

Characterization of genes expressed in *Casuarina equisetifolia* in response to elicitation by cell wall components of *Trichosporium vesiculosum*

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Abstract

Casuarina equisetifolia has the widest distribution of all *Casuarina* species and is a nitrogen-fixing tree planted in tropical/subtropical littoral zones of Asia, the Pacific and Africa for coastal reclamation, charcoal making, pulp and timber. *Trichosporium vesiculosum*, the causal agent of blister bark disease, is a serious pathogen of *C. equisetifolia*. The present study was undertaken to understand the molecular mechanisms involved during pathogen cell wall elicitation in this hardy tree species. Transcript profiling during elicitation induced by cell wall components of *T. vesiculosum* revealed expression of resistance genes; cytochrome oxidase; trans membrane proteins; genes involved in programmed cell death like 26S proteasome and ubiquitin activating enzyme; early nodulin gene, wound inducible metallocarboxy peptidase inhibitor, glucanase, metal binding protein and signal recognition particle. The fold expression of selected transcripts including glucanase, 26 S proteasome, signal recognition particle, cytochrome oxidase and the metal binding protein using RT-qPCR revealed 12-59 fold increase in expression after 48 hours of elicitor treatment. The expression of these transcripts during abiotic stresses like heat, mechanical wounding, salt (NaCl) and drought (PEG) was also analyzed. Glucanase was up-regulated significantly during wounding and heat stress while proteasome was up-regulated 1-4 fold during NaCl, PEG and wounding stress. The novel transcript CeHMA was up-regulated under all the stress conditions.

This is the first report on molecular defense in *C. equisetifolia* and has provided a pool of candidate genes for detailed molecular dissection to further broaden the knowledge on the response of woody perennials during pathogen cell wall elicitation.

Key words: Abiotic stress; elicitor treatment; transcript profiling; tree defense.

Introduction

The genus *Casuarina* is a member of the Casuarinaceae family which is phylogenetically distinct, with no close relatives and hence assigned to an order of its own, the Casuarinales (BEADLE, 1981). *C. equisetifolia*, a major species under this family is a nitrogen-fixing multipurpose tree used for landscaping, timber, medicine, dye, pulp, tannin, wood fuel production, soil stabilization, reforestation of marginal ecosystems, amenity planting and land fertilization (PAN et al., 1996).

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One of the major disease reported in *C. equisetifolia* is the blister bark or wilt disease caused by the hyphomycete fungus *Trichosporium vesiculosum* (synonym *Subramanianospora vesiculosa*) (TITZE and VAN DER PENNE, 1983; MOHANAN and SHARMA, 1993). The disease is characterized by foliage yellowing, rapid wilting followed by desiccation, browning and dieback of trees either singly or in groups. The disease incidence ranges from 40 percent in India in pockets (SHARMA, 1995) to 90 percent in Binh Thuan provenance in Vietnam (SHARMA, 1994) and has been reported from India, China, Vietnam, Thailand, Mauritius and Sri Lanka (MOHANAN and SHARMA, 1993; PONGPANICH et al., 1996; CHONGLU, 2000). It is reported mostly from regions where the species has been introduced. Until recently, *T. vesiculosum* was not categorized based on the mechanism of infection, however the mode of infection indicates that the pathogen behaves as an obligate biotroph with limited host range (as the pathogen is reported to infect only *Casuarina* sp.) and requires living cells to complete its life cycle (VELUTHAKKAL and GHOSH DASGUPTA, 2012).

Research on management of this disease has been limited to assessment of provenances for tolerant phenotypes, etiology and epidemiological studies (NARAYANAN et al., 1996; KARTHIKEYAN et al., 2011).

Plant – microbe interactions are among the most dynamic and complex biological phenomena as they involve host contact with multiple microbes including pathogens, myriad of symptoms and interaction with unlimited number of pathogen molecules at every perceivable cellular levels (SCHNEIDER and COLLMER, 2010). Fungal plant pathogens are classified into two major groups (biotrophs and necrotrophs) based on their mode of infection. Hemibiotrophy has been defined by PERFECT and GREEN (2001) including pathogens with initial period of biotrophy followed by necrotrophic phase. Molecular signaling to different classes of pathogens has been widely studied and involves three major pathways, the salicylic acid (SA) dependent pathway predominant in biotrophs and the jasmonic acid (JA) and ethylene (ET) dependent pathways operative during necrotrophy and herbivory (THOMMA et al., 2001; KESSLER and BALDWIN, 2002; GLAZEBROOK, 2005). However, extensive cross-talk between the signaling pathways involving antagonistic and synergistic interactions has been reported (NORMAN-SETTERBLAD et al., 2000; DE VOS et al., 2005). The defense mechanisms triggered by both pathways are usually overlapping but the outcome (resistance or susceptibility) depends on the nutritional preference of the invading pathogen (FRIESEN et al., 2008; WOLPERT et al., 2002).

The SA-dependent pathways operative during biotrophic interactions is widely documented during host-pathogen interaction. The initial response to pathogen invasion occur rapidly resulting in local gene activation causing hypersensitive reaction (HR) and cell death (SOMSSICH and HAHNBROCK, 1998). Subsequently, signal transduction cascades through altered cytoplasmic Ca^{2+} levels, reactive oxygen species, nitric oxide and post-translationally regulated mitogen-activated protein kinase results in transcriptional activation of genes involved in systemic acquired resistance (SAR) (ZHANG and KLESSIG, 2001; MUR et al., 2006; FRAIRE-VELÁZQUEZ et al., 2011). SAR is characterized by the expression of pathogenesis-related (PR) proteins and induction of the phenyl propanoid pathway. This results in an extensive reprogramming from primary to secondary pathways with down-regulation of non-essential cellular activities (SOMSSICH and HAHNBROCK, 1998).

Recent studies have revealed that the physiological and biochemical responses of trees in response to invading pathogens are homologous to the herbaceous crops, but variations are likely to occur at gene regulation and signaling pathways (VELUTHAKKAL and GHOSH DASGUPTA, 2010). Studies on molecular responses of trees to compatible/incompatible pathogens are reported from *Pinus* spp. (ADOMAS et al., 2007; MORSE et al., 2004; RICHARDSON et al., 2010), *Castanea* spp. (BARAKAT et al., 2009), *Populus trichocarpa* (DUPLESSIS et al., 2009; HACQUARD et al., 2011), Douglas-fir (STURROCK et al., 2007), *Fagus sylvatica* (SCHLINK, 2009; 2010; VALCU et al., 2009), *Eucalyptus* spp. (FURTADO et al., 2007), hybrid poplar (MIRANDA et al., 2007; RINALDI et al., 2007; AZAIEZ et al., 2009) and Norway Spruce (NAGY et al., 2004). However, there has been no systematic study in the family casuarinaceae on the molecular aspects of disease resistance. The only study on the molecular interaction is from *C. glauca*, phylogenetically close taxa to *C. equisetifolia*, during symbiotic association with *Frankia* (HOCHER et al., 2006; FRANCHE et al., 2011). Thus, the molecular event during host-pathogen interaction has not been addressed in this genus. Hence, the present study was taken up to understand the defense response during *C. equisetifolia* when challenged with the cell wall elicitors derived from its pathogen, *T. vesiculosum*.

Materials and Methods

Fungal strain and elicitor preparation

Fungal isolate of *Trichosporium vesiculosum* was obtained from the culture collection of the Division of Plant Protection, Institute of Forest Genetics and Tree Breeding, Coimbatore, India and maintained on potato dextrose agar medium. Hyphal mass was grown in potato dextrose broth for 30 days. The mycelial mat was harvested, rinsed with sterile water several times and re-suspended in sterile water and homogenized. The slurry was filtered and the residue was extracted thrice with water followed by chloroform: methanol (1:1) and finally with acetone. The preparation was air dried and considered as mycelial wall. The extract was suspended (1gm in 10ml) in water and autoclaved twice. The autoclaved

suspension was clarified by centrifugation and used as elicitor. One ml of elicitor was used for further studies.

Plant material and Stress treatments

Single tree cuttings were collected from *Casuarina equisetifolia* subsp. *equisetifolia* (CSIRO seed lot number 19129 from Lakei/sibur Bako, Malaysia) maintained by the Institute of Forest Genetics and Tree Breeding, Coimbatore at Panampally Research Station, Kerala, India. The cuttings were rooted and maintained in the vegetative propagation complex for bioassay studies. One month old rooted cuttings were used for further studies.

Elicitor treatment was given to rooted cuttings based on the protocol described by MOHAN and MANOKARAN (2001) where in one month old rooted cuttings were subjected to the cell wall elicitor treatment till 48 hours and observations were made for appearance of wilting symptom in the needles, characteristic of blister bark disease. Needle tissues from each replication were harvested after 24 h and 48 h post treatment. A water control was also maintained for analysis.

Besides fungal elicitor, the temporal expression of selected transcripts were evaluated in response to vari-

Table S1. – Primers used for transcript profiling.

Primer ID	Primer Sequence
P1:	5'ATTAACCCCTCACTAAATGCTGGGA3'
P2:	5'ATTAACCCCTCACTAAATCGGTCATAG3'
P3:	5'ATTAACCCCTCACTAAATGCTGGTGG3'
P4:	5'ATTAACCCCTCACTAAATGCTGGTAG3'
P5:	5'ATTAACCCCTCACTAAAGATCTGACTG3'
P6:	5'ATTAACCCCTCACTAAATGCTGGGTG3'
P7:	5'ATTAACCCCTCACTAAATGCTGATG3'
P8:	5'ATTAACCCCTCACTAAATGGAGCTGG3'
P9:	5'ATTAACCCCTCACTAAATGTGGCAGG3'
T1	5'-CATTATGCTGAGTGATATCTTTTTTTTAA-3'
T2	5'-CATTATGCTGAGTGATATCTTTTTTTTAC-3'
T3	5'-CATTATGCTGAGTGATATCTTTTTTTTAG-3'
T4	5'-CATTATGCTGAGTGATATCTTTTTTTTCA-3'
T5	5'-CATTATGCTGAGTGATATCTTTTTTTTCC-3'
T6	5'-CATTATGCTGAGTGATATCTTTTTTTTCG-3'
T7	5'-CATTATGCTGAGTGATATCTTTTTTTTGA-3'
T8	5'-CATTATGCTGAGTGATATCTTTTTTTTGC-3'
T9	5'-CATTATGCTGAGTGATATCTTTTTTTTGG-3'

ous other environmental stimuli including mechanical wounding, salinity stress, osmotic stress and heat stress for 24 h. Needles were cut into small pieces with sterile razor blade and were kept in water for 24 h for inducing transcript expression during mechanical wounding while salinity stress was imposed by transferring the rooted cuttings to Hoagland solution containing 1.5 M NaCl. Osmotic stress was given by incubating the cuttings in solution of 40% (w/v) polyethylene glycol (PEG) 6000, while heat stress was given by incubating the rooted cuttings at 50°C for 24 h. The control rooted cuttings were maintained in Hoagland solution at room temperature and sampled at the same time as the stressed plants. The needles were harvested at indicated time intervals, frozen directly in liquid nitrogen and used for RNA isolation.

Transcript profiling during pathogen elicitation

Total RNA was isolated from the water treated (control) and elicitor treated needle tissues using an in-house protocol (patent pending). Subsequently, total RNA was treated with RNase free DNase I (Fermentas, USA) according to the manufacturer's protocol and first strand cDNA was synthesized from both control and

elicitor treated RNA using cDNA synthesis kit (Fermentas, USA). The cDNA pools were amplified using nine arbitrary primers and nine Oligo d (T) primers provided in the Delta Differential display kit (Clontech Laboratories Inc., Palo Alto, CA) individually and in pair – wise combination (*Table S1*). Further, gene specific primers (both degenerate and non degenerate) were designed and synthesized for chitinases, thaumatin-like proteins, polygalacturonase inhibiting protein (PGIP), defensins and β -1,3-glucanases. They were also amplified in the cDNA pools and amplicons were resolved on a 4% denaturing PAGE and stained with silver nitrate (BASSAM et al., 1991). A 50bp DNA ladder (Fermentas, Hanover, MD, USA) was used to determine the size of the amplicons. Differentially expressed fragments were re-amplified and cloned in pDrive vector (Qiagen, Hilden, Germany) following the manufacturer's procedure and sequenced using automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sequence assembly and analysis

The sequences were edited and their similarity to existing sequences was analyzed by BLASTn and

Table 1. – Primers synthesized for RT-qPCR of selected transcript.

Genes Targeted	Primer Code	Primer Sequence
26S Proteasome (<i>CeProt</i>)	QPROTFP	GAAAGAGACATCCGGTTTGAACCTT
	QPROTRP	TGAACTTCTGGTATCCCTTGATGA
Novel Transcript with metal binding domain (<i>CeHMA</i>)	QHMAFP	AGAAAGCTGCACGTAATCTGGAAG
	QHMARP	CATCTTCTCTGCAGGCTTCTTGTC
Signal Recognition Particle (<i>CeSRP</i>)	QSRFPF	TGAGATGAGTGCCTTATCACGAAA
	QSRPRP	TCGAAGTCGCGTTTACTTATCTCC
Nodulin (<i>CeNod</i>)	QNODFP	CCTGCGTTGTCAATTTGTTCACG
	QNODRP	GCCCTTAATGGCAGTGGCAATACT
Cytochrome Oxidase (<i>CeCox</i>)	QCOXFP	GTACTGGCAGGGGCTATTACAATG
	QCOXRP	ATCCAGGCAGAAATGGGAATATACA
Glucanase (<i>CeGlu</i>)	QGLUFP	ATTCATCTGCACAGTTCCTCTTCC
	QGLURP	ACTTGAATGAGCCTTTTGATGGAG
Resistant gene (<i>CeR</i>)	QCERFP	CCATAGCACACCATCACCAC
	QCERRP	CACTGACTACTCCAGCGTCCA
Ubiquitin (Internal reference) (<i>CeUbi</i>)	UBIFP	GAAAAACCATAACCTTGGAAGTTG
	UBIRP	GATTCCTTTTGGATGTTGTAATCC

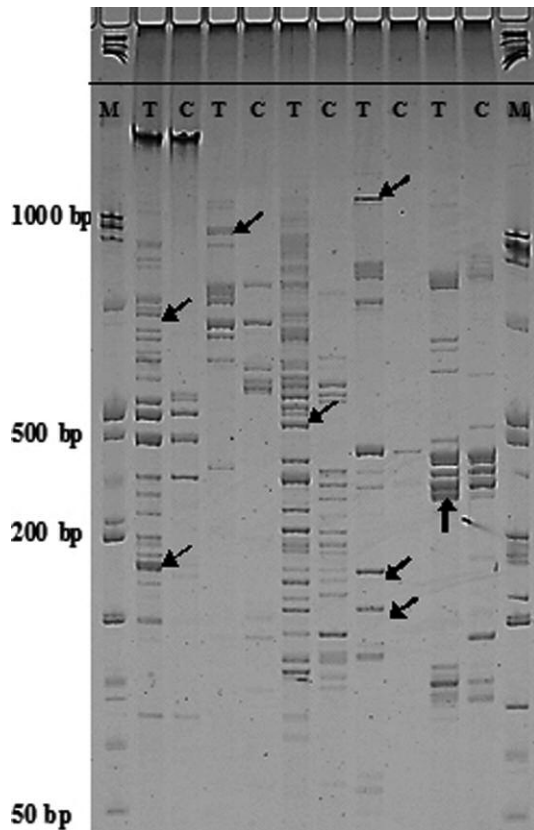


Figure 1. – Transcript profiles of control and elicitor treated samples using arbitrary/anchored primer pairs. Denaturing PAGE (4%) showing differential amplification of transcripts using P5 and T₁₋₅ primers in control and elicitor treated cDNA pools. The gels were visualized by silver staining.

M: 50 bd Ladder (Fermentas).

C: Amplification in cDNA pool derived from needle tissues harvested from cuttings maintained in sterile water.

T: Amplification in cDNA pool derived from needle tissues harvested from pathogen cell wall elicitor treated cuttings.

Arrows indicate differentially amplified amplicons.

BLASTx (www.ncbi.nlm.nih.gov/blast/Blast.cgi) against the GenBank non-redundant database for nucleotide and translated sequences.

RT-qPCR analysis of gene transcripts during stress conditions

Total RNA was isolated from 1g of control and treated needles after 24 h and 48 h as described earlier. The quality of RNA was checked on a 1% agarose gel and concentration was determined spectrophotometrically. mRNA was isolated from ten microgram of both control and treated total RNA and first strand cDNA was synthesized using first strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) following the manufacturers' protocol. The cDNA pools were quantified using Picodrop spectrophotometer (Picodrop Limited, Saffron Walden, UK) and 100ng of each cDNA sample was used for RT-qPCR amplification.

Primer pairs for RT- qPCR assays were designed from the transcript sequence using PRIMER3 program (ROZEN and SKALETSKY, 2000). Primers targeting ubiqui-

tin was used as internal reference for RT-qPCR analysis based on earlier reports from its closely related taxa, *C. glauca* (LAPLAZE et al., 2000; HOCHER et al., 2006; SANTOS et al., 2010) (Table 1). All primer pairs were amplified prior to RT-qPCR and validated in 1% agarose gel.

RT-qPCR was carried out in Applied Biosystems ABI 7700 sequence detection system using the SYBR green chemistry. The PCR efficiency was optimized for annealing temperature, cDNA concentration and primer concentration. Melting curve analyses were performed after the 40 cycles of RT-qPCR program by a constant increase in temperature between 60 and 95°C. RT-qPCR was performed in a final volume of 10 µL containing 5 µL of 2X SYBR Green Jumpstart Taq Ready Mix (Sigma Aldrich, USA), 500 nM each of forward and reverse primers and 100 ng of cDNA template. After an initial activation step of the DNA polymerase at 95°C for 10 min, samples were subjected to 40 cycles of amplification (denature at 95°C for 15 s, annealing and extension together at 58°C for 1 min). Quantification of the target gene expression was done with comparative CT method (LIVAK and SCHMITTGEN, 2001). The relative expression level of the gene of interest was computed with respect to ubiquitin to account for any variance in the amount of input cDNA. Average CT values from triplicate PCRs were normalized to average CT values for ubiquitin from the same cDNA preparations.

Statistical analysis

The fold expression of transcripts under different stress conditions were statistically analyzed using the T-Test using SPSS software (version 20.0) and difference between treatments were considered statistically significant when $P < 0.05$.

Results

Transcript profiling during pathogen cell wall elicitation

Expression profiling of the elicitor treated cDNA and untreated cDNA was conducted using eighty one arbitrary/anchored primer pairs and nine gene specific primer pairs (Figure 1). Most of the gene specific primers did not amplify except for chitinase and glucanase specific primer pairs. Approximately, 125 amplicons which differentially expressed in the elicitor treated sample were re-amplified, cloned and sequenced. The sequences were trimmed and those below 100bp were not considered for further analysis. Fifty two sequences were submitted to the GenBank (Accession number GR228669 to GR228718; GR312926 and GR312925). Sequences which showed significant similarity to known genes included disease resistance (*CeR*) genes involved in HR; genes involved in aerobic metabolism like cytochrome oxidase (*CeCox*); trans membrane proteins; genes involved in programmed cell death like 26S proteasome (*CeProt*) and ubiquitin activating enzyme; symbiosis related early nodulin gene (*CeNod*), transcripts involved in signal transduction like protein kinase; wound inducible metallocarboxy peptidase inhibitor and PR genes like class I chitinase (*CeChi1*) and glucanase (*CeGlu*). Two transcription factor includ-

ing class III homeodomain leucine zipper TF and CCR4-NOT were documented in the study. A novel transcript showing similarity to metal ion binding protein (*CeHMA*) was also expressed during elicitation. The study also revealed the expression of transcript showing significant similarity to signal recognition particle (*CeSRP*), a ribonucleoprotein involved in protein targeting (Table 2).

RT-qPCR analysis of gene transcripts during pathogen cell wall elicitation

The expression pattern of selected transcripts was analyzed using RT-qPCR and the transcripts included *CeGlu*, *CeCox*, *CeProt*, *CeSRP* and *CeHMA*. PCR efficiency was optimized and the specificity of the amplicons was confirmed by the presence of a single peak (data not shown). Electrophoretic separation of the

Table 2. – Identification of pathogen elicitor-responsive transcripts from *Casuarina equisetifolia*.

Sl No.	Clone ID	GenBank Accession No.	Primer Pairs	Amplicon Size (in bp)	Best database match (Species and E-value)	Annotation
1	CEDD1	GR228669	5'-CATTATGCTGAGTGATATCTTTTTCG-3' 5'-ATTAACCCCTCACTAAATGCTGGTG-3'	414	NA	Hypothetical protein
2	CEDD10	GR228670	5'-ATTAACCCCTCACTAAATGCTGGTG-3' 5'-CATTATGCTGAGTGATATCTTTTTCG-3'	233	NA	No significant hit
3	CEDD11	GR228671	5'-ATTAACCCCTCACTAAATGCTGGTG-3' 5'-CATTATGCTGAGTGATATCTTTTTCG-3'	313	<i>Sisymbrium officinale</i> (2e-05)	Maturase K
4	CEDD12	GR228672	5'-ATTAACCCCTCACTAAAGATCTGACG-3' 5'-CATTATGCTGAGTGATATCTTTTTCG-3'	275	NA	Hypothetical protein
5	CEDD2	GR228673	5'-CATTATGCTGAGTGATATCTTTTTCG-3' 5'-ATTAACCCCTCACTAAATGCTGGTG-3'	220	<i>Arabidopsis lyrata subsp. lyrata</i> (2e-05)	kinase family protein
6	CEDD3	GR228674	5'-CATTATGCTGAGTGATATCTTTTTCG-3' 5'-ATTAACCCCTCACTAAATGCTGGTG-3'	205	NA	No significant hit
7	CEDD4	GR228675	5'-CATTATGCTGAGTGATATCTTTTTCG-3' 5'-ATTAACCCCTCACTAAATGCTGGTG-3'	222	<i>Arabidopsis lyrata subsp. lyrata</i> (2e-05)	kinase family protein
8	CEDD5	GR228676	5'-CATTATGCTGAGTGATATCTTTTTCG-3' 5'-ATTAACCCCTCACTAAAGATCTGACG-3'	131	<i>Medicago truncatula</i> (6e-15)	ADP-ribosylation factor GTPase-activating protein
9	CEDD6	GR228677	5'-CATTATGCTGAGTGATATCTTTTTCG-3' 5'-ATTAACCCCTCACTAAATGCTGGTG-3'	205	NA	No significant hit
10	CEDD7	GR228678	5'-CATTATGCTGAGTGATATCTTTTTCG-3' 5'-ATTAACCCCTCACTAAATGCTGGTG-3'	466	NA	No significant hit
11	CEDD8	GR228679	5'-ATTAACCCCTCACTAAATGCTGGTG-3'	486	<i>Vitis vinifera</i> (3e-11)	Putative disease resistance protein RGA4-like
12	CEDD9	GR228680	5'-ATTAACCCCTCACTAAATGCTGGTG-3'	187	NA	Predicted protein
13	CEDDP3a	GR228681	5'-ATTAACCCCTCACTAAATGCTGGTG-3'	505	<i>Micromonas</i> sp. (2e-08)	Ribonucleoside-diphosphate reductase
14	CEDDP3b	GR228682	5'-ATTAACCCCTCACTAAATGCTGGTG-3'	505	<i>Glycine max</i> (2e-07)	Ribonucleoside-diphosphate reductase large subunit
15	CEDDP5a	GR228683	5'-AGTCAGATCTTTAGTGAGGGTTAATA-3'	269	<i>Brachypodium distachyon</i> (5e-05)	Predicted: CCR4-NOT transcription complex subunit 1-like
16	CEDDP5b	GR228684	5'-CAGTCAGATCTTTAGTGAGGGTTAATA-3'	311	NA	No significant hit
17	CEDDP7	GR228685	5'-ATTAACCCCTCACTAAATGCTGGTG-3' 5'-ACAGCATTTAGTGAGGGTTAATA-3'	777	<i>Vitis vinifera</i> (4e-06)	UDP-glycosyltransferase 75D1-like
18	CEDDP7a	GR228686	5'TCCCCAGCATTTAGTGAGGGTTAATA 3'	387	NA	No significant hit
19	CEDDP7b	GR228687	5'-ATTAACCCCTCACTAAATGCTGGTG-3' 5'-CATACAGCATTTAGTGAGGGTTAATA-3'	776	<i>Vitis vinifera</i> (4e-06)	UDP-glycosyltransferase 75D1-like
20	CEGSP 58-2	GR228688	5'-GGGTCGAGAGAAGCCAGGTATT-3' 5'-GCCCTTAATGGCAGTGGCAATACT-3'	215	<i>Vitis vinifera</i> (7e-11)	Early nodulin-93
21	CEGSP1	GR228689	5'AGG AGA AAY RIT CTY ITW GGS YTW GGS 5'CAT CCO RTC SAC RWT IGM RTG GTG-3'	276	<i>Arabidopsis thaliana</i> (6e-06)	Pto kinase interactor, putative
22	CEGSP2	GR228690	5'-AGGTAGGTGGGGCCATGTGA-3' 5'GAC CAT GTC TGG CTT GCC TTC TCA CA3'	342	<i>Zea luxurians</i> (6e-05)	cytochrome c biogenesis
23	CEGSP3	GR228691	5'-AGCAGCACAGTTCACAGTG-3' 5'GAC CAT GTC TGG CTT GCC TTC TCA CA3'	245	NA	Hypothetical protein
24	CEGSP4	GR228692	5'GCI ACI TTT ACI ATA GAI AAT CA3' 5'-ATA IGC CTG IGG ACA CTG ICC-3'	366	NA	Hypothetical protein

Table 2. – Continued.

SI No.	Clone ID	GenBank Accession No.	Primer Pairs	Amplicon Size (in bp)	Best database match (Species and E-value)	Annotation
25	CEGSP5	GR228693	5'-TGAGATGAGTGCCTTATCACGAAA-3' 5'-TCGAAGTCGCGTTTACTTATCTCC-3'	632	<i>Glycine max</i> (7e-87)	signal recognition particle 54 kDa protein 2-like
26	CEGSP58-8	GR228694	5'-GGN GAY GCN GTN YTN GAY GG-3' 5'-ATAIGCCTGIGGACACTG ICC3'	297	NA	Hypothetical protein
27	CEGSP6	GR228695	5'-TGACATTGAAGGAGGGACAA-3' 5'-CGCCAGAAAACCTACCGAACCCAC-3'	274	<i>Ricinus communis</i> (1e-42)	ptm1, putative
28	CEGSP7	GR228696	5'-CCATAGCACACCATCACCAC-3' 5'-CACTGACTACTCCAGCGTCCA-3'	226	<i>Vitis vinifera</i> (3e-18)	multiple C2 and trans-membrane domain-containing protein 2
29	CEGSP8	GR228697	5'-CACTGACTACTCCAGCGTCCA-3' 5'-AGGGTGTAAGACCGAGCCAAAG-3'	232	<i>Vitis vinifera</i> (5e-14)	Early nodulin-93
30	CEGSP9	GR228698	5'-GATCCCGCTGGAAGTGGAAA-3' 5'-GATGAAACACCAGATAGATGAGGAT-3'	516	<i>Abelia chinensis</i> (5e-87)	Cytochrome oxidase subunit 1
31	CEGSPGLU	GR228699	5'-GGCCATCCTGTCTCATCTAC-3' 5'-CTGTCTCTGATATAACGATCTCCAA-3'	123	<i>Solanum tuberosum</i> (2e-06)	Metalloproteinase inhibitor
32	CEGSPRP12	GR228700	5'-GCI ACI TTT ACI ATA GAI AAT CA-3' 5'-CTTCTCGCCCATATTTTAGTAGGT-3'	366	<i>Medicago truncatula</i> (3e-05)	Pentatricopeptide repeat-containing protein
33	CEGSPS1b	GR228701	5'-AGGTATGGAAGTTGGGCGCTAGTGT-3' 5'-CAGGATCCCTCGAGCTTGTTCGGA-3'	439	<i>Oryza sativa</i> (2e-05)	Regulatory protein-like protein
34	CEGSPS3-2	GR228702	5'-GCCCTCTGCTACATAGGAGCACT-3' 5'-CAAAATGTCTCGCAGGTGTGTA-3'	344	<i>Vitis vinifera</i> (6e-05)	Probable disease resistance protein At5g63020-like
35	CEGSPS3a	GR228703	5'-CGGGGAGCACAAAGATAGAT-3' 5'-TGAACCTCTGCTATCCCTTGATGA-3'	454	NA	Hypothetical protein
36	CEGSPS3b	GR228704	5'-GAAAAACCATAACCTTGGAAAGTTG-3' 5'-GATTCTTTTGGATGTTGTAATCC-3'	533	<i>Medicago truncatula</i> (2e-06)	Ubiquitin-activating enzyme E1
37	CEGSPS3d	GR228705	5'-GCCTCCATCCTTTTGACCTC-3' 5'-GTTGGGGAAGAATCCCATAGT-3'	469	<i>Micromonas pusilla</i> (5e-06)	Oxoprolinase family protein
38	CEGSPS5-6	GR228706	5'-YTG NAC CCA NAC GTA RTC RA-3' 5'-CGC GGC CAN CCN SWY TC-3'	270	NA	Hypothetical protein
39	CEGSPS5-7	GR228707	5'-AGAGGTACCGCTGGCTTACAGG-3' 5'-TCCAAACCCCACTTTTCAATAGTGGT	348	<i>Arabidopsis thaliana</i> (2e-05)	cysteine-rich repeat secretory protein 18
40	CERACEARF	GR228708	5'-CATCACACAGTGAACCTGCTT-3' 5'-AAAGGATGATGGAGAGAAGAAGG-3'	392	<i>Ricinus communis</i> (1e-24)	Metal ion binding protein, putative
41	CERACE1	GR228709	5'-CGAAATCCCGCCATCGCTAAGCT-3' 5'-TCGAAGTCGCGTTTACTTATCTCC-3'	472	<i>Medicago truncatula</i> (9e-08)	Eukaryotic translation initiation factor 3 subunit F
42	CERACE10	GR228710	5'-CGAATTCCCGCCATCGCTAAGCT-3' 5'-CATCTTCTCTGCAGGCTTCTTGTC-3'	342	NA	Hypothetical protein
43	CERACE2	GR228711	5'-GGGTGAGAGAGAAGCCAGGTATT-3' 5'-ACAAAGCTCGATACAATTCACCA-3'	566	<i>Oryza sativa</i> (8e-53)	26S protease regulatory subunit 7
44	CERACE3	GR228712	5'-AGAGGTACCGCTGGCTTACAGG-3' 5'-ATA GAC CTG TCT TAA GGG CAG C-3'	392	<i>Casuarina glauca</i> (3e-10)	chitinase class I
45	CERACE4	GR228713	5'-CGCCAGAAAACCTACCGAACCCAC-3' 5'-ATGGCCCTTAATGGCAGTG3'	462	<i>Physcomitrella patens</i> (8e-05)	Class III homeodomain-leucine zipper
46	CERACE5	GR228714	5'-TGCGAGCACAAAGATAGATCG3' 5'-CGCCAGAAAACCTACCGAACCCAC-3'	327	<i>Populus trichocarpa</i> (4e-05)	tr-nbs-1rr resistance protein
47	CERACE6	GR228715	5'-AGCAGCACAGTTCACAGTG-3' 5'-GGGCTTTCAGCGAAGTTG-3'	133	<i>Vigna radiata</i> (2e-05)	RNA polymerase beta' subunit
48	CERACE7	GR228716	5'-GGTCAGAGAGAAGCTGGAGCAG-3' 5'-AGAGGCCCTCCGAGAGCATAATA-3'	418	<i>Glycine max</i> (3e-05)	Biotin carboxyl carrier protein subunit precursor
49	CERACE8	GR228717	5'-CACATGACTACTCCAGCGTCCA-3' 5'-TGAACCTCTGCTATCCCTTGATGA-3'	600	<i>Glycine max</i> (4e-59)	60S ribosomal protein L23A-like
50	CERACE9	GR228718	5'-AGCAGCACAGTTCACAGTG-3' 5'-TTTGACAACCAAGTGGAGCAGTAA-3'	514	NA	Hypothetical protein
51	CEGSPGLU1	GR312925	5'-CGC GGN GTN TGY TAY GG-3' 5'-TTATGCCCTCTTCACAGCTCCTTC-3'	294	<i>Castanea sativa</i> (2e-58)	B-1,3-glucanase
52	CEGSPchi	GR312926	5'-CCCGGGTACGGTGTGTTTACGA-3' 5'-GTGGCCTACATGGTATCCACCAAGA-3'	439	<i>Casuarina glauca</i> (1e-08)	class I chitinase

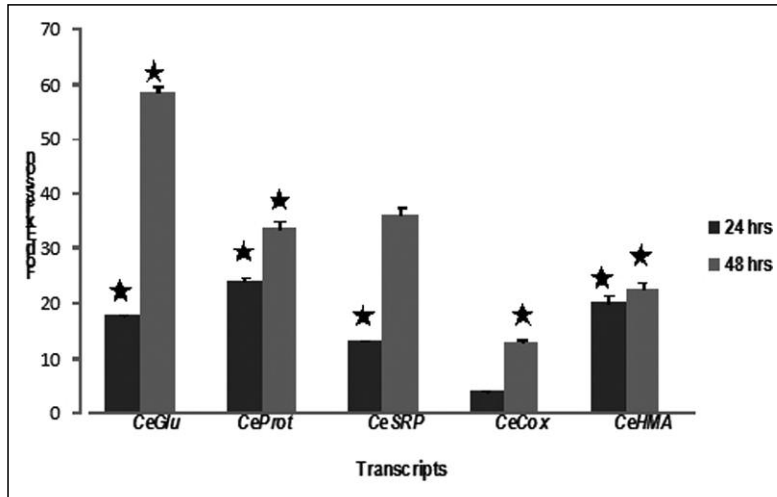


Figure 2. – Reverse transcriptase quantitative real-time PCR (RT-qPCR) analysis of transcript accumulation in *Casuarina* needles in response to cell wall elicitor from *T. vesiculosum*. Transcript levels of five genes (glucanase, 26S proteasome, cytochrome oxidase, signal recognition particle and transcript with metal binding domain) are expressed as fold increase normalized to the transcript level of ubiquitin gene used as internal reference. Results are means of triplicate data from independent replications.

* indicates $P < 0.05$.

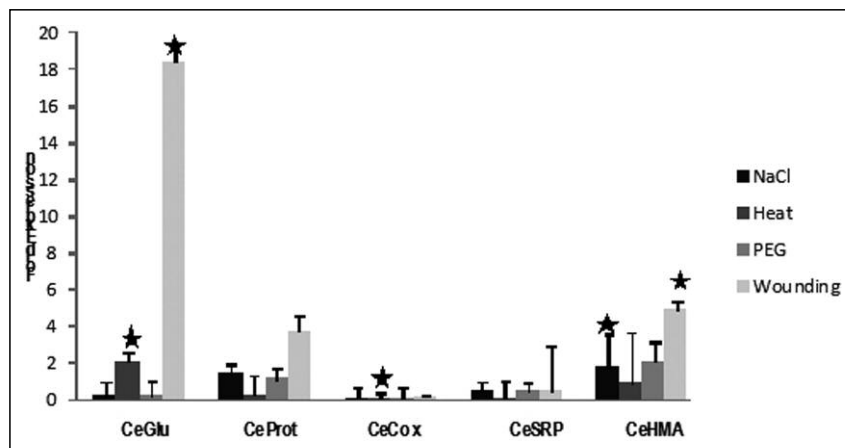


Figure 3. – Reverse transcriptase quantitative real-time PCR (RT-qPCR) analysis of transcript accumulation in *Casuarina* needles in response to abiotic stresses (heat, NaCl, PEG and mechanical wounding). Transcript levels of five genes (glucanase, 26S proteasome, cytochrome oxidase, signal recognition particle and transcript with metal binding domain) are expressed as fold increase normalized to the transcript level of ubiquitin gene used as internal reference. Results are means of triplicate data from independent replications.

* indicates $P < 0.05$.

amplicons produced a single fragment of the expected size, with no visible primer-dimer products. The expression of *CeChi1* under pathogen elicitation and abiotic stress conditions have been reported elsewhere (VELUTHAKKAL and GHOSH DASGUPTA, 2012).

All the five transcripts showed up-regulation subsequent to elicitation, with *CeGlu* showing the maximum expression of 18 fold and 59 fold increase after 24 hrs and 48 hrs respectively. Similar expression pattern was observed for *CeProt* (24 fold and 34 fold), *CeSRP* (13 fold and 36 fold), *CeCox* (4 fold and 13 fold) and *CeHMA* (20 fold and 23 fold) during elicitation (Figure 2). The

expression patterns of most of the transcripts were statistically significant with p value < 0.05 .

RT-qPCR analysis of gene transcripts during abiotic stress conditions

The expression of the five transcripts during abiotic conditions including NaCl, PEG, heat and mechanical wounding was also studied. The glucanase transcript was up-regulated significantly during wounding (18 fold) and moderately during heat stress (2 fold), while the novel transcript *CeHMA* was up-regulated in all the stress conditions. *CeCox* and *CeSRP* showed not signifi-

cant variation in expression under any stress conditions, while the transcript showing similarity to proteasome (*CeProt*) was up-regulated 1-4 fold during NaCl, PEG and wounding stress with no significant change in expression upon heat stress (*Figure 3*).

Discussion

Distinct defense –related responses observed following elicitor treatment in C. equisetifolia

The present study in *C. equisetifolia* identified the expression of defense – related genes involved in HR (R gene, 26S proteasome, and ubiquitin activating enzyme); signatures of SAR (chitinase and glucanase); defense genes like metallocarboxy peptidase inhibitor; protein kinases involved in signal transduction during pathogen defense and aerobic respiration related cytochrome oxidase which are typical response against biotrophic pathogens. Further, the induction of a class I chitinase (*CeChi1*) in *C. equisetifolia* in response to SA and *Trichosporium* elicitation supports the hypothesis that *Trichosporium vesiculosum* is a probable biotrophic pathogen eliciting SA – mediated defense responses (VELUTHAKKAL and GHOSH DASGUPTA, 2012).

Similar studies in tree – pathosystem have also revealed the up-regulation of 26S proteasome subunit, polyubiquitin and metallothionin-like protein in *Pinus sylvestris* infected with the root pathogen *Heterobasidion annosum*. However, the study revealed the down-regulation of protein kinase and resistance gene during the infection (ADOMAS et al., 2007). In chestnut-*Cryphonectria parasitica* interaction, the expression of kinases and CCR4-NOT transcription factor was reported by BARAKAT et al. (2009). In another study conducted on hybrid poplar (*Populus trichocarpa* × *P. deltoides*) infected with the rust fungus *Melampsora medusa*, transcripts including ADP-ribosylation factor 1 (ARF1), glucanase, 40S ribosomal protein, heavy metal transport/detoxification protein, 26S proteasome and Polyubiquitin were up-regulated after nine days post inoculation (MIRANDA et al., 2007). In *Populus nigra* × *P. maximowiczii*, the leucine rich repeat family protein and glucanase were up-regulated during interaction with two biotrophic pathogens *Melampsora larici-populina* and *M. medusae* f. sp. *deltoidae* (AZAIEZ et al., 2009). Interaction of *Fagus sylvatica* – *Phytophthora citricola* resulted in over expression of two distinct defense-related genes viz., glucanase and protein kinase (SCHLINK, 2009; VALCU et al., 2009).

Beta-1,3 glucanase classified under PR-2 play a direct role in fungal defense by hydrolyzing fungal cell walls and an indirect role by generating oligosaccharide elicitors (KLARZYNSKI et al., 2000). The expression profile of *CeGlu* during elicitation was characteristic of PR proteins with distinct up-regulation (18 fold and 59 fold) suggestive of systemic defense reaction. The up-regulation of the transcript during mechanical wounding to a highly significant 18 fold is in agreement with studies in *Ziziphus jujube* (TIAN et al., 2007) and *Castanea sativa* (SCHAFLEITNER and WILHELM, 2001). This suggests that casuarina glucanase can directly act on opportunistic pathogens invading through the wounds and mount a

defense response either by directly hydrolyzing the pathogen cell wall or indirectly by releasing oligosaccharide elicitors which in turn would induce a cascade of defense reactions (CHEONG et al., 2000).

In the recent years the critical role of protein degradation by Ubiquitin-26S proteasome (UPS) pathway has been recognized as a critical regulator in plant defense response (DELAURÉ et al., 2008; DIELEN et al., 2010; PAJEROWSKA-MUKHTAR and DONG, 2009). Detailed studies on the specific role of proteasome and ubiquitin complex during pathogen interaction was reported from crops like barley (AZEVEDO et al., 2002); *Nicotiana benthamiana* (PEART et al., 2002); *Medicago sativa* (WRZACZEK et al., 2007); *Arabidopsis thaliana* (BOYES et al., 1998; AUSTIN et al., 2002) and potato (BHASKAR et al., 2008). In trees, the expression of proteasome during pathogen interaction has been reported but their level of expression was not validated. The present study confirmed the role of protein degradation by proteasome unit in casuarina where in 24 fold and 34 fold increase in expression was observed in 24 hrs and 48 hrs post elicitation. The involvement of the UPS in regulation during abiotic stress conditions is well documented in crop plants and mutations in the regulatory particle subunit of 26S proteasome are reported to reduce the rate of ubiquitin-mediated proteolysis, thus altering the stress response in plants (SMALLE et al., 2003; SMALLE and VIERSTRA, 2004; KUREPA et al., 2008). Several abiotic stresses like heat and oxidative stress inhibit 26S proteasome activity due to increase in substrate load of mis-folded proteins (KUREPA et al., 2009), while the process of ubiquitination is rapidly induced during desiccation, cold and mechanical wounding (CHO et al., 2006; 2008). Contrarily, in the present study 24 hrs of abiotic stress induction revealed up-regulation of proteasome expression with maximum of 4 fold during mechanical wounding. This variance could be due to high abiotic stress tolerance exhibited by casuarina with efficient protein degradation mechanism against stress conditions. This is the first comprehensive report on expression of proteasome during abiotic stress tolerance from tree species.

The expression of metal detoxifying proteins including metallothionins during biotic stress in trees has been reported in *Pinus sylvestris* (ADOMAS et al., 2007) and hybrid poplar (RINALDI et al., 2007). In *Casuarina equisetifolia*, the novel *CeHMA* was up-regulated to the level of 20 and 23 fold post elicitation for 24 hrs and 48 hrs respectively. The role of metallothionin during infection is its involvement in regulating available metal ions, which subsequently affect the intracellular active oxygen species (AOS) produced in stressed plants and also as scavengers of AOS during oxidative burst (CHUBATSU and MENEHINI, 1993; CHOI et al., 1996). Metal detoxifying gene up-regulated during pathogenesis was reported from *Nicotiana glutinosa* during TMV infection (CHOI et al., 1996); *Arabidopsis* (BUTT et al., 1998) and *Abutilon theophrasti* (DAUCH and JABAHI-HARE, 2006). In plants, these genes are also known to confer abiotic stress tolerance by detoxifying AOS. They also expressed during PEG induced osmotic stress (SINGH et al., 2011). Salt and drought stress are reported to induce expression of

metal binding/ metallothionin like proteins in barley (OZTURK et al., 2002), rice (YANG et al., 2009), maize (ANDJELKOVIC and Thompson, 2006), *Mesembryanthemum crystallinum* (KORE-EDA et al., 2004) and *Tamarix hispida* (LI et al., 2009). The *CeHMA* identified in the present study was up-regulated under all the stress treatments confirming the significant role of AOS scavenging during both biotic and abiotic stress conditions.

The CCR4-NOT complex is involved in the control of diverse aspects of transcription and mRNA metabolism, including mRNA deadenylation and its subsequent degradation (DENIS and CHEN, 2003; COLLART et al., 2004). *AtCAF1a* and *AtCAF1b* which encodes CAF1 protein, a subunit of the CCR4-NOT were identified as JA induced in *Arabidopsis* and was also induced by mechanical wounding and pathogen infection (LIANG et al., 2009). The deadenylation of mRNA during stress response has been reported and its role in defense response was studied in tomato (SAROWAR et al., 2007) and Cocoa (LOPES et al., 2010). In *C. equisetifolia*, a transcript (accession number GR228683) with significant similarity to CCR4-NOT was identified from elicitor induced needle tissues. Further, the present study also identified the expression of homeodomain leucine zipper class III transcription factor in elicitor treated tissues (accession number GR228713). Homeobox proteins are ubiquitous in higher organisms and represent master control switches involved in developmental processes and cellular adaptation to changes in the environment. Functional characterization of some members of the homeobox family supports a role as key regulators of hormone signaling (HIMMELBACH et al., 2002), adaptive responses to environmental cues (STEINDLER et al., 1999; ZHU et al., 2004), and pathogen-derived signaling processes (MAYDA et al., 1999). In *Arabidopsis*, a mutant *ocp3* (for overexpressor of cationic peroxidase 3) with impaired homeodomain TF activity showed increased resistance to necrotrophic pathogens (COEGO et al., 2005). A similar report from transgenic tomato lines revealed that the HD Zip TF was involved in cellular protection by limiting PCD in infected plants (MAYDA et al., 1999).

In plants, mitochondria are a major site for AOS production and the generation of AOS by the respiratory chain is a physiological and continuous process (TIWARI et al., 2002). Abiotic stress is also demonstrated to significantly shift the cytochrome pathway to alternate respiratory pathway with the induction of alternate oxidase to scavenge the AOS (LAMBERS et al., 2005; RIBAS-CARBO et al., 2005). Salinity induced CAM transition resulted in increased COX activity in *Mesembryanthemum crystallinum*, which was suggested as an efficient energy conserving strategy in the halophyte (NIEWIADOMSKA et al., 2004). However, the study in casuarina revealed no marked change in the *CeCox* expression in response to any of the abiotic stresses posed on the needle tissues. However, pathogen elicitation up-regulated the transcript expression by 4 fold and 13 fold subsequent to 24 hr and 48 hr post treatment. There are limited reports on the expression pattern of *Cox* during pathogen infection. An earlier study in sweet potato showed an increase in cytochrome oxidase activi-

ty during wounding and infection by *Ceatocystis fimbriata* (ASAHI et al., 1965). Recently, up-regulation of COX from tomato infected with tomato bushy top virus was reported. The differential expression of the transcript was demonstrated where in higher levels of expression was observed in resistant cultivars in comparison to the susceptible cultivar (HAFEZ and MOUSTAFA, 2011). Increased level of expression of *CeCox* in casuarina may be indicative of a basic tolerant nature of the species to pathogenic microbes. The limited disease incidences of the species under natural habitat would suggest a highly proficient innate immune system of the species.

Up-regulation of unique transcripts during defense reaction

Signal recognition particle (SRP) is ubiquitous and abundant small cytoplasmic ribonucleoprotein particle (RNP) involved in targeting the translation of specific pre-secretory proteins to the endoplasmic reticulum. They play a critical role in the sorting of nascent secretory and membrane proteins. In *E. coli* they are reported to maintain protein homeostasis and general fitness of the cell (WICKSTRÖM et al., 2011). SRPs are also associated with degradation of mis-targeted cytoplasmic membrane proteins. SRP mediated degradation of secretory proteins like amylase and defense-related proteins like endochitinase and proteases were demonstrated in barley (BRODL and HO, 1991) and heat shock inhibited the release of SRP from ER in aleurone layers of barley (CHU et al., 1997). Direct evidence on role of SRP during pathogen defense is not reported to our knowledge. However, the present work revealed a distinct up-regulation 13 and 36 fold post 24 hrs and 48 hrs elicitation. Abiotic stresses did not show any significant change in expression. The probable up-regulation of the SRPs during elicitation could be associated with degradation of secretory proteins during localized apoptosis associated with HR.

In the present study, the molecular response of *C. equisetifolia* to cell wall elicitors derived from *Trichosporium* has provided an insight into the molecular mechanisms involved in host immunity in this tropical tree species. The induction of transcripts involved in HR, SAR and signal transduction highlights that the basic response to pathogen infection in trees could be similar to their annual counterparts. However, the significantly high level of induction of transcripts like proteasome, glucanase, cytochrome oxidase and metal binding proteins support a highly plastic immune system evolved to reduce the tissue damage by delimiting the pathogen movement. Further, the expression of SRP during pathogen elicitation adds a new realm to host-pathogen interaction, indicating the probable role of protein targeting and their subsequent degradation during immune responses.

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