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Short communication

Molecular cloning and characterization of a novel MAP kinase gene in *Chorispora bungeana*

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Abstract

Chorispora bungeana Fisch. and C.A. Mey (*Chorispora bungeana*) is a rare alpine subnival plant species that is highly capable of resisting freezing environment. Since it is a stress-tolerant plant, we investigated the participation of mitogen-activated protein kinases (MAPKs) as possible mediators of abiotic stresses. We have isolated from *Chorispora bungeana* a new MAPK cDNA *CbMAPK3* which encodes a 369 amino-acid protein with moderate to high nucleotide sequence similarity to previously reported plant MAPK genes. *CbMAPK3* contains all 11 of the MAPK conserved subdomains and the phosphorylation motif TEY. The transcripts of *CbMAPK3* were detected and no tissue-specific expression were observed in both roots and leaves, The transcripts of *CbMAPK3* accumulated highly and rapidly when *Chorispora bungeana* treated with cold (4 and –4 °C), ABA and salinity stress. These results indicate that the *CbMAPK3* may play an important role in response to environmental stresses.

Keywords: RACE; Mitogen-activated protein kinases; *Chorispora bungeana*; Environmental stresses

1. Introduction

To survive biotic and abiotic stresses, plants have developed elaborate mechanisms to perceive external signals and adjust metabolic pathways by modulating the expression of genes. Activation and/or inactivation of appropriate genes in response to particular stimuli are mediated through well-tuned signal transduction systems [1]. The phosphorylation cascades including mitogen-activated protein kinases (MAPKs), MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs) have been reported to function in various signal transduction pathways from yeasts to vertebrates [2]. MAPKs are activated when both tyrosine and threonine residues in the TXY motif are phosphorylated by dual-specificity kinases MAPKKs. MAPKKs are activated when serine and serine/threonine residues in the S/T–$X_{3–5}$–S/T motif are phosphorylated by serine/threonine kinases MAPKKKs [3].

In plants, a variety of genes encoding MAPKs have been identified [4–10]. The analysis of *Arabidopsis* genome sequence has revealed the presence of 24 MAPK genes in the genome, which suggests that the MAPK cascades in plants may be quite complex [9]. Compared with mammalian MAPKs, all plant MAPKs have highest homology to the extracellular signal-regulated kinase (ERK) subfamily [11]. Most of isolated plants MAPKs have the TEY motif as the dual-phosphorylation site. The predicted amino acid sequences of these plant MAPKs show high conservation over the entire length with highest similarity in the eleven domains that are necessary for the catalytic function of serine/threonine protein kinase.

Comparisons of deduced amino acid sequences indicate that plant MAPKs can be grouped into at least five distinct families (Fig. 3). MAPKs within one branch serve similar function in different species [12], the MAPKs in family A and B are

Abbreviations: ABA, Abscisic acid; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; ORF, Open reading frame; RACE, Rapid amplification of cDNA ends; RT-PCR, Reverse transcription polymerase chain reaction; UTR, untranslated region.

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mostly involved in environmental and hormonal responses, some of the MAPKs of family C are involved in cell cycle regulation and some involved in environmental stress responses, the function of MAPKs in family D are little known at present, family E MAPKs are all have the TDY motif instead of TEY in their T-loop and also have the C-terminal extension domain.

Chorispora Bungeana Fisch. and C.A. Mey. (*Chorispora bungeana*) is a representative alpine subnival plant which can survive under frequent temperature fluctuations, and freezing temperatures. *Chorispora Bungeana* is a perennial herb that belongs to Brassicaceae. It grows in the freeze-thaw tundra in border of glacier where almost all the other flowering plants have great difficulty for growing, and it mainly distributes in an ice free cirque (with a 3800–3900 m height above sea level) beside the Glacier No. 1 in the source area of Urumqi River in Tianshan mountains, Xinjiang province, China, where the average temperature is below subzero during the growth period from June to September [13]. Some works had been done for the exploration of its special cold-hardiness mechanism. Previous research showed that the alpine subnival plant did not possess special morphological characteristics that helped it survive under freezing environment [14]. Therefore, molecular and physiological mechanisms were assumed to account for its adaptation to the freezing environment.

In this report, we describe the cloning and characterization of a plant MAPK from *Chorispora bungeana*, *CbMAPK3*. The deduced protein sequences of *CbMAPK3* were most closely related to *Arabidopsis AtMPK3*, and all belonged to subgroup A. To get an insight into its behavior and regulation by environmental stimuli, we have characterized the mRNA expression profile of *CbMAPK3* against cold (4 and –4 °C), ABA and salinity stress. The results obtained in this study demonstrate that *CbMAPK3* is transcriptionally regulated by abiotic stress.

2. Results and discussion

2.1. Cloning and sequence analysis of the full-length cDNA of CbMAPK3

Based on cDNA sequences of the conserved regions of plant MAPK genes, primer P1 and P2 were designed and synthesized for the amplification of the middle region of MAPK-like cDNA from *Chorispora bungeana*. A single fragment of about 1030 bp showing high homology to *AtMPK3* was obtained. Based on the middle region sequences, a reverse specific primer CbRACE5 and two pairs of PCR primers were designed for the 5′RACE, a single and specific fragment of about 300 bp was obtained in which a 5′ untranslated region (UTR) of 91 bp was found upstream of the first ATG codon. A reverse specific primer CbRACE3 and a pair of PCR primers were designed and used in the 3′RACE, a single and specific fragment of about 400 bp was obtained, and a 3′ UTR of 218 bp was found downstream from the stop codon. Based on the sequences of the middle region sequences and the 3′,5′RACE products, the full-length cDNA was deduced and amplified using a pair of primer P3 and P4, which was confirmed by sequencing for three times. The cloned full-length cDNA of mitogen-activated protein kinase gene from *Chorispora bungeana* was 1419 bp with a polyA tail of 15 bp (Fig. 1). The cDNA contained a 1110-bp ORF encoding a protein of 369 amino acids with a calculated molecular weight of about 42.5 kDa and with a p*I* of 5.79. The protein exhibit closest homology to a subgroup of plant MAPKs containing *Arabidopsis AtMPK3* and we thus refer to them as *CbMAPK3* (GenBank accession number: *AY805424*).

An alignment of the predicted amino acid sequences of *CbMAPK3* with the cloned MAPKs from various plants were performed using DNAStar software shown in Fig. 2. The *CbMAPK3* protein contained all 11 conserved amino acid and peptide motifs characteristic of 11 subdomains of protein kinases with serin/threonine specificity. The TEY motif, which includes the threonine and tyrosine residues whose phosphorylation is necessary for MAP kinase activation and is a characteristic feature of MAP kinase, is also conserved in the *CbMAPK3* protein sequence (Fig. 2).

A phylogenetic tree based on the genetic distance of the protein sequences was constructed by the Clustal method using DANStar software, it has been proposed from the analysis of sequences homology of the predicted amino acid sequences that plant MAPKs can be grouped at least five distinct groups. Based on the relationship tree of cloned plant MAPKs, *CbMAPK3* can be grouped into subgroup A (Fig. 3). Comparison of the predicted protein sequences of the *CbMAPK3* with MAP kinases of other plants shows that CbMAPK3 is most homologous to the *Arabidopsis AtMPK3* (95%), *Nicotiana tabacum NtWIPK* (82%), *Capsicum annuum CaMPK1* (81%), *PsMAPK3* (80%), *Medicago sativa MsMMK4* (80%), *Petrosecinum crispum PARSLEY MAPK* (80%) (Fig. 2).

The secondary structure of the putative *CbMAPK3* protein was analyzed by SOPMA [http://npsa-pbil.ibcp.fr/cgi-bin] and the result showed that the putative *CbMAPK3* peptide contained 46% alpha helix, 13% extended strand, 6% beta turn, and 35% random coil (Fig. 4). The alpha helix and random coil constituted interlaced domination of the main part of the secondary structure. From the above sequence analyses, *CbMAPK3* was found to have many characteristics common to the MAPKs in plant and family A members tend to share highly similar sequences.

2.2. Expression patterns of CbMAPK3 in different tissue and under abiotic stresses

To investigate *CbMAPK3* expression pattern in various tissues of *Chorispora bungeana*, total RNA was isolated from 1-month-old leaves and roots, and subjected to RT-PCR analysis, the result shown that its expression has almost no tissue specificity (Fig. 5).

Chorispora bungeana was a rare and typical alpine subnival plant growing near the No. 1 glacier, the origin of Urumqi River on Tianshan Mountains in china. The annual average temperature therein was lower than 5 °C in daytime and

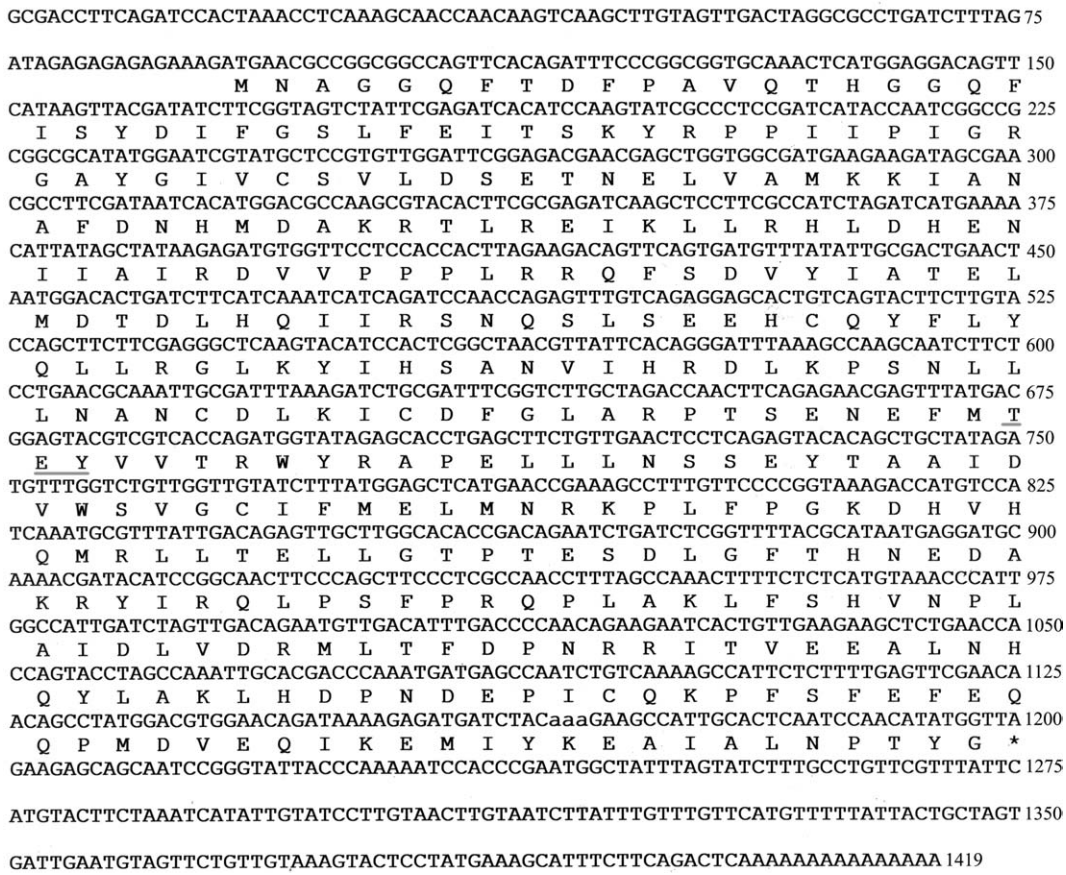

Fig. 1. The full-length cDNA sequence and deduced amino acid sequence of *Chorispora bungeana CbMAPK3* cDNA (GenBank Accession No. AY805424). The nucleotides are numbered on the right, the conserved motif TEY is underlined.

–4 °C at night. The temperature also underwent a big fluctuation from nearly 4 to –10 °C during favorable growth season from June to September and the plant could survive even embedded in snow. So the plant had an extraordinary resistant ability to freezing temperature. In order to research whether MAPK is involved in *Chorispora bungeana* defense mechanisms, *CbMAPK3* expression patterns under cold and other abiotic stresses were analyzed by semi-quantitative RT-PCR. Total RNA (1 μg) of *Chorispora bungeana* leaves was used as template to detect transcripts level at different time points after each treatment.

After exposure to 4 °C, transcript levels of *CbMAPK3* increased rapidly, maximum levels was observed 1–2 h after cold treatment, then, the *CbMAPK3* transcript declined gradually and fell to the basal level at 24 h (Fig. 6A). When *Chorispora bungeana* was exposure to –4 °C, transcript levels of *CbMAPK3* also increased rapidly, but the transcript maintained at a high level up to 24 h (Fig. 6B).

It has been widely reported that cold-inducible gene also respond to water deficit [15]. Evidence has also shown that an MAPK cascade is involved in several signal transduction pathways in plant [16]. Thus, we examined the effects of NaCl stress on the expression of *CbMAPK3*. The expression of *CbMAPK3* could also be induced by salt stress, under salt stress, the transcripts of *CbMAPK3* increased to a high level after 2 h and maintained high levels at 48 h after treatment (Fig. 6D).

The expression of *CbMAPK3* could also be induced by ABA treatment, when treated with ABA, the transcripts of *CbMAPK3* increased significantly after 30 min, and decreased after 12 h (Fig. 6C).

The ability to sense extreme changes of abiotic environmental conditions has been shown to be essential for a plant to launch the processes underlying acclimation [17]. To survive these conditions, Plants have developed specific mechanisms to withstand abiotic stress, such as the synthesis of stress hormones like abscisic acid (ABA) and the expression of specific sets of genes that result in changes in the composition of the major cell components [18,12]. Several MAPK have been shown to be involved in the transmission of abiotic stress. It is likely that the activation of the plant MAPK is achieved posttranslationally by MAPK kinases [19]. For instance, in alfalfa, a MAP kinase, MMK4, can be transiently activated by cold and drought stress in vitro phosphorylation assay [15]. However, *MMK4* transcript level also accumulate after cold and drought stress. The mRNA level of *AtMPK3* in *Arabidopsis thaliana* increased markedly within 5 min in response to cold, touch, and salinity stress [2]. Similarly, expression of genes of parsley *ERMK*, rice *OsBIMK1* and *BWMK1* are activated by various stresses [6,10]. The increase in expression of

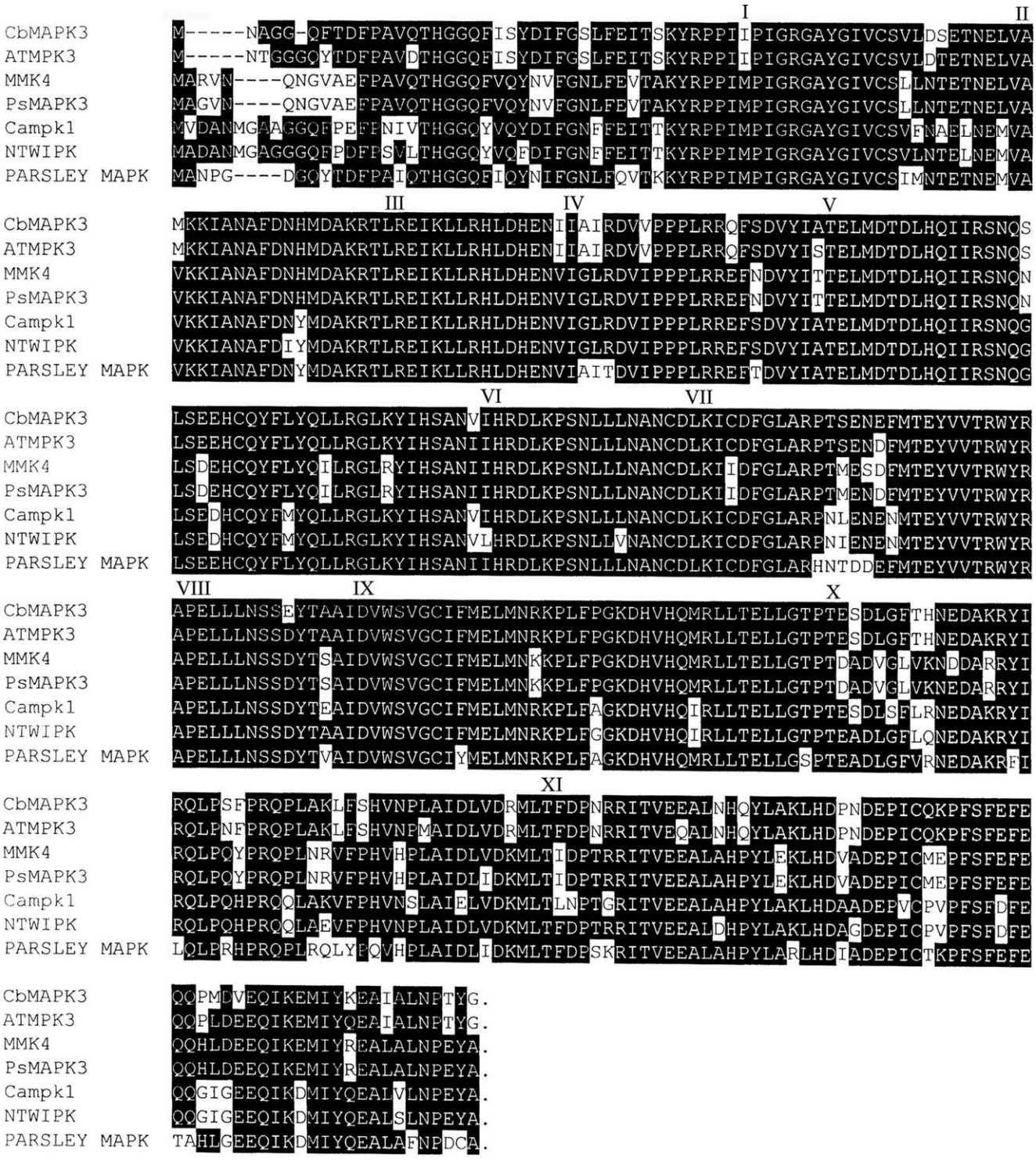

Fig. 2. Alignment of the *Chorispora bungeana* CbMAPK3 with MAPKs from other plant species. The plant MAPKs used for alignment are: *Arabidopsis thaliana* AtMPK3 (GenBank Accession No. D21839); tobacco (*Nicotiana tabacum*) NtWIPK (GenBank Accession No. D61337); pea *Pisum sativum*) PsMAPK3 (GenBank Accession No. AF153061); alfalfa (*Medicago sativa*) MsMMK4 (GenBank Accession No. X82270); *Capsicum annuum* CaMPK1 (GenBank Accession No. AF247135) and parsley (*Petrosecinum crispum*) parsley MAPK (GenBank Accession No. Y12785).

these MAPK genes may contribute to the stress response by increasing the amount of proteins available for activation [20]. Therefore, it can be concluded that MAPK not only become activated by posttranslational phosphorylation of the highly conserved threonine and tyrosine residue through upstream kinases, transcriptional control is also an important mechanism in MAPK signaling cascades in plant.

Expression of *CbMAPK3* was induced by cold (4 °C), the change of expression levels was similar with *AtMPK3* treated at 4 °C, but when *Chorispora bungeana* was treated at –4 °C,

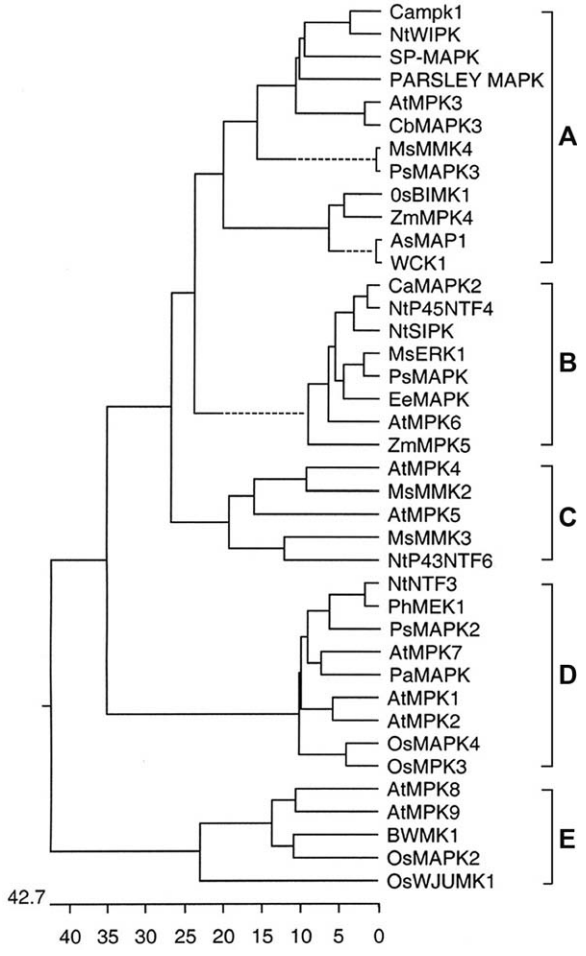

Fig. 3. The phylogenetic relationship of the *Chorispora bungeana* CbMAPK3 with other MAPKs from plant species. A phylogenetic tree based on the genetic distance of the protein sequences was constructed by the Clustal method using DNAStar software. The protein sequences of the MAPKs used for construction of the tree are listed in the GenBank database under the following accession numbers: CaMPK1 (AF247135), NtWIPK (D61377), SP-MAPK (AF149424), PARSLEY MAPK (Y12785), AtMPK3 (D21839), MMK4 (X82270), PsMAPK3 (AF153061), OsBIMK1 (AF332873), ZmMPK4 (AB016801), AsMAP1 (S56638), WCK1 (AF079318), NtP45NTF4 (X83880), CaMAPK2 (AF247136), NtSIPK (U94192), MsERK1 (L07042), PsMAPK (X70703), EeMAPK (AF242308), AtMPK6 (D21842), ZmMPK5 (AB016802), AtMPK4 (D21840), MsMMK2 (X82268), AtMPK5 (D21841), NtP43NTF6 (X83879), MsMMK3 (AJ224336), NtNTF3 (X69971), PhMEK1 (X83440), PsMAPK2 (AF154329), AtMPK7 (D21843), PaMAPK (AF134730), AtMPK2 (D14714), AtMPK1 (D14713), OsMAPK4 (AJ251330), OsMPK3 (AF216317), BWMK1 (AF177392), OsMAPK2 (AF194416), AtMPK9 (NM112686), OsWJUMK (AJ512643).

the Expression levers of *CbMAPK3* maintained high levers at 24 h, suggesting that an efficient system exists in *Chorispora bungeana* for perception of freezing stresses through *CbMAPK3*. To our knowledge, this is the first description of a plant MAPK gene whose expression is inducible at –4 °C treatment. C. LU reported that overexpression of the *AtMPK3* gene can increase ABA sensitivity [21]. Our study shows that *CbMAPK3* can be induced by ABA, indicating that the *CbMAPK3* gene was ABA dependent. On the whole, the *CbMAPK3* transcript levels were increased by cold (4 and –4 °C), salt and ABA treatment, so *CbMAPK3* is involved in several signal transduction pathways.

3. Conclusion

In conclusion, a novel MAPK gene *CbMAPK3* was identified and characterized. The expression of *CbMAPK3* was induced by cold, salt, and ABA but no tissue specificity. However, the biochemical mechanism for activation of *CbMAPK3* enzymatic activity in the defense response and whether induction of *CbMAPK3* gene expression is required for activation of its activity need to be studied further.

4. Materials and method

4.1. Plant material and stress treatment

The wild species of *Chorispora bungeana* were collected from an ice-free cirque (43°05′N, 86°49′E, with an altitude of 3800–3900 m) near the No. 1 glacier in the source area of Urumqi River in Tianshan Mountains, Xinjiang, China, and transplanted to the experimental field of botanical garden at Lanzhou University. In the second year, the regenerated plants were used for experiments. One-month-old leaves were used for treatment under different stress conditions. For cold treatment, plants were placed at 4 °C or –4 °C; for salinity stress treatment, plants were placed directly into a container of 150 mM NaCl. For ABA treatment, plants were sprayed with 0.1 mM ABA.

4.2. Cloning of the middle region of the CbMAPK3

Two primers, P1 (5′-TYTTCGGTARTYTWTTYG-3′) and P2 (5′-TAYKYWGGATTGAGTGCWA-3′) (where Y is A,

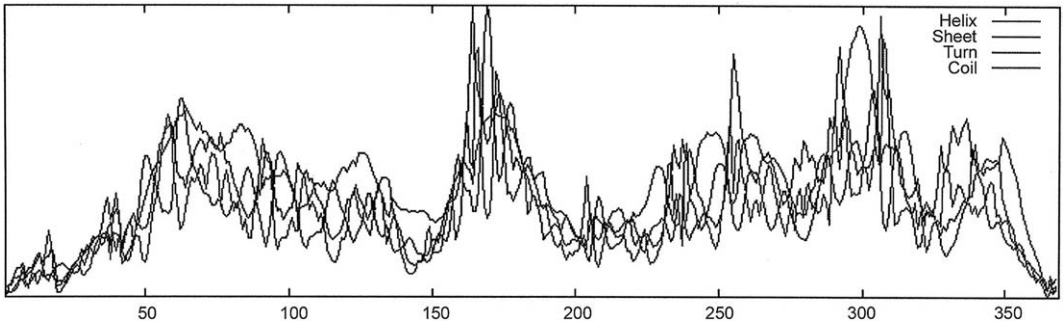

Fig. 4. Prediction of the secondary structure of CbMAPK3. The helix, sheet, turn and coil were indicated respectively with blue, red, green, and yellow lines.

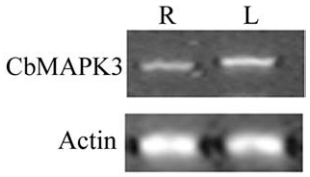

Fig. 5. Expression profile of *CbMAPK3* in different tissues. Total RNA (1 μg per sample) was isolated from leave and root, respectively, and subjected to one-step RT-PCR amplification with 25 cycles (upper panel). *Actin* gene (GenBank Accession No. AY825362) was used as the control to show the normalization of the amount of templates in PCR reactions (lower panel).

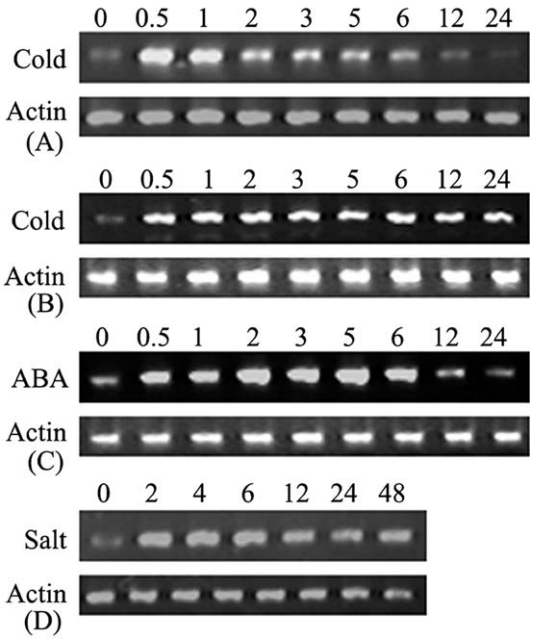

Fig. 6. Expression profiles of *CbMAPK3* under abiotic stresses treatment. Total RNA (1 μg per sample) was isolated at 0, 0.5, 1, 2, 3, 5, 6, 12, 24 h after cold treatment (4 °C) (A), (–4 °C) (B), ABA (C) and 0, 2, 4, 6, 12, 24, 36 h after salt treatment (D), respectively, and subjected to one-step TR-PCR amplification with 25 cycles (upper panel). *Actin* gene was used as an internal control (lower panel).

and T; R is A and G; W is A and T; K is G and T), which correspond to sequences of the conserved regions of plant MAPK genes were used to polymerase chain reaction (PCR) amplify the cDNA that was reverse transcribed from total RNA samples extracted from *Chorispora bungeana* leaves by the CTAB method [22] after 2 hours of cold stress treatment at 4 °C. The reverse transcription-PCR product of ~1030 bp was cloned into pGEM-T vector (Promega, USA) and sequenced.

4.3. 5′ RACE of CbMAPK3

The first strand cDNA was synthesized from total RNA according to the manufacture's guidelines of the 5′-full RACE core set kit (Takara) using the 5′ RACE gene-specific reverse transcription primer CbRACE5 (5′-(P) AGTGCTCCTCTGA CA-3′) designed and synthesized according to the middle region of the *CbMAPK3* sequenced above. Then the cDNA was ligated from 5′ end to 3′ end by 1 μl T4 RNA ligase at 16 °C for 24 h in a total reaction volume of 20 μl. In the PCR step, two pairs of gene-specific primers CBS1 (5′-AGCTCCTTCGC CATCTA-3′), CBA1 (5′-AGGCGTTCGCTATCTTCT-3′) and CBS2 (5′-TTGCGACTGAACTAATGGACACTG-3′), CBA2 (5′-CCGCGGCCGATTGGTATGAT-3′) was designed and synthesized according to the middle region of the *CbMAPK3*. 1st PCR was performed using CBS1 and CBA1 as the forward and the reverse primer in a total reaction volume of 50 μl containing 2 μl cDNA, 1 μl CBA1 (20 pmol/μl), 1 μl CBS1 (20 pmol/μl), 1 μl dNTPs (10 mM each), 1 × PCR buffer and 5 U Tag polymerase (Promega). PCR was performed using the following protocol: the cDNA was denatured at 94 °C for 4 min followed by 35 cycles of amplification (94 °C for 60 s, 53 °C for 60 s, 72 °C for 90 s) and by 7 min at 72 °C. The amplified product was diluted 10-fold and used as template in the second PCR using CBS2 and CBA2 as the forward and the reverse primer in a total reaction volume of 50 μl same as the first PCR under the following condition: 94 °C for 4 min, 35 cycles of 94 °C for 50 s, 56 °C for 50 s, 72 °C for 90 s and a final extension at 72 °C for 7 min. The product was purified and subcloned into pGEM-T vector and sequenced.

4.4. 3′ RACE and full-length cDNA cloning of CbMAPK3

The first strand cDNA was synthesized according to the manufacture's guidelines of the 3′-full RACE core set kit (Takara) using the oligo dT-3 sites adaptor primer provided within the kit. 3′RACE-PCR was performed with the gene-specific primer CbRACE3 (5′-GTTCGAACAACAGCCTATGGAC-3′) as the forward primer, and the three sites adaptor primer (5′-CTGATCTAGAGGTACCGGATCC-3′) provided within the kit as the reverse primer. PCR was carried out by denaturing the cDNA at 94 °C for 4 min followed by 35 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s) and by a final extension at 72 °C for 7 min. The PCR product was purified and cloned into pGEM-T vector followed by sequencing.

By comparing and aligning the sequences of 3′RACE, 5′ RACE and the middle region products, the full-length cDNA sequence of *CbMAPK3* was obtained, which was subsequence amplified via PCR using a pair of primers P3 (5′-GCGACCTT CAGATCCACTAA-3′) and P4 (5′-TGCTTTCATAGGAG TACTTTACAA-3′), and sequenced. The PCR amplification for the full-length *CbMAPK3* was repeated three times. The full-length *CbMAPK3* was subsequently analyzed for molecular characterization such as the presentation of conserved motifs, sequence homology, and the secondary structure.

4.5. Semi-quantitative RT-PCR analysis

To investigate the tissue-specific expression pattern of *CbMAPK3*, and the expression patterns of *CbMAPK3* under abiotic stresses, total RNA was extracted from *Chorispora bungeana* leaves and roots by the CTAB method and used in RT-PCR analysis. The RNA samples were quantified spectrophotometrically at 260 nm. Aliquots of 1 μg total RNA of each

treatment were used as template in RT-PCR reactions with the forward primer P5 (5′-TTGGTCGTGGAGCTTATGGAATC-3′) and reverse primer P6 (5′-CTAGCAAGACCGAAATCA CAAATC-3′) using one-step RT-PCR kit (Takara, Japan). Amplifications were performed at 94 °C for 4 min followed by 25 cycles of amplification (94 °C for 40 s, 53.5 °C for 40 s, 72 °C for 60 s). PCR products (5 μl) were separated on 1% agarose gels stained with ethidium bromide. The bands of PCR products were analyzed by Gene Tools software from Gene Company. The RT-PCR reaction for the housekeeping *actin* gene (GenBank Accession No. *AY825362*), using specific primers actin1 (5′-GCTCCGTGTTGCCCCTGAAGA-3′) and actin2 (5′-CTCGGCGGTGGTGGTGAACA-3′), was performed at the same condition as described above to estimate if equal amounts of RNA among samples were used in the RT-PCR reaction.

Acknowledgments

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