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Molecular Cloning and Sequence Analysis of a Phosphomevalonate Kinase Gene (*CnPMK*) from *Chamaemelum nobile*

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Abstract

Roman chamomile (*Chamaemelum nobile* L.) is renowned for its production of sesquiterpenoids, and specifically sesquiterpene lactones. Phosphomevalonate Kinase gene (*PMK*) is one of the core enzymes in the biosynthesis pathway of sesquiterpenoids, which catalyzes the formation ofmevalonate-5-diphosphate. To isolate and identify the key genes involved in the sesquiterpenoids biosynthesis of the *C. nobile*, a *PMK* gene designated as *CnPMK* (GenBank Accession No. KX894316) was cloned from *C. nobile*. The full-length cDNA of *CnPMK* was 1942bp and contained a 1482bp open reading frame (ORF), which encoding a 493 amino-acid protein. The molecular weight and pI of the CnPMK protein are 53.8KDa and 5.55, respectively. The encoding amino-acid sequence of the *CnGAS* showed high similarity to other plant PMK proteins. Phylogenetic tree analysis revealed that CnPMK clustered with the PMK of Asteraceae in the dicotyledons clade. The *CnPMK* gene was isolated from *C. nobile*, which will lay a foundation for further study of sesquiterpenoid biosynthesis pathway in *C. nobile*.

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Introduction

Roman chamomile (*Chamaemelum nobile* L.) is a perennial herbaceous plant of the Asteraceae family found in wild and cultivated in western Europe, North America and northern Africa and Asia (Ma et al., 2007). As a traditional medical plant, Roman chamomile is considered to be an antiseptic, antibiotic, disinfectant, bactericidal, fungicidal and vermifuge (Guimarães et al., 2013). This herb is often used against nausea, vomiting, indigestion, and loss of appetite (Srivastava et al., 2010). The biological activity of chamomile is mainly due to the flavonoids apigenin, luteolin, quercetin, patuletin and terpenoids chamazulene, sesquiterpene lactones, bisabolol and its oxides and azulenes (Hadaruga et al., 2009).

Terpenoids are synthesized by a variety of terpenoid synthases that constitutes a highly diverse gene family producing a wide range of cyclic and acyclic molecules consisting of isoprene (C5) residues. Over 60,000 terpenes and derivatives are found in nature (Cheng et al., 2007; Bohlmann and Keeling, 2008). Terpenoids are synthesized in plants through two independent pathways: the mevalonate (MVA) pathway in the cytoplasm and the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastids (Fig. 1, Vranová et al., 2013). During recent decades, there has been great progress in identification and functional characterization of terpenoid biosynthesis genes, enzymes and in metabolic engineering of terpenoid synthesis, and this has contributed greatly to improved understanding of basic mechanisms and variability of terpenoid biosynthesis (Chen et al., 2011; Rajabi Memari et al., 2013). In particular, proteomics data has previously shown that the fourth and fifth enzymes in the MVA

pathway—mevalonate kinase (MK) and phosphor-mevalonate kinase (PMK), respectively— are expressed at relatively low levels and may be targets for increasing overall isoprenoid production (Redding-Johanson et al., 2011; Singh et al., 2012). Previous study has also shown that substrate inhibition and feedback inhibition of MK may be responsible for limiting flux through the pathway (Ma et al., 2011).

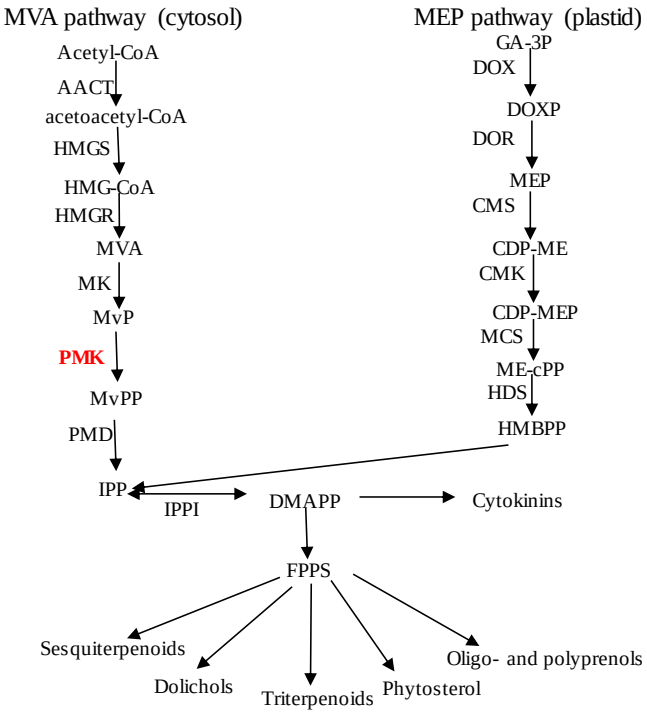

Fig. 1: Isoprenoid biosynthetic pathways in the plant cell.

As an important enzyme in the MVA pathway, PMK catalyzes the reaction of mevalonate-5-phosphate (MvP) and ATP to form mevalonate-5-disphosphate (MvPP) and ADP (Miziorko, 2011), which is further converted into isopentenyl diphosphate (IPP) by the mevalonate diphosphate decarboxylase (PMD). All the isoprenoids are derived from IPP precursors, and the IPP is generated by the pyrophosphorylation and decarboxylation of MVA; common precursors are used for the biosynthesis of terpenoids such as mono-, sesqui-, di-, and triterpenoids. Normally, sesquiterpene lactones are synthesized from MVA pathway. Given its importance in the terpenoids biosynthesis, some *PMK* genes have been studied for its function in the MVA pathway. PMK is found in eukaryotes and some Eubacteria. The amino acid sequence for animal and low homology invertebrate PMK proteins are not orthologous to those for PMK in plants, fungi, and bacteria (Smit and Mushegian, 2000). Thus, the proteins that catalyze the enzymatic reaction differ widely, depending on their source. Although Roman chamomile is an important medical plant, few studies focused on identifying the enzymes or genes involved in sesquiterpenoid biosynthesis. To investigate the whole biosynthesis pathway of terpenoids in Roman chamomile, each gene involved in this pathway should be identified and characterized. Here, we report the cloning of the full-length cDNA of the *PMK* gene from Roman chamomile for the first time. We also analyzed the structure of the sequence, aiming to add the gene source for increasing the content of sesquiterpenoids in *C. nobile* through the genetic engineering.

Materials and methods

Erect Roman chamomile (*C. nobile*) plants were grown under controlled conditions in a growth chamber (16 hrs day length and day/night temperature of 23 /18°C, incident quantum flux density of 400 µmol m^{-2} s^{-1}). Roman chamomile leaves were collected and immediately frozen in liquid nitrogen and stored at a -80°C for RNA extraction. MiniBEST Plant RNA Extraction kit, PrimeScript™ 1st Strand cDNA Synthesis Kit, Agarose Gel DNA purification Kit Ver.4.0 and pMD18-T vector kit were purchased from TaKaRa Company (Dalian, China). Both the primers synthesis and DNA sequencing were performed by Shanghai Sangon Biotechnology Company, in China.

Cloning the full-length cDNA of *CnPMK*

Total RNA was isolated from frozen plant tissues using the TaKaRa MiniBEST Plant RNA Extraction kit. The first strand cDNA was synthesized according to the instruction of PrimeScript™1st Strand cDNA Synthesis Kit. Five ESTS, showed a high similarity level with the beginning and the end of queries, respectively. Primers CnPMK-up (5'-ATGTCTGTGGTTGCTTCGGCTC-3') and CnPMK-dn (5'-TTACTTTTCCCGAGGATCACCACT-3') were designed on Group1_Unigene_BMK.5730 sequence to investigate the full length. CnPMK cDNA was amplified under the following conditions: 94°C for 4 min; 30 cycles of amplification at 94°C for 30 s, 63°C for 30 s, and 72°C for 90 s; and a final extension at 72°C for 10 min. The amplified products were analyzed by 1% gel electrophoresis and purified by Agarose Gel DNA purification Kit Ver.4.0. The purified products were ligated into the pMD18-T vector, and then cloned into the *Escherichia coli* strain DH5α followed by sequencing.

Bioinformatic analysis

The obtained nucleotide sequence and deduced amino

acid sequence were analyzed by the bioinformatics software on websites (http://www.ncbi.nlm.nih.gov/ BLAST/ and http://web. expasy.org/ protparam/). Plant PMK protein sequences were retrieved from NCBI GenBank. Multiple sequence alignments were performed using Vector NTI 11.5 program and phylogenetic analysis was conducted using MEGA 6.0 software. The neighbor-joining method was used to construct a guided tree, supported by boostrapping based on 1000 replicates.

Results and discussion

Cloning and characterization of *CnPMK*

The cDNA of *CnPMK* (GenBank Accession No. KX894316) was cloned by a pair of specific primers based on *CnPMK* unigene of transcriptome data. The PCR products were sequenced, and results showed that the cDNA sequence of the PCR products was 1942bp and contained a 1482bp open reading frame (ORF), which encoding a 493 amino-acid protein (Fig. 2).

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1    ATGTCTGTGGTTGCTTCGGCTCCGGGGAAGGTTTTAATGGCTGGTGGTTATTTGGTATTGGAGAGGCCTAATGCA
1     M  S  V  V  A  S  A  P  G  K  V  L  M  A  G  G  Y  L  V  L  E  R  P  N  A
76   GGAATCGTGCTTAGTACTAATGCTCGGTTTTACGCGATTCTTAAGCCTCTTTATGATGCACTTGAATCCGACAAT
26    G  I  V  L  S  T  N  A  R  F  Y  A  I  L  K  P  L  Y  D  A  L  E  S  D  N
151  TTGGCAGTGGAGTGGATGGATCTTAAAGTCAACTCTCCTCAAATGTCAAGAGAAACTACATACAAAATGTCACTA
51    L  A  V  E  W  M  D  L  K  V  N  S  P  Q  M  S  R  E  T  T  Y  K  M  S  L
226  AAAGATTTTACACTTCAGTGTTCGAGTGAATCAAGGAACCCCTTTGTCGAGTATGCAGTCCAATATTCCATAGCA
76    K  D  F  T  L  Q  C  S  S  E  S  R  N  P  F  V  E  Y  A  V  Q  Y  S  I  A
301  GCAGCATATGCCAAACTCGAGAAGTATAAAAGGAATAAATTGCTGAAATTACTGTCACAAGGTATGGATATTACA
101   A  A  Y  A  K  L  E  K  Y  K  R  N  K  L  L  K  L  L  S  Q  G  M  D  I  T
376  ATCCTAGGTAGCAACGACTTCTATTCATATCGGAATCAGATTGAAGCACGTGGCCTGCCTTTAGTCCCGGAATCA
126   I  L  G  S  N  D  F  Y  S  Y  R  N  Q  I  E  A  R  G  L  P  L  V  P  E  S
451  TTAGCCACTCTTCAACCATTCACATCAATTACTTTCAATGCCGAAGAATCAACTACAGAAAACTCCAAACCAGAA
151   L  A  T  L  Q  P  F  T  S  I  T  F  N  A  E  E  S  T  T  E  N  S  K  P  E
526  GTTGCAAAAACTGGATTAGGGTCGTCTGCAGCTATGACAACTGCTGTAGTAGCTGCTTTGCTAAATTATCTTGGA
176   V  A  K  T  G  L  G  S  S  A  A  M  T  T  A  V  V  A  A  L  L  N  Y  L  G
601  GTTGTTGATCTTCCACTTTATAGTACAAATCAAAAGTTAGATAAAAGTTTGGATCTTGATTTAGTGCATATGATC
201   V  V  D  L  P  L  Y  S  T  N  Q  K  L  D  K  S  L  D  L  D  L  V  H  M  I
676  GCTCAAACAGCGCACTGTATTGCTCAAGGGAAAGTCGGCAGTGGATTTGATGTTAGCTCTGCTGTTTATGGCAGT
226   A  Q  T  A  H  C  I  A  Q  G  K  V  G  S  G  F  D  V  S  S  A  V  Y  G  S
751  CATCGTTATGTTCGTTTTTCGCCAAATATCATTTCTTCAGCTCAGGAAGCTGTCGACACCACACCATTAGATGAA
251   H  R  Y  V  R  F  S  P  N  I  I  S  S  A  Q  E  A  V  D  T  T  P  L  D  E
826  GTTATTGGTGATGTCTTGAAAGGAAAGTGGGATCACGAAAGAACTAATTTCTCATTGCCACCATTAATGAATCTG
276   V  I  G  D  V  L  K  G  K  W  D  H  E  R  T  N  F  S  L  P  P  L  M  N  L
901  ATTTTGGGAGAACCAGGAAGTGGAGGATCATCTACACCGTCGATGGTTGGCGCTGTCAAGAAATGGCAAAAGTCT
301   I  L  G  E  P  G  S  G  G  S  S  T  P  S  M  V  G  A  V  K  K  W  Q  K  S
976  GACCCGCAAAAGTCAAAGGATACATGGAACAAGTTGTCAGAGGCAAATTCGGCACTTGAAACACAATTTAACTTG
326   D  P  Q  K  S  K  D  T  W  N  K  L  S  E  A  N  S  A  L  E  T  Q  F  N  L
1051 TTAAGTAAATTAGCAGCAGATAACTGGGACTCATACAAAGCTGTCATCACCGGCTGCAGCATGTTAAAATCAGAA
351   L  S  K  L  A  A  D  N  W  D  S  Y  K  A  V  I  T  G  C  S  M  L  K  S  E
1126 AAGTGGATGGAGCAATTCAGTGAAACAACTCATGTAGAAATTGTTAAAGCATTAACGGGAGCTAGAGATGCAATG
376   K  W  M  E  Q  F  S  E  T  T  H  V  E  I  V  K  A  L  T  G  A  R  D  A  M
1201 CTTAAGATCCGGTTCCACATGAGGCAGATGGGCGATGCTGCAGGCATTCCGATTGAACCTGAATCACAAACTCGA
401   L  K  I  R  F  H  M  R  Q  M  G  D  A  A  G  I  P  I  E  P  E  S  Q  T  R
1276 CTCTTGGACGTGACCATGGACACAGAAGGTGCCTTGTTAGGTGGAGTTCCTGGTGCAGGTGGATTTGATGCTGTT
426   L  L  D  V  T  M  D  T  E  G  A  L  L  G  G  V  P  G  A  G  G  F  D  A  V
1351 TTTGCTATTACTTTAAGTGACTCAAGTACAAATTTGACCAAAATATGGAGTTTCCATAATGTTTTGGCAATGTTA
451   F  A  I  T  L  S  D  S  S  T  N  L  T  K  I  W  S  F  H  N  V  L  A  M  L
1426 GTGAGGGAAGATCCGCGTGGTGTTTCTATAGAAAGTGGTGATCCTCGGGAAAAGTAA
476   V  R  E  D  P  R  G  V  S  I  E  S  G  D  P  R  E  K  *
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Fig. 2: Nucleotide sequence and deduced amino acid sequence of *CnPMK*. The primer sequences are underlined. Asterisks represent the termination codon.

The nucleotide sequence of *CnPMK* had a high similarity with other *PMK* genes. The nucleotide sequence of *CnPMK* was 85%, 76%, 76%, 76%, 75%, and 74% identical to those of the *PMK* genes from *Taraxacum kok-saghyz*, *Daucus carota* subsp. *sativus*, *Citrus sinensis*, *Citrus clementina*, *Ziziphus jujube*, and *Hevea brasiliensis* (Table 1). Thus, the results indicated that the gene we cloned might be a member of the *PMK* gene family.

Table 1. Nucleotide sequence of *CnPMK* similarity to the *PMK* genes from other plant species

Species	Accession No.	Homology
Taraxacum kok-saghyz	KT899411.1	85%
Daucus carota subsp. *sativus*	XM_017374652.1	76%
Citrus sinensis	XM_006471412.2	76%
Citrus clementina	XM_006432616.1	76%
Ziziphus jujuba	XM_016012840.1	75%
Hevea brasiliensis	JN036535.1	74%

Characterization of the deduced CnPMK protein

The deduced CnPMK protein contained 493 amino acids. The theoretical molecular weight and isoelectric point (pI) of the deduced CnPMK protein were predicted to be 53.8KDa and 5.55, respectively. Multiple sequence alignment of CnPMK amino acid sequences with PMKs from other species showed high sequence similarity (Fig. 3). The CnPMK protein showed 75%, 76%, 73%, 73%, 71%, 73%, 73%, and 71% identical to the counterparts of *D. carota* subsp. *sativus*, *Panax notoginseng*, *Populus euphratica*, *Ricinus communis*, *Tripterygium wilfordii*, *Morus alba*, *Z. jujube*, and *Siraitia grosvenorii*, respectively. The multiple alignments analysis revealed that CnPMK represents the plant and fungal forms of the ERG8 type of PMK (*Pmev_kin_ERG8* type, eukaryotic branch). So far, two nonorthologous genes encoding PMK have been described, the *Saccharomyces cerevisiae* ERG8 gene and the human PMK gene. Houten and Waterham (2001) reported that orthologues of ERG8 are present in Eubacteria, fungi, and plants, while orthologues of human PMK are found only in animals, indicative of a nonorthologous gene displacement early in animal evolution.

Molecular evolution analysis

To investigate the evolutionary relationships in CnPMK and PMK proteins from other species, we selected the typical PMK proteins from the GenBank. We constructed a phylogenetic tree using the software MEGA 6.0 with neighbor-joining (NJ) method to analyze the molecular evolution of CnPMK on the basis of the three groups

(Dicotyledoneae, Monocotyledoneae and bacteria) and ten families of Angiosperms. As shown in Fig. 4, CnPMK belonged to the Asteraceae in the branch of Dicotyledoneae; the CnPMK protein had a close relationship to TkPMK of *T. kok-saghyz*. Taken together, these results indicated that CnPMK shared common evolutionary originals with other PMK proteins based on conserved sequence structure and sequence characteristics.

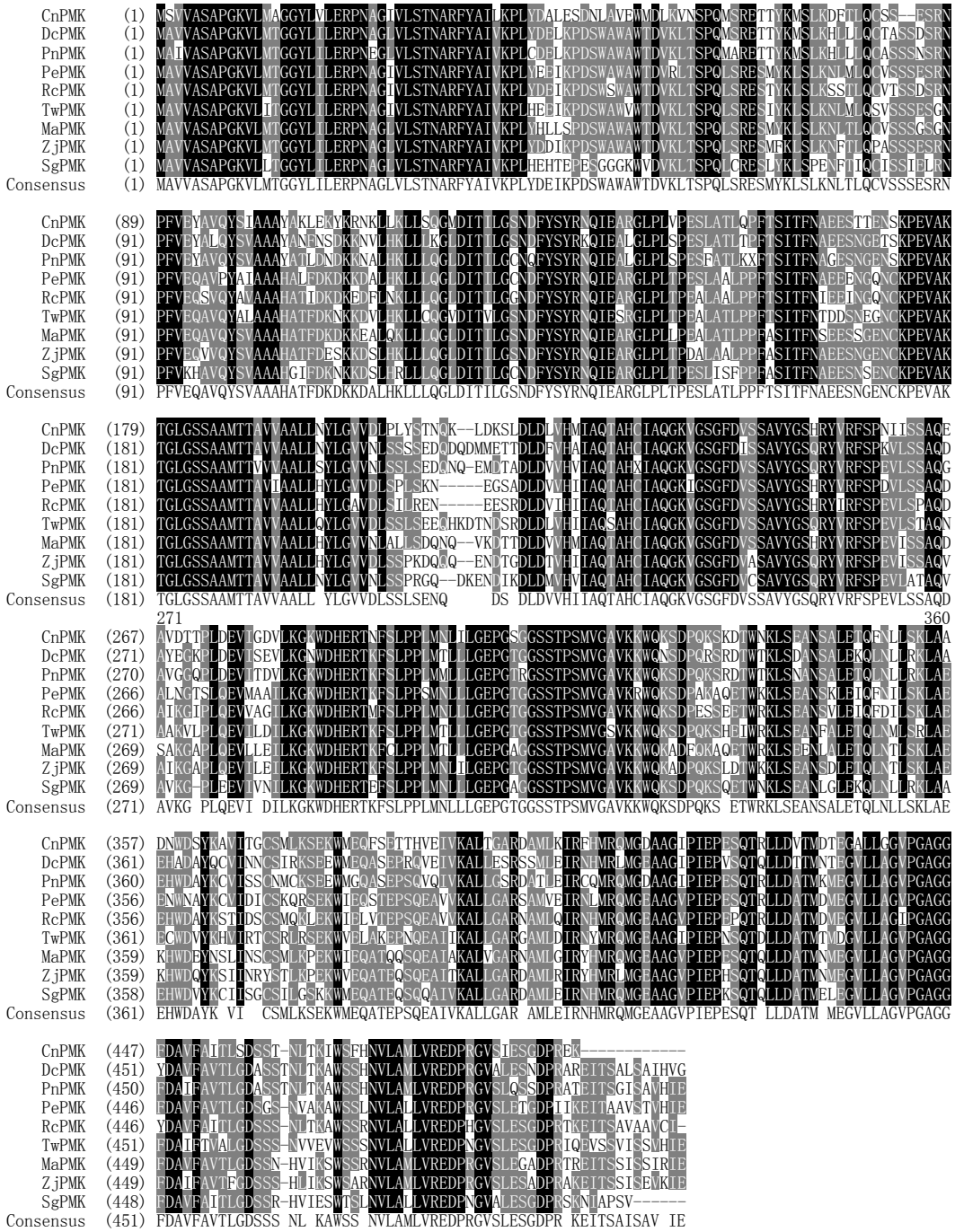

Fig. 3: Sequence multi-alignment of the deduced CnPMK protein with other PMK proteins. The species name and GenBank accession number are shown as following: CnPMK, *Chamaemelum nobile*; DcPMK, *D. carota* subsp. *sativus* (XP_017223228.1); PnPMK, *P. notoginseng* (AIK21784.1); PePMK, *P. euphratica* (XP_011028097.1); RcPMK, *R. communis* (XP_002520206.1); TwPMK, *T. wilfordii* (AMB15002.1); MaPMK, *M. alba* (ALD84323.1); ZjPMK, *Z. jujube* (XP_015868326.1); SgPMK, *S. grosvenorii* (AEM42973.1). Shaded in black are identical sequence. Shaded in gray are conservative sequence.

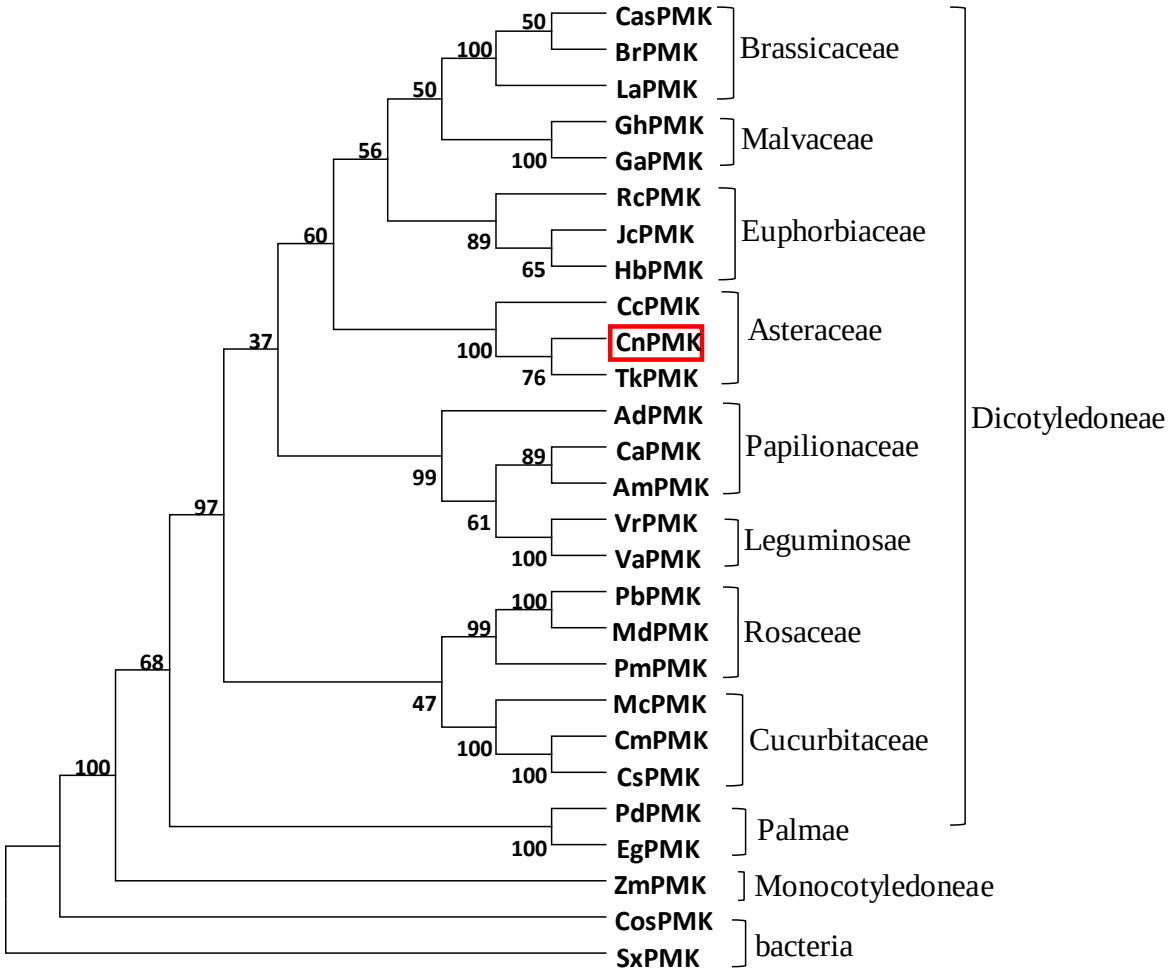

Fig. 4: The phylogenetic tree of phosphomevalonate kinase including CnPMK. Phylogenetic analysis of *CnPMK* with other PMK proteins from other plants and bacteria. Bootstrap values are expressed in percentages and placed at the nodes in the tree. The GenBank accession No. of the PMK proteins and species are as following: CasPMK, *Camelina sativa* (XP_010478612.1); BrPMK, *Brassica rapa* (XP_009108002.1); LaPMK, *Lepidium apetalum* (ALJ53305.1); GhPMK, *Gossypium hirsutum* (XP_016736729.1); GaPMK, *Gossypium arboretum* (XP_017638066.1); JcPMK, *Jatropha curcas* (XP_012092013.1); HbPMK, *H. brasiliensis* (AFJ74328.1); CcPMK, *Cynara cardunculus* (KVH95774.1); TkPMK, *Taraxacum kok-saghyz* (AMB19700.1); AdPMK, *Arachis duranensis* (XP_015934336.1); CaPMK, *Cicer arietinum* (XP_004502634.1); AmPMK, *Astragalus membranaceus* (AID51440.1); VrPMK, *Vigna radiate* (XP_014519068.1); VaPMK, *Vigna angularis* (XP_017422173.1); PbPMK, *Pyrus* × *bretschneideri* (XP_009341974.1); MdPMK, *Malus domestica* (XP_008368652.2); PmPMK, *Prunus mume* (XP_016651369.1); McPMK, *Momordica charantia* (AKO82483.1); CmPMK, *Cucumis melo* (XP_008466028.1); CsPMK, *Cucumis sativus* (XP_004136210.1); PdPMK, *Phoenix dactylifera* (XP_008788523.1); EgPMK, *Elaeis guineensis* (XP_010920023.1); ZmPMK, *Zea mays* (NP_001149345.1); CosPMK, *Colletotrichum sublineola* (KDN67694.1); SxPMK, *Staphylococcus xylosus* (KTW24759.1).

Conclusion

In this study, a *PMK* homologue, *CnPMK* was successfully cloned and characterized for the first time from a Asteraceae plant, *C. nobile*, which is involved in the sesquiterpenoids biosynthesis. Multiple sequence alignment showed that CnPMK had a high similarity with other plant PMK genes. The phylogenetic tree analysis also showed that the CnPMK might keep a strong conservation during the molecular evolution. The cloning and characterization of CnPMK will provide a theoretical basis for increasing the content of sesquiterpenoids in *C. nobile* through genetic engineering.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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