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马尾松 *PmPGK1* 和 *PmGPIC* 基因的克隆和表达分析

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摘要：为了解马尾松(*Pinus massoniana*)磷酸甘油酸激酶 1 (PGK1)与胞质溶胶葡萄糖磷酸异构酶(GPIC)的功能，采用 RACE 技术克隆了 *PmPGK1* 和 *PmGPIC* 基因，并进行了生物信息学分析与亚细胞定位，采用实时荧光定量 PCR 技术分析 *PmPGK1* 和 *PmGPIC* 的表达特性。结果表明，*PmPGK1* 和 *PmGPIC* 全长为 2 106 和 1 848 bp，分别编码 507 和 566 个氨基酸。PmPGK1 和 PmGPIC 分别定位于叶绿体和胞质溶胶。*PmPGK1* 表达量为新叶 > 老叶 > 新茎 > 根 > 花；而 *PmGPIC* 为老叶 > 花 > 新叶 > 新茎 > 根。低温胁迫 24 h，*PmPGK1* 和 *PmGPIC* 的表达量均随时间延长先降低后升高，且 *PmGPIC* 的表达量在处理 2 h 后即降至较低水平；高浓度 CO_2 胁迫 24 h，*PmPGK1* 的表达量随时间延长呈降低-升高-再降低的变化趋势，*PmGPIC* 的表达下调但变化较不显著。因此，推测 PmPGK1 主要参与卡尔文循环及叶绿体 / 质体糖酵解，PmGPIC 主要参与细胞质基质糖酵解；PmPGK1、PmGPIC 活性在低温胁迫下均受抑制；PmPGK1 活性在 CO_2 胁迫下受到显著抑制，而 PmGPIC 活性的影响不大。

关键词：马尾松；*PmPGK1*；*PmGPIC*；基因克隆；亚细胞定位
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Cloning and Expression Analysis on *PmPGK1* and *PmGPIC* Genes in *Pinus massoniana*

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Abstract: To understand the functions of phosphoglycerate kinase 1 (PGK1) and cytosolic glucose phosphate isomerase (CPIC) of *Pinus massoniana*, the cDNA of *PmPGK1* and *PmGPIC* were cloned by RACE, and the bioinformatic and subcellular localization of PmPGK1 and PmGPIC were analyzed, and then their expression patterns were performed by qRT-PCR. The results showed that the full-length cDNA of *PmPGK1* and *PmGPIC* were 2 106 and 1 848 bp, encoding 507 and 566 amino acids, respectively. PmPGK1 and PmGPIC proteins were located in chloroplast and cytosol, respectively. The expression of *PmPGK1* was in order of new leaf>old leaf> new stem>root>flower, while that of *PmGPIC* was old leaf>flower>new leaf>new stem>root. Under low temperature stress for 24 hours, the expression of *PmPGK1* and *PmGPIC* decreased at first and then increased, and the expression of *PmGPIC* decreased to a low level after 2 hours. Under high CO_2 stress for 24 hours, the expression of *PmPGK1* was significantly down-regulated, showing a trend of decrease-increase-decrease, while the down-regulation of *PmGPIC* was not obvious. Therefore, it was suggested that PmPGK1 mainly participated in the Calvin cycle and chloroplast/plast glycolysis, and PmGPIC was mainly involved in cytosolic glycolysis. The activities of PmPGK1 and PmGPIC were inhibited under low temperature stress, and PmPGK1 activity was

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significantly inhibited under high CO_2 stress, while PmGPIC activity was less affected.
Key words: *Pinus massoniana*; *PmPGK1*; *PmGPIC*; Gene clone; Subcellular localization

Phosphoglycerate kinase (PGK) is an important, soluble, rate-limited kinase used for basic metabolism in all organisms[1]. In plants, PGK participates in the Calvin cycle and glycolysis by catalyzing the release and transfer of high-energy phosphate groups between 3-phosphoglycerate (PGA) and 1,3-diphosphoglycerate (DPGA) and then catalyzing the reversible conversion of PGA and DPGA[2]. Glucose phosphate isomerase (GPI) is a multifunctional dimer protein in organisms that plays an important role in the carbohydrate metabolism cycle[3]. It catalyzes and breaks the molecular ring structure of fructose-6-phosphate (F6P) or glucose-6-phosphate (G6P) and transfers the intramolecular proton *via* its enzymatic acid-base catalytic mechanism and finally the two hexoses undergo reversible isomerization[2,4].

PGK exists in only one form in prokaryotes, while in most eukaryotes, there are 2–3 isozymes with different subcellular localizations[1,5]. PGK in plants is divided into two localization subtypes: cytosolic and chloroplast/plastidial PGK. Cytosol PGK mainly participates in cytoplasmic matrix glycolysis, while chloroplastic/plastidial PGK is mainly involved in the dual metabolic pathway of the Calvin cycle and chloroplastic/plastidial glycolysis[6]. GPI exists as a single form of cytosolic GPI in most animals and microorganisms, while there is also a plastidial GPI in plant cells[7]. Cytosolic GPI mainly participates in sucrose synthesis and glycolysis, and plastidial GPI is mainly involved in the metabolism of the oxidative pentose phosphate pathway (OPPP) and starch synthesis[8–9].

At present, research on PGK and GPI mainly focuses on clinical diagnosis[10–12]. There are relatively few studies on the protein subcellular localization, biological function and gene expression analysis of isoenzymes encoding different subtypes of PGK and GPI in plants, and the researches of the two enzymes in plants are mainly concentrated on annual plants, such as *Arabidopsis thaliana* or *Helianthus annuus*[13–16]. In this research, we cloned the *PGK1* and *GPIC* genes of masson pine, which encode phosphoglycerate kinase 1 (PGK1) and cytosolic glucose phosphate isomerase (CPIC), respectively. To explore the function of *Pm*PGK1 and *Pm*GPIC, bioinformatic analysis, subcellular localization analysis and quantitative real-time PCR (qRT-PCR) analysis based on tissue-specific expression, low temperature and high CO_2 stress were performed. The results of this research could advance our understanding of PGK and GPI in Masson pine and other plants.

1 Materials and methods

1.1 Test materials

The plant material used for rapid amplification of cDNA ends (RACE) analysis was derived from germinated seeds of masson pine (*Pinus massoniana*). The protoplast material used for subcellular localization was obtained from wild-type *Arabidopsis thaliana* (Columbia ecotype) at 3–4-week-old and not flowered. A laboratory-preserved pJIT166 transient expression vector was used for subcellular localization. Materials for tissue-specific expression were collected from 15-year-old masson pine in the arboreal garden of Nanjing Forestry University. The annual masson pine seedlings were used for expression analysis under low-temperature and elevated-CO_2 stress, which were provided by Fujian Baisha Forest Farm and then planted in soil (nutrient soil∶vermiculite∶perlite=1∶1∶1) in laboratory in September, 2018. The seedlings were grown in a naturally chamber with a cycle of 10 h light/14 h dark, day/night temperature of 30℃/26℃, and a relative humidity of 60%, with a slow seedling stage for about 15 days. Then the seedlings with the same growth state were selected for the following experiment.

1.2 Total RNA extraction and full-length gene clone

Total RNA was extracted from masson pine seedlings followed the RNAprep Pure RNA extraction

kit (Tiangen, Beijing). The concentration and quality of the RNA were detected by a NanoDrop fluorometer (ThermoFisher, MA, USA) and electrophoresis, respectively. First-strand cDNA was synthesized by using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing).

The *PmPGK1* and *PmGPIC* sequences were screened out after homology comparison between the *PGK1* and *GPIC* gene sequences of *Arabidopsis thaliana* obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/) and the masson pine transcriptome database (NCBI access No.: PRJNA561037). The cloning and amplification of intermediate fragments were carried out using the amplification primers (Table 1). Then, according to the intermediate sequence, the 5′/3′ RACE-specific primer was used to amplify the 5′/3′ end sequence according to the instructions of the SMARTer RACE 5′/3′ Kit (TaKaRa, Beijing). The full-length of *PmPGK1* and *PmGPIC* cDNA was obtained after sequence alignment and splicing predicttion, and conserved domains from the NCBI database were used for PmPGK1 and PmGPIC conserved domain analysis.

Table 1 Sequences of primers used in this study

Primer	Sequence (5′−3′)
PmPGK1-Mid	F: TGCCTTCTAATAGTTCCAAGCTTG
	R: CAGGTCCCATCATCGATCTTAAAG
PmGPIC-Mid	F: GGTCAATGATGGTAGTAAGATGCTTAG
	R: GACGGAATTATATTGGACTATTCTCGG
PmPGK1-5′ RACE	CCCAATCCGAACCTGGACTTGACCT
PmPGK1-3′ RACE	TCTTGTGCCCAGGCTTACAGAGTTGC
PmGPIC-5′ RACE	GAAACTTGCTAACAATAGGAAACCCATAC
PmGPIC-3′ RACE	CCCTTATCACTTCAGTATGGGTTTCCTA
pJIT166-*PmPGK1*	F*: tggagaggacagcccaagcttATGGCTTCAACAACCGCAGC
	R*: gcccttgctcaccatggatccAACTGTCACTGGGACAGCTTCAT
pJIT166-*PmGPIC*	F*: tggagaggacagcccaagcttATGGCAAGCACTAATCTGATCAGC
	R*: gcccttgctcaccatggatccTGATTTTGAGTGGAGAAAGCCC
pJIT166-35S-F*	atcccactatccttcgcaagacc
pJIT166-*GFP*-R	CACGAGGGTGGGCCAGGGC
PmACTIN2-qRT-PCR	F: GGTAGCTCCACCTGAGAGGAAGT
	R: GCCTTTGCAATCCACATCTGT
PmPGK1-qRT-PCR	F: CCTCCCTTGTTGAAGATGAC
	R: CTGGAATATCACTAGCAGGC
PmGPIC-qRT-PCR	F: GGAGGGAGCAAGAAGTATAG
	R: CCTTCCCATTACTCTCCATG

*: Lowercase letters are the carrier sequence.

1.3 Bioinformatic analysis

Blastn and Blastp from the NCBI were used to compare the homologous sequences of *PmPGK1* and *PmGPIC* cDNA and the amino acid sequences of their encoded proteins. Then, MEGA 7.0 was used to construct PmPGK1 and PmGPIC phylogenetic trees by the neighbor-joining method. WoLF PSORT (https://wolfpsort.hgc.jp/) was used to analyze the subcellular localization of PmPGK1 and PmGPIC proteins.

1.4 Subcellular localization

pJIT166 plasmid was double digested (restriction enzyme cutting sites: *Hin*d III and *Xba* I). The recombinant expression vector was constructed according to the ClonExpress II One-Step Cloning Kit (Vazyme Biotech, Nanjing). The recombinant plasmid was extracted according to the instructions of the Plasmid Maxi Kit (QIAGEN, Germany) after transformation into *Escherichia coli* and culture expansion. According to the manufacturer's instructions (Real-time Biotech, Beijing), approximately 10 large and plump wild-type *Arabidopsis thaliana* (Columbia ecotype) leaves were selected and cut into thin strips with a width of 0.5−1.0 mm to prepare protoplasts, and then, the pJIT166 recom-

binant plasmid (>1 μg/μL) was transformed into the prepared protoplast (10 μL). Finally, the above solution was put it in a 22℃–25℃ dark environment for 14–16 h of incubation, and the fluorescence reaction was recorded at 488 and 543 nm by fluorescence microscopy. Protoplasts with empty vector accompanied by green fluorescent protein (GFP) were used as the control group (CK group).

1.5 Expression patterns

For tissue-specific expression analysis, total RNA from 5 tissues, including new leaves (NL), old leaves (OL), new stems (NS), flowers (F) and roots (R), was extracted with three biological replicates for each treatment using a Plant RNA Isolation Kit (Tiangen Biotech, Beijing). Then, first-strand cDNA was synthesized with FastKing gDNA Dispelling RT SuperMix (Tiangen Biotech, Beijing) according to the manufacturer's instructions. For cold stress, annual seedlings were placed in a refrigerator at 4℃ and the seedling leaves were collected at 0, 2, 4, 8, 12 and 24 h, respectively. For elevated-CO_2 stress, the seedlings were moved into a growth chamber with 10 h light/14 h dark at 25℃. Air containing approximately 400–450 mg/m^3 CO_2 (approximately two times the ambient CO_2 concentration) was injected into the growth chamber constantly for at least 24 h. Then, the seedling leaves were sampled after 0, 6, 12 and 24 h of treatment. For qRT-PCR, the mixtures consisted of 10 μL of 2×ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme Biotech, Nanjing), 0.4 μL of forward primer and reverse primer, 2 μL of cDNA, and 7.2 μL of ddH_2O. The qRT-PCR program consisted of three stages: 95℃ for 30 s (preincubation), 95℃ for 10 s, 60℃ for 30 s, and 72℃ for 30 s, cycling 40 times (amplification), and 95℃ for 15 s, 60℃ for 1 min and 95℃ for 15 s (melting curves). QRT-PCR quality was estimated based on the melting curves. *PmActin2* (NCBI Accession No.: KM496525.1) was used as the internal control[17]. The gene-specific primers employed are shown in Table 1. Three independent technical replicates were performed for each treatment. Quantification was achieved using comparative cycle threshold (Ct) values, and gene expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method[17].

2 Results

2.1 Full-length clone of *PmPGK1* and *PmGPIC*

The intermediate fragments of *PmPGK1* and *PmGPIC* were 1 564 and 1 305 bp, respectively. The 5′/3′ RACE sequences of *PmPGK1* were 297 and 1 225 bp, respectively, and those of *PmGPIC* were 1 056 and 1 117 bp, respectively. After sequence alignment and splicing, it was found that the full-length of *PmPGK1* and *PmGPIC* were 1 848 bp (NCBI Accession No.: MT586614) and 2 106 bp (NCBI Accession No.: MT591683), respectively (Table 2). According to ORF Finder analysis, the 5′/3′ cDNA ends of *PmPGK1* had 93 and 324 bp untranslated regions (UTRs), respectively. The open reading frame (ORF) of *PmPGK1* covered 1 524 bp, encoding 507 amino acids. In contrast, the 5′/3′ cDNA ends of *PmGPIC* had 146 and 259 bp UTRs, respecttively. The ORF of *PmGPIC* covered 1 701 bp, encoding 566 amino acids. Conserved domain analysis showed that *PmPGK1* and *PmGPIC* were members of the phosphoglycerate kinase superfamily and PLN06249 superfamily, respectively (Fig. 1).

2.2 Sequence alignment and phylogenetic tree

The Blastn comparison results showed that the similarity of *PGK1* between *P. massoniana* and *P. pinaster* (the only species reported *PGK1* sequence from gymnosperms) was 98.53%, and that between *P. massoniana* and angiosperms was 73%–80%. The similarity of *PmGPIC* with *GPIC* in *Cedrus libani* reached 93.66%, and with those in *Ginkgo biloba*, *Encephalartos barteri*, and *Cryptomeria japonica* was 85.60%, 84.23% and 83.64%, respectively.

The similarity of PGK1 between *P. massoniana* and *P. pinaster* was 99.01%. In terms of known angiosperm plants, the similarity with the chloroplastic PGK of *Carica papaya*, *Asparagus officinalis* and *Prosopis alba* was 87.65%, 85.22% and 84.28%, respectively, and that with the cytosolic PGK of *Cajanus cajan*, *Glycine max* and *Dendrobium catenatum* were

Table 2 Sequence of full-length cDNA of *PmPGK1* and *PmGPIC*

Gene	Sequence (5′−3′)
PmPGK1	CAGGTCCCATCATCGATCTTAAAGCTACCCACTGAACGCATCTGGAAACCCCACGAGTCTTCCGAGTTCTGTCTGAAAACTGCG TAGGTAACAATGGCTTCAACAACCGCAGCTGCTGCAGCGCTCGGCCGAATTGCCTCGACACCAAATCTGCAAGCCGTGTCATCG TCGTCCACTTCCTCTCCCGTGGCCCGCCTCGCCATTGGATCCAGATCGTGGTCCGCCTCTCCTCACCGCCGGCTCCGTTCTATTGC CTCCTCGCCCCTGCTGGAGGTCAAGTCCAGGTTCGGATTGGGCGCCGCTTCCAACCTGTTTGCCTCTGAGAATGATCTCGCCTCT GCTGTAGCAGCGCAGGTTGGTGGCGCCAACAATGGCAGAGGCTGCCGAGGAGTTGTCTCCATGGCCAAGAGGAGCGTCGGGG ATTTGAAGGAATCCGATCTTAAGGGCAAGAAGGTTTTTGTCCGGGCCGATTTGAATGTCCCCCTCGATGAAAATCAGAACATCA CCGATGATACGCGTATCAGGGCCGCCGTTCCCACCATCAAGTACTTAATCAGCAACGGTGCCAAGGTTATTCTTACAAGTCACCT TGGTCGTCCTAAGGGTGTCACACCAAAGTACAGCTTGAAGCCTCTTGTGCCCAGGCTTACAGAGTTGCTTGGGGTGAATGTGGT AAAAGCAGATGATTGTGTTGGACCGGAAGTAGAAAAAGCAGTGGCATCGCTTCCAAATGGAGGAGTTTTGCTTCTTGAGAATG TTCGATTCTACAAGGAGGAAGAGAAAAATGATCCTGAATTTGCAAAGAAGCTGGCATCTATTGCAGACCTTTATGTTAATGATGC TTTTGGTACAGCTCACAGAGCACATGCGTCAACGGAAGGTGTTACTAAATATCTCAAGCCAGCTGTTGCTGGTTTCCTTCTGCAG AAGGAACTTGACTACCTTGTAGGCGCTGTGTCTATTCCTAAGAGACCTTTTGCTGCCATTGTGGGAGGGTCGAAGGTGTCATCC AAAATTGGAGTTATTGAGTCTTTGCTCGAGAAGGTGGATATTCTTCTCCTTGGAGGAGGGATGATTTTCACTTTCTACAAAGCCC AAGGGCTTAAAGTTGGATCCTCCCTTGTTGAAGATGACAAGATTGAGCTTGCCAAGACCCTGATGCAGAAAGCGAAAGAGAAG GGTGTCTCTCTTTTATTGCCAACAGATGTAGTAATTGCAGACAAATTTGCTGCTGATGCAAACAGCAAGGTTGTGCCTGCTAGTG ATATTCCAGATGGATGGATGGGTCTTGATATTGGAGCTGATTCAATCAAGACTTTTAGTGAAGCTTTGGATACAACTAAGACTGTT ATTTGGAATGGTCCCATGGGAGTTTTTGAATTTGACAAATTTGCCGCTGGAACCGAGTCAATTGCAAAGAAGCTCGCAGAGCTA AGTGGGAAGGGTGTGATCACAATCATTGGTGGAGGTGATTCCGTTGCTGCCGTGGAGAAAGTAGGTGTAGCTGAGAAGATGAG CCACATTTCCACAGGTGGTGGAGCAAGCTTGGAACTATTAGAAGGCAAAACCCTTCCTGGTGTCCTTGCCTTGGATGAAGCTGT CCCAGTGACAGTTTAATCTATTTCAGTAGGATAATTTTGTCATTTTTCATTTGGAATGAAATATTTAGTTTCAACCTTTCAGATGGG GAGTGGCACTAAGTGTGAAGAAAGAGTGTTGAGTTATCTTCATATCAGCCTAGTACGTTCATGGTTTTTGAGTCCTGTTCATTTGT AAAAGTTAAAAACTCGTCAATAAAATAGCAATTTTCGACTCGGGATTCAGGAGCTGTTGATCAAAAAAAAAAAA
PmGPIC	GAAACGCATTGGTATTAGTAGGCCTGGCAGCTGCTGGAGGATCAGTAATTGCGCTCTGTATCCATCGTTTCAGGATCCGTTTGCC TTCGTCCAAAGGGGCATTACGCTTGTACAGTCTGTCTGAAGCTGCAGCTGAAAGCCTTTCAATGGCAAGCACTAATCTGATCAG CGCAACTGAGGAATGGAAATGCTTAAGGGAACATGTTGCAGATGTTGATAAGACACACTTGAGAAATTTACTGAAGAATGAAGC TCGCTGTGAATCAATGACAGTAGAATTTGACGGAATTATATTGGACTATTCTCGGCAAAGGATAACTCAAGAGACTGTGAAGAA GCTTTTTAAGCTAGCAGAGGTGGCGCATCTGAAGGAGAAGATTGACAAAATGTACAATGGAGACCATATAAATGTCACAGAGAA TCGTTCTGTGCTTCATATTGCTCTAAGGGCTGCCAGGGATACAATCATCAATTGTGATGGGAAGAATGTAGTTGATGATGTCTGGC AAGTTCTCGATAAGATCGGTGAATTTTCTGAAAGGGTTCGGACTGGGGCTTGGGTAGGTGCAACAGGGAAGTCGCTTAAAGATG TTGTGGCAGTAGGCATTGGTGGCAGCTTTCTGGGTCCACTTTTTGTGCACAGTGCTCTTCAAACAGATCCTGAGGCAGCTCAAT GTGCAAATGGGCGTCAACTGCGATTTCTAGCAAATGTTGATCCAGTTGATGTCGCTCGGAGTATTAATGGGTTAAACCCGGAAAC TACATTAGTTGTTGTGGTATCAAAGACATTCACAACAGCAGAAACCATGCTAAATGCTCGAACATTAAGGACATGGATCACATCT GCCCTTGGTTCTGAAGCTGTTGCAAAGCACATGGTGGCAGTTAGTACTAACCTTAAGCTTGTAAAAGAATTTGGAATAGACCCA CAAAATGCTTTTGCATTCTGGGATTGGGTTGGCGGTCGCTACAGCGTGTGCAGTGCTGTGGGTGCCCTCCCCTTATCACTTCAGT ATGGGTTTCCTATTGTTAGCAAGTTTCTGGAGGGAGCAAGAAGTATAGATAACCATTTCCACACAACTCCATTTGAGAAAAATAT TCCTGTTTTGCTTGGTCTCCTCAGTGTGTGGAATGTTTCCTTTCTTGGGTATCCAGCTAGGGCTATCTTGCCATATACTCAGGCTCT GGAGAAGTTTGCACCGCACATCCAGCAGCTTAGCATGGAGAGTAATGGGAAGGGAGTTTCTATTGATGGTGTGCCCCTCAATTT TGAGGCTGGTGAAATAGATTTTGGAGAACCTGGTACAAATGGCCAGCATAGCTTTTACCAGTTAATTCATCAGGGTCGTGTAATT CCTTGTGATTTTATTGGCATCGTCAAGAGCCAGCAGCCCATTTATCTACAAGGGGAAGTTGTGAGTAACCACGATGAGCTCATGT CTAATTTTTTTGCACAGCCAGATGCTCTTGCCTGTGGGAAGACTCCTGAGGAGTTGAGGAATGAGAAAGTTTCTGATCATCTTGT TGCACACAAGACATTCTCTGGGAATCGACCATCACTAAGCATCTTACTACCATCATTGACCGCATATAACGTAGGCCAGTTGTTA GCAATGTATGAACACAGGATTGCAGTTCAGGGTTTCATTTGGGGCATTAATTCCTTTGACCAATGGGGCGTGGAGCTTGGCAAGT CACTGGCATCTCGTGTACGAGCTCAGCTGAACTTGTCTCGCACTGAAGGGAAGCCAGTTGAGGGCTTTAACCCCAGCACCACA ATGTTGATAAAACGATACTTGAAGGAAAATGCTCCAATTACTGCGGGCTTTCTCCACTCAAAATCATAGTACATACTTTTCTATTT GTTGAGCACCAGTCAGTCTTTTGATTTTGAATGCCAGTCCTTATTTTGTATTTGAATGAGAGAGATTCACTTATGTAGAAAGCTTT CTGTTAAAATGGAGGCACTTTTGATGATCAGGAATCGATATTTTTTTGTCATAAACGGGGAACCCAGTATTCGTATCTTCAGTTGA GTTGAAAATAACATGCATCTTCATAATAAAGAAACTGGTGAAATCTTTTCCAGTTTAGAAAAAAAAAAAA

87.92%, 87.89% and 83.56%, respecttively. For GPIC, among the known angiosperms, the similarities between Masson pine and *Ananas comosus*, *Elaeis guineensis* and *Apostasia shenzhenica* were higher than those with others, reaching 81.16%, 80.86% and 80.43%, respectively.

According to PGK phylogenetic analysis, *P. massoniana* clustered with *P. pinaster* firstly, showing the closest relationship, and then clustered with angiosperms (Fig. 2: A). For GPIC, masson pine, *Cedrus libani*, *Cryptomeria japonica* and *Ginkgo biloba* were clustered initially, with a confidence of 100%, and then sequentially clustered with monocotyledonous or dicotyledonous plants (Fig. 2: B). These results were consistent with the homology comparison results.

2.3 Subcellular localization

The localization results showed that the empty vector GFP fluorescence signal was expressed in the cell membrane, cytoplasm and nucleus (Fig. 3: a−d). WoLF PSORT predicted that PmPGK1 was located in the cytosol, which was different from the subcellular localization result that it was located in the chloroplast (Fig. 3: e−h). The fluorescence signal of PmGPIC filled the cytoplasmic matrix (Fig. 3: i−l), which was consistent with the prediction of WoLF PSORT.

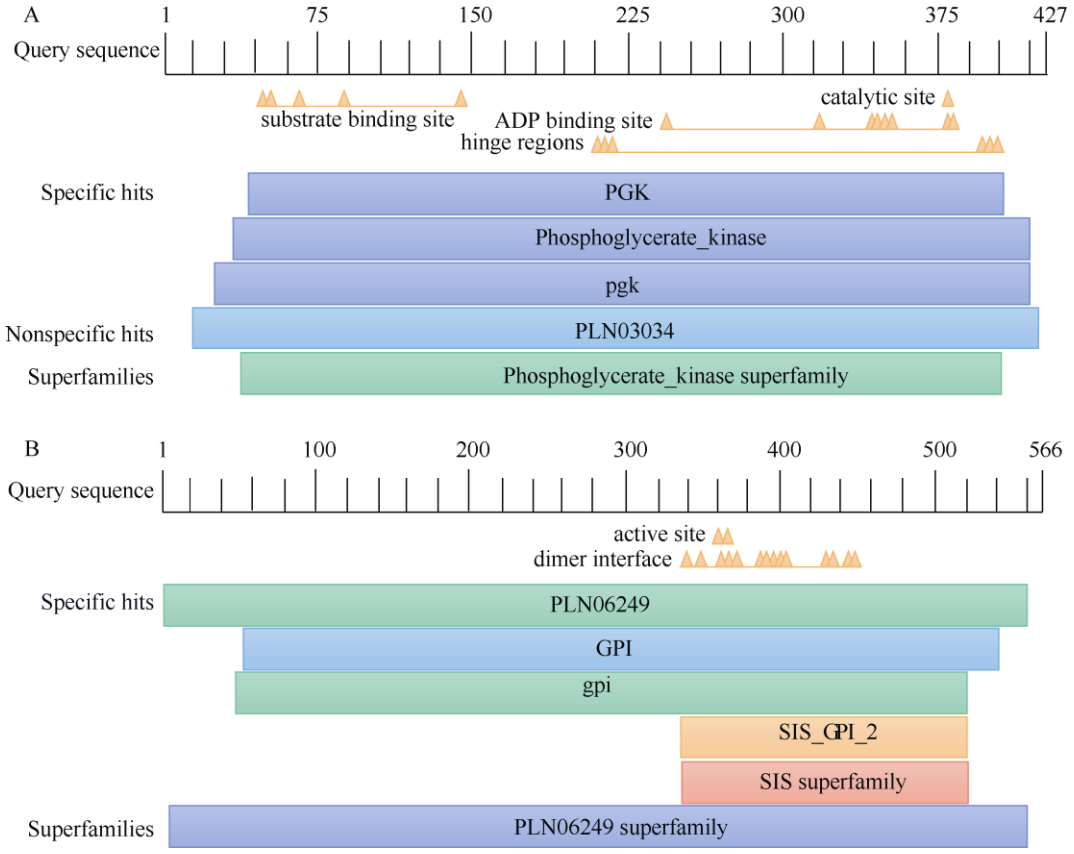

Fig. 1 Conserved domain of amino acids of PmPGK1 (A) and PmGPIC (B)

2.4 Gene expression pattern

Tissue-specific expression analysis revealed that the expression of *PmPGK1* in new leaf (NL) was the highest, followed by new stem (NS) and old leaf (OL). The expression difference among three tissues was relatively small. The lowest expression was observed in flower (F), which had significant difference from that in other tissues ($P<0.05$) (Fig. 4: A). For *PmGPIC*, the expression was the highest in old leaf and nearly zero in the roots (R). There were significant differences among all tissues except new leaf and flower (Fig. 4: A).

Under low-temperature stress within 12 h, the expression of *PmPGK1* and *PmGPIC* decreased with the time, and the decrease in *PmGPIC* was more obvious than that in *PmPGK1*. The expression level of *PmPGK1* decreased to the lowest level after 12 h stress, showing a significant difference from that in other groups (Fig. 4: B). On the other hand, along the time, there was no significant difference in expression of *PmGPIC* among the experimental groups (Fig. 4: B). Under elevated-CO_2 stress, *PmPGK1* showed significant differences among different time and showing a trend of decreasing-increasing-decreasing (Fig. 4: C). The expression of *PmGPIC* did not change signifycantly under high-CO_2 stress and within 24 h (Fig. 4: C).

3 Conclusion and discussion

Both of PGK and GPI have two localization subtypes in plant cells, i.e. cytoplasmic and plastidial. Isoenzymes of different localization types perform different functions in cell metabolism[1,7]. According to the subcellular localization, it was found that PmPGK1 was located in the chloroplast, belonging to chloroplastic/plastidial subtype, mainly involved in Calvin cycle and chloroplast/plastid glycolytic metabolism and catalyzes the reversible reaction between PGA and DPGA[6,13–14]. This result is the same as AtPGK1 in *Arabidopsis thaliana* by Rosa-Téllez et al.[13], but contrary to that of Huang et al.[18], who proved that PGK1 was localized in the cytosol.

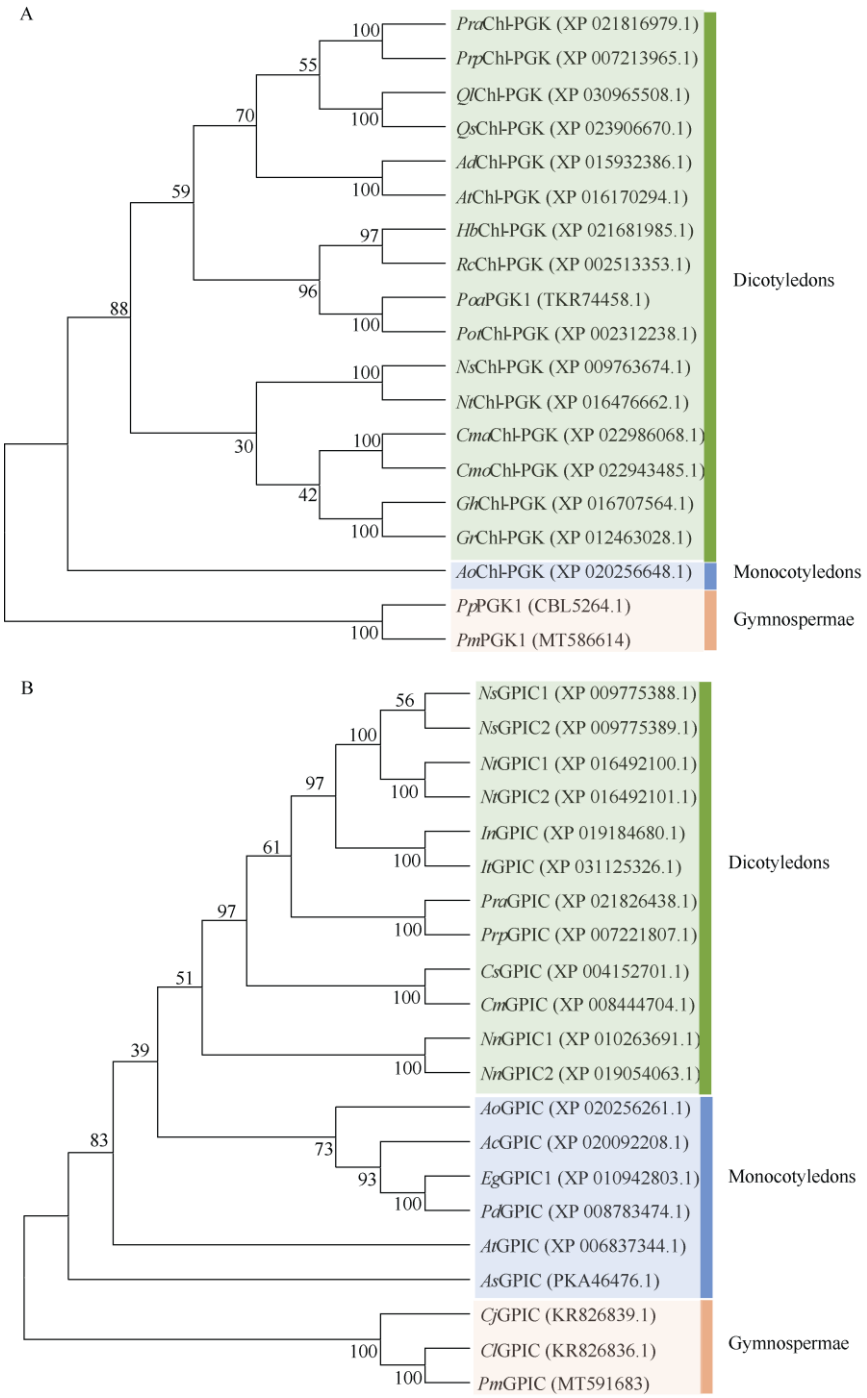

Fig. 2 Phylogenetic tree of PGK1 (A) and GPIC (B). *Pra*: *Prunus avium*; *Prp*: *P. persica*; *Ql*: *Quercus lobata*; *Qs*: *Q. suber*; *Ad*: *Arachis duranensis*; *Ai*: *A. ipaensis*; *Hb*: *Hevea brasiliensis*; *Rc*: *Ricinus communis*; *Poa*: *Populus alba*; *Pot*: *P. trichocarpa*; *Ns*: *Nicotiana sylvestris*; *Nt*: *N. tabacum*; *Cma*: *Cucurbita maxima*; *Cmo*: *C. moschata*; *Cs*: *C. sativus*; *Cm*: *C. melo*; *Gh*: *Gossypium hirsutum*; *Gr*: *G. raimondii*; *Ao*: *Asparagus officinalis*; *Pp*: *Pinus pinaster*; *Pm*: *P. massoniana*; *In*: *Ipomoea nil*; *It*: *I. triloba*; *Nn*: *Nelumbo nucifera*; *Ac*: *Ananas comosus*; *Eg*: *Elaeis guineensis*; *Pd*: *Phoenix dactylifera*; *At*: *Amborella trichopoda*; *As*: *Apostasia shenzhenica*; *Cj*: *Cryptomeria japonica*; *Cl*: *Cedrus libani*.

PmGPIC was located in the cytosol, which proved that it was mainly involved in sucrose synthesis and glycolytic metabolism in the cell matrix and catalyzed the reversible isomerization between F6P and G6P. Currently, there are no reports on the subcellular localization of plant GPI isoenzymes.

The expression of *PmPGK1* in leaf was higher than that in other tissues, which was consistent with

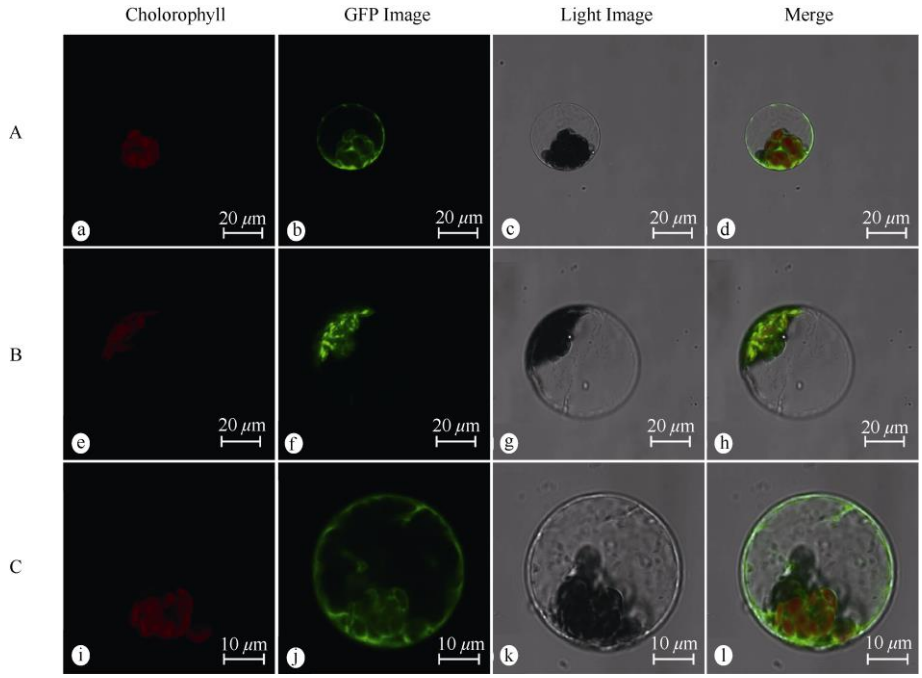

Fig. 3 Subcellular localization of PmPGK1 and PmGPIC proteins in protoplast of *Arabidopsis thaliana*. A: pJIT166-GFP; B: pJIT166-*PmPGK1*-GFP; C: pJIT166-*PmGPIC*-GFP; a, e, f: Chloroplast autofluorescence field; b, f, j: GFP field; c, g, k: Bright field; d, h, l: Merged pictures.

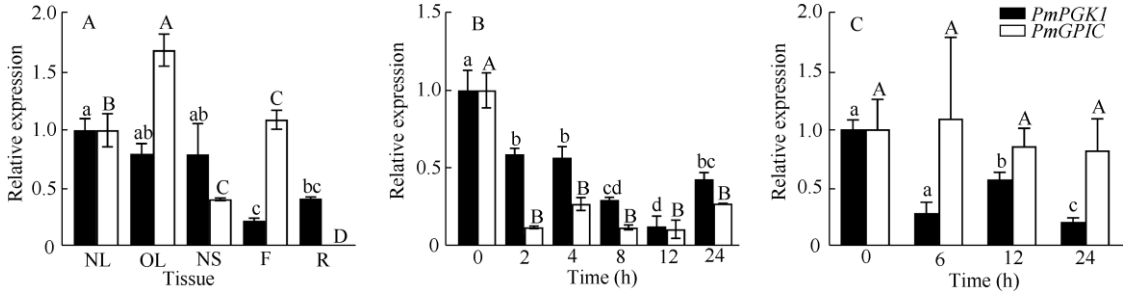

Fig. 4 Expression of *PmPGK1* and *PmGPIC* in different tissues (A), under low-temperature stress (B) and high-CO_2 stress (C). NL: New leaf; OL: Old leaf; NS: New stem; F: Flower; R: Root. Different letters upon column indicate significant differences at 0.05 level by Duncan test.

the results in *Arabidopsis thaliana*[13] and *Brassica napus*[19]. On the other hand, *PmPGK1* was also expressed in root, new stem and flower, suggesting that it is involved in glycolytic metabolism in these organs. Compared with *P. massoniana*, *PGK1* in *Arabidopsis thaliana*[13,18] and *Brassica napus*[19] showed the highest expression in flower. This discrepancy may be caused by some metabolic differences between perennial trees and annual herbs. *PmGPIC* was mainly expressed in leaves and flowers, and the expression level was highest in old leaves. Therefore, it is speculated that the transformation reaction between F6P and G6P catalyzed by *Pm*GPIC in old leaves was stronger than that in new leaves and flowers. This result was consistent with that of Troncoso-Ponce et al.[16].

Previous studies have proven that low temperature could reduce the stability of chlorophyll, the solubility of CO_2 in cells and the affinity of Rubisco to CO_2, directly affecting the integrity and activity of the photosynthetic system[20–21]. As important regulating enzymes in the Calvin cycle, RuBP carboxylase (RuBPCase), phosphoribulose kinase (PRK) and 1,6-fructose bisphosphatase (FBP) all showed a decreasing trend of expression under low temperature stress[22–25]. The reaction product of RuBPCase and PRK was the reaction substrate of PmPGK1; therefore, the influence of low temperature on both led to a decrease in the

activity of PmPGK1 and gene expression. Meanwhile, the respiration metabolism of plants under low temperature stress was dominated by the tricarboxylic acid cycle (TCA), at which time the glycolysis pathway was inhibited[26]. In addition, FBP activity was reduced under low temperature, resulting in a decrease in the catalytic substrate of PmGPIC[23,25]. Based on the above factors, the expression of *PmGPIC* was more downregulated after low temperature treatment than that of *PmPGK1*, showing a sharp decline.

Previous studies[27] have shown that the expression of all genes in the Calvin cycle, except GAPDH, decrease under a high CO_2 concentration in masson pine; therefore, it was speculated that the photosynthetic acclimation of masson pine could be completed within 6 h. In addition, it was found that an increased CO_2 concentration leads to a significant increase in hexokinase (HK) activity[27], while HK strongly inhibits the activity of Rubisco and Rubisco small subunit (RbcS)[28–29], in turn decreasing the content of PGA (the direct catalytic substrate for the transformation of RuBP to PGK, resulting in a significant decrease in the expression of *PGK1*). According to the changes in the expression levels of the two genes, PmGPIC was less affected by CO_2 stress than PmPGK1. The transcriptome sequencing results in a previous study[27] showed that the expression levels of *PmGPIC* were higher after treatment than those at 0 h, which was different from the finding in this experiment, but the overall trends of change were consistent. Invertase (INV) phosphorylates glucose and fructose to form the catalytic substrate of GPIC[2]. Under elevated CO_2 conditions, the expression levels of INV and HK in Masson pine were significantly increased[27], so the expression level of *PmGPIC* was slightly increased after 6 h. The decrease in *PmGPIC* after 12 h might have been caused by gradual decreases in the accumulation of photosynthetic products and the glucose metabolic rate. In addition, since photosynthetic adaptation is more obvious in annual needles than in that mature needles under elevated-CO_2 stress[30], more detection could be used before photosynthetic adaptation, and the response mechanism of *PmPGK1* and *PmGPIC* could be further explored by comparison with the responses in perennial Masson pine samples.

In this study, full-length *PmPGK1* and *PmGPIC* were cloned, and encoding proteins were belong to the plastidial and cytoplasmic subtypes, respectively. *PmPGK1* was mainly expressed in leaves, and *PmGPIC* was mainly expressed in leaves and flowers. The expression of *PmPGK1* and *PmGPIC* was inhibited under low-temperature stress, and the inhibitory effect on *PmGPIC* was stronger. Elevated-CO_2 stress signifycantly inhibited the expression of *PmPGK1* but had little effect on the expression of *PmGPIC*. The results of this study provide some references for subsequent studies on PGK and GPI in plants.

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