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Isolation and characterization of pathogen defence-related class I chitinase from the actinorhizal tree *Casuarina equisetifolia*

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Summary

Casuarina equisetifolia has the widest distribution of all *Casuarina* species and is a nitrogen-fixing tree of considerable social, economic and environmental importance. *Trichosporium vesiculosum*, a causal agent of blister bark disease, is a serious pathogen of *C. equisetifolia*. In this study, a cDNA clone encoding class I chitinase (*CeChi1*) belonging to PR-3 family was cloned and characterized from the needle tissues of *C. equisetifolia* challenged with the toxic exudate of the fungal pathogen *T. vesiculosum*. The *CeChi1* open reading frame comprised 966 nucleotides that encoded 321 amino acid residues with the molecular mass of mature protein being approximately 34 kDa. Analysis of the predicted amino acid sequence revealed the similarity of *CeChi1* protein to class I chitinase from other plant species containing a hydrophobic signal peptide domain and hinge domain. The sequence also harboured a cysteine-rich chitin-binding domain and lysozyme-like domain. A hydrophobic C-terminal domain similar to the vacuole targeting sequences of class I chitinases isolated from other plants was also detected. The genomic sequence of *CeChi1* indicated that the coding region contained three exons and two introns. *In silico* analysis of the untranslated regions revealed the presence of several *cis*-acting regulatory elements associated with hormonal regulation and stress responses. Quantitative real-time PCR analyses at different time points showed upregulation of the transcript during pathogen elicitation and salicylic acid signalling. However, no significant expression of *CeChi1* was observed during other abiotic stress condition including wounding, water deficit, salt and heat stress revealing the specific expression of the gene during pathogenesis. This is the first report on isolation of a gene from *C. equisetifolia*, and the detailed functional analyses of *CeChi1* will help in understanding its specific role in defence against pathogens in this tropical tree species.

1 Introduction

Plants have evolved intricate and orchestrated defence mechanisms and strategies that are either preformed or induced during stress to protect themselves from potential invaders present in their milieu. The inducible defence responses include cell wall reinforcement, lignification (Ride 1975), hypersensitive cell death (Levine et al. 1994), accumulation of anti-microbial secondary metabolites such as phytoalexins, (Kuc and Rush 1985), induction of oxidative burst (Baker and Orlandi 1995), cross-linking of wall glycoprotein (Bradley et al. 1992) and accumulation of pathogenesis-related (PR) proteins (Velazhahan et al. 1998). The initial response to pathogen invasion occurs rapidly resulting in local gene activation causing hypersensitive reaction (HR) and cell death (Somssich and Hahlbrock 1998). Subsequently, signal transduction cascades through altered cytoplasmatic Ca2+ 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).

Chitin, a polymer of *N*-acetyl-D-glucosamine, is one of the most abundant biopolymers and a major structural component of the cell wall of many pathogenic true fungi. Plant chitinases are hydrolytic enzymes (EC 3.2.1.14) that catalyse the hydrolysis of chitin and are believed to play important role in plant defence against infection by pathogens (Collinge et al. 1993). It has been hypothesized that the induction of chitinase activity is a part of the defence response in plants, as there are no apparent natural substrate for the enzyme in higher plants and also because purified chitinases show antifungal activity *in vitro* (Schlumbaum et al. 1986).

Plant chitinase genes have been classified into various classes belonging to the glycoside hydrolase families 18 and 19 (Henrissat 1991; Neuhaus et al. 1996) on the basis of primary structures and specific domains. Within these two families, chitinases are further grouped into seven classes based on their structure, enzymatic property and sub-cellular localization. Class I, II, IV, VI and VII chitinases make up family 19, whereas class III and V chitinases constitute family 18 (Neuhaus 1999). The class I chitinases are usually basic in nature and are mostly localized in vacuoles. They are similar to class IV chitinases because both classes harbour the chitin-binding domain. Class II chitinases lack the chitin-binding domain (Graham and Sticklen 1994) and are distinguished from class I chitinases by their acidic pI. The class III chitinases do not possess any sequence homology to class I or II chitinases, unlike class IV chitinases, which are presumed to have evolved from class I chitinases (Araki and Torikata 1995). The class V chitinases do not have similarities to the other classes of chitinases, but show a weak similarity to bacterial exochitinases (Melchers et al. 1994).

Both *in vitro* (Schlumbaum et al. 1986; Mauch et al. 1988) and *in vivo* (Broglie et al. 1991; Robert et al. 2002; Maximova et al. 2006; Xiao et al. 2007) investigations have reported the inhibition of fungal growth by chitinases. Upregulation of genes encoding chitinase following pathogen attack or in response to a variety of abiotic stress factors (Porat et al. 2001; Hong and Hwang 2002; Wu and Bradford 2003; Wiweger et al. 2003; Khan and Shih 2004; Bailey et al. 2005; Shinya et al. 2007; Keulen et al. 2008) and elicitors (Kasprzewska 2003; Bravo et al. 2003) like *N*-acetyl-chitooligosaccharide from

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fungal sources is reported in many plant species. Furthermore, some chitinase genes are expressed at low levels in certain organs and may also possess developmental and physiological functions, including processes related to somatic embryogenesis (Jong et al. 1995; Dong and Dunstan 1997; Passarinho et al. 2001).

In forest tree species, chitinases are reported from *Pinus* (Liu et al. 2005), Japanese cedar (Fujimura et al. 2005), Spruce (Johnk et al. 2005), Poplar (Rinaldi et al. 2007), *Casuairna glauca* (Fortunato et al. 2007) and Douglas-fir (Islam et al. 2010). The present study was undertaken to isolate and characterize the defence-related chitinase from needle tissues of fungal elicitor challenged rooted cuttings of the tropical tree *Casuarina equisetifolia*. To our knowledge, this is the first report on cloning and analysis of a pathogenesis-related gene from this species.

2 Materials and methods

2.1 Plant and fungal material

2.1.1 Plant material

Vegetative cuttings of *C. equisetifolia* subsp. *equisetifolia* (CSIRO seed lot number 19129 from Lakei/sibur Bako, Malaysia) were collected from the casuarina species trial maintained by the Institute of Forest Genetics and Tree Breeding, Coimbatore, at Panampally Research Station, Kerala, India, and were rooted and maintained in the vegetative propagation complex for bioassay studies. One-month-old rooted cuttings of *C. equisetifolia* were subjected to elicitor treatment, and a set of cuttings subjected to sterile water was used as control. Needle tissues from both elicitor-treated and untreated cuttings were used for subsequent studies.

2.1.2 Fungal isolate

Trichosporium vesiculosum was obtained from the culture collection of the Division of Plant Protection, Institute of Forest Genetics and Tree Breeding, Coimbatore, India, and was maintained on potato dextrose agar medium. The hyphal mass was grown in potato dextrose broth for 30 days, and the fungal exudate was filtered twice through muslin cloth, autoclaved, and the filtrate was used as elicitor for treatment of rooted cuttings of *Casuarina* based on the protocol described by Mohan and Manokaran (2001).

2.2 Total RNA isolation, cDNA synthesis and optimization of elicitor treatment

One-month-old rooted cuttings were subjected to the culture filtrate (elicitor) for 0, 24, 48 and 72 h to determine the time required for optimal elicitation. Total RNA was isolated from the control and treated needles using an in-house protocol (Patent pending). Elimination of genomic DNA from RNA was performed with rDNase I (Fermentas, , Hanover, MD, USA). The quality of RNA was checked by resolving the RNA on 1% agarose gel stained with ethidium bromide, and the quantification was performed using Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltman, MA, USA). First-strand cDNA was synthesized with template of total RNA using first-strand cDNA synthesis kit (Fermentas) following the manufacturer's instructions. Two micrograms of total RNA was converted to cDNA in a 20 μl reaction mixture containing 1 μM oligo dT primer and 20 units of MMuLV reverse transcriptase. The mixture was incubated at 37°C for 1 h in air incubator. The reaction was terminated by incubation at 65°C for 5 min followed by incubation on ice. The first-strand cDNA synthesized from all the four samples including control was amplified using an arbitrary primer P8 (5′-ATTAACCCTCACTAAATGGAGCTGG-3′) provided in the Delta Differential display kit (Clontech Laboratories Inc., Palo Alto, CA, USA) and resolved on a 4% polyacrylamide gel to determine the time required for optimal elicitation.

2.3 Isolation of chitinase transcript from elicitor-treated needles

Primers were designed from the protein sequences of *Quercus*, *Fagus* and *C. glauca* chitinase (Table 1), which were available in public domain database using Primer 3 (Rozen and Skaletsky 2000; http://frodo.wi.mit.edu/primer3/). The species chosen for primer design were based on their phylogenetic nearness to the family Casuarinaceae. The primer pairs were amplified in the cDNA pool of treated needle tissues. One microlitre of first-strand cDNA was amplified using 400 pM of each dNTP, 0.4 pM primer and 1.0 unit of Taq DNA polymerase (Genet Bio, Chungnam, Korea). The PCR was conducted in Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with thermal cycling conditions comprising of an initial denaturation step at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, annealing at x°C for 1 min (Refer Table 1 for annealing temperatures) and extension at 72°C for 2 min, with a final 10-min extension at 72°C. After agarose gel fractionation, the amplicons with the expected size were purified and cloned into pTZ57R/T vector using InstaClone[CR] PCR cloning kit (Fermentas) following the manufacturers' instructions and sequenced. The sequence obtained was compared against the sequences in NCBI GenBank database using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 3′ and 5′ rapid amplification of cDNA ends (RACE)

The data obtained after sequencing of the re-amplified fragments were used to design primers to isolate the 3′ and 5′ ends of the chitinase cDNA.

Table 1. Chitinase-specific primer pairs synthesized for transcript profiling.

Primer ID	Primer sequence (5–3′)	Annealing temperature (°C)
CHQ1F	GGNGAYGCNGTNYTNGAYGG	52.5
CHQ1R	YTGNACCCANACGTARTCRA	50.9
CHQ2F	GACTTTGATATTGAAGGAGG	51.2
CHQ2R	ATAGACCTGTCTTAAGGGCAGC	59.5
CHQ3F	AGCTGGAGGTGCTCTCTGTCTT3′	63.7
CHQ3R	GTGGCCTACATGGTATCCACCAAGA	64.6
CHCG1F	GCCAAAGGCATCAAAGTCAT	56.5
CHCG1R	CCTTTAATGGCTGGAAGCAC	57.4
CHCG2F	CGGGGAGCACAAAGATAGAT	59.2
CHCG2R	AAATGGACGCGAAGATGATT	54.8
CHCG3F	TGACATTGAAGGAGGGACAA	58.0
CHCG3R	ATGGCCCTTAATGGCAGTG	58.8

2.4.1 3′ RACE

The first-strand cDNA was synthesized with template of total RNA extracted from 48 h elicitor-treated needles using AMV Reverse Transcriptase and Oligo dT-3sites Adaptor Primer provided in 3′-Full RACE Core kit (Takara Bio Inc., Otsu, Japan) as per manufacturer's instructions. The synthesis was conducted in Veriti® Thermal Cycler (Applied Biosystems) by using the following programme: 1 cycle of 30°C for 10 min; 50°C for 20 min; 95°C for 5 min and 5°C for 5 min. A 10 µl reaction containing 2 µl of 1st strand cDNA was amplified using 400 pM of each dNTP, 0.4 pM of primers (three sites Adaptor Primer: 5′-CTGATCTAGAGGTACCGGATCC-3′) provided by the manufacturer and the synthesized gene-specific forward primer CECHI3LP: 5′- AGAGGTACCGCTGGCTTACACG -3′ and 1.0 unit of Taq DNA polymerase (Genet Bio). The PCR was conducted in Veriti® Thermal Cycler (Applied Biosystems) with thermal cycling conditions comprising of an initial denaturation step at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, annealing at 60°C for 30 s and extension at 72°C for 2 min, with a final 10-min extension at 72°C. The resulting amplicons were purified, cloned into pTZ57R/T vector using InstaClone^{CR} PCR cloning kit (Fermentas) by following the manufacturers' instructions and sequenced.

2.4.2 5′ RACE

Rapid amplification of 5′ cDNA ends was carried out using a First-Choice RLM-RACE kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions with few modifications. PCR was carried out with a gene-specific reverse primer 5Chieq RP2 (5′- TCACCCACTAGAAGCAAGGCTACA -3′) and the 5′ RACE outer primer (5′-GCTGATGGCGA TGAATGAACACTG-3′) provided in the RLM kit, to amplify the 5′ end of the chitinase gene, including the 5′-untranslated region (5′-UTR) and the N-terminal coding region. To increase the specificity and product yield of 5′ RACE, nested PCR was subsequently performed with 2 µl of the diluted initial PCR using internal gene-specific reverse primers (Table 2) and 5′ RACE inner primer (5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′) provided in the kit. The PCR cycling conditions were same as described for 3′ RACE. The PCR products of 5′ RACE designated as *5Chi1*, *5Chi2 and 5Chi3* were cloned into pTZ57R/T vector using InstaClone^{CR} PCR cloning kit (Fermentas) by following the manufacturers' instructions and sequenced.

Table 2. Primers synthesized for 5′ RACE.

Primer code	Primer sequence (5–3′)	Annealing temperature (°C)
CECH1RP	GGACCCAAAGTCCTCCATTT	59.9
CECHI2RP1	GGAAGGAAGATGCGCTCGTA	60.5
CECHI2RP2	CCCCCACCAGCCACTTCTATT	64.4
5ChigNRP1	AAACGTGGAGCTGGAGATGAGG	63.3
5Chieq RP1	GTGGCCTACATGGTATCCACCAAGA	64.6
CHIBRP1	TATCAAACACAGAGCTGGAGATGAG	59.3
CHIBRP2	TTTGACAACCAGTGGAGCAGTAA	59.7
CHIBRP3	TCCAAAACCTCATTTTCATAGTGGT	57.7
CE5UTRCRP1	TTTGACAACCAGTGGAGCAGTAA	59.7
CE5UTRCRP2	AGTGGTTTTTGCCACTGGTTTT	58.7
CE5UTRRP3	GAGAAGGGGAAAGTTTGTCTGA	59.1
CE5UTRRP4	GCATTATCAACAGAGGGTGAAA	56.0

2.5 Isolation of full-length *CeChi1* cDNA

The vector-trimmed high-quality sequences obtained from amplification of cDNA pool were selected for further clustering and alignment to form transcript assemblies (TAs) using the CAP3 program (Huang and Madan 1999). The entire coding region of *CeChi1* cDNA was predicted and amplified with a pair of gene-specific primers (ChiORFFP: 5′-AAAATGAGGTTTTG GATCTTTGC-3′; ChiORFRP: 5′-CATGGTATCCACCAAGAGTCCATTG– 3′). The PCR conditions were same as described for 3′ RACE. The PCR product was purified and cloned into pTZ57R/T vector using InstaClone^{CR} PCR cloning kit (Fermentas) by following the manufacturers' instructions and sequenced.

2.6 Bioinformatic analysis

The DNA sequence data were converted to single-letter code in text file format using the Chromas 1.56 program (Technelysium Pty. Ltd, Tewantin, Qld, Australia). Sequence similarity search was performed with Blastn program of NCBI (National centre for Biological Information) (Altschul et al. 1990). The conceptual translation of the nucleotide sequence was made using open reading frame finder program (www.ncbi.nlm.nih.gov/projects/gorf/). Computation of the various physical and chemical parameters of the predicted protein like molecular weight, theoretical pH, amino acid composition, estimated half-life, instability index were carried out using the ProtParam tool (http://expasy.org/tools/protparam.html). Typical domains were analysed using the web tool (smart) from EMBL (http://smart.embl.de/smart/set_mode.cgi/GENOMIC=1), and similarities to important amino acid motifs in proteins with known function were determined using the online protein motif search programs InterPro (www.ebi.ac.uk/InterProScan) and Motif (www.motif.genome.jp). Prediction of post-translational modifications like the presence of signal peptides and its cleavage sites was identified with SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011). The deduced amino acid sequence was assessed for potential glycosylation and phosphorylation sites using the NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) (Gupta et al. 2004) and NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al. 1999) programs. The sub-cellular localization of the protein was predicted using the TargetP v1.01 (Emanuelsson et al. 2000; www.cbs.dtu.dk/services/TargetP/). Sequence alignments were carried out using the ClustalW method (Thompson et al. 1994) using the EMBL server (www.ebi.ac.uk/clustalw/). *In silico* analysis of the 5′ and 3′ untranslated regions was conducted using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al. 2002) and place (http://www.dna.affrc.go.jp/PLACE/signalscan.html/; Higo et al. 1999).

2.7 Phylogenetic analysis

A phylogenetic tree based on the amino acid sequence alignment was constructed to decipher the evolutionary relationships of *C. equisetifolia* class I chitinase. The molecular evolutionary and phylogenetic analyses were conducted using the software mega Version 5.05 (Tamura et al. 2011). A phylogenetic tree was constructed based on amino acid sequences of members of chitinases from each class *viz.* Class I, II, III, IV, V and VII available in the GenBank and *CeChi1* to elucidate the relationship of it with class I chitinase of other species deposited previously. The Litchi thaumatin-like protein was introduced as the out-group. The molecular distances of the aligned sequences were calculated according to the parameter of p-distance, and the phylogenetic trees were generated using the rooted neighbour-joining method from the aligned amino acid sequences. Pairwise deletions were used to deal with gaps. The reliability of the tree was established by conducting 1000 neighbour-joining bootstrap sampling steps, and nodes with <50% bootstrap confidence were collapsed.

2.8 Isolation of *CeChi1* from *Casuarina equisetifolia* genomic DNA

Genomic DNA was isolated from 100-mg needles of *C. equisetifolia* using DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). The integrity of DNA was checked using 0.8% agarose gel with ethidium bromide following the protocol described by Sambrook et al. (1989) and viewed under UV-transilluminator and documented using Kodak-DC290 digital camera (Kodak, Rochester, NY, USA). The quantity and quality of DNA were measured by comparing band intensity with standard lambda DNA, and DNA was also quantified using NanoDrop-ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Full-length genomic clone of *CeChi1* was subsequently isolated by PCR with 20 ng of genomic DNA as template using the forward primer ChiORFFP (5′-AAAATGAGGTTTTGGATCTTTGC-3′) and reverse primer ChiORFRP (5′-CATGGTATCCACCAAGAGTCCATTG– 3′). PCRs were performed in a Veriti® Thermal Cycler (Applied Biosystems) with a denaturation step at 94°C for 5 min, followed by 30 cycles (denaturation at 94°C for 1 min, annealing at 60°C for 30 s, extension at 72°C for 2.0 min), and a final extension step at 72°C for 10 min. PCR product designated as g*CeChi1* was purified and cloned into pTZ57R/T vector using InstaClone^{CR} PCR cloning kit (Fermentas) by following the manufacturers' instructions and subsequently sequenced. Finally, the DNA sequence was analysed using Spidey (www.ncbi.nlm.nih.gov/spidey/) program of NCBI.

2.9 Differential analysis of *CeChi1* using RT–qPCR

One–month-old cuttings were subjected to fungal elicitor treatment as described earlier. Total RNA was isolated from 1 g of control, 24 and 48 h pathogen elicitor-treated needle tissues as described earlier. The quality of RNA was checked on a 1% agarose gel, and concentration was determined spectrophotometrically. mRNA was isolated using GenElute mRNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) from 10 μg of both control and treated total RNAs to avoid contamination of genomic DNA. First-strand cDNA was synthesized from both control and treated RNAs using first-strand cDNA synthesis kit (Fermentas) by following the manufacturers' instructions. Similarly, the expression of *CeChi1* was evaluated during salicylic acid (SA) signalling. The cuttings were subjected to 5 mM SA for 24 and 48 h, and total RNA, mRNA and cDNA were synthesized from needle tissues as described above. Three biological replications were conducted for each treatment.

Besides fungal elicitor, an attempt was also made to evaluate the temporal expression of *CeChi1* transcript in response various 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 expression during mechanical wounding while salinity stress was imposed by transferring the rooted cuttings to Hoagland solution containing 1500 mM 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 under normal conditions and sampled at the same time as the stressed plants. The needles were harvested at indicated time intervals, frozen directly into liquid nitrogen and were used for RNA extraction and subsequent analysis. Total RNA isolation, mRNA isolation and cDNA synthesis were performed as described above. The cDNAs were quantified by spectrophotometry in a NanoDrop-ND1000 spectrophotometer (NanoDrop Technologies). Finally, 100 ng of each cDNA sample was used for real-time RT-PCR amplification.

2.10 Real-time primer design and amplification

Primer pair designing for real-time PCR assays was carried out using the PRIMER3 program. As there is no reference genomic DNA sequence available for *C. equisetifolia*, primers was designed from sequences obtained from mRNA regions of the class I chitinase gene. A set of *C. equisetifolia* ubiquitin primers were also designed (Table 3) for use as an endogenous control to normalize the data for differences in input RNA and efficiency of reverse transcription between the various samples. qPCR was performed in StepOne plus Sequence Detection System (Applied Biosystems) and associated software using the SYBR green chemistry. PCR was performed in a final volume of 10 μl containing 5 μl of 2X SYBR Green Jumpstart Taq Ready Mix for Quantitative PCR (Sigma-Aldrich), 500 nM each of forward and reverse primers, and 100 ng of cDNA template. After an initial activation step of the DNA polymerase at 94°C for 2 min, samples were subjected to 40 cycles of amplification (denature at 94°C for 15 s, annealing and extension together at 60°C for 1 min). The primer specificities were further confirmed with the melting curve generated after amplification. PCRs containing cDNA and 'no template' control (NTC; sterile water only) were run in parallel for each template and primer combination.

Relative quantification of the target gene expression was performed with comparative C_t method (Livak and Schmittgen 2001). The C_t used in the real-time PCR quantification is defined as the PCR cycle number that crosses an arbitrarily chosen signal threshold in the log phase of the amplification curve. The relative expression level of the gene of interest was computed with respect to ubiquitin to account for any variance in expression of the target transcript. All experiments were independently conducted in triplicate, and average C_t values from all PCRs were normalized to average C_t values for ubiquitin from the same cDNA preparations. The expression level was calculated by the formula $2^{-\Delta\Delta C_T}$ that represents the x-fold difference from the calibrator.

3. Results

3.1 Amplification of chitinase from cDNA pool

A 900-bp fragment was amplified from 48 h (optimal elicitation time determined) fungal elicitor-treated needle cDNA using degenerate primer designed from conserved regions of chitinase genes. BLAST analysis showed that this fragment shared high sequence similarity with class I chitinases from several species such as *C. glauca*, *Ricinus communis*, *Hevea brasiliensis* subsp. *brasiliensis*, *Ulmus pumila*, etc. The 5′ RACE and 3′ RACE resulted in three DNA fragments with approximate size of 250, 500 and 400 bp, and CAP3 generated a contig of 1549 bp (designated as f*CeChi1*). The f*CeChi1* cDNA sequence consisted of a 966-bp open reading frame (ORF) encoding a protein of 321 amino acids, a 293-bp 5′ untranslated region and a 290-bp 3′-untranslated region containing a polyadenylation signal. The complete CDS of *fCeChi1* amplified with specific primer pairs resulted in a fragment of size approximately 1.0 kb, designated as *CeChi1*. The *CeChi1* sequence (Fig. 1) was deposited in GenBank database with accession number HQ414236.1.

We analysed the upstream (partial 5′ UTR) and downstream region (3′ UTR) of *CeChi1* using PLANTCARE and PLACE and identified several functionally significant *cis*-acting regulatory elements that are associated with hormonal regulation (ABRECE1HVA22, GCCCORE, GT1CONSENSUS, TCA-element, GARE-motif, and JERE), pathogen stress response (AGCBOXNPGLB,

Table 3. Sequences of primers used for quantitative real-time RT-PCR.

Gene targeted	Primer ID	Sequence
Ubiquitin	UbiFP	5′-GAAAAACCATAACCTTGGAAGTTG-3′
	UbiRP	5′-GATTCCTTTTGGATGTTGTAATCC-3′
Chitinase	QChiFP	5′-TGACCCTGTCGTTTCCTTTAAGTC-3′
	QChiRP	5′-TGATGTTCGTAACAACACCGTACC-3′

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1      ATGAGGTTTTGGATCTTTGCCATTCTCTCTTTGTTGCTGTCCTCCTTCCGAGAAGGCTCA 60
       M  R  F  W  I  F  A  I  L  S  L  L  L  S  S  F  R  E  G  S
61     GCTGAGCAGTGTGGAAGGCAAGCTGGAGGTGCTCTCTGCCCTGGTGGGCAGTGCTGTAGC 120
       A  E  Q  C  G  R  Q  A  G  G  A  L  C  P  G  G  Q  C  C  S
121    CAGTATGGCTGGTGCGGCTCCACATCTGATTACTGCTCCACTGGTTGTCAAAGCCAATGT 180
       Q  Y  G  W  C  G  S  T  S  D  Y  C  S  T  G  C  Q  S  Q  C
181    GGTGGAGGAGGTGGTGGTGGAGGTGGCGGTGGGGACATTGGTGGCCTCATCTCCAGGTCT 240
       G  G  G  G  G  G  G  G  G  G  G  D  I  G  G  L  I  S  R  S
241    GCGTTTAATAATCTGCTGAAACATCGGAACGATGGTGCTTGCCCAGCCAAGGGATTCTAC 300
       A  F  N  N  L  L  K  H  R  N  D  G  A  C  P  A  K  G  F  Y
301    GCCTATGATGCTTTCATCGCTGCTGCAAAGGCTTTTCCCAACTTTGCTACCACAGGGGAC 360
       A  Y  D  A  F  I  A  A  A  K  A  F  P  N  F  A  T  T  G  D
361    AGTGCCACCCGGAAAAGAGAGATTGCTGCTTTCTTAGCCCAAACATCCCATGAAACTACC 420
       S  A  T  R  K  R  E  I  A  A  F  L  A  Q  T  S  H  E  T  T
421    GGGGGATGGGCAACTGCTCCTGATGGCCCATACGCATGGGGATATTGTTATCTCAGGGAA 480
       G  G  W  A  T  A  P  D  G  P  Y  A  W  G  Y  C  Y  L  R  E
481    CAAAACCCTGGATCATACTGCGCTTCGGATCCGACTTACCCTTGTGCTGCAGGCAAGCAG 540
       Q  N  P  G  S  Y  C  A  S  D  P  T  Y  P  C  A  A  G  K  Q
541    TACTATGGCCGAGGTCCCATCCAACTATCATGGAACTATAACTATGGAAGGTGCGGAAAA 600
       Y  Y  G  R  G  P  I  Q  L  S  W  N  Y  N  Y  G  R  C  G  K
601    GCCATAGGAGTGGACCTATTGAACAACCCAGACCTCGTGGCGACTGACCCTGTCATTTCC 660
       A  I  G  V  D  L  L  N  N  P  D  L  V  A  T  D  P  V  I  S
661    TTTAAGTCGGCTCTCTGGTTCTGGATGACTCCACAGTCACCAAAGCCATCATGCCATGAT 720
       F  K  S  A  L  W  F  W  M  T  P  Q  S  P  K  P  S  C  H  D
721    GTCATCACCGGAAGATGGAACTCATCCGCTGCTGACAAGGCAGCCGGTCGGAATTCCGGG 780
       V  I  T  G  R  W  N  S  S  A  A  D  K  A  A  G  R  N  S  G
781    TACGGTGTTGTTACGAACATCATCAACGGCGGGCTCGAGTGTGGTAAGGGCTGGAACGCG 840
       Y  G  V  V  T  N  I  I  N  G  G  L  E  C  G  K  G  W  N  A
841    AAAGTGGAGGATCGCATCGGGTTCTTCAAGAGGTACTGTGACATACTCGGGGTTGGCTAC 900
       K  V  E  D  R  I  G  F  F  K  R  Y  C  D  I  L  G  V  G  Y
901    GGCAGCAACCTTGACTGCTATAACCAGAGATCTTTTGGCAATGGACTCTTGGTGGATACC 960
       G  S  N  L  D  C  Y  N  Q  R  S  F  G  N  G  L  L  V  D  T
961    ATGTAG 966
       M  *
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Fig. 1. Nucleotide and deduced amino-acid sequences of *CeChi1*. Nucleotides are numbered in the 5′–3′ direction, beginning with the initiation codon ATG. The predicated amino-acid sequence of *CeChi1* is shown below the nucleotide sequence. The termination codon TAG is marked with an asterisk. The initiation codon and the termination codon are marked in bold.

ELRECOREPCRP1, GT1GMSCAM4, TC-rich repeats, box E, WBOXATNPR1, WBOXNTERF3, WRKY71OS) and water stress response (MYB1AT, MYB2AT, MYB2CONSENSUSAT, MYBCORE, MYCCONSENSUSAT and EBOXBNNAPA). In addition, many other cis-elements involved in light responsiveness (GATA-box, IBOX, IBOXCORE, IBOXCORENT, SORLIP1AT, Sp1, TCT-motif and Box 4) and symbiotic genes-related (NODCON1GM, NODCON2GM, OSE1ROOTNODULE, OSE2ROOTNODULE) elements were also predicted. TATA and CAAT motifs are also identified close to the translational start site.

3.2 Analysis of the deduced amino acid sequence of *CeChi1*

The *CeChi1* open reading frame comprised 966 nucleotides that encoded 321 amino acid residues with approximate molecular mass of mature protein being 34.11 kDa and theoretical isoelectric point of 8.25, which was consistent with the properties of most previously published plant class I chitinases. The instability index (II) was computed at 39.91, which classifies the protein as stable and the aliphatic index, regarded as a positive factor for increased thermostability, and was calculated at 59.41 by using ProtParam. The total number of negatively (Asp + Glu) and positively charged residues (Arg + Lys) in the deduced amino acid sequence of *CeChi1* was 23 and 27, respectively. The N-terminal 21 amino acid residues exhibited the characteristics of a signal peptide from secreted eukaryotic proteins with a positively charged stretch of amino acids (the n-region), a central hydrophobic core (the h-region), followed by a polar amino acid stretch that included the cleavage site (the c-region). A signal peptide was predicted using the program SignalP, and the most likely cleavage site was between positions 21 and 22: GSA-EQ. A short C-terminal signal sequence similar to that of the tobacco chitinase (GLLVDTM) required for localization in the vacuole was also identified in *CeChi1.*

Multiple alignment of the predicted *CeChi1* amino acid sequence with other class I chitinases shows that amino acid sequences share highly conserved region in the chitin-binding domain and catalytic domain but not in the signal peptide and hinge region (Fig. 2). Like a typical class I chitinase, no deletion of residues was found in the catalytic domain of *CeChi1*. Furthermore, the amino acid residues important for chitin binding and chitinolytic activities in plant class I chitinases (Garcia-Casado et al. 1998; Ohnuma et al. 2004) including W43, E138, E160, Q188, S 190, N269 were all conserved in *CeChi1*.

A potential glycosylation site (NSSA) was located at amino acid position 247 of *CeChi1* as predicted by NetNGlyc program. In addition, the *CeChi1* deduced protein also contained multiple potential sites for phosphorylation at serine, threonine or tyrosine residues. There were 12, 4 and 5 phosphorylation sites for serine, threonine and tyrosine on *CeChi1* as

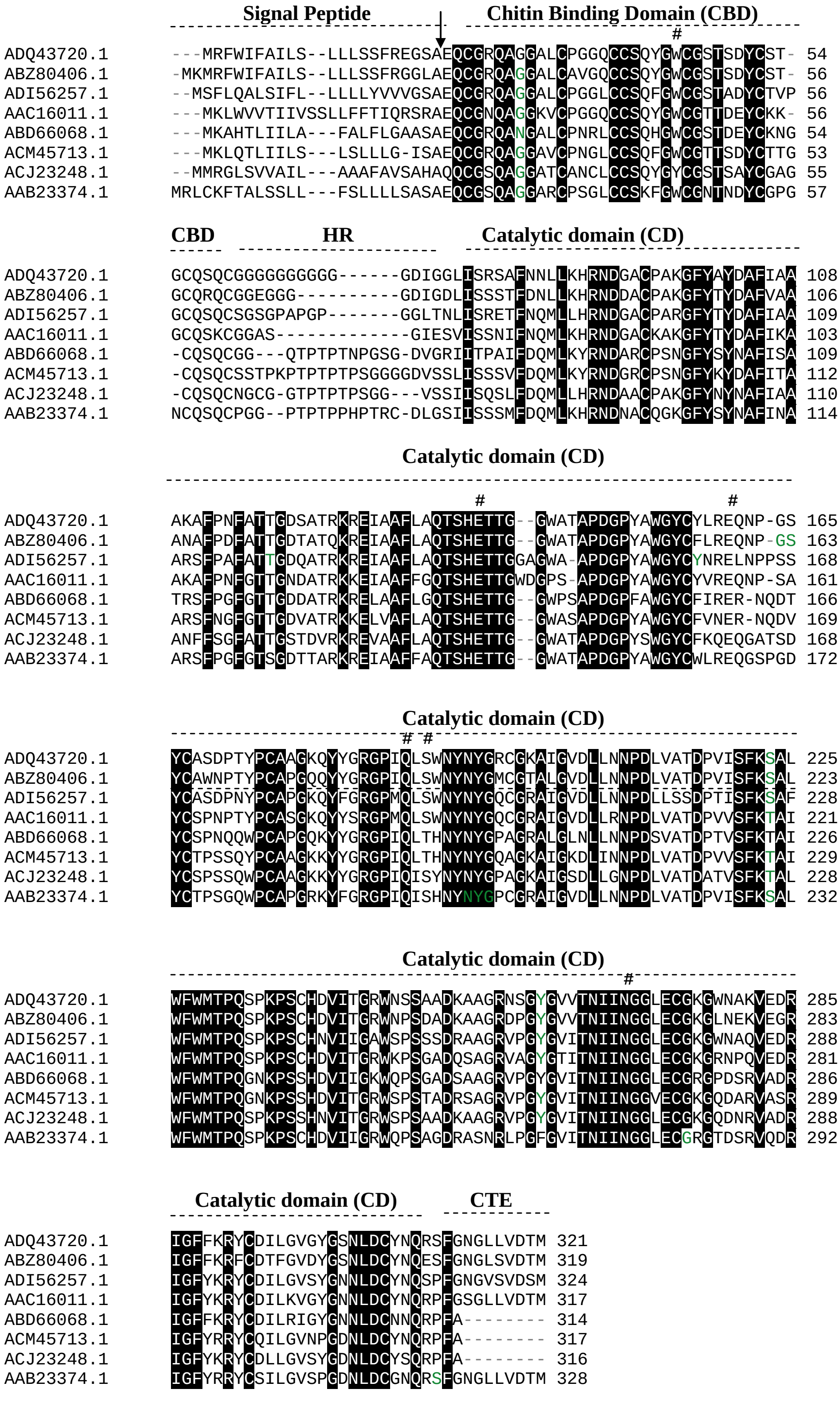

Fig. 2. Multiple alignment of amino acid sequences of *CeChi1* with other class I chitinases *viz. Casuarina equisetifolia* (ADQ43720.1), *Casuarina glauca* (ABZ80406.1), *Gossypium hirsutum* (ADI56257.1), *Elaeagnus umbellta* (AAC16011.1), *Momordica charantia* (ABD66068.1), *Pyrus pyrifolia* (ACM45713.1), *Festuca arundinacea* (ACJ23248.1), *Nicotiana tabacum* (AAB23374.1). The symbol # marks the residue important for chitin binding and chitinolytic activities of plant class I chitinases. The conserved chitin binding domain (CBD), catalytic domain (CD) and the C- terminal extension (CTE) are indicated by dotted lines. Gaps introduced for optimal alignment are indicated by dashes. The amino acids conserved in all the sequences are shaded in gray. The numbers on the right refers to amino acid residue positions. A putative cleavage site of the N-terminal signal peptide is indicated by an arrow.

predicted by NetPhos. The TargetP program predicted that *CeChi1* is secreted via the secretory pathway. An analysis for conserved protein domains performed using smart, motif and InterPro Search indicated that the predicted amino acid sequence of *CeChi1* contained a glycoside hydrolase family 19 domain (from position 72 to 314), a chitin-binding, type I domain (from position 23 to 60) and a lysozyme-like superfamily domain (from position 129 to 268).

3.3 Phylogenetic analysis

The phylogenetic tree grouped different classes of chitinases into six clusters with few exceptions and *CeChi1*grouped with class I chitinases from other plant species. *CeChi1* grouped with its orthologue from *C. glauca* with 100% confidence level as indicated by the bootstrap value (Fig. 3). The horizontal lengths of branches were proportional to the relative homologies between chitinase sequences.

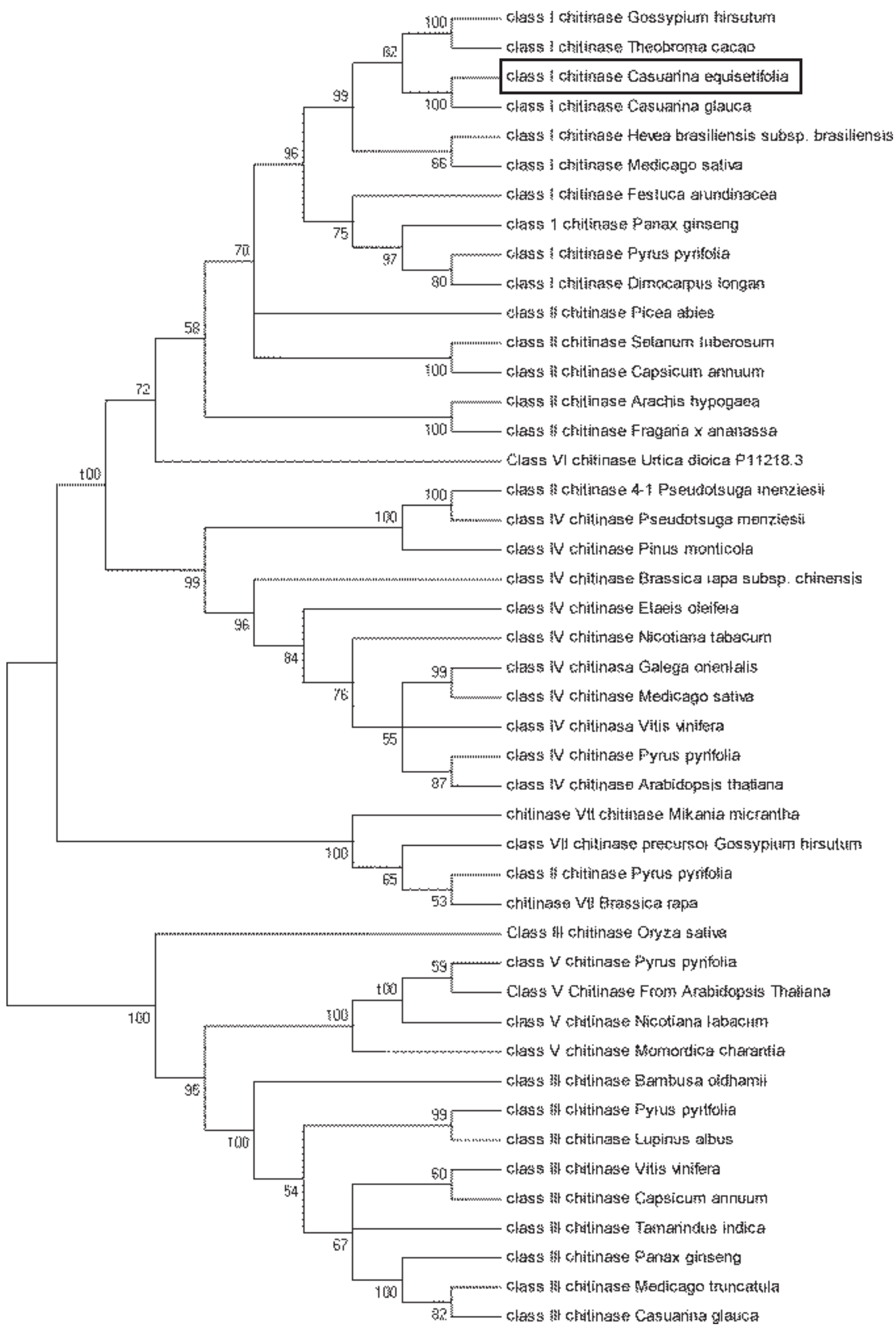

Fig. 3. Phylogenetic tree constructed using deduced amino acid sequence of *CeChi1* along with members of chitinases from each class *viz.* Class I, II, III, IV, V, and VII.

3.4 Analysis of the genomic *CeChi1* sequence

The 1184-bp DNA fragment of g*CeChi1* was amplified from the start codon to the termination codon, which encompassed two introns and three exons. The intron had the conserved G/GT and AG/G motifs at the splice junctions (Shapiro and Senapathy 1987). The genomic DNA of g*CeChi1* contained two introns with a size of 132 and 86 bp, respectively. The size of *CeChi1* exons ranged from 149 to 422 bp with an average exon size of 321 bp. The intron pattern of *CeChi1* was compared other chitinases and was found similar to the pattern reported from other dicots like *Arabidopsis thaliana* with amino acid sequence surrounding the introns at their 5′ and 3′ being HETT and GGWA for intron2 and QLSW and NYNY for intron 3 (Wiweger et al. 2003). The nucleotide sequence around the predicted start codon region, AAAATG, is in agreement with the Kozak consensus initiator ANNATGG (Lutcke et al. 1987) proposed for the translation start of plant genes. The coding region of the polypeptide was followed by a TAG stop codon.

3.5 Differential expression of *CeChi1* using qRT-PCR

Quantitative real-time PCR was performed to elucidate the expression profiles of *CeChi1* in *C. equisetifolia* needles treated with *T. vesiculosum* fungal elicitor, SA and various abiotic stresses at different time points. A single product-specific melting curve was obtained for primers of *CeChi1* and *ubi* (Fig. 4), indicating that primers were designed with optimal efficiency and were effective at targeting and amplifying only the genes of interest. In response to the elicitor treatment, the expression of *CeChi1* was up-regulated by 1.2- and 2-fold at 24 and 48 h post-treatment in expression when compared with the background level (control). Similarly, the expression of the gene was upregulated by approximately 1.1-fold after both 24- and 48-h treatment with SA (Fig. 5a). The expression of the gene in response to the abiotic stresses like NaCl, wounding, PEG and heat was not highly significant revealing pathogenesis-specific expression of *CeChi1* (Fig. 5b).

4 Discussion

Plants being sessile are often exploited by a variety of organisms for food and shelter. They have developed intricate mechanisms to combat such stress by activating a complex network of signal transduction pathways, which results in the expression of a large number of defence genes including chitinases (Somssich and Hahlbrock 1998; Glazebrook 2001). There has been growing evidence on the dynamism of tree defence, and studies have revealed the involvement of both constitutive and induced (direct and indirect) mechanisms in tree resistance (Veluthakkal and Ghosh Dasgupta 2010).

Plants synthesize various chitinases, and they are divided into seven classes on the basis of their primary structures. Class I chitinases have an N-terminal cysteine-rich chitin-binding domain and a C-terminal catalytic domain and, these are connected by a short linker peptide of about 10–20 amino acid residues (Collinge et al. 1993). Analysis of deduced *CeChi1* amino acid sequence showed an N-terminal motif directing protein to the secretory pathway, a cysteine-rich chitin-binding domain, a glycine-rich hinge domain and a catalytic domain. The sequence of the first 21 amino acids at the N-terminal end is characteristic of a eukaryotic signal peptide with a highly hydrophobic core and a typical amino acid composition near the putative cleavage site. Class I chitinases from other species are also synthesized with a signal peptide for translocating the polypeptide into the endoplasmic reticulum (Shinshi et al. 1990; Zhu and Lamb 1991; Rasmussen et al. 1992). The 37 amino acids of *CeChi1* that are distal to the putative signal peptide form a

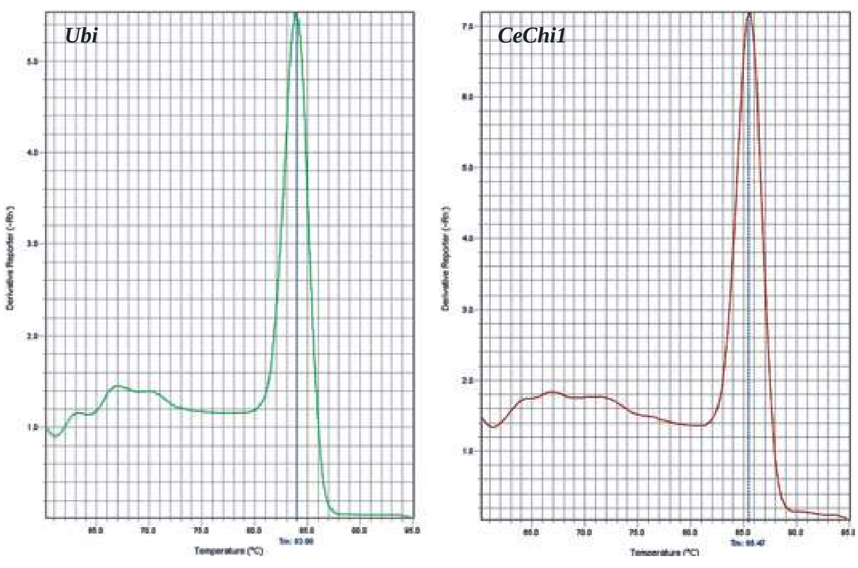

Fig. 4. Melting curve analysis (MCA) of Ubiquitin reference gene (*Ubi*) and class I chitinase (*CeChi1*).

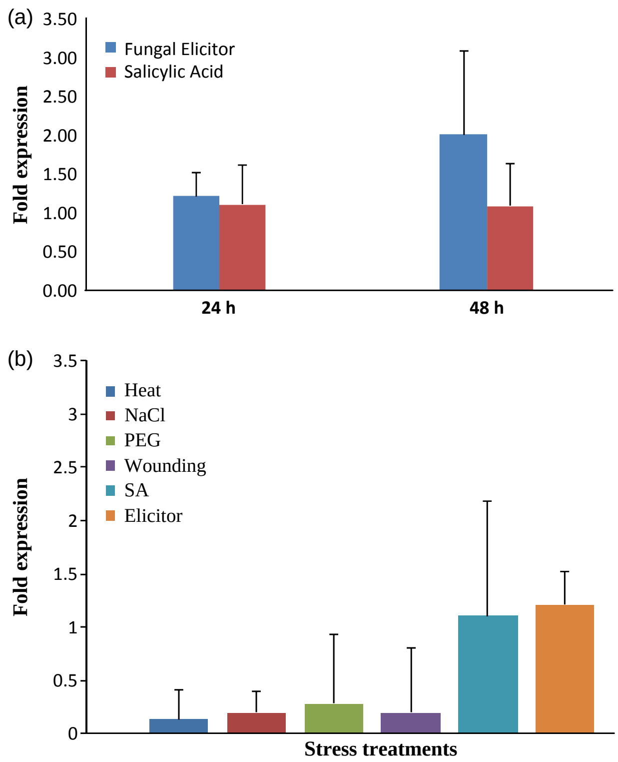

Fig. 5. (a) Fold changes in accumulation of *CeChi1* in needle tissue of *Casuarina equisetifolia* at specified hours subsequent to treatment with fungal elicitor of *Trichosporium vesiculosum* and 5 mM salicylic acid. (b) Expression pattern of *CeChi1* after 24 h in response to various biotic and abiotic stress treatments.

cysteine-rich domain linked by a 15 amino acid-long glycine-rich hinge region to the carboxy-terminal catalytic domain. This primary structure is characteristic of basic chitinases including class I chitinases (Shinshi et al. 1990). The calculated isoelectric point of *CeChi1* was 8.25, suggesting it encodes a basic protein, which also comply with the characteristics of class I chitinases. Further, a C-terminal extension has been found to be essential for targeting the mature chitinase protein to the vacuole in tobacco and other class I chitinases reported previously (Broglie et al. 1986; Gaynor 1988; Samac et al. 1990; Shinshi et al. 1990). A C-terminal region with the same characteristics (GLLVDTM) has been found in *CeChi1*, implying vacuolar localization.

In the present investigation, we have also cloned and characterized the corresponding full-length genomic clone of the chitinase coding region. The protein coding region of this gene encompasses three exons with size of 422, 151 and 393 bp interrupted by two small introns of 132 and 86 bp. These two introns were AT rich (64.0 and 66.0% respectively) and possess a consensus splice junction of 5′-G|GT and 3′-AG|G. Generally, the genes for class I chitinases are relatively small, which may or may not be interrupted by an intervening sequences. The Arabidopsis class I chitinase gene has one intron (Samac et al. 1990) and tobacco chitinase genes have two introns (Shinshi et al. 1990; Van Buuren et al. 1992), while bean (Broglie et al. 1989) and rice chitinase genes (Zhu and Lamb 1991) are intronless.

Studies on the presence of cis-elements in the untranslated regions of the genes and its role in post-transcriptional regulation have been reported for genes from humans (Liu et al. 2008). Earlier studies have demonstrated that cis-regulatory elements located in both the 5′- and 3′-untranslated regions (UTR) or even the coding regions of transcripts can enhance or regulate gene expression in plant and animals by interacting with trans-acting factors (Gallo-Meagher et al. 1992; Caspar and Quail 1993; Bolle et al. 1994; Zhang and Mehdy 1994; Kertesz et al. 2006). The attributes of the 5′UTR and the 3′UTR of mRNAs are known to be involved in the translation of genes in response to environmental stresses (Floris et al. 2009). However, to our knowledge, there are no published reports of cis-acting elements in the untranslated regions of the PR genes. However, analysis of cis- elements in promoters is well document in plant genes (Mongkolsiriwatana et al. 2009; Ibraheem et al. 2010) including pathogenesis-related genes (Matton et al. 1993; Zarei et al. 2011). In the present study, using the PLACE and PLANTCARE database, we have identified several functionally significant *cis*-acting regulatory elements that are associated with hormonal regulation, pathogen stress response and water stress response. Plant hormones such as ethylene, SA, jasmonic acid (JA) and abscisic acid (ABA) are important regulators of stress responsive pathways. The importance of these hormones as primary signals in the regulation of plant's immune response is well established (Pozo et al. 2004; Loake and Grant 2007; Van Dahl and Baldwin 2007). Many stress associated cis-acting regulatory elements that activate transcription of genes in response to salinity, drought, wounding and pathogen infection have been identified in plants (Higo et al. 1999; Singh et al. 2002; Rani 2007).

The regulatory effect of the 5′ untranslated region on the gene expression can be positive or negative depending upon the sequence features such as the presence of regulatory *cis*-elements, intron sequence, upstream open reading frame (uORF) or uATG, and the secondary structures (Zheng et al. 2009). Furthermore, it is not mandatory that the function of the predicted cis-acting regulatory sequences will comply with experimental expression data, and hence, a comprehensive analysis on the role of these predicted cis-elements and their interaction with the corresponding *trans*-acting factors would give a picture on the transcriptional regulation of *CeChi1* expression.

Real-time PCR was used to characterize the expression profile of *CeChi1* gene during fungal elicitor and SA treatment. The increase in expression fold of *CeChi1* transcript is a strong indication that the fungal elicitor stimulated a systemic accumulation of chitinase gene, suggesting the role in plant defence. This observation is in compliance with the explanation that chitinolytic enzymes have been stimulated against pathogenic organisms, such as insects, nematodes and fungi (Sahai and Manocha 1993). The hydrolytic products of the fungal cell wall can also act as elicitors, which may induce other types of defence reactions in plants (Keen and Yoshikawa 1983). The insignificant expression pattern of the gene during other abiotic stresses including wounding, salt, drought and heat suggests its specific role during pathogen defence. Generally, members of class II and IV chitinases are known to be expressed during abiotic stresses, while class I chitinases are usually specific against pathogen defence. However, in Norway Spruce, Hietala et al. (2004) reported the downregulation of class I chitinase (*PaChi1*) during wounding and infection by *Heterobasidion annosum*, while several fold higher expression of class II and class IV chitinases (*PaChi2* and *PaChi4*) suggesting a possible non-defence developmental role of the *PaChi1*. The low C_t value of *PaChi1* also suggested a constitutive expression of the gene. In the present study, also the C_t value of *CeChi1* in untreated control tissues was low, indicating high constitutive expression of this gene. Further characterization of the protein encoding *CeChi1* for antifungal activity would provide an evidence of its direct role during defence-reaction in this tree species.

The major disease reported in *C. equisetifolia* is the blister bark or wilt disease caused by the hyphomycete fungus *T. vesiculosum* (synonym *Subramanianospora vesiculosa*) (Titze and van der Pennen 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 and disease incidence range from 40 to 90% in plantations (Sharma 1994, 1995). The pathogen is yet to be 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. In the present study, the induction pattern of *CeChi1* during SA signalling further confirms it as a biotroph, which is characterized by the induction of the gene by salicylate dependent defence pathways (Hammond-Kosack and Parker 2003). Chitinases are known to be induced by wounding, SA, JA and ethylene signalling (Clarke et al. 1998; Wu and Bradford 2003; Rakwal et al. 2004; Fossdal et al. 2006) indicating its overlapping expression during biotic interactions with both biotrophic and necrotrophic pathogens. Further, wounding is reported to be under ethylene and/or jasmonate control, which is operative during infection by necrotrophs (Glazebrook 2005). In the present study, the insignificant expression of *CeChi1* during wounding and its up-regulation during SA signalling suggests the biotrophic nature of the pathogen. Thus, the study has provided insight to classify *T. vesiculosum* as an obligate biotroph based on the expression of *CeChi1*.

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