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Cathepsin B cysteine protease gene is upregulated during leaf senescence and exhibits differential expression behavior in response to phytohormones in *Picrorhiza kurrooa* Royle ex Benth.

Jai Parkash [a,b], Sanjeeta Kashyap [a], Shruti Kirti [a], Anil Kumar Singh [a,b,*], Som Dutt [a,*,1]

[a] Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, Himachal Pradesh 176061, India
[b] Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

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ABSTRACT

Medicinal importance of Picrorhiza (*Picrorhiza kurrooa* Royle ex Benth — an herb of western Himalayan region) and its endangered status in Red Data Book presses an urgent need for intensive R&D interventions towards ensuring its availability for the medicinal use, its sustainability and improvement. The present study was conducted on cathepsin B cysteine protease in *Picrorhiza*. Cathepsin B cysteine protease has been reported to function in diverse processes such as senescence, abscission, programmed cell death, fruit ripening and in response to pathogen and pest attacks. A full-length cDNA-*Pk-cbcp* encoding cathepsin B-like cysteine protease was cloned from *Picrorhiza*. The full length *Pk-cbcp* cDNA consisted of 1369 bp with an open reading frame of 1080 bp, 80 bp 5′ untranslated region and 209 bp 3′ untranslated region. The deduced Pk-cbcp protein contained 359 amino acids with a molecular weight of 39.981 kDa and an isoelectric point of 5.75. Secondary structure analysis revealed that Pk-cbcp had 28.97% α-helices, 14.48% β-turns, 19.50% extended strands and 37.05% random coils. Semi-quantitative PCR analysis revealed 157% higher expression of *Pk-cbcp* during senescence compared to that of pre-senescence. Further, application of phytohormones abscisic acid, jasmonic acid and cytokinin influenced the temporal expression status of *Pk-cbcp*. Abscisic acid and jasmonic acid increased the expression level whereas cytokinin reduced the expression. The findings suggest the role of *Pk-cbcp* in leaf senescence in Picrorhiza which may be differentially mediated through phytohormones.

1. Introduction

Picrorhiza (*Picrorhiza kurrooa* Royle ex Benth.), a small perennial herb (family Plantaginaceae), grows primarily in the northwestern Himalayan region at an altitude of 3000–5000 m above mean sea level. Its underground parts, rhizomes and roots, are widely used in traditional systems of medicine due to its antioxidative, hepatoprotective, antiproliferative, immunomodulatory, antibacterial and antiviral activities (Banerjee et al., 2008). The plant is self-regenerating but unregulated overharvesting has caused it to be threatened to near extinction and thus *Picrorhiza* has been listed in the Red Data Book as an endangered plant species (Kala, 2000). The presence of picrosides, the main medicinally active compounds, was reported in the leaves of *Picrorhiza* (Dutt et al., 2004). It was observed that in addition to rhizome and roots, leaves are also a good source of picrosides. However, the contents of these picrosides decrease sharply during the senescence phase (Singh et al., 2011). Thus, deeper insights into the understanding of leaf senescence phenomena in *Picrorhiza* may be of vital importance to devise and utilize the molecular strategies for delaying leaf senescence and increasing biomass production, and thereby improving the picroside contents.

In plants, senescence-associated proteolysis is a crucial process to relocalize nutrients from leaves to growing or storage tissues. Plant proteolytic enzymes are thought to be associated with developmentally programmed cell death in developing flowers, organ senescence and tracheary element differentiation (Beers et al., 2000). The degradation of leaf proteins by proteases provides a large pool of cellular nitrogen for recycling during senescence (Makino and Osmond, 1991). C1A cysteine proteases, grouped as cathepsin L, B, H, and F like cysteine proteases are the most abundant enzymes responsible for the proteolytic activity during leaf senescence (Martinez and Diaz, 2008). The role of individual C1A proteases has been reported in diverse processes such as senescence, abscission, programmed cell death, fruit ripening,

Abbreviations: Pk-cbcp, *Picrorhiza kurrooa* cathepsin B cysteine protease; RACE, rapid amplification of cDNA ends; CDD, conserved domain database; RT-PCR, reverse transcription-polymerase chain reaction; SOPMA, self-optimized prediction method with alignment; ABA, abscisic acid; JA, jasmonic acid; 6-BAP, 6-benzylaminopurine.

* Corresponding authors at: Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh 176061, India.
E-mail addresses: anil@ihbt.res.in, anils13@gmail.com (A.K. Singh), sd_bio@yahoo.com (S. Dutt).
[1] Present address: Crop Physiology and Biochemistry Division, ICAR — Central Potato Research Institute (ICAR-CPRI), Shimla, 171001 Himachal Pradesh, India.

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mobilization of proteins in seeds and tubers and in local and systemic defense in response to biotic stress (Grudkowska and Zagdanska, 2004; Van der Hoorn, 2008; Shindo and Van der Hoorn, 2008; McLellan et al., 2009). Although cysteine proteases have been reported to be associated with senescence in various plant species (Sugawara et al., 2002; Wagstaff et al., 2002; Martinez et al., 2008; Esteban-Garcia et al., 2010; Fan et al., 2009) the exact roles are yet to be fully understood. In the present study, we cloned the gene encoding cathepsin B cysteine protease from *Picrorhiza* (hereinafter referred to as *Pk-cbcp*) and analyzed its expression in relation to leaf senescence and three phytohormones: abscisic acid, jasmonic acid and cytokinin treatments.

2. Materials and methods

2.1. Plant material

Picrorhiza (*P. kurrooa*) plants used in the present study were collected from its natural habitat at Rohtang Pass (4000 m altitude, 32°23′N, 77°15′E, India) during December when the plants were dormant, and brought to the institute at Palampur (1300 m altitude; 32°06′N, 76°33′ E, India). These were transplanted in plastic pots and maintained in the experimental farm of the institute as described previously (Gangola et al., 2013; Parkash et al., 2014a,b; Sanjeeta et al., 2014).

2.2. Cloning of cDNA of Pk-cbcp

Total RNA was isolated from *Picrorhiza* leaf tissue using PureLink™ RNA Mini Kit (Invitrogen, USA) and treated with DNase I (RNase free) (Fermentas Inc., USA). Complementary DNA (cDNA) was synthesized from 2 μg of DNase treated total RNA as a template in 20 μl reaction volume by using cDNA synthesis kit (Invitrogen, USA) as described previously (Parkash et al., 2014b). Degenerate primers (*Pk-cbcp-dF1*, *Pk-cbcp-dR1*) for *Pk-cbcp* were designed from the conserved regions of corresponding gene reported from different plant sources, and the partial gene sequence was amplified by PCR as detailed in Table 1. The amplicon was cloned in pGEM-T Easy Vector (Promega, USA). Plasmids were isolated using Fermentas GeneJET™ Plasmid Miniprep Kit (Fermentas Inc., USA), and sequencing was performed using Big Dye terminator cycle sequencing mix (Version 3.1; Applied Biosystems, USA) using an automated DNA sequencer (ABI 3130 xl Genetic Analyzer, Applied Biosystems, USA). Protocols were followed essentially as described by the respective manufacturer. Full-length cDNA was cloned by performing rapid amplification of cDNA ends (RACE; SMARTer™ RACE cDNA Amplification Kit; Clontech, USA) as per the manufacturer's instructions using the gene specific primers (*Pk-cbcp-5′ RACE R1*, *Pk-cbcp-5′ N RACE R2*, *Pk-cbcp-3′ RACE F1* Table 1). These primers were designed based on the partial sequence of the gene as cloned above. After aligning the sequences obtained by 5′ and 3′ RACE, full-length cDNA was amplified using the end sequences (*Pk-cbcp-FlF1*, *Pk-cbcp-FlR2*), cloned in pGEM-T Easy Vector (Promega, USA) and confirmed by sequencing. Blastx analysis of full-length protein sequence of Pk-cbcp showed sequence similarity with different plant species available at NCBI database. Multiple sequence alignment of 29 full-length protein sequences along with three representatives of Arabidopsis cbcp proteins was performed using ClustalW2 program with default parameters. A phylogenetic tree was plotted using MEGA5.05 software by the Neighbor-joining method with 1000 bootstrap replicates.

2.3. In silico characterization of Pk-cbcp

Aliphatic index and hydropathy index were calculated using protein analysis toolbox ProtParam. Conserved domains were identified using the conserved domain database (CDD) available at NCBI website (http://www.ncbi.nih.gov/structure/ccdd/wrpsb.cgi). Secondary structure of the deduced protein was analyzed using Self-Optimized Prediction Method with Alignment (SOPMA; http://www.npsa-pbil.ibcp.fr/).

2.4. Search and expression analysis of cbcp protein using Arabidopsis microarray data

The hidden Markov Model profile of cbcp domain [Propeptide_C1 (PF08127)] was retrieved from Pfam (http://www.sanger.ac.uk/Software/Pfam). The profile was utilized to identify all putative cbcp proteins by searching against the annotated proteins in the whole *Arabidopsis* genome (*TAIR* v10). The microarray data for *Arabidopsis* cbcp under different developmental stages were taken from the AtGenExpress (http://jsp.weigelworld.org/expviz/expviz.jsp). The raw Affymetrix values were $\log_{10}$ transformed. A heatmap was generated, and hierarchical clustering was done using MeV software package (Eisen et al., 1998).

2.5. Application of phytohormones

The effect of exogenous application of abscisic acid (ABA), jasmonic acid (JA) and cytokinin on the expression status of *Pk-cbcp* gene was analyzed using leaf disc experimentation. For treatments, fully expanded, green leaves were detached and leaf discs (diameter 8 mm) were cut with a cork borer, each from different detached green leaves (12th week after transplantation) of *Picrorhiza* at active growth phase and floated abaxial side up in sterile water (in Petri plates) containing different concentrations of ABA, jasmonic acid and cytokinin (Sigma-Aldrich) hormones (50 μM, 100 μM and 500 μM) at different time intervals (6, 12,

Table 1
Oligonucleotide sequences and PCR conditions used in cloning and expression analysis of *Pk-cbcp* gene.

Name	Sequence (5′-3′)	PCR condition
Degenerate primers		
Pk-cbcp-dF1	GGTTCTTGYTGGGCWTTTGGTGCTGTTG	Initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C, 30 s; 53 °C, 40 s; 72 °C, 50 s. Final extension at 72 °C for 7 min
Pk-cbcp-dR2	TCCTCGTARACAGTRAARGMRACCTC	
Primers for RACE PCR		
Pk-cbcp-3′ RACE F1	GGTTCTTGYTGGGCWTTTGGTGCTGTTG	Primary PCR
Pk-cbcp-5′ RACE R1	GTAGTCCCCCATCCAATTAGCTTCAC	5 cycles of 94 °C, 30 s; 72 °C, 3 min, followed by 5 cycles of 94 °C, 30 s; 70 °C 30 s; and 30 cycles of 94 °C, 30 s; 68 °C 30 s 72 °C, 3 min
Pk-cbcp-5′ N RACE R2	GACAGTGAAAGAGACCTCAACTGGTC	Secondary PCR
Pk-cbcp-5′ N RACE R3	GGATCACACTCTTCAGTCACAACACC	30 cycles of 94 °C, 30 s; 68 °C, 30 s; 72 °C, 3 min. Final extension at 72 °C for 7 min
Primers for full length cloning of Pk-cbcp		
Pk-cbcp-Fl F1	ATGGGCAGGGCTAGGATATGTTCGTT	Initial denaturation at 94 °C for 3 min., followed by 33 cycles of 94 °C, 30 s; 53 °C, 45 s; 72 °C, 40 s. Final extension at 72 °C for 7 min
Pk-cbcp-Fl R1	TCAGAATGACGCATAACGTGAAACAT	
Primers for expression studies		
Pk-cbcp-expF1	GACTGAAGAGTGTGATCCTTACTT	Initial denaturation at 94 °C for 3 min., followed by 32 cycles of 94 °C, 30 s; 53 °C, 45 s; 72 °C, 40 s. Final extension at 72 °C for 7 min
Pk-cbcp-expR1	TAGACAGTGAAAGAGACCTCAAC	

Primers name with "F" and "R" represent forward primers and reverse primers, respectively.