway is highly influenced by the *E. vexans* infection in both SA6 and TES34 cultivars (Fig. S1). In the present study, *Cs4CL*, the gene involved in the early steps of flavonoid synthesis demonstrated to be highly induced during disease induction in cultivar SA6 in contrast to TES34 (Fig. 6b). This could also be seen as an induction of resistance in bean (Cramer et al. 1985).

Further, 4CL plays a important role in plant defense because of its position joining the phenylpropanoid pathway with lignin and flavonoid branch pathways. The expression of *CsCHI*, *CsF3H* and *CsANS* were induced in TES34 during various stages of infection whereas it showed induction to a lesser extent and was even suppressed in SA6 (Fig. 6c, e, g). The mere activation of the core gene of the flavonoid pathway *CsF3H* as seen in TES34, cannot be considered to confer resistance as the induction of the downstream steps of the pathway involving the genes *DFR*, *ANS* and *ANR* are required for the increased accumulation of proanthocyanidins which are known to have antimicrobial properties and involved in plant resistance. Thus the co-ordinated induction of the three afore-said genes along with *F3H* may confer the cultivar resistant to the fungal pathogen.

Polymerisation of catechins to oligomeric proanthocyanidins and 2,3-*cis* isomerisation occurs in tea during *Exobasidium vexans* infection (Punyasiri et al. 2004). Thus oligomeric proanthocyanidins might function as important growth-limiting factor towards endophytic and pathogenic fungi.

Further studies relating to the changes in protein accumulation pattern of the flavonoid pathway genes in the resistant SA6 and susceptible TES34 cultivar would be required to address the down- and up-regulation of these genes during infection, respectively. This preliminary investigation into the expression pattern of the flavonoid pathway genes during different infection stages shows that the fungal infection influences all of the eight genes under study. Various stages of disease development had a varied impact on the expression of these genes suggesting the possible role of lignins, flavonoids, anthocyanins and other compounds synthesized via the pathway involving these genes in acting as anti-microbial/fungal agents.

Conclusion

Based on our studies, it is identified that the resistant cultivar responds to the infection by increased accumulation of CsCHIT which on purification documented strong antifungal activities against the spores of *E. vexans* and *P. thea* (unpublished data) and ROS which in turn aids in the activation of varied defence pathways. SSH library results indicate the up-regulation of metabolic pathway genes to meet up the increased energy demand of the plant to fight the infection and genes to burst out the stress signals during the infection. Further our observations show that engineering resistance of tea plants with synergistic expression of *CsCHIT* and *CsGLUC* will be a valid tool towards raising resistant cultivars. Various stages of pathogen development had a varied impact on the expression of flavonoid pathway genes suggesting the possible role of lignins, flavonoids, anthocyanins and other compounds synthesized via the pathway in acting as anti-microbial/fungal agents. Put together, this study has generated data that unveils the changes taking place in resistant and susceptible cultivars that would be directly involved with the resistance/susceptibility of host towards the *Exobasidum vexans* pathogen. The findings of this study have identified promising tools to modulate the resistance of tea cultivars towards *E. vexans* through biotechnological approach.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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