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Isolation and characterization of *Calcineurin B-like* gene (*PbCBL1*) and its promoter in birch-leaf pear (*Pyrus betulifolia* Bunge)

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ABSTRACT. Calcium plays a critical role in regulating abiotic stress responses in plants. Calcineurin B-like (CBL) proteins are calcium sensors in calcium signaling pathways. However, the molecular mechanisms underlying calcium signaling remain to be elucidated. In this study, the *CBL1* gene, which codes for the CBL protein, was isolated from the birch-leaf pear. One 2,969-bp sequence was cloned using PCR, and using the cloned 2,027-bp sequence was isolated from pear genomic DNA via genome walking. Sequencing analysis revealed that the 4,996-bp sequence was a *PbCBL1* gene consisting of eight exons and seven introns, and the 2,027-bp sequence was identified as the promoter of the *PbCBL1* gene, which contains the basic promoter elements TATA and CAAT boxes. In addition, some other *cis*-acting elements including heat, cold, drought, and hormone responsive elements were also present. To further investigate the activity of this promoter, the sequence was used to drive a *GUS* fusion gene into leaf discs of tobacco (*Nicotiana benthamiana*) with *Agrobacterium*-mediated transformation method. *GUS* gene expression could be regulated by the *PbCBL1* promoter following induction by GA, ABA,

SA, and MeJA. Furthermore, the results of real-time RT-qPCR indicate that the *PbCBL1* gene can respond to changes in the intracellular calcium concentration, and that it can be induced by cold, heat, drought, and stress by several hormones including GA, ABA, SA, and MeJA. *PbCBL1* gene may be involved in several signal transduction pathways, and play an important role in the condition of adversity stress in pear.

Key words: Environmental stress; Expression characteristic; *GUS*; *PbCBL1*; *Pyrus betulifolia* Bunge; Promoter

INTRODUCTION

Plants have evolved complex signaling pathways to respond to stressors, and have acquired plasticity in both their metabolic functions and developmental switches in order to adapt to changing environmental conditions (Huang et al., 2012). The study of stress-related genes and their upstream regulatory mechanisms is essential to reveal how plants respond to stress.

The responses of plants to stress signals are encoded by different Ca^{2+} signatures. Calcium plays a crucial role as a secondary messenger and mediates various defensive responses to environmental stimuli (Batistič and Kudla, 2012). Calcineurin B-like protein (CBL) is a primary calcium sensor that has been isolated and identified in several higher plants (Luan, 2009; Weinl and Kudla, 2009). Recent studies indicate that *CBL* is an upstream regulator stress gene expression and that overexpression of *CBL* may confer tolerance to multiple kinds of stress in plants (Wang et al., 2007; Gu et al., 2008; Guo et al., 2010). Within the *CBL* gene family, *CBL1* can be induced by diverse stress signals in *Arabidopsis*, including drought, cold, injury, and salinity (Albrecht et al., 2003). Many previous studies on the expression of *CBL* gene have demonstrated distinct or even opposite patterns when under different abiotic stresses, including *Populus* (Zhang et al., 2008). We predicted that such variation in expression patterns might be caused by the upstream regulatory effect.

Gene promoters are located upstream of the coding region in DNA sequences, and often direct the transcription of a gene. The constitutive cauliflower mosaic virus (CaMV) 35S promoter has been used widely in genetic manipulation of plants because it is highly transcribed in a variety of plant tissues (Wu et al., 2008; Monika et al., 2010). However, the use of such constitutive promoters - i.e., those that direct expression in nearly all tissues, across a wide range of taxa, may have certain limitations, such as a lack of temporal and spatial regulation. This precludes their use in driving the expression of genes that are harmful to plants when chronically over- or under-expressed (Wang et al., 2013). Any differential expression of target genes is likely due to the activity of regulatory elements in their promoters regions (Wu et al., 2012). Compared to a constitutive promoter, a gene-specific promoter affects the growth and development of transgenic plants more mildly, making them a better option for use in the study of target gene expression (Wei et al., 2006).

The birch-leaf pear tree (*Pyrus betulifolia* Bunge) originates from China and is one of the most widely used rootstocks for pear production. It is highly adaptable and is tolerant to cold, drought, flooding, and fertile land (Chang et al., 2012). To date, biological studies on the pear plant have focused mainly on genetic diversity, reproductive physiology, and cell signal

transduction (Wu et al., 2011). Although the *CBL1* gene is an important part of calcium signaling pathways, little is known about its regulatory mechanisms. Isolating the upstream regulatory region of *PbCBL1* in the pear, should contribute to our understanding of the molecular mechanisms of calcium signaling cascades in plants (Guo et al., 2010).

The aim of this study was to assess the structural and molecular characteristics of the *CBL1* gene in *P. betulifolia* Bunge. The complete *PbCBL1* gene and its promoter sequence were isolated directly from genomic DNA using routine PCR and genome walking. We also gained further insight about the activity of the *PbCBL1* gene promoter from transient expression of a *GUS* fusion reporter gene transfected into tobacco plants under hormone induction, including gibberellin (GA), abscisic acid (ABA), salicylic (SA), and methyl jasmonate (MeJA). Moreover, profiles of *PbCBL1* gene expression were verified by RT-qPCR under various experimental conditions. Our results augment current knowledge of the structural and functional characteristics of *PbCBL1*, and thus facilitate a better understanding of the molecular mechanisms underlying the response to various environmental stresses in pear.

MATERIAL AND METHODS

Plant materials and DNA preparation

Birch-leaf pear (*P. betulifolia* Bunge) seedlings growing at the National Pear Germplasm Repository in Nanjing, China were used throughout this study. Seeds were washed with distilled water and germinated in soil in a green house. For RT-qPCR analysis, the seedlings of *P. betulifolia* Bunge at the five leaf stage were grown in 1/2 MS nutrient solution containing $CaCl_2$ solution at varying concentrations (0, 100, 200, 300, and 400 mM) at room temperature for 6h, or 10% (w/v) PEG6000 solution at varying timepoints (0, 2, 6, 12, and 24 h) to simulate drought stress. Seedlings were also exposed to varying temperatures (24°, 4°, and 42°C for 6h). For different hormone treatments, seedlings of *P. betulifolia* Bunge at the five leaf stage were grown in 1/2 MS nutrient solution containing different concentrations of hormones [GA (100 μM), ABA (50 μM), SA (1 mM), and MeJA (50 μM)] for various duration (0, 2, 6, 12, and 24 h). Seedlings grown in 1/2 MS nutrient solution without hormone or stress treatment were used as control plants. Leaves were collected in triplicate, frozen in liquid nitrogen, and stored at -80°C (except for fruits) until total RNA extraction.

Genomic DNA was isolated using the CTAB method and directly used for PCR amplification (Liu et al., 2003). Total RNA was extracted with a Simply P total RNA extraction kit (BioFlux, China) according to the manufacturer procedure.

Tobacco leaves were disinfected in 10% bleach with a few drops of Tween 20 for 10 min using a stirrer. The leaves were rinsed five times with sterile distilled water with agitation; 0.5-cm disks were cut, taking care to avoid the midrib in preparation for *Agrobacterium* tumefaciens transfection (Burow et al., 1990).

Cloning of the 5'-flanking DNA sequence of *PbCBL1* coding segment

To clone the DNA and cDNA sequences of *PbCBL1* gene, the primer pair PbCBL1-F and PbCBL1-R was designed based upon the cDNA sequence of the *PbCBL1* gene in NCBI

(Accession No: KF578142.1). PCR was performed in a total volume of 25 µL containing 2.0-mM Mg^{2+}, 0.15-mM dNTPs, 0.4-mM of each primer, 0.8-U Taq DNA polymerase (TAKARA), and 15-ng genomic DNA or cDNA under the following conditions: an initial denaturation step at 94°C for 3 min, 35 cycles at 94°C for 50 s, 56°C for 50 s, and 72°C for 100 s, a final extension at 72°C for 10 min, with a final hold at 4°C. The PCR products were separated, recovered from agarose gels, and sequenced with an ABI 3730 after T-A cloning. The PMD18-T simple vector (TAKARA) was used to clone the PCR fragment.

Isolation of the upstream regulatory region was conducted with the BD Genome-Walker Universal Kit User Manual (Clontech, USA). Aliquots (2.5 µg) of genomic DNA from pear leaves were digested with four restriction endonucleases, *Dra*I, *Eco*RV, *Ssp*I, and *Pvu*II to produce blunt ends, at 37°C for 14-16 h, and were then placed at 70°C for 15 min to stop the reaction. To prepare the adaptor, a total volume of 50 µL containing 50-µM Adaptor 1 (AD1) and Adaptor 2 (AD2) was denatured at 95°C for 5 min, transferred to the 70°C water bath, and then kept at room temperature for 1 h until further use. The digested fragments were ligated to the adaptor at 16°C overnight, and the ligation products were diluted 10-fold for further use.

The outer adaptor primer 1 (AP1) and gene-specific primer (PbCBL1R1) were used in primary PCR, while the inner adaptor primer 2 (AP2) and nested gene-specific primer (PbCBL1R2) were used in a second round of amplification (Table 1). The primary PCR involved a total volume of 50 µL, containing 100-ng DNA-ligase, 2.5-mM dNTP, 10X PCR buffer (with Mg^{2+}), 2.5-U Taq DNA polymerase, and 10-pm primers (AP1 and PbCBL1R1). The primary PCR amplification products were diluted 50-fold and used as templates in the second round of PCR (thermal cycling conditions are listed in Table 2). The second round PCR products were resolved on a 1.2% agarose gel, and the longest and distinct bands were recovered and sequenced after T-A cloning. The promoter's transcription start sites (TSS) were predicted using the Softberry databases (http://linux1.softberry.com/berry.phtml?topic=tssp&group=program&subgroup=promoter) (Liu et al., 2011). The TFSearch program was used for searching putative transcription factor-binding sites (Heinemeyer et al., 1998). PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and PLACE (http://www.dna.affrc.go.jp/PLACE/) databases were used to determine *cis*-acting regulatory elements for plants (Higo et al., 1999; Lescot et al., 2002).

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')
PbCBL1-F	TAGATGCTCTAAATGGGTTG
PbCBL1-R	TCATGTAGCAATCTCATCA
AD1	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'
AD2	5'-PO4-ACCAGCCC-NH2
PbCBL1R1	GGAGAAACGAAACAAGTAGG
PbCBL1R2	ACAAGCTGTTTGCGAAGC
PbCBL1F3	TTAATTAAATTAAGGTCATGTGGTATGCTTCA
PbCBL1R3	GGCGCGCCTTAGAGCATCTACCTACCAACACC
PbCBL1F4	AAGATAGACAAGTATGAATGGAAC
PbCBL1R4	TGTAGCAATCTCATCAACCT
ACT2/7-F	CTCCCAGGGCTGTGTTTCCTA
ACT2/7-R	CTCCATGTCATCCCAGTTGCT

Table 2. Cycling conditions for touchdown PCR used to amplify the *PbCBL1* gene promoter.

Program No.	No. of cycles	Cycle conditions
1	2	94°C 25 s, 72°C 3 min
2	2	94°C 25 s, 70°C 3 min
3	2	94°C 25 s, 68°C 3 min
4	2	94°C 25 s, 66°C 3 min
5	32* (25)**	94°C 25 s, 72°C 3 min
6	1	64°C 10 min

*Number of cycles of touchdown PCR for the primary PCR. **Number of cycles of touchdown PCR for the secondary PCR.

Construction of *PbCBL1* promoter-*GUS* gene fusion vector

Based on the 5'-upstream regions of *PbCBL1*, the primers PbCBL1F3 and PbCBL1R3 (Table 1), which harbor *Asc* I and *Pac* I recognition sites, were designed to amplify the genomic DNA of pear. Amplified fragments were cloned into the pMD18-T simple vector, and confirmed by restriction enzyme digestion and DNA sequencing. The confirmed fragments were then digested with *Asc* I and *Pac* I, and cloned directionally into the binary vector PS1aG-3, obtained from the Plant Industry division of CSIRO, Australia (http://www.pi.csiro.au) with the β-glucuronidase (*GUS*) reporter gene to construct the *PbCBL1*::*GUS* fusion vector (Ai et al., 2009) (Figure 1).

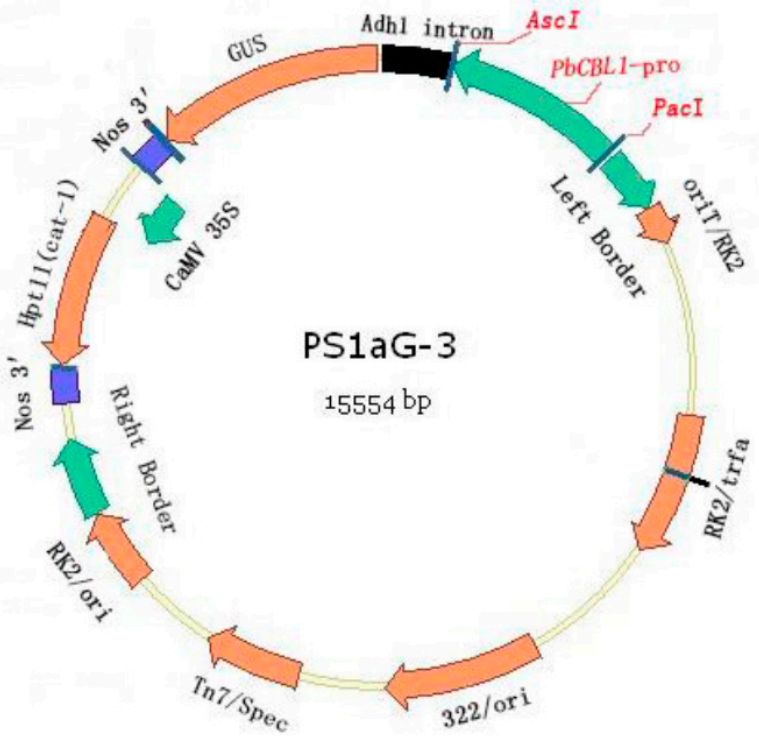

Figure 1. Structure of the *PbCBL1*-promoter sequence that was inserted into binary vector PS1aG-3.

Genetic transformation and histochemical localization of *GUS* expression

The *PbCBL1*:*GUS* fusion vector was verified by sequencing and transferred into *A. tumefaciens* strain GV3101 using the freeze-thaw technique (Burow et al., 1990). The culture media used, and procedures for *A. tumefaciens* suspension transformation were previously published methods (Li et al., 2009). The *GUS* fusion gene was then inserted into the prepared tobacco leaf discs using *Agrobacterium*-mediated transformation (Horsch et al., 1985). GA (100 µM), ABA (50 µM), SA (1 mM), and MeJA (50 µM) were added into co-culture medium to induce expression, and transformation of the binary vector without the promoter insert was used as a negative control. The incubated tobacco leaf discs were used for histochemical staining and *GUS* analysis: *GUS* activity was assessed histochemically using the chromogenic staining substrate 5-bromo-4-chloro-3-indyle-β-D-glucuronide (X-Gluc) (Raghothama et al., 1997). The samples were immersed in the GUS staining solution [1-mM X-Gluc, 2-mM $Fe^{2+}CN$, 2-mM $Fe^{3+}CN$, 10-mM Na_2EDTA, 0.1% (v/v) Triton X-100, 100-mg/mL chloramphenicol in 50-mM sodium phosphate buffer at pH 7.0] for 2 to 3 days at 37°C. The stained tissue sections were immersed in 95% ethanol to remove chlorophyll, and then photographed with an Olympus MVX10 stereomicroscope (Olympus Instrument, Japan).

Real-time quantitative RT-PCR

To evaluate the expression profiles of the *PbCBL1* gene under various experimental conditions, its relative transcript levels were normalized to *ACT2/7* in this study. Primers were designed using the Beacon Designer 7.0 software and are listed in Table 1. RT-qPCR was carried out on 96-well plates with an ABI7500 Real-Time PCR Detection System (Applied Biosystems, USA) using SYBR Green Master ROX (Roche, Japan), following the manufacturer instructions. Reaction mixtures and conditions were performed as described by Xu et al (2012). All reactions were performed with three replicates and the relative expression levels of *PbCBL1* were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical differences were assessed with Duncan's test at a significance level of $P < 0.05$ with the DPS software (Hangzhou, China). Calculations were carried out with the Microsoft Excel software.

RESULTS AND DISCUSSION

DNA sequence isolation of *PbCBL1* gene

The DNA sequence was 3.0-kb long (GenBank accession no. KF578143.1) (Figure 2), and it comprised eight exons and seven introns, with the length of exons ranging from 53 bp to 113 bp (Table 3). Analysis of the amplified DNA sequences revealed that eight exons encode a protein that is 213 amino acids long, with a theoretical isoelectric point of 4.63 and a molecular mass of ~24.55 kDa. By aligning the amino acid sequence of PbCBL1 with AtCBL1, we found that the structure was fairly conserved and the protein similarity was 87.5%. Comparison to the EF-hand motifs of CBL1 in *Arabidopsis*, along with motif scanning in PROSITE, revealed that the PbCBL1 protein possesses four EF-hand motifs, with six amino acid differences among them. Furthermore, the number of EF-hand motifs and spacing do not vary between AtCBL1 and PbCBL1 (Figure 3). There are 23 amino acids between EF1 and EF2, 25 amino acids between EF2 and EF3, and 32 amino acids between EF3 and EF4, which is identical to the PtCBL proteins of *Populus trichocarpa* (Albrecht et al., 2003; Zhang et al., 2008).

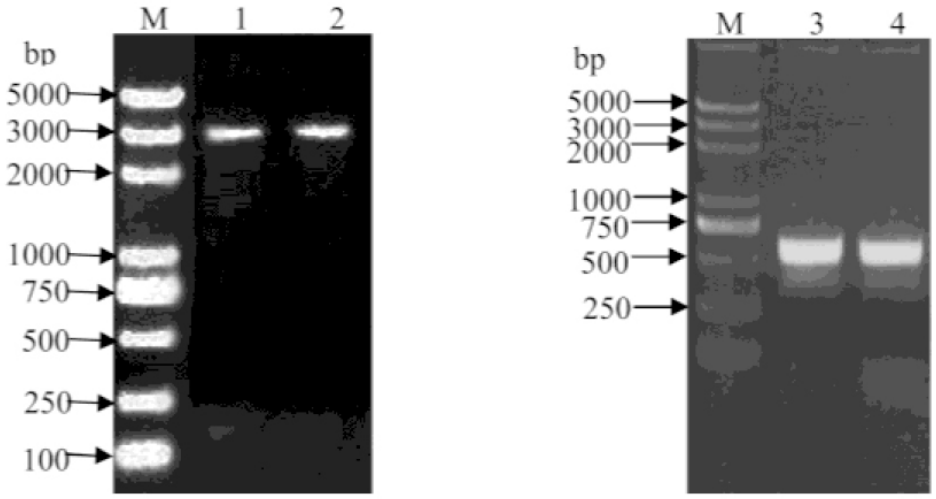

Figure 2. Detection of *PbCBL1* DNA and cDNA by agarose gel electrophoresis. *Lanes 1* and *2* represent the amplified cDNA products and *lanes 3* and *4* represent the amplified DNA products. *Lane M* = molecular marker.

Table 3. Description of *PbCBL1* exons compared with *AtCBL1* exons.

Character	Genomic coordinate	mRNA coordinate	mRNA length	Similarity (%)	Mismatches
Exon 1	1-79	1-79	79	67.1	26
Exon 2	545-627	80-162	83	79.5	17
Exon 3	1044-1103	163-222	60	65.0	21
Exon 4	1560-1668	223-331	109	83.5	18
Exon 5	1802-1854	332-384	53	73.6	14
Exon 6	2278-2358	385-465	81	87.7	10
Exon 7	2701-2813	466-578	113	70.8	33
Exon 8	2906-2969	579-642	64	84.4	10

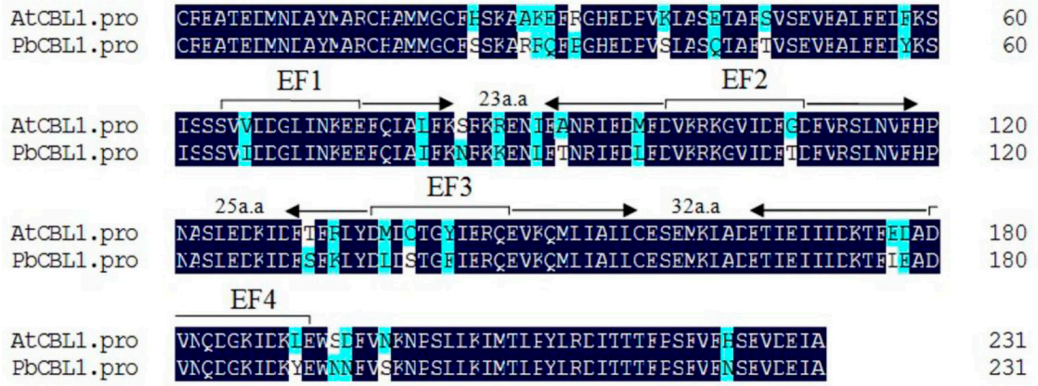

Figure 3. Amino acid sequence alignment of AtCBL1 and PbCBL1. White letters on a dark background represent amino acids with complete homology, while those sharing a non-identical conserved identity are shadowed in blue. Both the size of the linking regions between the EF-hands and their numbers are indicated. Alignment was performed with the DNAMAN 6.0 software program.

Isolation of the 5'-flanking regions of *PbCBL1*

Gene promoters are one of the most important regulatory elements of transcription, and the association of RNA polymerase and transcription factors ensures accurate and effective transcription initiation. Promoters control expression of a given gene in a particular organism, at a specific developmental stage, and/or under certain environmental conditions (Wu et al., 2008; Butler and Hannapel, 2012). Moreover, the gene promoter is a key factor required for the timely and spatially accurate control of exogenous gene expression in transformed plants (Lightfoot et al., 2013). Therefore, the identification of a suitable promoter is essential in the investigation of the regulatory mechanisms of gene expression when using transgenic technology.

In this study, improved genome walking kits that are based on ligation-mediated PCR have now been designed as a powerful method to search unknown genomic DNA sequences that are adjacent to known sequences, including promoter regions (Chang et al., 2009; Liu et al., 2011; Fang et al., 2013). For the experiments reported here, success absolutely depended on the appropriate design of the DNA walker adaptor and on the selection of suitable restriction enzymes. The design of specific primers is also important for genome walking progress, and are usually designed derived from the first exon. In this experiment, specific primers were selected using a touchdown PCR program to have a melting temperature of 65°-75°C, a length of 18-24 bp, and a GC content of 45-55%. Touchdown PCR typically uses an annealing/extension temperature that is 3°-10°C higher than the melting temperature of the primers that are used during the initial PCR cycles (typically 5-10 primers). At this higher temperature, although the primer is much more specific, it is less efficient. The temperature is then reduced to that of the primer melting temperature for the remaining cycles, and repeated cycles are used to ensure that digestion is complete (Fang et al., 2013). We digested the genomic DNA of pear using four restriction endonucleases and then used the adaptor for ligation. Some smears and nonspecific bands were obtained from the primary PCR, while the secondary PCR performed with specific primers generated distinct DNA fragments from 0.2 to 2.0 kb in length (Figure 4) and the longest fragment was sequenced after T-A cloning. Analysis of the 5'-upstream region of the obtained *PbCBL1* gene revealed that the previously known regions of our sequence shared 99% homology with the cDNA of *PbCBL1* in GenBank (Accession No. KF578142.1).

The 2,027-bp product isolated from pear genomic DNA was identified as the promoter region of *PbCBL1*. Prediction by the Softberry databases showed that TSS was located 175 bp upstream of the ATG codons in the *PbCBL1* gene promoter (Figure 5). To predict the functional elements present in the promoter region of *PbCBL1*, the PLACE and PlantCARE databases were used to identify matches with the *cis*-regulatory elements of the *PbCBL1* promoter. This revealed the presence of several *cis*-acting enhancer elements and DNA motifs commonly associated with functional specific promoters. These included the CAAT and TATA boxes (TTT AAAAA) 127 bp upstream of the ATG code and the 5'UTR Py-rich stretch, which is a *cis*-acting element that confers high levels of transcription (Yamaguchi-Shinozaki and Shinozaki, 1993; Diaz-De-Leon et al., 1993; Daraselia et al., 1996). In addition, multiple light-responsive motifs including the G-box, GAG-motif, Gap-box, I-box, Box4, Sp1, TCCC-motif, as-2-box, ACE, MRE, and several other regulatory elements that are involved in growth and development were identified (Kuhlemeier et al., 1987; Sibéril et al., 2001). Furthermore, other *cis*-acting elements that are involved in defense and in the response to stress, such as TC-rich repeats, the abscisic acid responsiveness element (ABRE), anaerobic induction element (ARE), gibberellin-responsiveness element (GARE-motif and P-box), heat stress responsiveness

element (HSE), low-temperature responsiveness (LTR), drought-inducible element (MBS), salicylic acid responsiveness (TCA) element, and MeJA-responsiveness (CGTCA-motif and TGACG-motif) were also identified (Yamaguchi-Shinozaki and Shinozaki, 1993; Manjunath and Sachs, 1997; Venter, 2007) (Table 4). The discovery of elements that are involved in stress responses, including the action of hormones, may indicate that the *PbCBL1* promoter plays an important role in regulating the expression of anti-adversity genes in pear. These results are also consistent with previous studies that have demonstrated that the *CBL1* gene is strongly expressed under various environmental stresses and also in response to hormone induction in several plant species (Zhang et al., 2008).

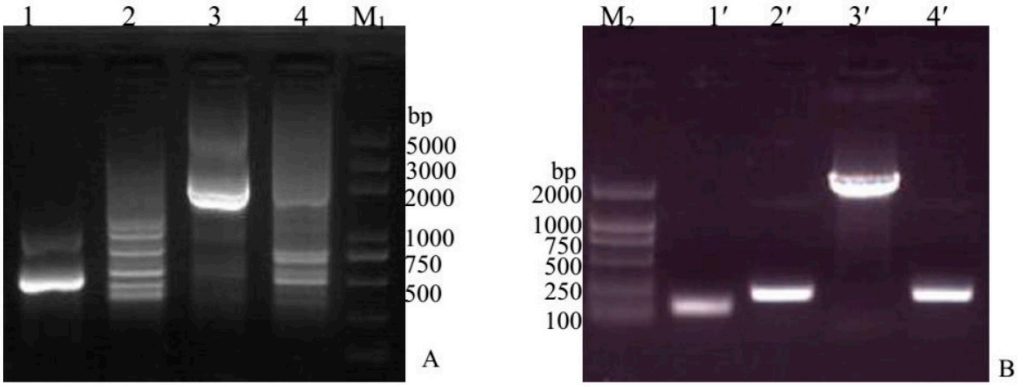

Figure 4. Amplified products of the *PbCBL1* promoter following nested PCR. **A.** Primary nested PCR amplification products. Genomic DNA of *Pyrus betulifolia* Bunge were digested with *Dra*I (*Lane 1*), *Eco*RV (*Lane 2*), *Ssp*I (*Lane 3*), or *Pvu*II (*Lane 4*). **B.** Gel patterns showing bands generated by secondary PCR, the reaction mixture contained 1-µL of each primer (*Lane 1*, *2*, *3*, and *4* in A) and 49-µL of deionized H_2O as a template. *Lane 1*' : *Dra*I library, *Lane 2*' : *Eco*RV library, *Lane 3*' : *Ssp*I library, or *Lane 4*' : *Pvu*II library. *Lane* M_1: DL2000 plus DNA ladder. *Lane* M_2: DL2000 DNA ladder.

Identification and confirmation of the activity of the *PbCBL1* gene promoter

Transient β-glucuronidase (*GUS*) expression has proven effective for evaluating the function of plant promoter sequences (Ramli and Abdullah, 2010; Fang et al., 2013). In this study, we found that the promoter contains important *cis*-acting elements including GA, ABA, SA, and MeJA. Therefore, *GUS* expression was used to assess the transcriptional activity of the *PbCBL1* gene promoter in response to GA, ABA, SA, and MeJA in tobacco leaf disks. The results of *Agrobacterium*-mediated transformation of tobacco leaf discs, using a PS1aG-3 vector, under GA, ABA, SA, and MeJA induction, led to high levels of *GUS* expression in transformed cells throughout the surfaces of the tobacco leaf discs (Figure 6A-D). However, no *GUS* expression was detected in tobacco leaf disks after transformation by *Agrobacterium* without insertion of the promoter or in the absence of hormone induction (Figure 6E and F). Therefore, the 2.0-kb fragment that we isolated from the 5'-upstream region of *PbCBL1* conferred a high level of gene expression under GA, ABA, SA, and MeJA induction in tobacco. Thus, the isolated *PbCBL1* gene promoter may play a key role in the gene expression that underlies the responses of pear to stress. Based on these results, we could speculate that the *PbCBL1* gene may also be involved in signal transduction of GA, ABA, SA, and MeJA, and

that it participates in the regulation of adversity stress. This result will be useful for teasing apart the regulatory mechanisms of gene expression in future research with pear cultivars.

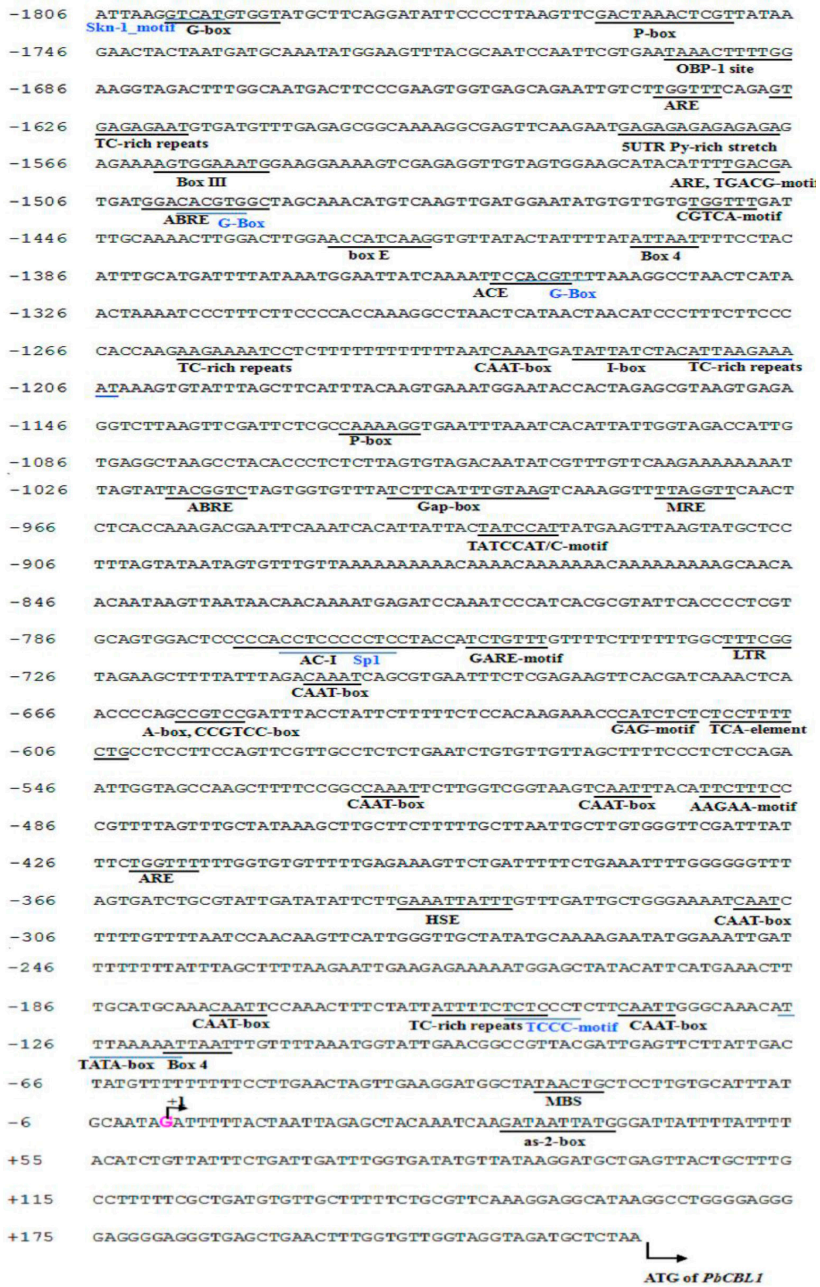

Figure 5. Analysis of functional elements of the *PbCBL1* gene promoter. Putative TSS is indicated with +1. All putative *cis*-elements are underlined.

Table 4. Functional predictions of *cis*-elements identified in the PbCBL1 promoters from '*Pyrus betulifolia* Bunge'.

Site name	Organism	Matrix score	Sequence	Function
A-box	*Petroselinum crispum*	6	CCGTCC	*cis*-acting regulatory element
AAGAA-motif	*Avena sativa*	7	GAAAGAA	Unknown
ABRE	*Triticum aestivum*	11	GGACACGTGGC	*cis*-acting element involved in the abscisic acid responsiveness
AC-I	*Phaseolus vulgaris*	9	CCCACCTACC	Unknown
ARE	*Zea mays*	6	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction
Box 4	*Petroselinum crispum*	6	ATTAAT	Part of a conserved DNA module involved in light responsiveness
CAAT-box	*Brassica rapa*	5	CAAAT	Common *cis*-acting element in promoter and enhancer regions
	Hordeum vulgare	5	CAAT	Common *cis*-acting element in promoter and enhancer regions
	Glycine max	5	CAATT	Common *cis*-acting element in promoter and enhancer regions
CCGTCC-box	*Arabidopsis thaliana*	6	CCGTCC	*cis*-acting regulatory element related to meristem specific activation
GAG-motif	*Spinacia oleracea*	7	AGAGATG	Part of a light responsive element
GARE-motif	*Brassica oleracea*	7	AAACAGA	Gibberellin-responsive element
Gap-box	*Arabidopsis thaliana*	9.5	CAAATGAA(A/G)A	Part of a light responsive element
HSE	*Brassica oleracea*	9	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
I-box	*Solanum tuberosum*	10	TATTATCTAGA	Part of a light responsive element
LTR	*Hordeum vulgare*	6	CCGAAA	*cis*-acting element involved in low-temperature responsiveness
MBS	*Arabidopsis thaliana*	6	TAACTG	MYB binding site involved in drought-inducibility
MRE	*Petroselinum crispum*	7	AACCTAA	MYB binding site involved in light responsiveness
P-box	*Pisum sativum*	11	GACCAAACTCGT	Gibberellin-responsive element
Sp1	*Zea mays*	5	CC(G/A)CCC	Light responsive element
TATA-box	*Zea mays*	8	TTTAAAAA	Core promoter element around -30 of transcription start
TATCCAT/C-motif	*Oryza sativa*	7	TATCCAT	Unknown
TC-rich repeats	*Nicotiana tabacum*	9	ATTTTCTTCA	*cis*-acting element involved in defense and stress responsiveness
TCA-element	*Brassica oleracea*	10	CAGAAAAGGA	*cis*-acting element involved in salicylic acid responsiveness
TCCC-motif	*Spinacia oleracea*	7	TCTCCCT	Part of a light responsive element
as-2-box	*Nicotiana tabacum*	9	GATAatGATG	Shoot-specific expression and light responsiveness
circadian	*Lycopersicon esculentum*	6	CAANNNNATC	*cis*-acting regulatory element involved in circadian control
5UTR Py-rich stretch	*Lycopersicon esculentum*	14	TTTCTCTCTCTCTC	*cis*-acting element conferring high transcription levels
ACE	*Petroselinum hortense*	7	ACGTGGA	*cis*-acting element involved in light responsiveness
Box III	*Pisum sativum*	9	CATTTACACT	Protein binding site
CGTCA-motif	*Hordeum vulgare*	5	CGTCA	*cis*-acting regulatory element involved in the MeJA-responsiveness
G-Box	*Pisum sativum*	6	CACGTG	*cis*-acting regulatory element involved in light responsiveness
G-box	*Brassica napus*	9	ACACGTGGC	*cis*-acting regulatory element involved in light responsiveness
OBP-1 site	*Arabidopsis thaliana*	10	TACACTTTTGG	*cis*-acting regulatory element
Skn-1_motif	*Oryza sativa*	5	GTCAT	*cis*-acting regulatory element required for endosperm expression
TGACG-motif	*Hordeum vulgare*	5	TGACG	*cis*-acting regulatory element involved in the MeJA-responsiveness
box E	*Petroselinum crispum*	9	ACCCATCAAG	Unknown

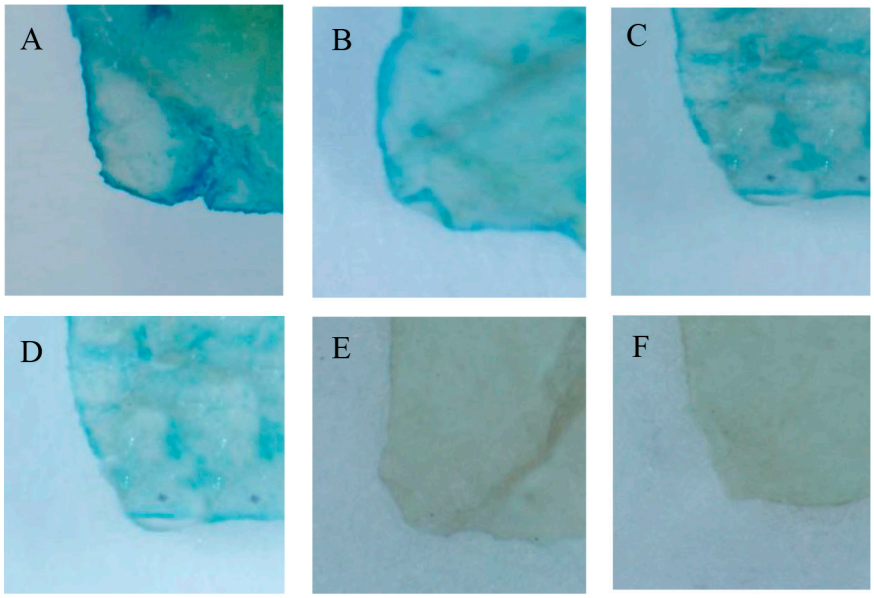

Figure 6. Transient *GUS* expression in transformed tobacco leaves. **A-D** represent transient expression using the fusion vector of PS1aG and the *PbCBL1* gene promoter under induction by GA, ABA, SA, and MeJA, respectively. **E** represents transient expression without hormone induction. **F** represents the negative control (the transformation vector without the *PbCBL1* gene promoter).

Validation and expression analysis of *PbCBL1* gene

Plant signaling under stress is accompanied by changes in cellular calcium concentration, since the activity or expression of CBL protein is mediated by calcium ions, the transcript levels of *PbCBL1* in response to exogenous calcium ions were analyzed in pear under different concentrations of $CaCl_2$ (Zhang et al., 2008). This study showed that the transcript level of *PbCBL1* was significantly upregulated in response to $CaCl_2$; the result indicates that the *PbCBL1* gene could respond to changes in the intracellular calcium concentration (Figure 7A). In *Arabidopsis*, *CBL1* functions in abiotic stress-specific signaling cascades, loss of *CBL1* function specifically and simultaneously affects the plant's capability to cope with cold and drought, but the effects of plant hormones such as ABA on *cbl1* mutation, was not detected (Albrecht et al., 2003; Cheong et al., 2003). In this study, the transcript level of *PbCBL7* was higher under cold- or heat-stress than at room temperature (Figure 7B). *PbCBL7* was upregulated in response to PEG6000, reaching maximum levels at 12 h before decreasing, a pattern that is similar to that observed under ABA treatment (Figure 7C and D). *PbCBL7* was consistently upregulated under GA treatment (Figure 7E), while SA and MeJA treatments, resulted in the maximum *PbCBL7* level being reached at 2 h and 6 h, respectively, before decreasing (Figure 7F and G). However, expression of the *PbCBL1* gene was stable in the controls in the absence of hormone or stress treatment (Figure 7H). These results verify that *PbCBL1* has an important role in the response to specific external stimuli. Our data will provide important evidence for further study that functionally dissects the CBL signaling network in pear.

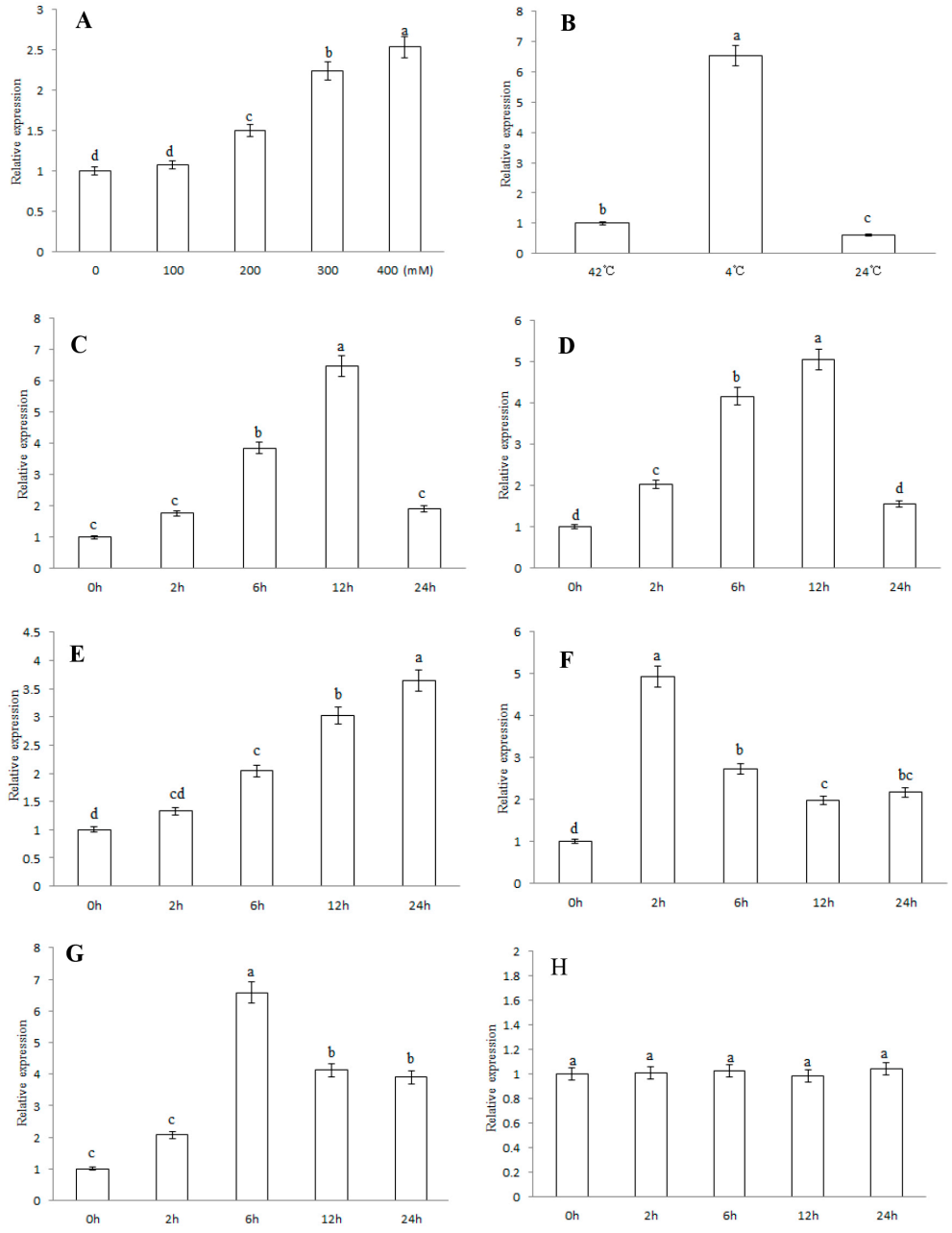

Figure 7. Effect of $CaCl_2$, low or high temperature, drought, ABA, GA, SA, and MeJA treatments on the expression level of *PbCBL1* in pear. **A:** $CaCl_2$ (0, 100, 200, 300, and 400 mM at room temperature for 6h). **B:** low or high temperature (24°, 4°, and 42°C for 6h). **C:** drought (PEG6000). **D:** ABA (50 µM). **E:** GA (100 µM). **F:** SA (1 mM). **G:** MeJA (50 µM). **H:** H_2O. Data are reported as means of three quantitative real-time PCR replicates ± SD (N = 3). Different letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests.

CONCLUSIONS

We discovered a *Calcineurin B-like1* gene from the genomic DNA of *P. betulifolia* Bunge, based on similarities in the sequence of amino acids, and designated it as *PbCBL1*. The *PbCBL1* gene sequence of a 2.0 kb 5′-upstream region was also isolated. Transient expression analysis indicated that the region directly upstream of the *PbCBL1* coding sequence was capable of directing high levels of *GUS* gene expression in tobacco leaves under the induction of GA, ABA, SA, and MeJA. Furthermore, real-time RT-qPCR revealed that *PbCBL1* responds to changes in intracellular calcium concentration and that it can be induced by cold, heat, drought, and hormone stress including that induced by GA, ABA, SA, and MeJA. These results may facilitate a better understanding of the structural and functional characteristics of the *PbCBL1* gene in pear plants.

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