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Full Length Article

Transcriptome-Guided Gene Isolation, Characterization and Expression Analysis of a Phosphomevalonate Kinase Gene (*GbPMK*) from *Ginkgo biloba*

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Abstract

Terpenoids are main active ingredients of *Ginkgo biloba*. Phosphomethoxylate kinase (PMK) is one of the core enzyme in the mevalonate pathway, one of the two pathways in plants that can synthase terpenoids. In this study, a novel *PMK* gene (designated as *GbPMK*) was cloned from *G. biloba*. The expression profile of *GbPMK* in different tissues (roots, stems, leaves, male strobili, female strobili, and fruits) and under the treatments of hormones and stresses were investigated by qRT-PCR. Bioinformatics analysis indicated that the cDNA sequence of *GbPMK* contained a 1557-bp open reading frame encoding 519 amino acids. Protein structure analysis showed that the GbPMK protein had four conserved domains and one conserved region of the GHMP kinase family. Phylogenetic tree analysis revealed that GbPMK possessed a conserved sequence structure and sequence characteristics. The qRT-PCR analysis results showed that *GbPMK* exhibited tissue-specific expression, with the highest expression in leaves and the lowest expression in male strobili. *GbPMK* exerted different degrees of response to treatments with MeJA, ABA, Eth, SA, dark and low temperature but did not respond to wound treatment. The characterization and expression analysis of *GbPMK* contributed to understanding the biosynthesis of terpenoids at the molecular level. This study provides molecular information for the future through genetic engineering techniques to improve the yield of terpenoids in *G. biloba*.

Keywords: *Ginkgo biloba*; Terpenoids; *PMK*; Gene cloning; Abiotic stresses; Expression pattern

Introduction

Ginkgo biloba L., also known as "living fossil", is the oldest relict plant in the gymnosperm after the quaternary glacier movement. *G. biloba* is a unique plant, which is a sole member of one family, genus, and species preserved in China, and has high medicinal value (Wang *et al*., 2004). The extracts of *G. biloba* (EGB) contain more than 160 kinds of chemical constituents, including flavonoids, terpenoids, phenols, alkaloids, and ascorbic acid (Mou, 2008). The EGB promotes blood circulation and brain metabolism and protects against cardio-cerebrovascular diseases, such as coronary heart disease and hypertension, in middle-aged and elderly patients (Smith and Luo, 2004). Among the EGB, terpenoids are one of the most important bioactive constituents.

Terpenoids are the largest family of plant secondary metabolites and are referred to as a group of compounds composed of several isoprene units. Depending on the number of isoprene units, terpenoids can be divided into monoterpene (C10), sesquiterpene (C15), diterpene (C20), triterpene (C30), tetraterpene (C40), and polyterpene (Thulasiram *et al*., 2007). To date, more than 25000 types of terpenoids have been identified from plants (Yonekura-Sakakibara and Saito, 2009). Sesquiterpene, monoterpene and sesquiterpene compounds are volatile (Huang *et al.*, 2012) and the main components responsible for floral fragrance (Wang *et al*., 2013), attracting pollinators (Pichersky and Gershenzon, 2002), regulating plant growth and development (Piccoli and Bottini, 2013), and resisting stress (Schnee *et al*., 2006). In addition, terpenoids are abundant in resin secretions of gymnosperms, such as giant fir, and are the major component of their defensive substances (Parveen *et al*., 2015). Terpenoids not only are important in plants but also have high commercial value for humans. Nowadays, Terpenoids are widely used in spices, cosmetics, food, drugs, and pesticides (Yu and Utsumi, 2009). In addition, sesquiterpenoids, such as farnesene and bisphenol A, are potential precursors of advanced biofuels (Lee *et al*., 2008; Peraltayahya *et al*., 2011).

Terpenoids are derived from two basic five-carbon general precursors, namely, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Schwarz and Arigoni, 1999). Biosynthesis of IPP and DMAPP in two independent pathways: the first pathway is situated in the cytoplasm of mevalonate (MVA), and the other pathway is

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newly discovered in the plastids of 2-C-methyl-D-erythritol-4-phosphate (MEP) (Laule *et al*., 2003) (Fig. 1). In the MEP pathway, pyruvate and 3-glyceraldehyde phosphate are converted into IPP and DMAPP includes eight enzymes; of which, monoterpenes and diterpenoids are mainly derived from MEP (Lange *et al*., 2000). The MVA pathway exists in most living organisms (Vranová *et al*., 2013). In MVA pathway, IPP is synthesized with acetyl-CoA synthase as raw material over acetyl CoA-acetyl C-acetyltransferase (AACT), hydroxymethylglutaryl-CoA synthase (HMGR), hydroxymethylglutaryl-CoA synthase (HMGS), mevalonate kinase (MVK), phosphomethanolate kinase (PMK), and pyrophosphate mevalonate decarboxylase (MVD). IPP is then converted into DMAPP by isobutenylpyrophosphate isomerase (IPPI). In recent years, several genes participated in the MVA and MEP pathways have been isolated from *G. biloba*. Previous researches demonstrated that the expression of these genes is related to synthesis of terpenoids, such as *HMGR* (Shen *et al*., 2006), *AACT* and *MVK* (Chen *et al*., 2017), *MVD* (Pang *et al*., 2006; Liao *et al*., 2015a), 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MECS) (Gao *et al*., 2006; Kim *et al*., 2006), and levopimaradiene synthase (LPS) (Kim *et al*., 2012). Therefore, up-regulating the expression of these genes can promote the biosynthesis of terpenoids in *G. biloba*.

Phosphomethoxylate kinase (PMK), a member of the GHMP kinase superfamily (Herdendorf and Miziorko, 2006), is the fifth enzyme involved in one of the three speed limiting reactions of the MVA pathway (Fig. 1). In the reaction catalyzed by PMK, γ-phosphate groups on ATP transfer to meglumine phosphate, producing diphosphate mevalonate (Yuan *et al*., 2012). *PMK* gene was first discovered in the 1950s. Since then, the *PMK* gene and encoded protein have been isolated in different bacteria and various animals and plants (Lombard and Moreira, 2011; Miziorko, 2011). Given the progress in biotechnology, previous studies revealed that *PMK* is important in prevention of human diseases and biosynthesis of multiple secondary metabolites (Pilloff *et al*., 2003; Boonsri *et al*., 2013). However, the *PMK* gene has been rarely reported in plants. Yuan *et al*. (2013) found that the expression level of *PMK* significantly affected the yield of downstream products in *Paeonia lactiflora* Pall. Furthermore, proteomics studies revealed the low expression of the PMK protein in the MVA pathway, leading to low terpenoid yield (Singh *et al*., 2012).

Although no literature reported *PMK* genes or enzyme in *G. biloba* up to now, several genes involved in sesquiterpenoids have been cloned in *G. biloba*. For example, two enzymes encoding sesquiterpenoid synthase are identified as TPS1 and TPS2, which belongs to the TPS family (Zeng *et al*., 2013), and one enzyme encoding farnesyl diphosphate synthase (FPS) was reported by Wang *et al*. (2004). At present, research progress on enzymes or genes in synthetic sesquiterpenoids has been less advanced. Therefore, whether or not the use of sesquiterpenoids is similar to that of other gymnosperms remains unknown (Parveen *et al*., 2015). In this study, a novel *PMK* gene (designated as *GbPMK*) was cloned from *G. biloba*. The structure, characteristics, and function of *GbPMK* were studied through bioinformatics analysis. The expression patterns of *GbPMK* in different tissues (roots, stems, leaves, fruits, and male and female strobili) and under the treatments of hormones (MeJA, SA, ABA, and Eth) and stresses (dark, low temperature, and wound) were investigated by qRT-PCR analysis. Our findings provide a solid theoretical foundation for future use of biotechnology to increase the yield of terpenoids in *G. biloba*.

Materials and Methods

Plant Material

Experimental materials were collected from Ginkgo Botanical Garden of Yangtze University, Jingzhou, Hubei province, China. All materials were quickly frozen with liquid nitrogen and stored in a refrigerator at -80°C. One-year-old seedlings of *G. biloba* leaves were sprayed on both sides with 100 μM MeJA, 100 μM ABA, 10 mM SA, 10 mM Eth, and sucrose dissolved in 0.01% Tween-20. For control, the leaves for each treatment were sprayed with 0.01% Tween-20. The treated leaves were collected at 0, 1, 2, 3, 4 and 5 d. The seedlings were treated at 4°C in an incubator for cold treatment, and the control was placing at 25°C. For dark treatment, in order to kept in complete darkness, the seedlings were covered with a shading box, and the control was treated in light with a 16/8 light-dark photoperiod. Wound treatment was conducted by cutting 6 cm of the leaf edge, and normal leaves were used as control. All controls were placed in a culture room at 25°C with 16/8 light-dark photoperiod. Leaves treated with cold, dark, wound, and control were harvested at 0, 8, 12, 24 and 48 h.

Experimental Reagents

Mini BEST Plant RNA Extraction kit, Prime Script™ First-strand cDNA Synthesis Kit, Agarose Gel DNA Purification Kit Ver.4.0, pMD19-T vector, RNasin, dNTPs, and Taq DNA polymerase were purchased from Takara Company (Dalian, China). PrimeScript™ RT reagent Kit Perfect Real Time with a gDNA (genomic DNA) Eraser and AceQ qPCR SYBR Green Master Mix was purchase from Vazyme Biotech Co. (Nanjing, China). Primer synthesis and DNA sequencing were conducted by Sangon Biotech. Co. (Shanghai, China).

Cloning of the Full-Length cDNA of *GbPMK*

RNA was extracted from the leaves, roots, stems, fruits, female and male strobili of *G. biloba* by using the TaKaRa MiniBEST Plant RNA Extraction Kit (Dalian, China) according to the manufacturer's instructions.

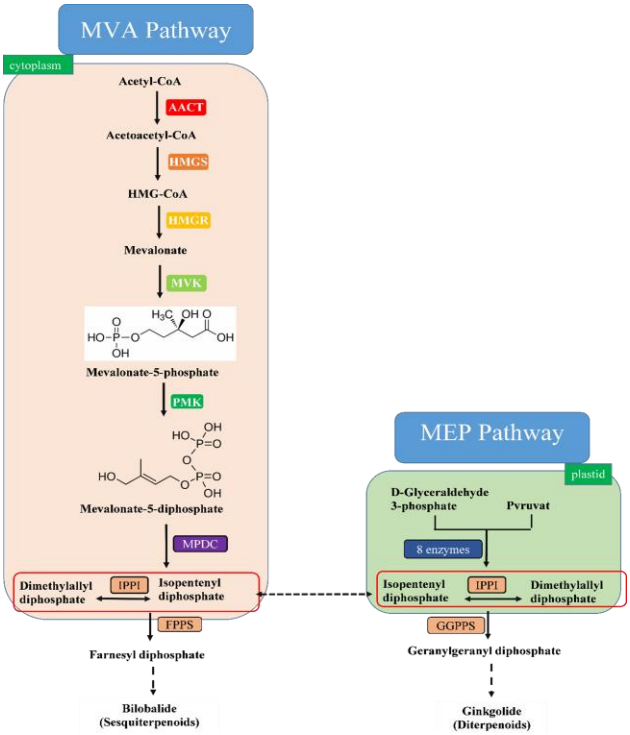

Fig. 1: Scheme of biosynthetic pathway of terpenoids in *Ginkgo biloba*

RNA concentration was determined using agarose gel electrophoresis or Eppendorf BioPhotometer. First-strand cDNA was synthesized following the instructions of PrimeScript™ First-strand cDNA Synthesis Kit. Primers UpPMK (5'-AGCTGATTATCTGCATTTGCTATCC-3') and DnPMK (5'-CGCATTTGTTAATTGTTTCTGAGTC-3') were designed on the basis of PMK unigene sequence of *G. biloba* transcriptome data (GenBank accession numbers SRX1982952 and SRX1983270). *GbPMK* cDNA was amplified under the following conditions: 94°C for 4 min; 32 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 90 s; and 72°C for 10 min. The amplified products were tested by 1% gel electrophoresis and purified by Agarose Gel DNA Purification Kit Ver.4.0. The purified products were ligated into the pMD19-T vector. The recombinant products were transformed into *Escherichia coli* DH5α competent cells (Nanjing, China) for sequencing.

Bioinformatics Analysis

Sequences were assembled, and their respective the open-reading frames (ORFs) were analyzed with Vector NTI 11.5.1. PMK protein sequences were retrieved from NCBI. The protein sequences were analyzed by software on http: //www.ncbi.nlm.nih.gov/BLAST/and http://web.expasy.org. Multiple sequence alignments were performed by DNAMAN6.0. InterPro Scan (http://www.ebi.ac.uk/interpro/) was used to predict the conserved domains of proteins. The secondary structure of the proteins was also predicted using online tools (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl). Multiple sequence alignments were conducted by MEGA 6.0 software. Phylogenetic tree of the PMK protein in the plants was constructed with NJ method using Clustalx 2.0 and MEGA 6.0.

GbPMK Transcript Analysis by Quantitative Real-time PCR

Different tissues (roots, stems, leaves, fruits, male and female strobili) of *G. biloba* and leaves subjected to the treatments of hormones (MeJA, SA, ABA and Eth) and stresses (dark, low temperature, and wound) were sampled to analyze gene expression patterns. RNA was reverse transcribed using PrimeScript RT reagent Kit, and 100 ng of RNA was used to synthesize single-strand cDNA. Based on the cDNA sequence of *GbPMK*, real-time fluorescent quantitative PCR primers were designed with Primer Premier 5.0. The upstream primer was PMKUp (5'-GCATAGTAGCACAGAGTCCCATAGC-3'), and the downstream primer was PMKDn (5'-CAGGCTCAAACTCTCAAGGGATAA-3'). The GAPDH gene was selected as internal reference gene, with forward primer of GAPDH-F and reverse primer of GAPDH-R. Single-strand cDNA as template (2 μL) in a 20 μL reaction mixture containing 10 μL of AceQqPCR SYBR Green Master Mix, forward and reverse primers were 0.4 μL, and 7.2 μL of ddH_2O. qPCR thermal cycling was conducted in accordance with the following conditions: 95°C for 5 min, 95°C for 10 s and 60°C for 30 s for 40 cycles, and 95°C for 15 s and 60°C for 60 s and 95°C for 15 s. Relative gene expression of transcripts in different tissues was determined by $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). All experimental procedures were carried on ice, and all samples were measured in biological triplicate. Data were technically replicated with error bars and represented as means ± SD (n = 3).

Results

Isolation and Characterization of the cDNA of *GbPMK*

Primers were designed on the foundation of the raw data of *PMK* unigenes of *G. biloba* transcriptions. Sequencing results demonstrated that the cDNA sequence of the gene amplified by RT-PCR was 1776 bp. Sequencing was performed using the online analysis tool of the Blast program on NCBI. The results showed that the cDNA sequence had homology with the PMK of other plants and named as *GbPMK* (GenBank: MG012793). The cDNA sequence of the *GbPMK* gene was 1776 bp and

contained a 1557-bp ORF encoding 519 amino acid protein (Fig. 2). The predicted molecular weight, and isoelectric point (pI) of the deduced GbPMK protein were 55.96 kDa and 5.74, respectively.

The conserved domain of the GbPMK protein was predicted by the online analysis tool InterPro. The GbPMK sequence was similar to the sequences of proteins belonged to two families, phosphomevalonate kinase 2 at position 2–508 of the amino acid sequence (IPR035102) and phosphomevalonate kinase Erg8 at positions 1–510 and 5–506 (IPR016005). The results of conserved domain analysis showed that GbPMK possessed four conserved domains: ribosomal protein S5 2-type (IPR020568) at positions 3–61, 89–140, and 189–294; ribosomal protein S5 2-type (subgroup, IPR014721) at 1–314; GHMP kinase N-terminal domain (IPR006204) at 185–263; and GHMP kinase C-terminal domain (IPR013750) at 412–485 and 315–491 (Fig. 3). In addition, the GbPMK protein contained a conserved region of the GHMP kinase family. Hence, our data indicated that GbPMK belonged to the ATP-dependent enzyme superfamily of GHMP kinase.

BLASTp search in GenBank database showed approximately 60% similarity between the GbPMK protein and the PMK protein of other plant species. Multiple sequence alignment revealed that GbPMK was 82.01% similar to the PMK protein sequences of other plants (Fig. 3). The secondary structure mainly consisted of four structure types, including a-helix (46.82%), extended strand (15.80%), β-turn (8.29%), and random coil (29.09%) (Fig. 4). The a-helices were represented by blue Hh; the extended strands were represented by red Ee; the β-turns were represented by green Tt; and the random coils were represented by purple Cc. The a-helix and the random coil accounted for the majority of the folding subordinate structures. In summary, these results suggested that GbPMK has a similar catalytic function to other plant PMKs.

Molecular Evolution Analysis

To gain the evolutionary relationship of GbPMK and PMKs from other species, we selected PMK protein from NCBI. A phylogenetic tree of GbPMK and other plant PMKs was constructed by CLUSTALX 2.0 and MEGA 6.0 with NJ method. As shown in Fig. 5, the PMK phylogenetic tree was divided into three branches, monocotyledoneae, dicotyledoneae, and gymnospermae. Specifically, monocotyledoneae was composed of gramineous plants, including *Aegilops tauschii subsp. Tauschi*, *Oryza sativa*, *Setaria italic*, and *Zea mays*. Dicotyledoneae was composed of rosaceae and araliaceae. Rosaceae included *Prunus mume* and *Malus domestica*, araliaceae included *Panax ginseng* and *Panax notoginseng*. Gymnospermae included *G. biloba* and *Picea glauca*. Based on the evolution of the phylogenetic tree, plants of the same genus were clustered together, indicating the relative closeness of the PMK gene of the same family. According to the conserved sequence characteristics and sequence structure, GbPMK shared common evolutionary origins with other PMK proteins.

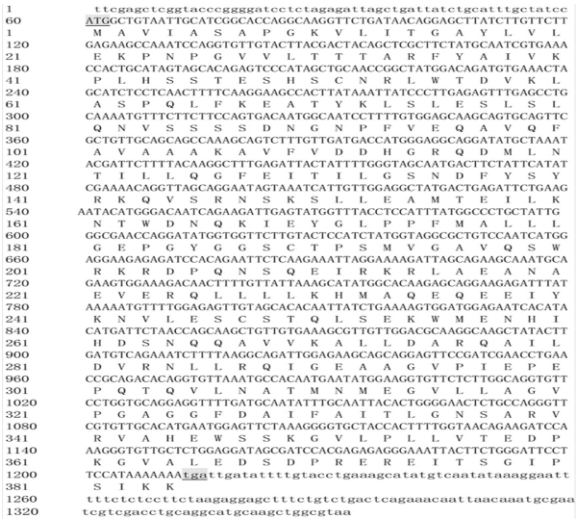

Fig. 2: The nucleotide acid sequence and deduced amino acid sequence of GbPMK. The underline is the start codon and the stop codon

Expression Patterns of *GbPMK* in Different Tissues of *G. biloba*

To investigate the expression pattern of *GbPMK* genes in different tissues of *G. biloba*, we extracted RNA from the roots, stems, leaves, fruits, male and female strobili. The qRT-PCR analysis showed that *GbPMK* gene was expressed in all tested tissues. However, *GbPMK* was expressed highest in the leaves, followed by the roots. A lowest expression level of *GbPMK* was observed in male strobili, which was significantly lower than that in other tissues (Fig. 6).

Expression Profile of *GbPMK* under the Treatments of Hormones and Stresses

To understand the expression profile of *GbPMK* subjected to MeJA, SA, ABA and Eth treatments, we extracted RNA from the leaves at different treatment times. Under MeJA treatment, the expression level of *GbPMK* decreased in different degrees with prolonged treated time. After 1 d, the expression level of *GbPMK* was reduced by 0.45-fold compared that in the control (Fig. 7A). MeJA significantly decreased the expression level of *GbPMK*. Meanwhile, SA generally increased the expression level of *GbPMK*; the expression first increased, then decreased, and finally reached the highest level 2 d after the treatment. The expression level was 1.21-fold higher than that in the control 2 d after treatments (Fig. 7B). For ABA and Eth treatments,

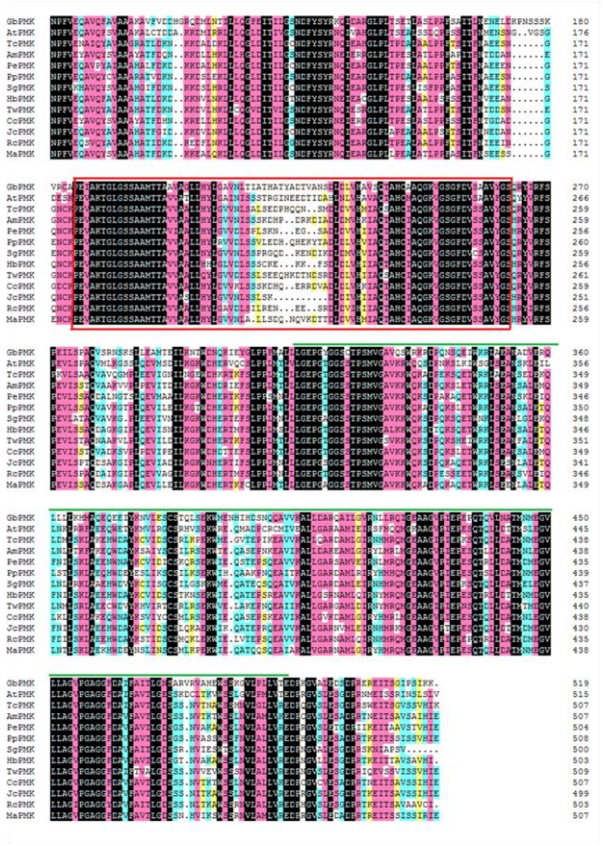

Fig. 3: The multiple alignments of GbPMK amino acid sequence with other plant PMK proteins. The completely identical amino acids are indicated with white foreground and black background. The conserved amino acids are indicated with black foreground and red background. Non-similar amino acids are indicated with black foreground and white background. The GHMP kinase N-terminal conserved domains in red frame, the GHMP kinase C-terminal conserved domains is marked with green line. The species, protein abbreviation and GenBank accession numbers are as following: *Ginkgo biloba*: GbPMK MG012793; *Amborellan trichopoda*: AtPMK, XP_011621878.1; *Theobroma cacao*: TcPMK, XP_007040735.2; *Astragalus membranaceus*: AmPMK, AID51440.1; *Populus euphratica*: PePMK, XP_011028097.1; *Prunus persica*: PpPMK, XP_020419647.1; *Siraitia grosvenorii*: SgPMK, AEM42973.1; *Hevea brasiliensis*: HbPMK, AFJ74327.1; *Tripterygium wilfordii*: TwPMK, AMB15002.1; *Cajanus cajan*: CcPMK, XP_020212065.1; *Jatropha curcas*: JcPMK, XP_012092013.1; *Ricinus communis*: RcPMK, XP_002520206.1; *Morus alba*: MaPMK, AOV62771.1

the expression level of *GbPMK* reached the peak after 1 d. The expression levels in ABA and Eth treatments were 3.03 and 1.56-fold higher than that in the control. After 4 d of

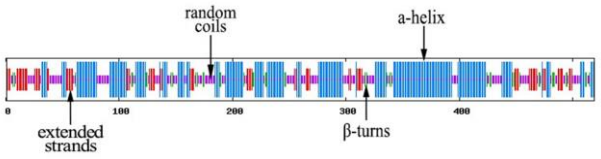

Fig. 4: The secondary structure of GbPMK. The a-helixes were represented by the blue Hh, the extended strands were represented by the red Ee, the β-turns were represented by green Tt, and the random coils were indicated by purple Cc

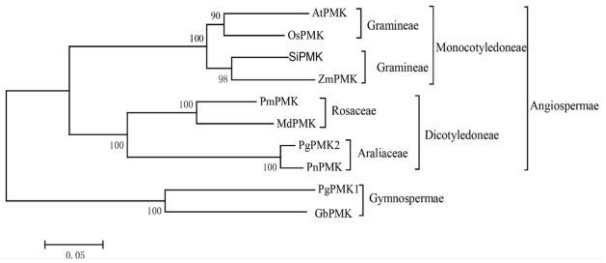

Fig. 5: Phylogenetic tree analysis of protein encoded by PMK genes. The number of Bootstrap replications was 1000. The species, protein abbreviation and GenBank accession numbers were as following: *Ginkgo biloba*: GbPMK MG012793; *Aegilopstauschii subsp. Tauschii*: AtPMK XP_020156382.1; *Oryzasativa Japonica Group*: OsPMK XP_015632523.1; *Setaria italic*: SiPMK XP_004984987.1; *Zea mays*: ZmPMK AQL08005.1; *Prunusmume*: PmPMK XP_016651369.1; *Malusdomestica*: MdPMK XP_008368652.2; *Panax ginseng*: PgPMK2 AGZ15314.1; *Panaxnotoginseng*:PnPMK AIK21784.1; *Piceaglauca*: PgPMK1 BT119875.1

treatment, the expression levels decreased in varying degree. Meanwhile, the expression level of *GbPMK* was higher in ABA treatment (Fig. 7C) but lower in Eth treatment than that in the control (Fig. 7D).

The expression pattern of *GbPMK* was analyzed by qRT-PCR under abiotic stress treatments with low temperature, dark, and wound. As shown in Fig. 8B, the expression level of *GbPMK* first increased and then decreased under dark. Meanwhile, an upward trend in *GbPMK* expression was observed after 48 h of treatment at 4°C (Fig. 8A). The expression level of *GbPMK* in the treatment at 4°C and dark were lower than that in the control and gradually decreased under prolonged treatment. However, there was no significant difference in the *GbPMK* expression under the wound treatment compared to the control during the whole times (Fig. 8C). Taken together, these results indicated that the expression of *GbPMK* was affected by low temperature and dark treatment but not by wound treatment. As a result, we speculated that *GbPMK* play a crucial role in the response of *G. biloba* to abiotic stresses.

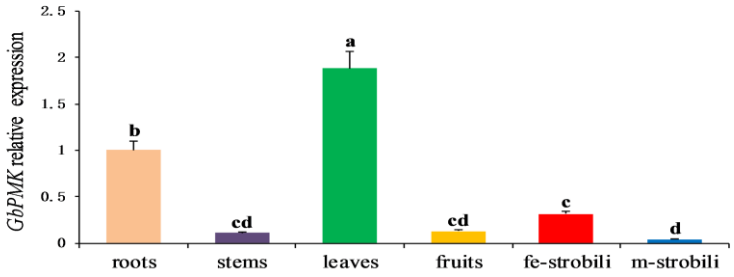

Fig. 6: Expression patterns of *GbPMK* gene in different tissues (roots, stems, leaves, fruits, male and female strobili). The gene level of roots was set to 1. Data from qRT-PCR were shown as means ± SD from triplicate experiments (n = 3). The test data were analyzed with single-factor ANOVA using SPSS11.0 software and methods were compared with Duncan multiple and the level of test at p<0.05

Fig. 7

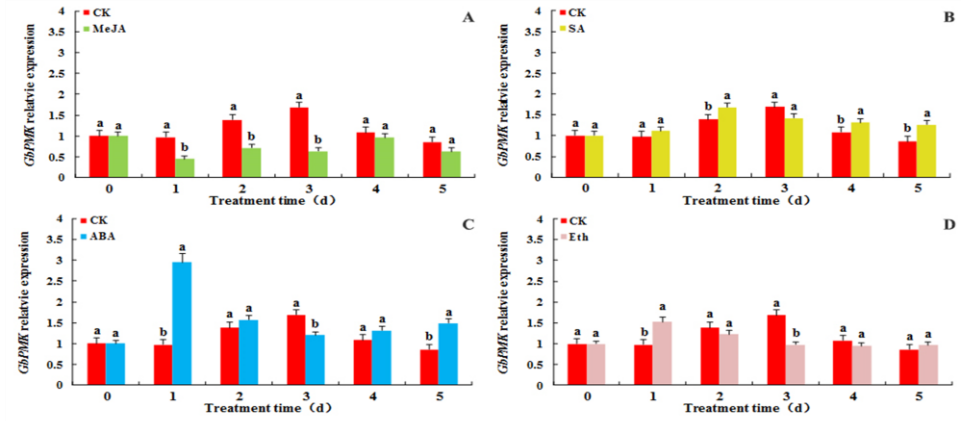

Fig. 7: Expression profiles of *GbPMK* under thehormone induction (MeJA, SA, ABA, and Eth).The gene expression level at the onset of hormone induction was set to 1, and the data analysis were compared with the expression ratio to the control level (CK).At least three biological replicates were carried out for each time point, and data were technically replicated with error bars and represented as means ± SD (n = 3). The test data were analyzed with single-factor ANOVA using SPSS11.0 software and methods were compared with Duncan multiple and the level of test at $p < 0.05$

Discussion

Ginkgo *GbPMK* had Similar Function with PMKs from other Plants

PMK, as one of the rate-limiting enzyme in the MVA pathway, plays important role in sesquiterpenoid biosynthesis pathway. However, little literature reported PMK enzymes or genes in plants at the molecular level. In the present work, a *PMK* gene (*GbPMK*) was cloned from *G. biloba* at first time. The results of sequence characteristics, structure, and conserved domain analysis suggested that GbPMK had a similar function with the PMKs of other species. It has been reported that overexpression of *PMK* can significantly increase the biosynthesis of plant terpenoids (Singh *et al*., 2012; Yuan *et al*., 2013). Therefore, it is reasonable to believe that the cloning and functional prediction of *GbPMK* may provide important target gene resources to increase the content of terpenoids using genetic engineering in *G. biloba*.

Expression Pattern of *GbPMK* in Different Organs of *G. biloba*

The terpenoids of *G. biloba* are mainly ginkgolides (diterpenoids) and bilobalide (sesquiterpenoids). Thus for some organs for biosynthesis of sesquiterpenoids have not been clearly reported. The tissue expression analysis show high *GbPMK* expression in the leaves and roots. Previous studies reported similar results, that is the gene expression was the highest in the leaves and roots in the MVA pathway (Strømgaard and Nakanishi, 2004; Yu and Utsumi, 2009).

Fig. 8

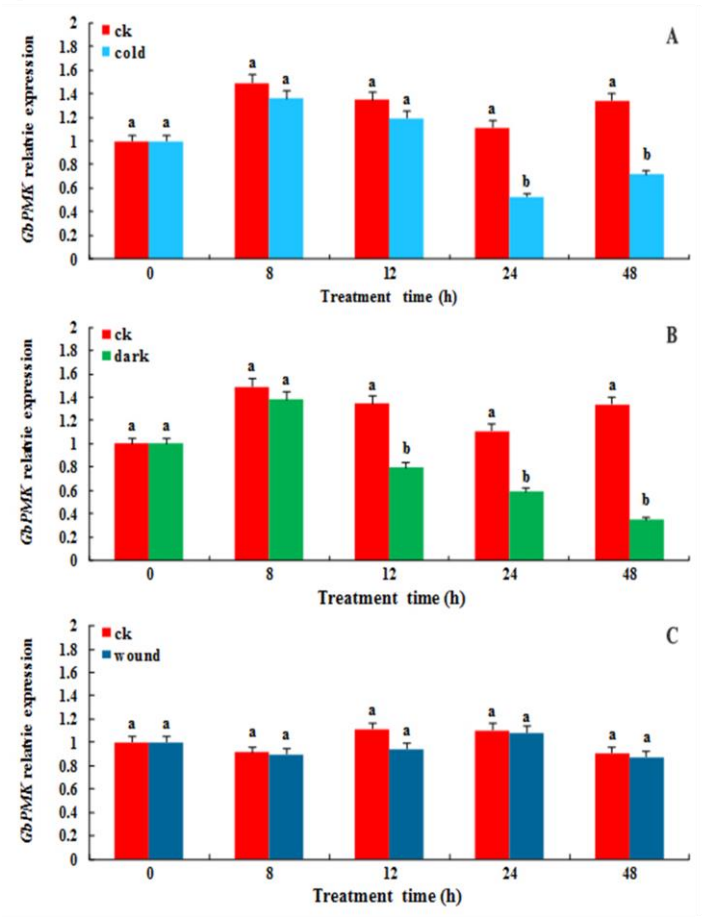

Fig. 8: The expression patterns of *GbPMK* under the low temperature, dark environment, and wound treatments. For each treatment, the expression levels of each time point were normalized to *GAPDH* gene. The gene expression level at the onset of stress treatments was set to 1, and the data analysis were compared with the expression ratio to the control level (CK). At least three biological replicates were carried out for each time point, and data were technically replicated with error bars and represented as means ± SD (n = 3). The test data were analyzed with single-factor ANOVA using SPSS11.0 software and methods were compared with Duncan multiple and the level of test at $p<0.05$

Given the expression of the *GbPMK* gene was related to the biosynthesis of the sesquiterpenoid, we speculate that leaf and root might be the main organs for sesquiterpenoid biosynthesis. The relationship between *GbPMK* gene expression and sesquiterpene biosynthesis pathway was also established to provide a reference for further studies.

Expression Level of GbPMK Changes under Different Elicitor Treatments in *G. biloba*

MeJA and SA are hormones that regulate the response of plants to stress (Robertseilaniantz *et al*., 2011) and have been applied as exogenous elicitor in plant secondary metabolism (Ketchum *et al*., 1999; Endt *et al*., 2007; Ketchum *et al*., 2015; Liao *et al*., 2016). MeJA could activate the overexpression of functional genes or transcription factors in the synthesis pathway of secondary metabolites (Endt *et al*., 2007). Exogenous application of SA can increase the accumulation of secondary metabolites in plants, induce plant resistance to various pathogens and promoting osmotic regulation (Conrath *et al*., 2002; Gharib, 2007). In this study, the expression level of *GbPMK* decreased in different degrees under prolonged MeJA treatment (Fig. 7A), while the expression level of *GbPMK* generally increased under SA treatment (Fig. 7B). As a result, *GbPMK* expression induced by SA but repressed by MeJA.

ABA is an important signaling molecule that is responsible for plant responses to stress (Guo *et al*., 2018); treatment of exogenous ABA enhanced the ability of plants to resist freezing injury and salt damage (Han *et al*., 2013; Yang *et al*., 2005). ABA treatment also increased the *GbPMK* expression level in *G. biloba*, consistent with previous studies (Xiao *et al*., 2015). Hence, we suggested that *GbPMK* was likely to be involved in ABA signal transduction in Ginkgo.

Ethephon (Eth) is a substitute of natural ethylene that regulates plant metabolism processes, including seed germination, root and plant growth, and plant development (Morgan and Gausman, 1966; Bingham and Mccabe, 2006). In the present work, Eth treatment increased the *GbPMK* expression level after 1 d, afterwards, Eth decreased the expression of *GbPMK* on the whole (Fig. 7D). We surmised that the *GbPMK* transcript level may be increased by spraying Eth at an appropriate temperature for an appropriate processing time.

Temperature is the main environmental factor that regulates plant metabolism. Low-temperature stress affects the normal growth and development of plants and can lead to death (Ji *et al*., 2010). In this study, the *GbPMK* expression first increased after 48 h and then decreased. The expression was lower than that in the control and gradually decreased under prolonged treatment at 4°C (Fig. 8A). This result in accord with previous reports that the "cold acclimation" response mechanism can alleviate and reduce damages in plants (Thomashow, 1990). The up-regulated expression profile of *GbPMK* by low temperature confirmed its role in response to cold stress.

Light is an essential factor for the growth and development of green plants (And and Chory, 1997). We observed that the expression of *GbPMK* was down-regulated by dark treatment. Our recent work also showed that the expression of another MVA pathway-related gene *GbHMGR* were induced by light (Liao *et al*., 2015b) due to presence of light-response motif in the gene promoter region. Therefore, we need to clone the promoter of *GbPMK* to classify the regulatory mechanism of effect of light on gene expression in further study.

Conclusion

In this study, anovel *GbPMK* gene was cloned from *G. biloba*. The tissue expression analysis showed that the expression level *GbPMK* was the highest in the leaves and the lowest in the male strobili. In addition, *GbPMK* exerted different responses to MeJA, ABA, Eth, SA, dark and low-temperature treatments. Therefore, *GbPMK* might play a significant role in the response of *G. biloba* to abiotic stresses. This work elucidates the biosynthesis of sesquiterpenoids in *G. biloba* at the molecular level and provides basis to increase the yield of terpenoids using genetic engineering techniques.

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