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A novel class I Chitinase from *Hippophae rhamnoides:* Indications for participating in ICE-CBF cold stress signaling pathway

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ABSTRACT

Plant chitinases are the members of PR (Pathogenesis related) proteins family and protect plants from biotic and abiotic stress. A novel chitinase HrCHI1 (Accession number JQ289153) of 954 bp ORF encoding 317 amino acids protein was cloned, expressed and characterized from seabuckthorn, a cold/freeze tolerant shrub. The 3D structure (predicted with I-TASSER server) showed highest homology with *Oryza sativa* class I chitinase (PDB 2dkvA). Putative promoter region (obtained by genome walking) showed GCC box, E-boxes, the binding site for bHLH proteins and DRE elements, the CBF (C-repeat binding factor) binding site besides TATA and CAAT boxes. The gel shift assay with the nuclear extract indicated that the HrCHI1 might be participating in CBF/ERF dependent cold stress signaling pathway. The quantitative transcript profiling supported this observation as cold induced expression of HrCBF peaked earlier (at 1 h) while HrCHI1 peaked latter (after 3 h) indicating HrCHI1 expression might be induced by HrCBF. Further, HrCHI1 expression was methyl jasmonate (MeJa) dependent and salicylic acid (SA) independent. HrCHI1 was expressed in *E. coli* and purified using chitin affinity chromatography. It showed 512 U/mg chitinase hydrolytic activity and resolved as a 34 kDa spot with a slightly basic pI (8.5) on a 2-D gel. The *E. coli* cells containing recombinant chitinase showed higher rate of growth in cold in comparison with the cells containing the empty vector. In conclusion, we have isolated and characterized a cold responsive basic class I chitinase which is regulated by MeJa and seems to be functioning *via* CBF/ERF dependent cold stress signaling pathway.

1. Introduction

Chitinases are present ubiquitously from bacteria to higher plants and animals, performing diverse physiological roles in defense, nutrition, parasitism, morphogenesis and immunity [1,2]. Plant chitinases are the members of pathogenesis related (PR) protein family, produced during pathological or related conditions. These enzymes catalyze cleavage of β-1,4-glycosidic bond of biopolymers containing *N*-acetylglucosamine "mainly chitin" to protect the plants from fungal attack. Plant chitinases have been broadly categorized into six classes (I to VI) [3,4]. Classes I, II, IV and VI belong to family 19 glycosidases while classes III and V belong to family 18 glycosidases. Chitinases have either an N-terminal or C-terminal targeting sequence that directs these first to the ER and then to the vacuole or the apoplast, respectively. Class I chitinases have three domains: a Cys rich chitin-binding domain, a Pro-rich hinge region, and a highly conserved catalytic domain. Class II chitinases lack the hinge region and chitin-binding domain, but have the catalytic domain nearly identical to Class I chitinases.

Chitinases show constitutive as well as induced expression. Belonging to PR proteins, many of them are induced by a plant pathogen or a biocontrol agent. Abiotic elicitors like mannitol, H_2O_2, NaCl, MeJa, ABA and SA can induce their expression [5]. During cold acclimation, plant secretes proteins in the extracellular space, which acts as first line of defense. These mainly include antifreeze proteins and PR proteins. In winter rye, dual functioning antifreeze proteins homologous to PR proteins like chitinase, β-1,4-glucanase and thaumatin like protein were observed [6]. Chitinase of winter rye [7], bromograss [8] and seabuckthorn [9] also possess antifreeze activity in addition to the hydrolytic activity. Jasmonic acid activates the expression of chitinases. Jasmonic acid and salicylic acid are defense related phytohormones [10]. Jasmonic acid accumulates during defense response and operates jasmonic acid signaling pathway either by MYC transcription factors binding to E-box or ERFs (Ethylene responsive factors) binding to GCC core [11–16]. Moreover, Hu et al. [17] showed that jasmonic

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acid activates ICE-CBF/DREB1 (Inducer of CBF expression-C-repeat binding factor/Dehydration responsive element binding proteins 1) transcription pathway in Arabidopsis. This suggests a cross talk between defense and cold stress signaling. ICE-CBF pathway is the most investigated cold stress signaling pathway till date. The CBFs accumulate within 15 min of cold stress and CBF expression is induced by an upstream regulator ICE. CBFs in turn activate the downstream target genes, which manifest the multiple physiological and biochemical changes leading to increase in the cold tolerance ability. As chitinase and CBF both accumulate under cold stress their regulatory networks might be overlapping.

Freezing stress induced the accumulation of chitinase unigene (detected from EST-SSR) in *Hippophae rhamnoides* (seabuckthorn) [18]. Moreover, chitinase protein accumulated in cold stress modulated secretome [19] indicating that cold responsive chitinases might contribute to the cold hardiness of seabuckthorn. Two antifungal class I and class III chitinases were earlier isolated from seabuckthorn [20]. Recently, Gupta and Deswal [9] purified two class I chitinases with constitutive antifreeze activity but regulation of these needs to be understood. Therefore, in the present study a cold responsive class I chitinase (HrCHI1) was cloned and characterized. In order to see if the HrCHI1 protein participates in providing cold tolerance, it was expressed in bacteria and purified. The growth of bacterial cells expressing chitinase was monitored under cold stress to confirm its role during cold stress. The three dimensional structure was predicted using I-TASSER server *in silico* to understand the sequence to structure paradigm. Furthermore, to get an insight about its regulation, genome walking was done and the *cis* acting regulatory elements were analyzed. As per the information gathered after *in silico* promoter analysis, the transcript profiling with plant hormones abscisic acid (ABA), methyl jasmonate (MeJa), salicylic acid (SA) as well as cold/freeze stress was performed. Interestingly, CBF binding site was present in the promoter. Therefore, the expression of HrCBF was analyzed under cold stress to relate its co-expression with HrCHI1 as its downstream target.

2. Materials and methods

2.1. Plant growth conditions and stress treatments

Seeds were isolated from berries of *Hippophae rhamnoides* collected from Keylong, Spiti valley of Himachal Pradesh, India. These were washed, dried and stored in a desiccator at RT and were germinated as previously described by Gupta and Deswal [19]. Seeds were grown at $24 \pm 2\,^{\circ}C$ with 270 $\mu mol/m^2/s$, 16 h light/8 h dark for 20 days. Cold treatment was given to seedlings at 4 °C (0, 0.5, 1, 3, 6, 24 h, 1 week) and freezing stress −10 °C (0, 1, 3, 6, 24 h). Seedlings were transferred to a solution containing 100 μM methyl jasmonate (MeJa) (Sigma Aldrich) and 1 mM salicylic acid (SA) (Sigma Aldrich) for phytohormone treatment (0, 3, 6, 24 h).

2.2. Nucleic acid isolation and cDNA preparation

DNA was isolated from the seedlings (1 g) using Cetyl- trimethylammonium bromide-based procedure. For RNA isolation, method described by Muoki et al. [21] was followed. RNA quality was checked spectrophometrically and by denaturing agarose gel electrophoresis. cDNA was prepared from DNaseI treated RNA (2 μg) using MMuLV-RT following manufacturer's instructions.

2.3. Cloning of Class I Chitinase and in silico analysis

H. rhamnoides belongs to the family Elaeagnaceae. Class I chitinases from plant systems were compared on homology basis and primers were designed from the sequence of basic chitinase of *Elaeagnus umbellata* (Accession No. AAC16011.1) [22]. The full length class I chitinase gene was amplified using 5′- CAC CAT GAA GTT ATG GGT AGT AAC AAT AAT TG-3′ and 5′-TTA CAT TGT ATC CAC CAA GAG TCC AGA CC -3′ as forward and reverse primers respectively using Advantage polymerase mix (Clontech) with DNA and cDNA as template. The PCR program included initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing 65 °C for 45 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The amplicon was excised from agarose gel (1.2%) and cloned in a pGEMT-Easy vector (Promega) as per the instructions of manufacturers. Plasmid with the insert was isolated and sequenced using an automated sequencer (Applied Biosystems). To search for protein functional sites, amino acid sequences were analyzed by Prosite online software (http://kr.expasy.org/cgi-bin/prosite/). Trans-membrane signal analysis and signal peptide analysis were also performed using amino acid sequences by TMHMM 2.0 online software (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/).

2.4. Three-dimensional structure prediction and validation

The secondary and three dimensional structure was predicted using the iterative threading assembly refinement (I-TASSER) server [23]. The stereochemical quality of the predicted structures was inspected using PROCHECK analysis. The phi/Psi Ramachandran plot obtained by PROCHECK server (http://www.eb.ac.uk/thronton-srv/databases/pdbsum/Generate.html) helped to check the residues in allowed and disallowed regions. The PDB file of modelled HrCHI1 was analyzed for structural motif analysis using PDBsum server (http://www.eb.ac.uk/thronton-srv/databases/pdbsum/Generate.html).

2.5. Construction of genome-walking libraries and cloning of promoters

DNA (2.5 μg) isolated from the seedlings using Cetyl- trimethylammonium bromide-based procedure was used for constructing libraries for genome walking (GenomeWalker Universal Kit; BD Bioscience, Clontech, USA). Genomic DNA was digested for 16 h with restriction enzymes *DraI, EcoRV, PvuII* and *StuI* separately to create blunt-end fragments and purified using phenol/chloroform. The restricted fragments were ligated to adaptors supplied by the manufacturer to produce four separate libraries. Promoter sequences were amplified by PCR using the Advantage Genomic Polymerase Mix (BD Bioscience, Clontech, USA) in a thermocycler. Primary PCR was performed with gene-specific primers 5′ CTTTCAATTCCGCTACCACTACCGCCAC 3′ and nested gene-specific primers 5′ CACCTGGGCAGACTTTGCCCCCAGCTTG 3′ and the nested adaptor primer (AP2) following manufacturer's instructions. The amplified PCR fragments were analyzed on 1.5% TAE agarose gel. The major amplicons were eluted and purified using PureLink Quick Gel Extraction Kit (Invitrogen) following manufacturer's instructions. The purified PCR fragments were sequenced and analyzed for prediction of putative promoter sequences using "plant *cis*-acting regulatory DNA elements" PLACE database (http://www.dna.affrc.go.jp/) [24].

2.6. Quantitative real time PCR

The transcript profiling of HrCHI1 was done under cold and freezing stress conditions at 4 °C and −10 °C respectively. The expression was also analyzed after salicylic acid (1 mM) and methyl jasmonate (100 μM) treatments by real-time quantitative PCR using Roche 480 Light Cycler (Roche diagnostics) using SYBR green dye (Thermo-Scientific). HrActin was used as internal control for quantitative real time PCR [18] and primer pair 5′ GCAAAG-