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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.

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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.

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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.

18 and 24 h). The ABA solutions of different concentrations were prepared in 0.4% ethyl alcohol (95% v/v) (Asghar and Ebrahimzadeh, 2006) and jasmonic acid solution was initially prepared as 0.1 M stock solution in dimethylformamide (DMF) (w/v). Further dilutions of different concentrations (50 μM, 100 μM and 500 μM) were made by diluting the appropriate amount of stock solution in water (Creelman and Mullet, 1995; Chen et al., 2002). Similarly, for cytokinin treatment, 6-BAP was initially prepared as 0.1 M stock solution in DMSO. Dilutions were made by dissolving the appropriate amount of stock solution in a 0.5% (v/v) Tween 20 solution (Suttle, 1986). Control leaf discs were handled similarly but placed only in sterile water. All treatments were carried out at room temperature for 24 h. Total RNA was isolated from leaf discs (100 mg) using PureLink™ RNA Mini Kit (Invitrogen, USA). The cDNA was synthesized from DNA-free RNA using SuperScript® III Reverse Transcriptase (Invitrogen, USA). This cDNA was to be used as template for semi-quantitative RT-PCR analysis for the expression level of *Pk-cbcp*. For each treatment three biological replicates were used.

### 2.6. Semi-quantitative expression analysis by RT-PCR

Semi-quantitative RT-PCR based expression analysis was performed to study the expression level of *Pk-cbcp* mRNA during active growth stage versus senescence stage. To study the expression pattern of *Pk-cbcp* at four different stages of leaf development, sampling was carried out from 4th leaf (from the top) of *Picrorhiza* harvested at four different stages of plant development. The four stages were: (i) active growth stage (12th week after transplantation), (ii) pre-senescence stage (16th week after transplantation), (iii) early senescence stage (18th week after transplantation), and (iv) advance senescence stage (20th week after transplantation). The harvested samples were immediately stored at −80 °C. Total RNA was extracted from the harvested samples. To analyze the expression level of the cloned gene, total RNA was isolated from leaf tissue (100 mg) using PureLink™ RNA Mini Kit (Invitrogen, USA). The cDNA was synthesized from DNA-free RNA using SuperScript® III Reverse Transcriptase (Invitrogen, USA) as described previously (Parkash et al., 2014b). This cDNA was to be used as template for Reverse transcription-PCR reaction using gene specific primers (*Pk-cbcp-expF1*, *Pk-cbcp-expR1*) as mentioned in Table 1. Cycling conditions were optimized to obtain amplification under the exponential phase. *26S rRNA* based primer pair was used as internal control for expression studies (Singh et al., 2004). Amplicons were analyzed and quantified using the Alpha Digi Doc Gel Documentation and Image analysis system (Alpha Innotech, USA). Three biological replicates were used at each time point and the representative figure of one experiment is shown in the manuscript.

### 2.7. Estimation of chlorophyll contents

For measuring the chlorophyll content, leaf tissue (100 mg; leaf at 4th position from the top of 3 individual plants) was ground in liquid nitrogen

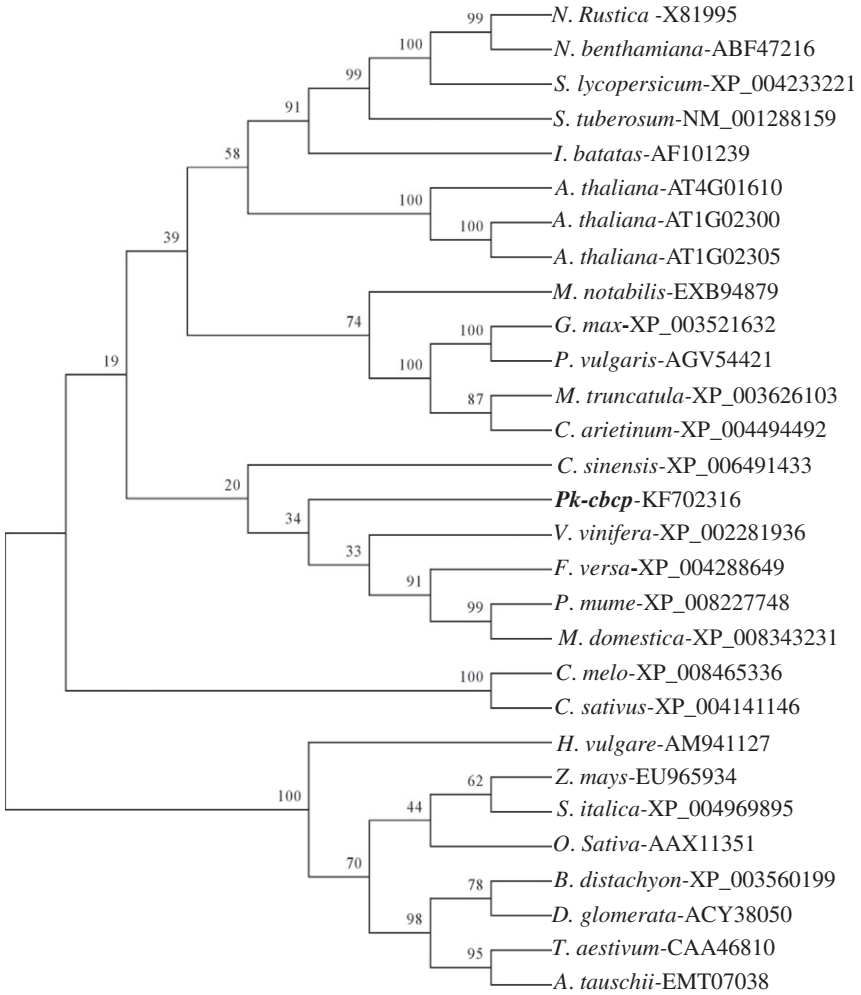


**Fig. 1.** Phylogenetic analysis of Pk-cbcp protein and cbcp proteins of other plant species. The phylogenetic tree was constructed by the neighbor-joining method using MEGA5.05 software. Branches were labeled with the different plant species and GenBank accession numbers. The tree was displayed as a phylogram in which branch lengths are proportional to distance. Numbers on the branches represent bootstrap values (for 1000 replicates).

separately and chlorophyll was extracted in 80% acetone. Chlorophyll was estimated according to the method of Porra et al. (1989) using the following equation: total chlorophyll (μg/ml) = 17.76 (A646.6) + 7.34 (A663.6), wherein A646.6 and A663.6 are absorbance at 646.6 and 663.6 nm respectively.

## 3. Results

### 3.1. Cloning of Pk-cbcp full length cDNA

Degenerate primers (Table 1) were designed using conserved regions of the reported *cbcp* from other plants. A partial cDNA fragment composed of 365 nucleotides of *Pk-cbcp* was amplified by RT-PCR. Blast analysis revealed strong homology of the cloned fragment with *cbcps* available in the NCBI database. Using the RACE method, full length cDNA of *Pk-cbcp* was amplified, cloned and subsequently confirmed by sequencing. The full length *Pk-cbcp* cDNA was 1369 bp long with an open reading frame (ORF) of 1080 bp (Supplementary Fig. S1). The ORF was flanked by an 80 bp 5′ untranslated region (UTR) and a 209 bp 3′ UTR. Sequence data from this study has been deposited in the GenBank database under the accession number KF702316. The deduced Pk-cbcp protein contained a total of 359 amino acids with a molecular weight of 39.98 kDa and an isoelectric point (pI) of 5.75. In Pk-cbcp, a conserved domain was detected between amino acid (aa) positions 100 and 333 (Supplementary Fig. S2). Analysis of Pk-cbcp using protein analysis toolbox Protparam revealed an aliphatic index of 69.81, a grand average of hydropathicity (GRAVY) as (−) 0.269 and the instability index was computed to be 38.53. In Pk-cbcp, SOPMA analysis revealed 28.97% α-helices, 14.48% β-turns, 19.50% extended strands and 37.05% random coils (Supplementary Fig. S3). A phylogenetic tree, constructed using the MEGA5.05 software (Tamura et al., 2011), was used to investigate the evolutionary relatedness of the Pk-cbcp amino acid sequence to cbcp proteins of other dicot and monocot plants. The phylogenetic tree was divided into five phylogenetic clades designated as I to V. The first four clades cover all dicot species while the fifth clade includes monocot species. Pk-cbcp was found to be clustered in the third clade along with that of *Vitis vinifera*-XP_002281936 (family Vitaceae), *Fragaria versa*-XP_004288649 (family Rosaceae), *Prunus mume*-XP_008227748 (family Rosaceae) and *Monodelphis domestica*-XP_008343231 (family Rosaceae) proteins (Fig. 1). The phylogenetic tree analysis showed a close relationship of *Picrorhiza* with Rosaceae family member plant species.

### 3.2. Expression of Pk-cbcp was higher during leaf senescence

To gain insights into the role of *Pk-cbcp* in regulating leaf senescence, the temporal expression of *Pk-cbcp* was investigated. The four stages of leaf development analyzed were: (i) active growth stage, (ii) pre-senescence stage, (iii) early senescence stage and (iv) advanced senescence stage; chlorophyll contents at these four stages were estimated to be 1.34 ± 0.39, 1.14 ± 0.30, 0.92 ± 0.33 and 0.46 ± 0.22 mg/g leaf fresh weight, respectively. Semi-quantitative RT-PCR based expression analysis was performed to study the expression level of *Pk-cbcp* mRNA during the abovementioned four developmental stages of Picrorhiza. *Pk-cbcp* transcript was found to be expressed during all the four stages (Fig. 2). However, 157% and 171% increases in transcript levels (*Pk-cbcp*) were observed in the advanced senescence stage as compared to pre-senescence and early senescence stage, respectively.

### 3.3. Microarray data analysis of Pk-cbcp gene isoform in Arabidopsis

In order to know the dynamics of *Pk-cbcp*-like transcripts in *Arabidopsis*, the microarray data for *Arabidopsis* under different vegetative and reproductive developmental stages at different time points were searched. Detailed analysis of *Arabidopsis* revealed three isoforms of *cbcp* gene [AT-*cbcp* 1 (AT1G02300), AT-*cbcp* 2 (AT1G02305) and AT-*cbcp* 3 (AT4G01610)] to be differentially regulated at different time points in response to various vegetative developmental and reproductive stages. AT-*cbcp* isoforms showed higher expression on 35 days senescing leaves, 21+ days flowers stage 15, sepal, 21+ days flowers stage 15, petals and 21+ days flowers stage 15, stamen (Supplementary Fig. S4).

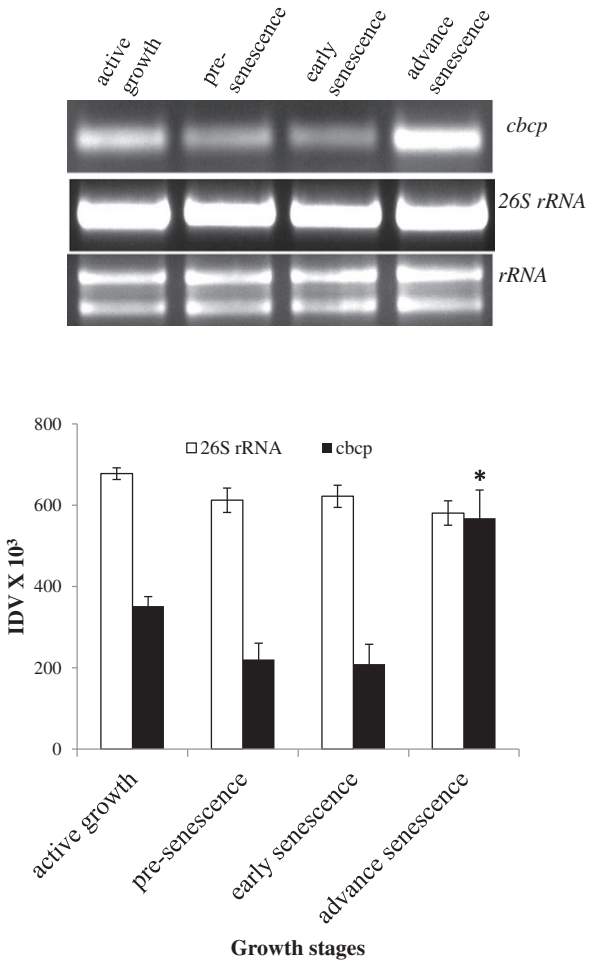


**Fig. 2.** Expression pattern of *Pk-cbcp* mRNA during active growth stage versus senescence stages. Total RNA was extracted from leaves of *Picrorhiza* that were harvested at different developmental stages [active growth stage (12th week after transplantation), pre-senescence (16th week after transplantation), early senescence (18th week after transplantation), advance senescence stage (20th week after transplantation)] and subjected to semi-quantitative RT-PCR based expression analysis. A constitutive 26S rRNA gene was used as an internal control for equal loading. Asterisks above the bar indicate statistically significant differences in expression at $P < 0.05$ using Student *T*-test.

### 3.4. ABA and jasmonic acid induce expression of Pk-cbcp

Exogenous application of ABA exhibited concentration dependent effects on the expression of *Pk-cbcp* gene in leaves of *Picrorhiza* (Fig. 3). With 50 μM ABA treatment, 23.1 ± 5.2%, 6.2 ± 10.5% and 11.4 ± 5.9% increases in the transcript level of *Pk-cbcp* were observed at 6 h, 12 h and 18 h of the treatment as compared to 0 h control. However at 24 h of 50 μM ABA treatment, a reduction by 18.5 ± 1.1% was observed in the transcript abundance of *Pk-cbcp* as compared to control. Application of 100 μM ABA resulted in change in the expression of *Pk-cbcp* by (+)30.7 ± 8.0, (−)34.4 ± 4.9, (−)16.2 ± 2.8 and (−)9.9 ± 6.2% at 6, 12, 18 and 24 h of ABA treatment, respectively. With 500 μM ABA, change of (+)27 ± 19, (+)108 ± 46, (+)146 ± 22 and (+)6 ± 11% in expression level of *Pk-cbcp* was observed at 6, 12, 18 and 24 h, respectively. The results indicate a positive regulation of *Pk-cbcp* by higher concentrations of ABA.

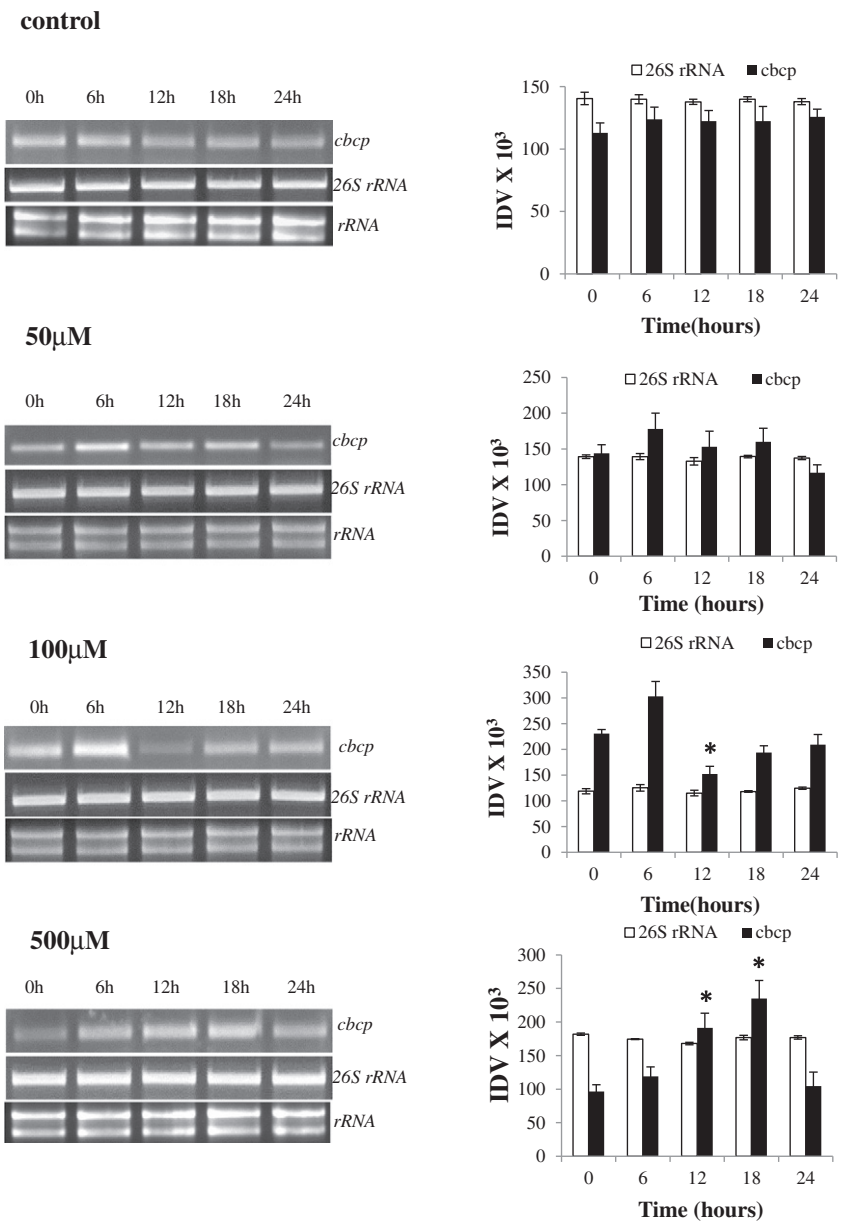


**Fig. 3.** Analysis of *Pk-cbcp* expression in response to ABA treatment. Leaf discs were treated with 50 μM (a), 100 μM (b), and 500 μM (c) ABA for the indicated time intervals. Total RNA was isolated from leaf discs treated with different concentrations of ABA and subjected to semi-quantitative RT-PCR analysis. A constitutive 26S rRNA gene was used as an internal control to normalize differences in template concentrations. Asterisks above the bar indicate statistically significant differences in expression at $P < 0.05$ using Student *T*-test.

In response to application of 50 μM jasmonic acid, (+)60 ± 10%, (−)14 ± 12%, (+)114 ± 14% and (−)2 ± 13% change was observed at 6, 12, 18 and 24 h of the treatment. Likewise, exogenous application of 100 μM jasmonic acid resulted in change in the expression of *Pk-cbcp* by (+)17.2 ± 6.3%, (+)66.8.0 ± 19.9%, (+)76.1 ± 15.2 and (+)36.3 ± 3.5% at 6, 12, 18 and 24 h of treatment, respectively. Also, (+)49.8 ± 12.9%, (+)36.1 ± 20.4% and (+)82.6 ± 29.2% and (−)19.3 ± 7.4% change in expression level of *Pk-cbcp* was observed at 6, 12, 18 and 24 h of 500 μM jasmonic acid treatment, respectively (Fig. 4). The overall results indicate the positive correlation in jasmonic acid treatment application and expression levels of *Pk-cbcp*.

### 3.5. Cytokinin decreased expression of Pk-cbcp

Leaf discs treated with cytokinin (50 μM) showed changes in *Pk-cbcp* expression by (+)4.3 ± 5.3%, (−)25.9 ± 2.7%, (−)6.0 ± 6.2% and (−)11.3 ± 10.8% at 6, 12, 18 and 24 h of treatment, respectively (Fig. 5). Likewise, exogenous application of 100 μM cytokinin resulted in changes in the expression of *Pk-cbcp* by (+)1.5 ± 12.8%, (−)6.0 ± 7.7%, (−)11.3 ± 15.4%, and (−)23.1 ± 12.1% at 6, 12, 18 and 24 h of treatment, respectively. In the case of 500 μM cytokinin treatment, (+)3.7 ± 3.0%, (−)38.7 ± 7.8%, (−)34.8 ± 4.7% and (−)25.7 ± 12.7% at 6,12, 18, and 24 h, respectively. This indicates an overall negative correlation in cytokinin and the expression levels of *Pk-cbcp*.

## 4. Discussion

*P. kurrooa* is a medicinal plant of northwestern Himalayan region and its medicinal properties are attributed to picrosides, which are iridoids with an iridane skeleton of monoterpene origin. Picroside-I and Picroside-II have been found to affect the activity of P-glycoprotein, which is one of the xenobiotic transport proteins implicated in multi-drug resistance in neoplastic tissues; thus also provide antioxidative,

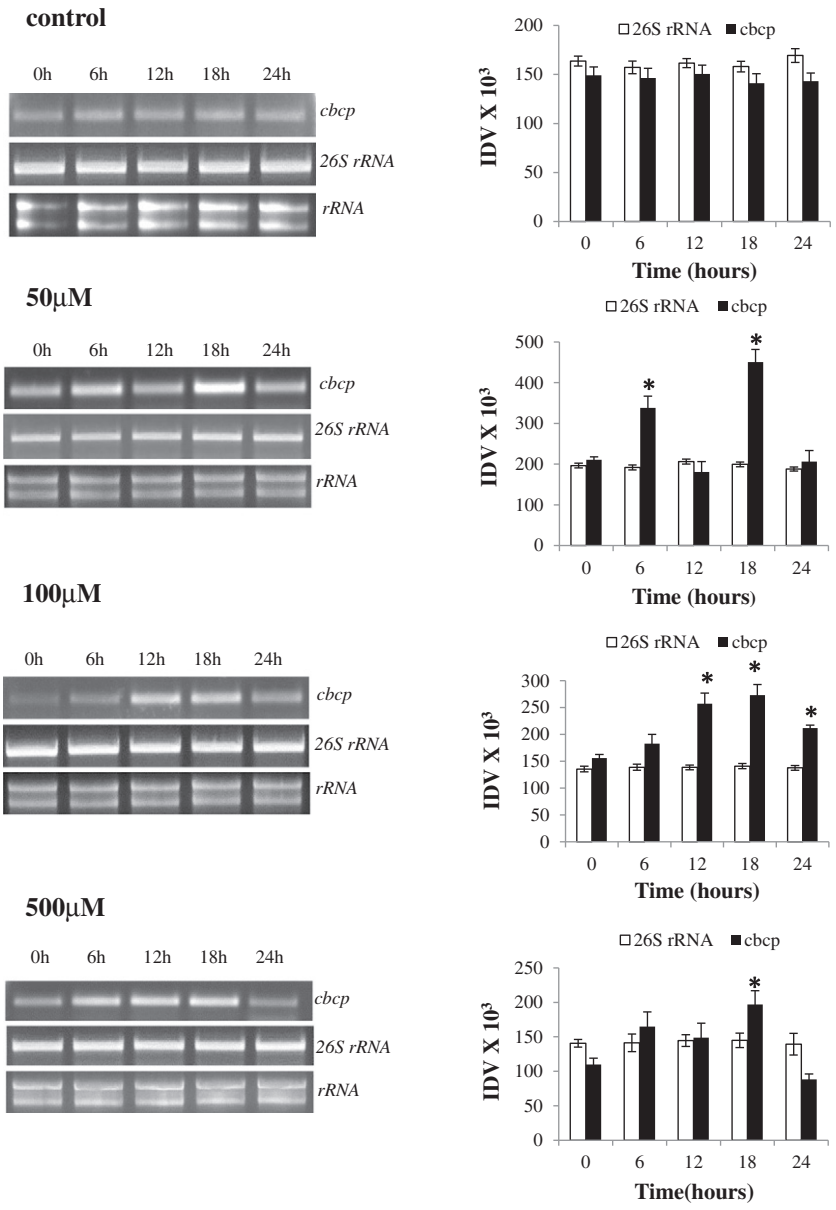


**Fig. 4.** Analysis of *Pk-cbcp* expression in response to jasmonic acid treatment. Leaf discs were treated with 50 μM (a), 100 μM (b), and 500 μM (c) jasmonic acid for the indicated time intervals. Total RNA was isolated from leaf discs treated with different concentrations of jasmonic acid and subjected to semi-quantitative RT-PCR analysis. A constitutive 26S rRNA gene was used as an internal control to normalize differences in template concentrations. Asterisks above the bar indicate statistically significant differences in expression at $P < 0.05$ using Student *T*-test.

hepatoprotective, antiproliferative, immunomodulatory, antibacterial and antiviral activities (Ansari et al., 1988; Najar et al., 2010; Banerjee et al., 2008). For the extraction of picrosides, roots and the rhizome of *Picrorhiza* are used, thus leading to complete uprooting of the plant. However, for the first time, we reported the presence of picrosides in leaves of *Picrorhiza* (Dutt et al., 2004) and suggested the use of *Picrorhiza* leaves as a source of picrosides which may also be helpful in the conservation of *Picrorhiza*, an endangered plant species (Singh et al., 2011). As picroside levels decrease during senescence, studies on leaf senescence of *Picrorhiza* become important. The precise biological function of the cathepsin-like proteases in plants is unknown at present, but it seems likely that they play an important role in regulating protein turnover in vivo (Lidgett et al., 1995).

In the present study we focused on cathepsin B cysteine protease, an enzyme reported to be associated with leaf senescence in other plant species. We cloned a complete cDNA of *cbcp* from *P. kurrooa*. The cloned *Pk-cbcp* showed sequence similarity with *cbcp* previously reported from various other plants like *V. vinifera*-XP_002281936, *F. versa*-XP_004288649, *P. mume*-XP_008227748, and *M. domestica*-XP_008343231 The high degree of similarity of *Pk-cbcp* with *cbcp* of *V. vinifera* and *F. versa* suggests that they may have a similar function. Semi-quantitative expression analysis revealed the presence of *Pk-cbcp* transcripts in the active growth stage as well as in the advanced senescence stage of *Picrorhiza*. However, 157% and 171% increases in transcript levels (*Pk-cbcp*) were observed in the advanced senescence stage as compared to pre-senescence and early senescence stage, respectively. These data therefore suggest the association of *Pk-cbcp* with leaf senescence in *Picrorhiza*. Previously, the role of cysteine proteases in regulating leaf senescence has been documented in various senescing tissues of plants. In sweet potato, higher expression of cysteine protease SPCP2 gene in natural senescent leaves has been reported (Chen et al., 2010). Similarly, Xiao et al. (2014) and McLellan et al. (2009) have reported the induction of cysteine protease transcripts during leaf senescence in *Capsicum annuum* and *Arabidopsis*, respectively. Three CathB

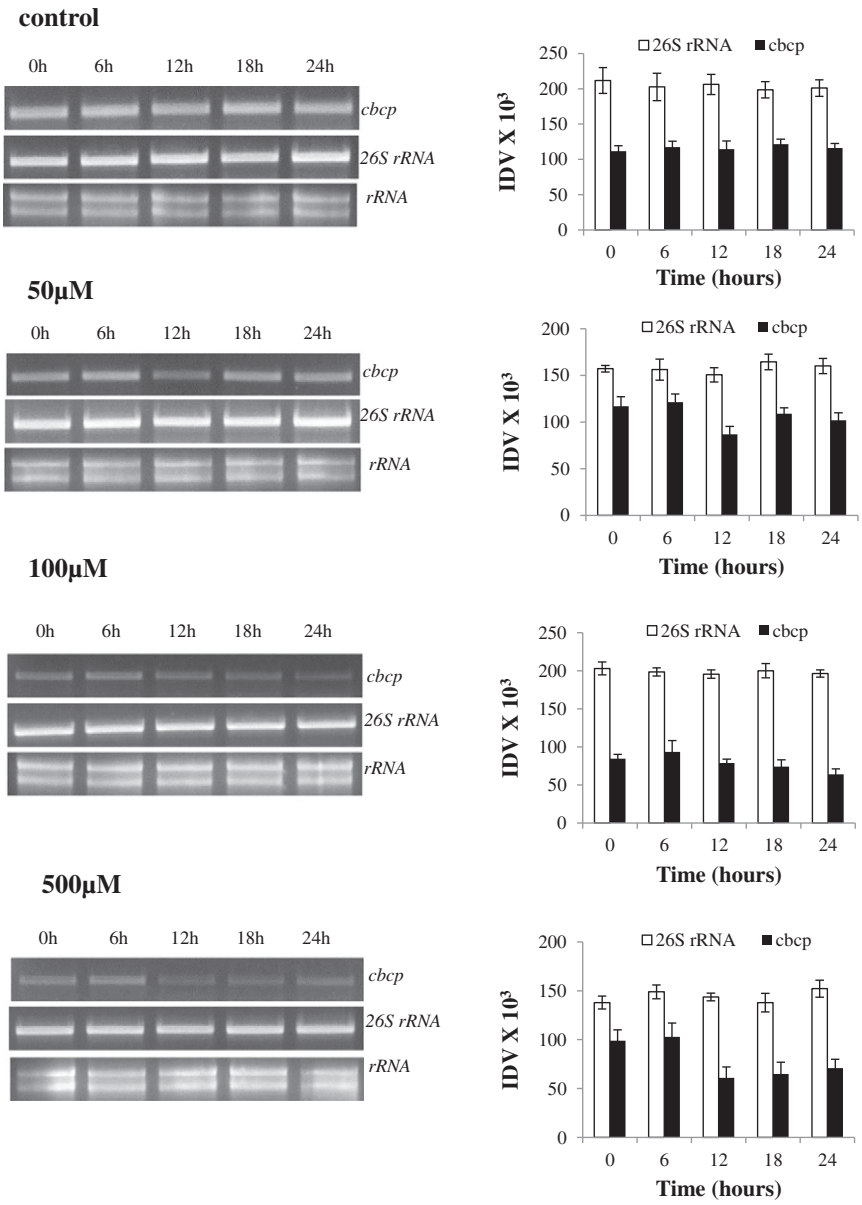


**Fig. 5.** Analysis of *Pk-cbcp* expression in response to cytokinin treatment. Leaf discs were treated with 50 µM (a), 100 µM (b), and 500 µM (c) cytokinin for the indicated time intervals. Total RNA was isolated from leaf discs treated with different concentrations of cytokinin and subjected to semi-quantitative RT-PCR analysis. A constitutive 26S rRNA gene was used as an internal control to normalize differences in template concentrations.

homologues (AtCathB1, AtCathB2, and AtCathB3) were identified in *Arabidopsis* and showed significant increases in expression after 2 days of dark-induced senescence. In contrast to these findings the transcripts of some cysteine protease genes have also been reported to be downregulated in senescent leaves such as *Nicotiana tabacum* NtCP2 and *Hemerocallis* spp. SEN102 (Guerrero et al., 1998; Beyene et al., 2006). In the present study, *Arabidopsis* microarray data analysis revealed higher expression of *cbcp* isoforms in senescing leaves. Recently, Iglesias-Fernandez et al. (2014) have reported that the transcripts of AtcathB1, AtcathB2 and AtcathB3 appeared throughout flower development in *Arabidopsis* while AtcathB3 isoform showed higher expression during flower development stage compared to other isoforms.

The initiation and progression of leaf senescence are known to be influenced by various internal and external factors (Smart, 1994; Nam, 1997; Park et al., 1998; Weaver et al., 1998). Among internal cues, hormones play a major role in regulating leaf senescence. For this reason, we examined the possibility of hormones in controlling the expression of *Pk-cbcp*. Exogenous application of ABA and jasmonic acid resulted in increased levels of *Pk-cbcp* transcript suggesting that *Pk-cbcp* may participate in positive regulation of ABA and jasmonic acid induced leaf senescence in *Picrorhiza*. ABA induced over-expression of a range of senescence associated genes have been reported in various plants (Woo et al., 2001). Oh et al. (1996) reported a marked increase in the mRNA level of a senescence associated gene, sen1, in *Arabidopsis* upon exogenous application of 100 mM ABA. Similarly, Zhang and Gan (2012) reported induced expression of senescence associated gene SAG113 in response to 1 h and 3 h treatment of ABA in *Arabidopsis*. Also, Yap et al. (2003) have reported ABA mediated over-expression of senescence associated gene SPA15 in sweet potato. NAC transcription factor *OsNAP* is induced specifically by 50 µM ABA in rice, with expression levels increasing approximately threefold after 2 h of ABA treatment and reached up to 16-fold after 4 h, 68-fold after 6 h, and 102-

fold after 12 h of treatment (Liang et al., 2014). The present results suggest the involvement of *cpcb* gene in ABA mediated regulation of leaf senescence in *Picrorhiza*.

External application of jasmonic acid exhibited positive correlation with expression levels of *Pk-cbcp*. Exogenous application of jasmonic acid has been reported to influence the expression of various senescence associated genes. For instance, in response to jasmonic acid application an increase in the trypsin inhibitory activity in barley leaves was reported by Casaretto et al. (2004). He et al. (2002) reported that exogenous application of jasmonic acid (30 μM) caused premature senescence in attached and detached rosette leaves in *Arabidopsis*. Cao et al. (2006) has reported the involvement of jasmonic acid in the induction of leaf senescence by potassium deficiency in *Arabidopsis*. Shan et al. (2011) identified a COI1-dependent jasmonic acid-repressed protein, Rubisco activase, in *Arabidopsis* and loss of Rubisco activase led to typical senescence-associated features. In contrast to these reports Chen et al. (2002) reported that methyl jasmonate did not induce the senescence associated gene SPG31 expression in leaves of sweet potato.

In the present study external application of cytokinin resulted in a reduction in expression levels of *Pk-cbcp* transcript. Shah et al. (2013) analyzed the expression of GhNAC transcription factor genes (GhNAC8–GhNAC17) in response to exogenous cytokinin (6-BAP) at different time intervals (0, 2, 4, 6, 8, 12 and 24 h) and found that exogenous cytokinin for 6–12 h triggered the expression of GhNACs significantly beyond control. On the other hand, the expression of GhNAC9 and GhNAC10 stayed nearly the same or decreased compared to control. Goldthwaite and Laetsch (1967) analyzed the regulation of senescence of bean leaf discs in response to 6-BAP and found that 6-BAP reduced the loss of chlorophyll and protein from the leaf discs. Similarly, cytokinins (kinetin and 6-BAP) were effective in retarding senescence in leaf discs of Rumex (Goldthwaite and Laetsch, 1968) and *N. tabacum* (Pathak et al., 2006). Thus the present findings suggest that the association of *cpcb* with leaf senescence in *Picrorhiza* might also be regulated through cytokinin.

## 5. Conclusions

A full length cDNA encoding cathepsin B-like cysteine protease was cloned from *Picrorhiza*. An expression profile of *Pk-cbcp* during leaf development revealed that *Pk-cbcp* was associated with leaf senescence. The cloned *Pk-cbcp* was found to be significantly up-regulated in response to phytohormones, especially abscisic acid and jasmonic acid, suggesting the role of *Pk-cbcp* in leaf senescence which might be mediated through ABA and jasmonic acid signaling. Thus, *Pk-cbcp* may be an important candidate to knock out or suppress for delaying leaf senescence in *Picrorhiza*. *Pk-cbcp*-silencing transgenic plant can be used to study the precise function of *cbcp* in *Picrorhiza*.

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