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# CLONING OF PGPP2C2 GENE AND ANALYSIS OF ITS EXPRESSION IN RESPONSE TO NITROGEN AND PHOSPHORUS DEFICIENCY, AND EXOGENOUS GR24 TREATMENT IN PANAX GINSENG

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**Abstract**

Type 2C protein phosphatases (PP2Cs) are the most abundant members of the plant phosphatase family and perform vital functions in response to various environmental stressors. To explore the role of *Panax ginseng* C.A. Meyer (*P. ginseng*) *PgPP2C*, a gene identified from a previously assembled transcriptome database, under adverse conditions, we created a clone of the gene and named it *PgPP2C2*. The length of its open reading frame was 1269 base pairs, and it coded for a 422-amino-acid protein that contained a 2C family serine/threonine phosphatase catalytic domain characteristic of the phosphatase 2C family proteins. Phylogenetic analysis of PgPP2C2 protein sequences from multiple species revealed that the *P. ginseng* sequence was similar to homologous sequences from *Macleaya cordata*, *Malus domestica*, *Nicotiana tomentosiformis*, *Solanum lycopersicum*, and *Handroanthus impetiginosus*. Transient expression analysis identified a PgPP2C2–GLosgfp fusion protein in the nucleus. The expression of *PgPP2C2* was detected by transcriptional analysis in all investigated tissues, but at a higher level in the roots. Moreover, nitrogen (N) and phosphorus (P) deficiency treatments were observed to trigger *PgPP2C2* expression. Specifically, the downregulation of *PgPP2C2* and the accumulation of abscisic acid–glucose ester (ABA–GE) were induced under N deficiency. The synthetic strigolactone analog GR24 restored the expression of *PgPP2C2* under N deficiency, but had no obvious effect on the ABA–GE content. The findings enhance our understanding of the genetic regulatory mechanism of *P. ginseng* in response to N and P deficiencies, as well as provide a potential strategy for improving the quality of cultivation of *P. ginseng* through hormone-mediated nutritional regulation.

**Key words:** *Panax ginseng, PgPP2C2*, Molecular cloning, Expression, Strigolactone, Nitrogen (N) and phosphorus (P).

**Abbreviations:** PP2Cs, Type 2C protein phosphatases; ABA, abscisic acid; SnRK2, SNF1-related kinase 2; ORF, open reading frame; MAPK, mitogen-activated protein kinases; HOG1, high osmolarity glycerol 1; NBP2, NAP1-binding protein 2.

## Introduction

The perennial herb ginseng (*Panax ginseng* C.A. Meyer) is a member of the Araliaceae family. It is known as the "king of herbs" and is the most prized Chinese medicinal material in East Asia and North America (Wen & Zimmer, 1996). Ginsenosides are the primary medicinal ingredients of ginseng and are used to treat cardiovascular diseases, age-related diseases, obesity, and stress (Chu & Zhang, 2009; Yang *et al*., 2012; Kim *et al*., 2014). However, in recent years, the cultivation of ginseng has encountered adverse conditions, such as climate warming, drought, and nutrient shortage, which have limited its growth. Consequently, it is imperative to identify stress resistance genes and breed ginseng for higher stress tolerance to increase overall yield and meet the ever-increasing market demand.

Protein kinases phosphorylate proteins, whereas protein phosphatases dephosphorylate them. During the regulation of plant metabolism, the two protein types work together to regulate the phosphorylation of substrate proteins. Type 2C protein phosphatases (PP2Cs) comprise a multi-member protein family with distinctive N-terminal extensions and are an important subtype of plant phosphatases. PP2Cs are serine/threonine protein phosphatases that exist in the form of monomeric enzymes. Their dephosphorylation activity depends on $Mn^{2+}$ and $Mg^{2+}$, and they are widespread in animals, plants, bacteria, and fungi (Hu *et al*., 2020); in particular, higher plants produce a large number of diverse PP2C enzymes. These proteins are involved in a variety of stress signaling pathways, such as those that respond to drought, salinity, and nutrient deficiency, and perform critical functions in plants under stress. Moreover, as regulators of the signal transduction pathway, PP2Cs are involved in abscisic acid (ABA) signal transduction. The interaction between the receptor and PP2C releases downstream SNF1-related kinase 2 (SnRK2) that further phosphorylates downstream proteins (Fujii *et al*., 2009; Umezawa *et al*., 2009), resulting in ABA transcription and ion channel activation (Hubbard *et al*., 2009; PriTal *et al*., 2009; Weiner *et al*., 2010). After the ABA receptor receives a molecular signal, it allosterically inhibits PP2C activity, thereby reducing or eliminating the inhibition of PP2C on the SnRK2 kinase and enhancing the phosphorylation of the substrate protein by SnRK2 kinase to regulate the overall response of the plant to ABA (Rigoulot *et al*., 2019). PP2Cs have been categorized into ten major groups (A-J) with six members that fall outside these main groups and are encoded in the *Arabidopsis* genome (Chu *et al*., 2021). There are 82 species? of PP2C in *Arabidopsis thaliana*, 90 in rice (*Oryza sativa* L.), 88 in pepper (*Capsicum annuum* L.), and 91 in tomato (*Solanum lycopersicum* Mill.) (Jiang, 2020). PP2C proteins regulate seed germination (Rovira *et al*., 2021; Yu *et al*., 2020), root growth (Miao *et al*., 2020), stress resistance (Zhang *et al*., 2021), hormone responses (Hu *et al*., 2020; Wang *et al*., 2021), and other processes. The expression profiles of PP2Cs have been shown to fluctuate significantly as a result of biotic and abiotic factors, such as drought, cold, light, salt, and plant hormones (Chu *et al*., 2021; Yu *et al*., 2020; Miao *et al*., 2020; Zhang *et al*., 2021; Wang *et al*., 2021; Wang *et al*., 2020a).

Currently, the *PP2C* gene sequences and expression profiles in *P. ginseng* are unclear. In our previous study, the *PgPP2C* gene was identified by high-throughput sequencing (Lei *et al*., 2023). To the best of our knowledge, the present study is the first to demonstrate the first isolation of a cDNA clone for the *PP2C* gene (*PgPP2C2*). Bioinformatic analyses were performed on the identified polypeptide product to gain insight into its gene homology, phylogeny, amino acid sequence, structure, and physiochemical characteristics. This study also offers the first detailed examination of PgPP2C2 subcellular localization and distribution analysis of *PgPP2C2* expression in various tissues. Moreover, it demonstrates the activity of *PgPP2C2* under N and P deficiency in *P. ginseng*. Currently, *P. ginseng* is being

promoted for ecological planting, however, its yield and quality under this system are limited by the unavailability of nutrients in the soil (Ma *et al*., 2021). The findings of our study provide insight into the regulation of *PP2C* by exogenous hormones in response to N and P nutrient deficiency during the growth of *P. ginseng*, with implications for addressing the issue of soil nutrient limitation in the ecological planting of *P. ginseng* through nutritional management.

## Material and Methods

**Plant materials, cultivation, and stress treatments:** *P. ginseng* seeds (*P. ginseng* collected and preserved in the *P. ginseng* planting base of Baishan City, Jilin Province, China) were germinated on wet Whatman filter paper in a rectangular culture tray (34 × 25 cm) under darkness for 3 days at 25°C. Germinated seeds were transferred to a hydroponic tank supplemented with pure water for 3 days at 70% relative humidity and 22–25°C ambient temperature. The seedlings were then cultured for 5 days in Hoagland's solution (Hoagland & Arnon, 1950). Subsequently, 40 uniform seedlings were selected and transplanted into each hydroponic tank, and the nutrient solution in the hydroponic tank was composed of 5 mmol $L^{-1}$ $Ca(NO_3)_2{\cdot}4H_2O$, 5 mmol $L^{-1}$ $KNO_3$, 1 mmol $L^{-1}$ $NH_4NO_3$, 2.5 mmol $L^{-1}$ $MgSO_4{\cdot}7H_2O$, 2 mmol $L^{-1}$ $KH_2PO_4$, 0.4 mmol $L^{-1}$ Fe-Na-EDTA, and 5 mL of trace elements (Hoagland & Arnon, 1950). The pH of the solution was adjusted to 5.8. For N and P treatments, the nutrient solution was also supplemented with 0 mmol $L^{-1}$ (−N) or 5 mmol $L^{-1}$ (+N) $Ca(NO_3)_2{\cdot}4H_2O$ and $KNO_3$, and 0 mol $L^{-1}$ (−P) or 2 mmol $L^{-1}$ (+P) $KH_2PO_4$. $CaCl_2$ and KCl were separately supplied to complement the $Ca^{2+}$ and $K^+$ concentrations under N- and P-deficient conditions. The nutrient solution was replaced every 2 days. After 72 h, 20 seedlings were randomly selected from each hydroponic tank, and their root, stem, and leaf organs were collected, rapidly frozen in liquid nitrogen, and preserved at –80°C for subsequent analyses. For the remaining 20 seedlings in each hydroponic tank, 10 mM synthetic strigolactone analog GR24 (Yuanye Bio-Technology Co., Ltd, Shanghai, China) was applied to the plants in the −N, −P, and −N−P groups every 8 h for 24 h. The experimental design included four groups, namely −N+GR24, −P+GR2, −N−P+GR24, and CK (without GR24). After treatment, roots were collected, rapidly frozen using liquid nitrogen, and preserved at −80°C for further use. Each treatment had three biological replicates.

**RNA isolation and cDNA cloning:** We used the RNAsimple Total RNA Kit (Tiangen, Beijing, China) following the manufacturer's instructions for RNA isolation. The RNA quality was assessed using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and 1.0% (w/v) agarose gel electrophoresis. Following the protocol provided by the manufacturer of RNase H-Reverse Transcriptase FastKing gDNA Dispelling RT SuperMix (Tiangen, Beijing, China), 9 μL of total RNA (1 μg/mL) was utilized to synthesize first-strand cDNA. Primers for *PgPP2C2* were designed based on a transcriptome database produced by our laboratory in a previous study (Lei *et al*., 2023): forward primer PgPP2C2, 5′-ATGATGATCAA TACCAACCTAAAA-3′ and reverse primer PgPP2C2, 5′-CGCCTAGTATTCCTCCTGATG-3′. The thermal cycling program for amplification was 95°C for 4 min; 35 cycles of 95°C for 30 s, 51°C for 35 s, and 72°C for 90 s; and a final extension at 72°C for 7 min. After PCR amplification, the target fragment was purified and subcloned using the TIANgel Midi Purification Kit (Tiangen, Beijing, China) and pEASY-T5 Zero Cloning Kit (TransGen Biotech, Beijing, China), respectively, following the manufacturer's instructions. Sequencing of the resulting product was conducted at Sangon Biotech (Shanghai, China), and the *PgPP2C2* cDNA sequence was obtained using the DNAman 6.0 Software based on fragment assembly.

**Bioinformatic analysis of PgPP2C2:** The open reading frame (ORF) of the *PgPP2C2* coding sequence, as well as the identity and similarity of *PgPP2C2* to other sequences, was determined using tools available on the NCBI website (https://www.ncbi.nlm.nih.gov/). Its protein structure and conserved domains were predicted using Prosite tools, including ProtParam (physicochemical properties), Protscale (http://web.expasy.org/protscale), SOPMA (https://npsa-prabi.ibcp.fr/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), and SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/). Interaction proteins were predicted by STRING (https://string-db.org/cgi/input), and protein structures were analyzed using the Swiss Model (https://www.swissmodel.expasy.org/). The NCBI database was searched for protein homologs, and an online alignment tool (http://www.uniprot.org/align) was used to construct multiple sequence alignments of protein sequences from diverse species. To determine the relationships among *PgPP2C2* from *P. ginseng* and *PP2C* genes from other species, we performed a phylogenetic analysis with MEGA6.0 software using the JTT substitution model and the G + I model for rates among sites (Tamura *et al*., 2013). In total, 1000 bootstrap replicates were used to determine the reliability of the internal branches. The neighbor-joining approach was used to generate a phylogenetic tree of PP2C homologs from various species.

**Subcellular localization of PgPP2C2:** To analyze the subcellular localization of PgPP2C2, the *PgPP2C2* ORF (minus the termination codon) was amplified from *PgPP2C2* cDNA with specific primers (Sl-F:cagtGGTCTCacaacatgatgatcaataccaacct and Sl-R: cagtGGTCTCatacagtattcctcctgatgatact). Subsequently, the *PgPP2C2* sequence was separately cloned into the pBWA(V)HS-ccdb-GLosgfp vector fused to the 5′ terminus of GFP and driven by the 35S promoter. The pBWA(V)HS-PgPP2C2-Glosgfp fusion vectors and GFP empty constructs were introduced into the *Agrobacterium tumefaciens* strain GV3101. Transformed GV3101 cells were grown for 2 days at 30°C and inoculated into 10 mL of YEB liquid medium cultured at 170 rpm/min for 1 h. It was suspended with 10 mM $MgCl_2$ (including 120 μMAS) when the absorbance at 600 nm was 0.6. One-month-old *Nicotiana benthamiana* plant with good growth was selected and the leaf lower epidermis was injected using a 1 mL syringe without the gun head. The plants were cultured for 2 days under low light. The leaves injected with *Agrobacterium tumefaciens* were placed on slides, and confocal microscopy (Nikon C2-ER; Nikon, Tokyo, Japan) was performed to determine the subcellular localization of PgPP2C2 based on the associated GFP fluorescence intensity. For co-localization, the marker plasmid was transformed into *Agrobacterium tumefaciens*, suspended with the constructed vector plasmid *Agrobacterium tumefaciens*, mixed in a 1:1 ratio before injection, and then injected into *Nicotiana benthamiana* leaves.

**Expression analysis of *PgPP2C2* by quantitative real-time PCR (qPCR) in P. *ginseng*:** The template for qPCR was the cDNA obtained by reverse transcription with the primers *PgPP2C2*-F (5′-TCAGTTAATCTCGCGGAAAGC-3′) and *PgPP2C2*-R (5′-GTCTCGTCTTCGTTCTCAAGTT-3′). *GAPDH* served as the reference gene for normalization with the primers *GAPDH*-F (5′-AATGACCTTGCCGACAGCCTTG-3′) and *GAPDH*-R (5′-TACACCGCCACCCAGAAGACC-3′) (Zhang *et al*., 2022). The 20 μL qPCR amplification system contained 0.5 μL of template cDNA, 0.5 μL of Primer F (10 μM, Sangon), 0.5 μL of Primer R (10 μM, Sangon), and 8.5 μL of ddH2O mixed with 10 μL of 2× qPCR Mix (Takara, Japan). All reactions were performed with three biological replicates. The

qPCR assay was performed following the instructions of the SYBR Premix ExTaq II (Takara, Japan) on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). The qPCR protocol consisted of a 2 min initial denaturing step at 94°C, followed by 40 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 30 s, and finally a 7 min extension at 72°C. Utilizing the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008), we detected the *PgPP2C2* gene relative expression levels in roots, stems, leaves, different N and P treatments, and exogenous GR24 application.

**ABA content assay:** The *P. ginseng* roots were harvested, immediately frozen using liquid nitrogen, ground into powder (30 Hz, 1 min), and preserved at –80°C for further use. In addition, 50 mg of *P. ginseng* roots were weighed into a 2 mL plastic microtube before freezing in liquid nitrogen and eventually dissolving in 1 mL of methanol/water/formic acid (15:4:1, V/V/V). To facilitate the quantification process, 10 µL of an internal standard mixed solution (100 ng/mL) was introduced to the extract. After 10 min of treatment in a vortex mixer, the mixture was centrifuged at 13,400 ×g for 5 min at 4°C. The supernatant was transferred to sterilized plastic microtubes, dried by evaporation, and diluted in 100 µL of 80% methanol (V/V) before filtering through a 0.22 µm membrane filter (Floková *et al*., 2014; Li *et al*., 2016). Then, using MetWare (http://www.metware.cn/), we detected the ABA content in the sample based on the Sciex QTRAP 6500 LC–MS/MS system (Sciex, Framingham, MA, USA).

**Statistical analysis:** All data are presented as the mean ± standard deviation (SD). The histogram was constructed with R (The R Programming Language) software. The images were merged using Adobe Illustrator CC 2018.

## Results and Discussion

**Cloning of *PgPP2C2* and encoded protein sequence analysis:** *PP2C* has been cloned and studied in detail in many plants, such as *Arabidopsis thaliana*, rice, and wheat (Chu *et al*., 2021; Xue *et al*., 2008; Yu *et al*., 2008). However, few studies are conducted on medicinal plants. In this study, the sequencing of *PgPP2C2* from *P. ginseng* (Fig. 1) revealed a 1269 bp ORF predicted to encode a 46.62-kDa protein of 422 amino acids with an isoelectric point of 5.28 (Fig. 2). *PgPP2C2* was predicted to contain a serine/threonine phosphatase catalytic domain characteristic of the protein phosphatase 2C family (Fig. 3). The protein structure and inferred catalytic mechanism of PP2C were similar to those of proteins from the PP1, PP2A, and PP2B families of serine/threonine phosphatases, which catalyze the dephosphorylation of phosphoserine and phosphothreonine residues on specific protein substrates. Dephosphorylation and phosphorylation are reversible protein-modification processes that may alter how plants respond biologically and developmentally (Shi, 2009; Uhrig *et al*., 2013). PP2C may inactivate mitogen-activated protein kinases (MAPKs) by catalyzing their dephosphorylation (Smékalová *et al*., 2013). In the present study, PgPP2C2 was predicted to interact with high-osmolarity glycerol 1 (HOG1, a MAPK involved in osmotic regulation) and NAP1-binding protein 2 (NBP2, a protein involved in the HOG pathway) on the STRING website, and the scores for these interactions were 0.996 and 0.984, respectively (Fig. 4). The PgPP2C2 protein structure was further analyzed by aligning its model templates with the aid of the Swiss Model (Fig. 5). The predicted results included a clear folding layer containing magnesium ions and showing the magnesium ion-dependent characteristic of 2C protein phosphatase activity. Protein phosphatase 2C is a $Mn^{2+}$- or $Mg^{2+}$-dependent serine–threonine phosphatase. Bivalent metal ions can activate or inhibit its serine–threonine phosphatase activity upon combination. Therefore, this prediction result can provide a reference for the further study of the phosphatase activity of PgPP2C2.

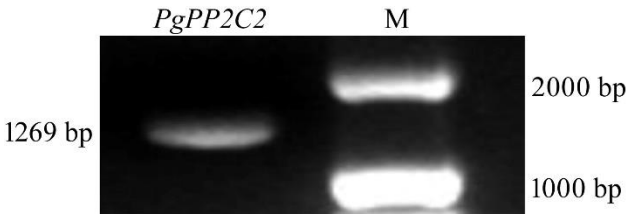


Fig. 1. *PgPP2C2*-related PCR product separated by electrophoresis on 1% agarose gel (M, DL2000 molecular weight marker).

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1    ATGATGATCAATACCAACCTAAAACGTAAATCGTCGGAGCCGGGGAATTGCCGGAGAACG
1     M  M  I  N  T  N  L  K  R  K  S  S  E  P  G  N  C  R  R  T
61   TCATTTTCAGATGCCGGCAAGCTGAATAATCAGTTAATCTCGCGGAAAGCTGCTAAACAT
21    S  F  S  D  A  G  K  L  N  N  Q  L  I  S  R  K  A  A  K  H
121  GTTCGTCAAAAAAGACTGGAACTCCGGCGACTTAAATCCCTGTTCTCAAATAAGACTAAA
41    V  R  Q  K  R  L  E  L  R  R  L  K  S  L  F  S  N  K  T  K
181  CTTGAGAACGAAGACGAGACTATGAACCGAATTTGCTCGCCGTTGGTGTTATCGCTGTCT
61    L  E  N  E  D  E  T  M  N  R  I  C  S  P  L  V  L  S  L  S
241  TACAATACGATGGAAGCTTCTTCGTCGTCAGAGATTGACGTCAGTGTTTCCCGTGTTACA
81    Y  N  T  M  E  A  S  S  S  S  E  I  D  V  S  V  S  R  V  T
301  AGTTCCCGAAGCGACACAACGCAAACGGCGAGAGCGCATTCACTAGCGGATGCCTGCGGG
101   S  S  R  S  D  T  T  Q  T  A  R  A  H  S  L  A  D  A  C  G
361  TCATACGGGAAAATATCGGTGATCGGACGGAGGAGAGAGATGGAGGATGCGGTGACGGTG
121   S  Y  G  K  I  S  V  I  G  R  R  R  E  M  E  D  A  V  T  V
421  GAGCTAGGGTTTGTTTCGGCAAGTTCGAGAAAATATGACTTTTTCGGTGTGTATGATGGT
141   E  L  G  F  V  S  A  S  S  R  K  Y  D  F  F  G  V  Y  D  G
481  CATGGCGGGTCACGTGTGGCGCACGCTTGCCGTGATCGGTTGCATCATTTGGTGGTGAAA
161   H  G  G  S  R  V  A  H  A  C  R  D  R  L  H  H  L  V  V  K
541  GAGGTGGAGGAAGAGGAGACGGTGGATTGGGAGAAGGTGATGGTAGCGAGTTTTGGGAAG
181   E  V  E  E  E  E  T  V  D  W  E  K  V  M  V  A  S  F  G  K
601  ATGGATGAGGAGGTGATTGCTAGTGGGGCGGTCACGGGGTCTATTGGCTCGACGGCGGTG
201   M  D  E  E  V  I  A  S  G  A  V  T  G  S  I  G  S  T  A  V
661  GTGGCAGTCGTAGGGGAGGAGGTGGTGGTGGTTGCTAATTGCGGGGATTCTAGAGCTGTG
221   V  A  V  V  G  E  E  V  V  V  V  A  N  C  G  D  S  R  A  V
721  TTGTCACGCGCCCGTGTCCCCATGCCATTGTCTAATGATCACAAGCCTGACAGACCAGAT
241   L  S  R  A  R  V  P  M  P  L  S  N  D  H  K  P  D  R  P  D
781  GAGTTGGAGAGAATTGAAGTTTCCGGTGGAAGAGTCATCAACTGGAATGGGCAACGGGTG
261   E  L  E  R  I  E  V  S  G  G  R  V  I  N  W  N  G  Q  R  V
841  TTAGGAGTACTTGCTACTTCAAGATCAATAGGGGATCAGTACCTGAAACCATATGTAATA
281   L  G  V  L  A  T  S  R  S  I  G  D  Q  Y  L  K  P  Y  V  I
901  GCTCAACCAGAGGTGACAGTGAGCAAAAGAAATGATAGGGATGAATTCTTGATACTAGCG
301   A  Q  P  E  V  T  V  S  K  R  N  D  R  D  E  F  L  I  L  A
961  AGTGATGGACTATGGGATGTTATCACAAATGAGCTTGCTTGCCGGGTAGTGAGAAAATGT
321   S  D  G  L  W  D  V  I  T  N  E  L  A  C  R  V  V  R  K  C
1021 TTGGATGGTCGACTGAGGAGGAGATTTTCGCAGGGAATTGTGAATGAAGAAACATCAGGT
341   L  D  G  R  L  R  R  R  F  S  Q  G  I  V  N  E  E  T  S  G
1081 GGTCATGATGATGATGATGATGATGATGATGATGATGAGATTAATATTGTAAATGAT
361   G  H  D  D  D  D  D  D  D  D  D  D  D  E  I  N  I  V  N  D
1141 GAAAGTCGAGCTATAGAGGCGGCAGCAGTGCTGGCTGAATTAGCAGTGGCTCGGGGTAGC
381   E  S  R  A  I  E  A  A  A  V  L  A  E  L  A  V  A  R  G  S
1201 GAGGATAATATTAGTGTGGTAGTAGTGGAGTTAAATAAACCCGGTCAGTATCATCAGGAG
401   E  D  N  I  S  V  V  V  V  E  L  N  K  P  G  Q  Y  H  Q  E
1261 GAATACTAG
421   E  Y  *
```

Fig. 2. Deduced amino acid sequence of *PgPP2C2* and overall length of the cDNA sequence. The red boxes represent the initiation and termination codons.

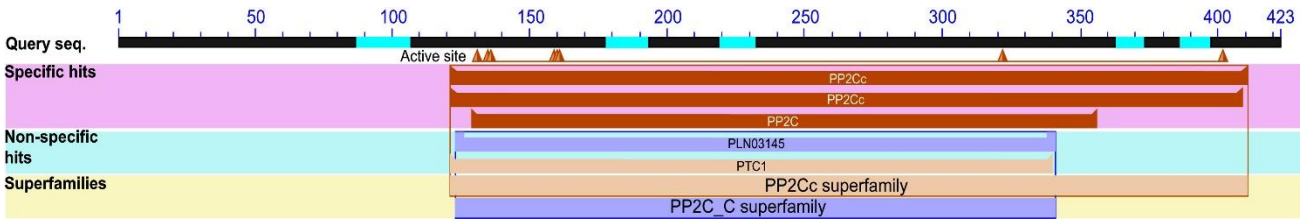


Fig. 3. Domain prediction of the PgPP2C2 protein.

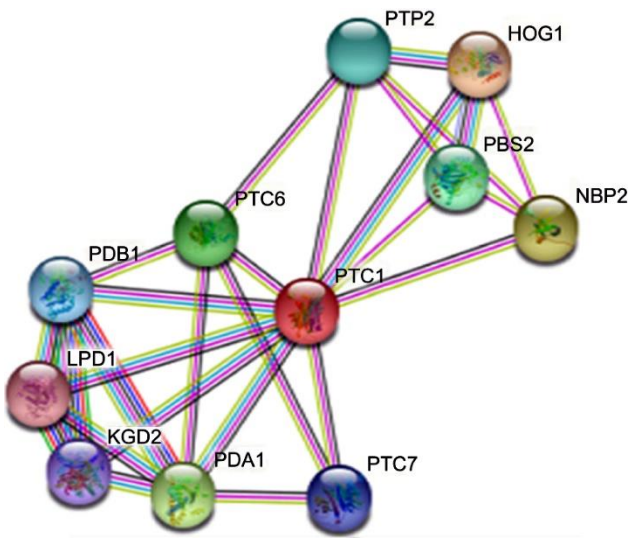


Fig. 4. Interaction prediction of the PgPP2C2 protein.

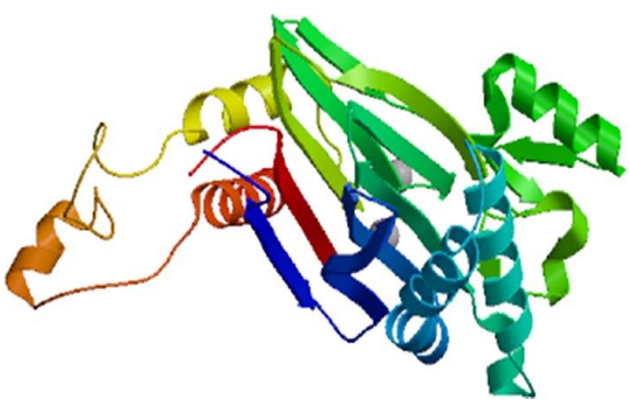

Fig. 5. Tertiary structure prediction of the PgPP2C2 protein.

**Phylogenetic analysis of protein sequences homologous to PgPP2C2:** To elucidate the structure of the *PgPP2C2*-encoded protein sequence, we constructed multiple sequence alignments of PgPP2C2 and other plant homologs obtained from the NCBI database. The primary structure of PgPP2C2 was quite similar to that of PgPP2C2-like proteins from five other species, all of which exhibited a substantial degree of sequence conservation (Fig. 6). According to previous studies, PP2C protein phosphatase is a $Mg^{2+}$/$Mn^{2+}$-dependent monomeric enzyme that is highly conserved in numerous diverse plant species (Yu *et al*., 2008; Cao *et al*., 2013). Thereafter, we used the neighbor-joining method in MEGA 6.0 to generate a phylogenetic tree of the PgPP2C2 and homologous protein sequences from 20 related plant species obtained from the NCBI database. PgPP2C2 was the most closely related to sequences from *Macleaya cordata* (OVA01953.1), *Malus domestica* (XP_028962043.1), *Nicotiana tomentosiformis* (XP_009592346.1), *Solanum lycopersicum* (NP_001333898.1), and *Handroanthus impetiginosus* (PIN20518.1), and was more distantly related to sequences from other species (Fig. 7). Currently, the PP2C genes of *Malus domestica* and *Solanum lycopersicum* have been sequenced (Yang *et al*., 2022; Chen *et al*., 2016). The *PYL* gene in *Malus domestica* and *Solanum lycopersicum* interacts with PP2CAs in ABA-dependent and -independent manners and is affected by characteristics of the PP2C protein (Yang *et al*., 2022; Chen *et al*., 2016). Our analysis suggests that PgPP2C2 may have functions similar to that of PP2C proteins in *Malus domestica*, *Solanum lycopersicum*, and *Nicotiana tabacum*. These results laid a foundation for the analysis of the relationship between *PgPP2C2* expression and the ABA content in subsequent experiments.

**PgPP2C2-related subcellular localization:** We developed a pBWA(V)HS-PgPP2C2-GLosgfp vector and transiently expressed it in tobacco leaves via *Agrobacterium tumefaciens*-mediated transformation to validate the specific subcellular location of PgPP2C2. *Nicotiana benthamiana* cells transformed with the empty pBWA(V)HS-ccdb-GLosgfp vector exhibited a uniform distribution of GLosgfp fluorescence (Fig. 8A-B). In contrast, PgPP2C2-GLosgfp fluorescence was localized to the nucleus. The PP2C protein is expressed differently in different plants. For example, AcPP2C3 and PbrPP2C are expressed in the nucleus (Wang *et al*., 2021; Zhai *et al*., 2023), BpPP2C1 is expressed in the nucleus and cell membrane (Xing *et al*., 2021), and AtPP2C49 is expressed in the cytoplasm and nucleus (Chu *et al*., 2021). These discrepancies in expression localization reflect differences in the functions of PP2C proteins and are also likely related to the diversity of PP2C proteins, which represent the largest protein phosphatase family.

**Analysis of *PgPP2C2* expression in various tissues and under different treatments:** We extracted RNA from the roots, stems, and leaves of *P. ginseng* cultured for 72 h, reverse transcribed it into cDNA, and detected the tissue-specific expression of *PgPP2C2* via qPCR. We discovered that *PgPP2C2* expression varied among different organs of *P. ginseng* under normal conditions, with higher expression in the roots than in the aboveground tissues (Fig. 9A). The tissue specificities of *PgPP2C2* gene expression were similar to those of *AtPP2CG1* and *AtPP2C49* in *Arabidopsis thaliana* (Chu *et al*., 2021; Liu *et al*., 2021). The root is the main medicinal part of *P. ginseng*. Our result suggests that *PgPP2C2* may have an important function in the highly prized *P. ginseng*. The gene expression of *PP2Cs* is widespread in different plants and their tissues. For example, the cotton *PP2C* gene, *GhDRP1*, has high expression in the cotyledons and anthers (Chen *et al*., 2021), whereas the oilseed rape *PP2C* gene *BcABI1* is expressed at high levels in the pods, flowers, and leaves (Kong *et al*., 2018). In the present study, the broad expression profile of the *PgPP2C2* gene across multiple organs suggests that it may also have regulatory functions in a variety of tissues throughout *P. ginseng* development.

To analyze the effect of N and P deficiencies on *PgPP2C2* expression, we used cDNA isolated from ginseng roots in the −N, −P, −N−P, and CK groups and performed PCR analysis. The expression of *PgPP2C2* in the −N, −P, and −N−P, groups was lower than that in the control group, with the lowest expression observed in the −N group (Fig. 9B).

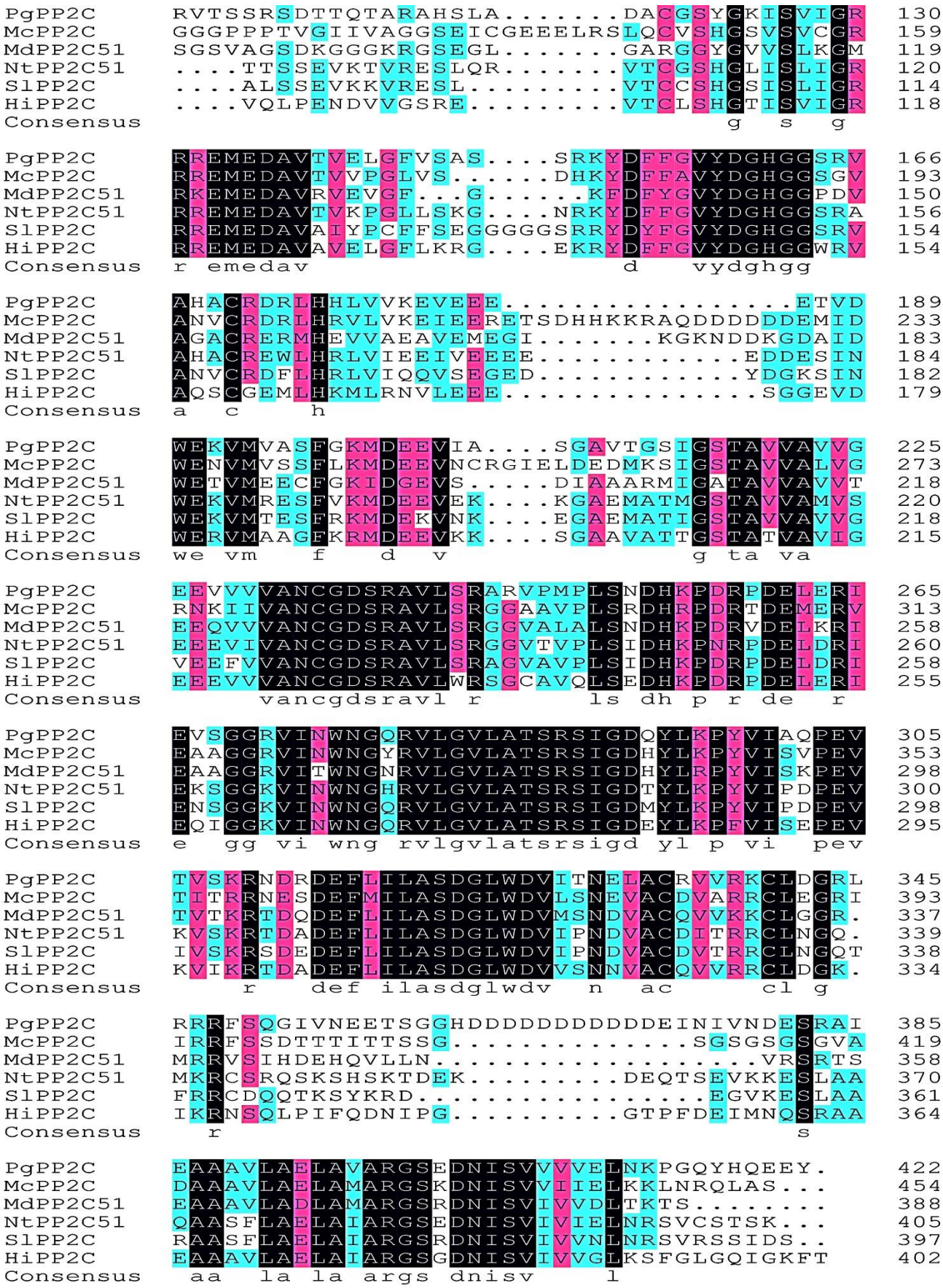


Fig. 6. Multiple alignments of PP2C2 protein sequences from *Panax ginseng* and other plant species according to DNAman 6.0 analysis. The aligned proteins were *Macleaya_cordata* (OVA 01953.1), Malus_domestica (XP 028962043.1), *Nicotiana_tomentosiformis* (XP 009592346.1), *Solanum_lycopersicum* (NP 001333898.1), and *Handroanthus_impetiginosus* (PIN 20518.1). The dark blue color denotes identical amino acids, whereas teal and pink colors indicate conserved amino acids.

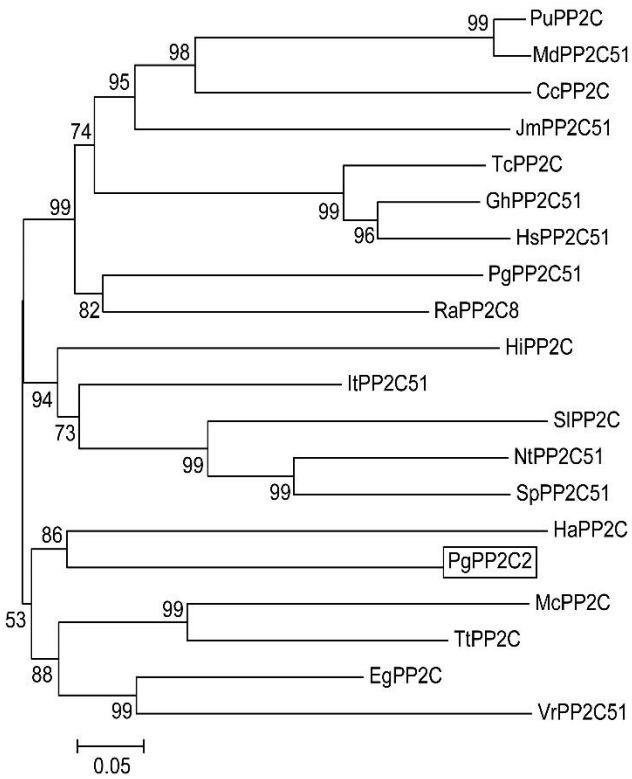


Fig. 7. Phylogenetic analysis of PP2C2 proteins derived from several species generated in MEGA 6.0 using the neighbor-joining technique with 1000 bootstrap replicates. The bootstrap values are provided next to each node.

Notably, the expression of *PgPP2C2* in the −N group was substantially lower than that in the control group ($p<0.05$), indicating that N deficiency had the greatest impact on *PgPP2C2* expression. N-limited conditions could enhance the expressional up-regulation of the *PP2C* genes (Effah *et al*., 2018; Wen *et al*., 2018), and a protein phosphatase gene that is rapidly induced by −N is Os09g0325700, which encodes protein phosphatase 2 C 68 (PP2C68) in rice (Hsieh *et al*., 2018). In contrast, our research showed that −N induced the downregulation of *PgPP2C2*, which may be affected by abundant medicinal substances in *P. ginseng* root. However, *PP2C* overexpression in the medicinal plant *Ammopiptanthus mongolicus* facilitates N acquisition in *Arabidopsis thaliana* (Han *et al*., 2018). Therefore, further functional studies of *PgPP2C2* are still needed. PP2C is crucial for plants to adapt to stress, and most researchers have focused on how it responds to salt (Chu *et al*., 2021; Wang *et al*., 2021), light (Rovira *et al*., 2021), drought (Yu *et al*., 2020) and hormones (Miao *et al*., 2020). However, few studies have focused on its roles in nutrient stress responses, especially multi-element synergistic stress. *AtPP2C49* is expressed at high levels in root vascular tissues when exposed to NaCl stress, and the salt-induced sensitivity of *AtPP2C49* is mediated by the *Arabidopsis thaliana* high-affinity potassium transporter (AtHKT1;1). By negatively regulating AtHKT1;1 activity, *AtPP2C49* mediates the systemic $Na^+$ distribution under salt stress (Chu *et al*., 2021). Moreover, the overexpression of *AmPP2C* (*Ammopiptanthus mongolicus PP2C*) facilitates K and N acquisition driven by transpiration (Han *et al*., 2018). The *PP2C* synergistic response to several nutrient elements is an important feature. In our results, *PgPP2C2* expression in the −N−P group was higher than that in the −N and −P groups. Therefore, we speculate that, under −N and −P stress, N and P transport-related proteins promote or inhibit the expression of *PgPP2C2*, but further experiments are needed to confirm this. Our previous studies revealed the role of the *P. ginseng* PP2C protein in response to drought and water stress (Lei *et al*., 2023), and it was shown that N deficiency could also induce *PgPP2C2* expression in this study, indicating that PP2Cs can participate in a variety of stress signal regulation. Although the role of *PgPP2C2* in response to −N and their relationship have not been determined, our findings contribute to the study of these potential N regulatory genes and may provide a novel approach to enhance N use efficiency in *P. ginseng*.

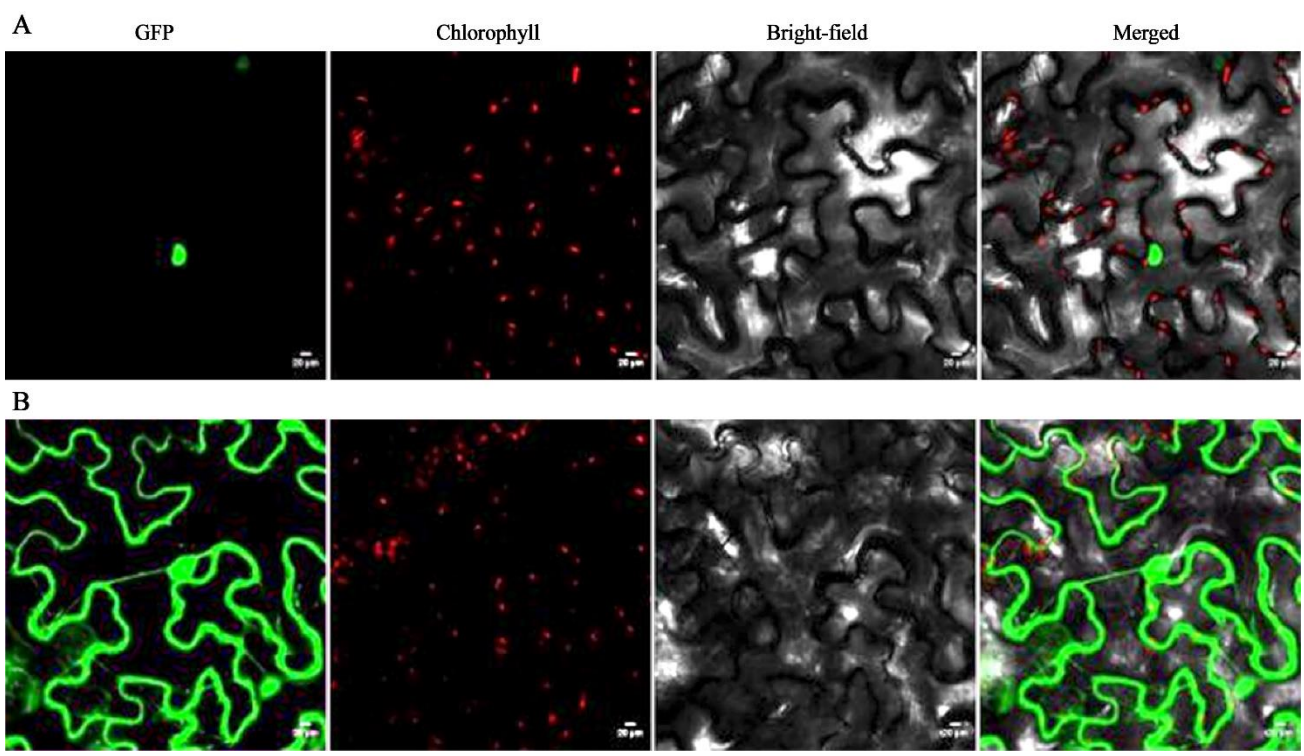


Fig. 8. Subcellular localization of PgPP2C2. (A) From left to right: target protein fluorescence signal, chlorophyll fluorescence signal, bright-field image, and merged image. Scale bar, 20 μm. (B) Empty vector (control). Panels are as described in (A). Scale bar, 20 μm.

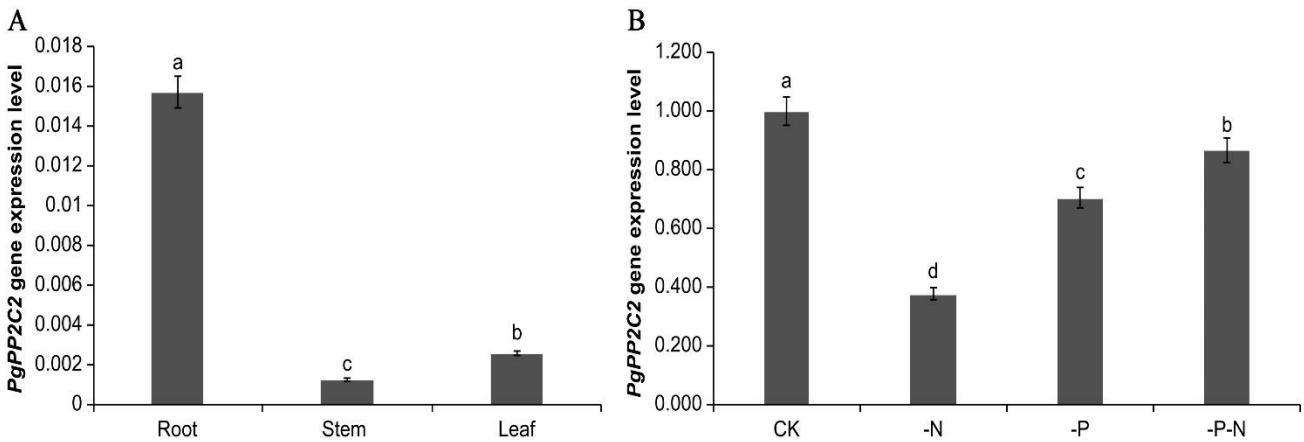


Fig. 9. *PgPP2C2* expression in various tissues and under different treatments. (A) Expression of *PgPP2C2* in distinct tissues of *P. ginseng*. (B) *PgPP2C2* expression in *P. ginseng* roots in response to N and/or P deficiency. Each bar represents the mean of six replicates with standard error. Different letters indicate significant differences ($p<0.05$, Duncan's test).

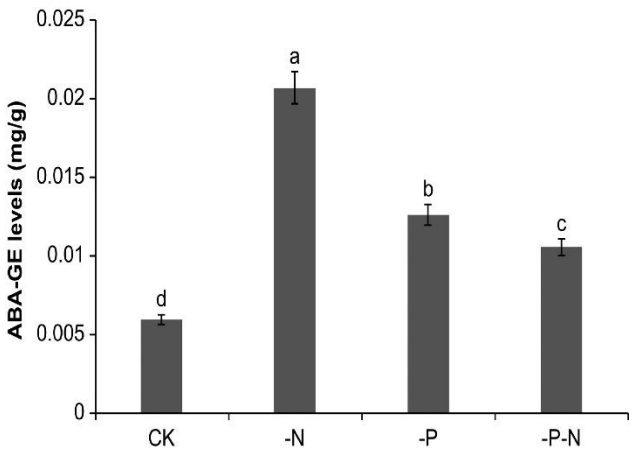


Fig. 10. Effects of different N and P deficiencies on ABA–glucose ester (ABA-GE) content in *P. ginseng* roots. Each bar represents the mean of six replicates with standard error. Different letters indicate significant differences ($P < 0.05$; Duncan's test).

**Effect of N and P deficiency on ABA content:** The total ABA content in the roots of *P. ginseng* was measured in response to N and P deficiencies. The levels of ABA–glucose ester (ABA–GE), the main form of ABA, were significantly higher ($p<0.05$) in the −N group than in the control group, and were higher in the −N group than in the −P and −N−P groups, indicating that −N treatment had the greatest effect on the ABA–GE content (Fig. 10). Plants accumulate ABA upon stress exposure and activate either ABA-independent or -dependent defensive stress response pathways (Yoshida *et al*., 2006). Drought (Xie *et al*., 2006; Zhang *et al*., 2006), salt (Song *et al*., 2023), and heat (Itai & Ben-Zioni, 1974) stress can induce ABA accumulation in plants. Notably, the ABA content also increases in plants that lack minerals, especially N (Xiong & Zhu, 2003). Similarly, we found that N deficiency induced a significant increase in the ABA–GE content of *P. ginseng* roots. N plays a role in regulating the production and transport of ABA, particularly during root development. Elevated concentrations of $NO_3^-$ in roots are positively related to ABA biosynthesis (Signora *et al*., 2001). In Wheat, $NO_3^-$ may upregulate β-glucosidase 1 (BG1), which, in turn, de-conjugates the inactive ABA–GE to release bioactive ABA (Wang *et al*., 2020b; Ondzighi *et al*., 2016). Arabidopsis PP2Cs have been shown to act as negative modulators of ABA (Gosti *et al*., 1999; Merlot *et al*., 2001; Schweighofer *et al*., 2004; Wasilewska *et al*., 2008). When ABA binds to the pyrabactin resistance1/PYR1-like/regulatory components of ABA receptors (PYR/PYL/RCARs), it attenuates the suppression of PP2Cs on protein kinase SnRK2s by inhibiting PP2Cs, phosphorylates transcription factors, such as ABI5 and RAV1, and activates the downstream ABA response gene (Li & Li, 2019). Through PCR analysis, we discovered that the expression level of the *PgPP2C2* gene under −N treatment was considerably reduced compared with that in the control group. Therefore, we speculate that, when plants are under N deficiency stress, they may downregulate *PgPP2C2* expression by accumulating ABA–GE. Research has shown that P deprivation induces the expression of the ABA biosynthesis genes, promoting ABA accumulation and activating the ABA signal pathway (Castro *et al*., 2023; Ribot *et al*., 2008). −N-induced genes, *PSY3* (phytoene synthase 3) and *BCH1* (beta-carotene hydroxylase 1) are associated with ABA biosynthesis (Hao *et al*., 2000; Li *et al*., 2008; Vallabhaneni & Wurtzel, 2009; Welsch *et al*., 2008). −N can rapidly and strongly induce the expression of *PSY3* and *BCH1* in rice roots (Hsieh *et al*., 2018). Similarly, our results showed that N deficiency triggered both the downregulation of *PgPP2C2* and the accumulation of ABA–GE. Therefore, *PgPP2C2* may act as a mediator of ABA modulation under N deficiency.

**Exogenous GR24 restored *PgPP2C2* expression and ABA–GE levels:** We conducted PCR analysis with cDNA derived from the roots of ginseng plants grown under various conditions to examine the influence of exogenous GR24 on *PgPP2C2* expression under N and P deficiency: −N+GR24, −P+GR24, −N−P+GR24, and CK. The results showed that exogenous GR24 restored *PgPP2C2* expression under N and P deficiency, to some extent (Fig. 11A-C). It has been reported that a variety of exogenous hormones regulate plant nutrition. Strigolactones play a fundamental role in plant responses to N and P nutrient (Marro *et al*., 2022). Regarding the effect of exogenous GR24 on *PP2C* gene expression, a study on alfalfa showed that *MsPP2C50* expression was down regulated by GR24 treatment under normal conditions, whereas *MsPP2C1* expression was upregulated after GR24 treatment under drought stress (Yang *et al*., 2022). In this study, plants treated with exogenous GR24 under N and P deficiency did not show ABA–GE recovery to CK levels (Fig. 11D-F). Therefore, GR24 may not have affected the synthesis of ABA–GE. However, −N+GR24 treatment resulted in attenuation of the *PgPP2C2* expression under −N treatment alone, with levels similar to those in the CK group (Fig. 11A). In contrast, the expression of *PgPP2C2* was lower after −P+GR24 and −N−P+GR24 treatments than that under −P and −N−P treatments (Fig. 11B-C), respectively. It has been reported that strigolactones regulate stomatal opening and closing by affecting the activity of the S-type anion channel SLAC1, which is independent of the ABA pathway (Lv *et al*., 2018). Our results were consistent with

this in that, under N deficiency, GR24 appeared to alleviate stress by modulating the activity of *PgPP2C2* in an ABA-independent manner, but further research is still needed. Interestingly, the administration of GR24 in the −P and −N−P treatment groups did not restore the expression of *PgPP2C2*, but rather further downregulated it. N and P may, therefore, have a synergistic effect in this process that, in turn, affects the expression of *PgPP2C2*. Similar studies have illustrated that soybean P homeostasis may be regulated in response to N deficiency by GmNLA1-1 and GmNLA1-3 (Zhou *et al*., 2022); unlike our study, these did not mention whether the genes were related to GR24. Therefore, *PgPP2C2* may also exhibit redundant and diversified functions in the coordinated response to N and P. Although further experiments are needed to confirm this, our study shows that *PgPP2C2* is involved in the response to N and P deficiency, and its expression is affected by exogenous GR24.

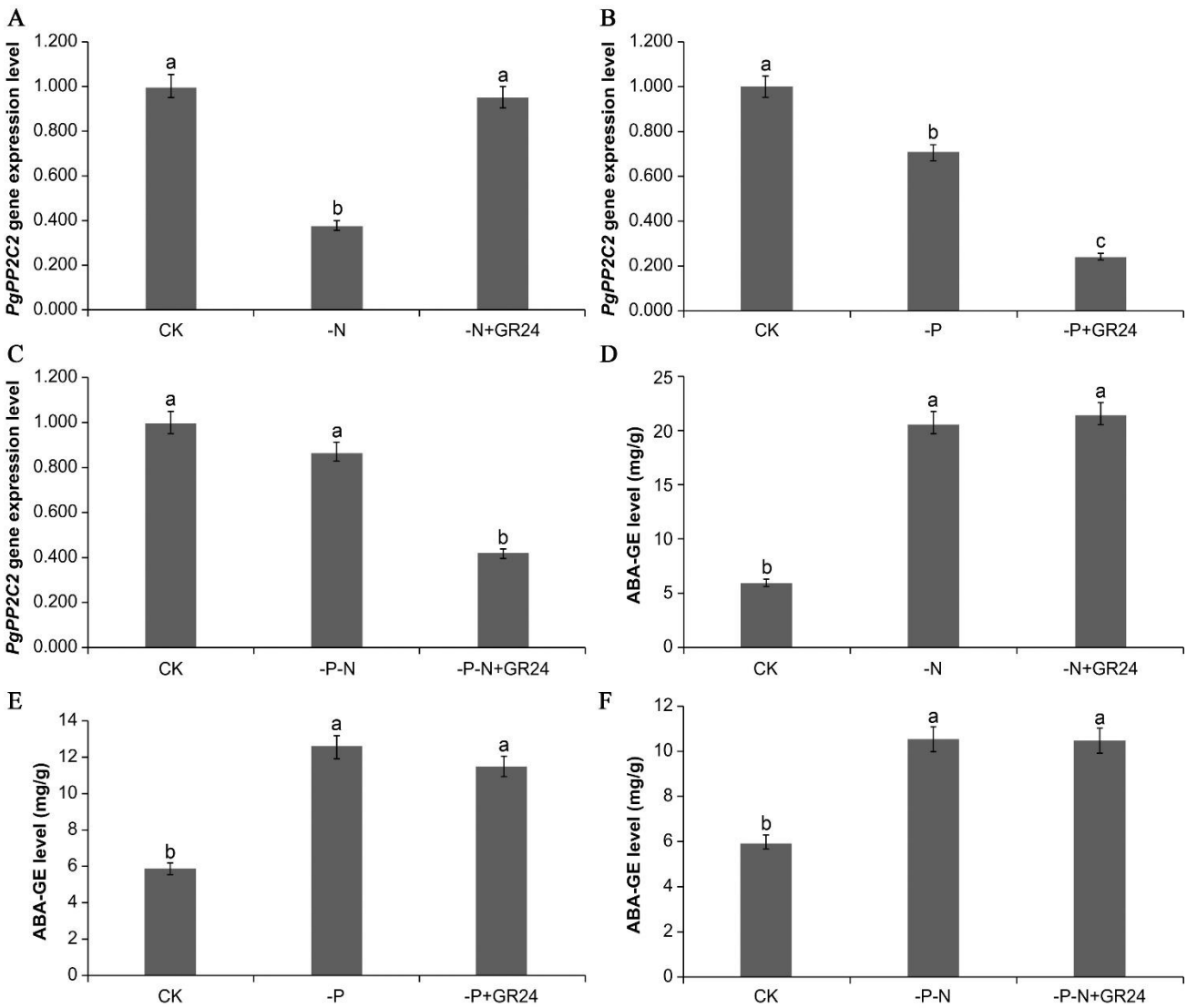


Fig. 11. Effects of exogenous GR24 treatment on *PgPP2C2* expression (A-C) and ABA–glucose ester (ABA-GE) content (D-F) under N and/or P deficiency. Different letters indicate significant differences ($P < 0.05$; Duncan's test).

**Conclusions**

To elucidate the tolerance mechanisms of *P. ginseng* under diverse stressors, it would be useful to examine the characteristics and expressional profiles of resistance genes, as these play an active role in various biotic and abiotic stress responses. In this study, the cDNA of the *PP2C* gene in *P. ginseng*, *PgPP2C2*, was effectively cloned and characterized for the first time. Our research showed that the PgPP2C2 protein exhibited nuclear localization, and *PgPP2C2* was spatially modulated at the transcription level and expressed primarily in the roots. This gene was also found to be stimulated by N and P deficiency, as was the accumulation of endogenous ABA. These findings suggest that *PgPP2C2* may represent an N and P nutrient stress gene that participates in ABA-associated pathways. Furthermore, exogenous GR24 restored or enhanced the expression of *PgPP2C2* under N and P deficiency treatment. Therefore, *PgPP2C2* may represent a potential target gene to regulate the growth of *P. ginseng* under N and P deficiency through the application of GR24. This is different from the traditional regulation of ginseng growth through soil nutrients, but similar to plant internal genes and exogenous hormones in regulating ginseng growth, which may help farmers to optimize *P. ginseng* cultivation; however, this method still requires validation through actual application in a production setting. Briefly, the cloning and characterization of *PgPP2C2* shown in this study have remarkable implications. It establishes a foundation for further investigations of the mechanisms by which *P. ginseng* adapts to environmental stress, and cultivate "excellent shaped and high-quality" ginseng, as well as represents a valuable reference for future molecular biology research of medicinal plant cultivation.

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