# Overexpression of a novel cytochrome P450 monooxygenase gene, CYP704B1, from Panax ginseng increase biomass of reproductive tissues in transgenic Arabidopsis

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ORIGINAL ARTICLE

# Overexpression of a novel cytochrome P450 monooxygenase gene, *CYP704B1*, from *Panax ginseng* increase biomass of reproductive tissues in transgenic *Arabidopsis*

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## Abstract

Cytochrome P450 monooxygenase 704B (CYP704B), a member of the *CYP86* clan, was found to be needed in *Arabidopsis* and rice to biosynthesize precursors of sporopollenin through oxidizing fatty acids. In the present study, we cloned and characterized a *CYP704B* gene in *Panax ginseng*, named *PgCYP704B1*. It shared high sequence identity (98–99%) with *CYP704* of *Arabidopsis*, *Theobroma cacao*, and *Morus notabilis*. The phylogenetic comparison of ginseng and higher plants between the members of *CYP86* clan revealed that ginseng *CYP704* was categorized as a group of *CYP704B* with dicot plants. The expression of *PgCYP704B1* is low in the stem, leaf, and fruit, and high in flower buds, particularly detected in the young gametic cell and tapetum layer of the developing anther. *Arabidopsis* plants overexpressing *PgCYP704B1* improved plant biomass such as plant height, siliques and seed number and size. A cytological observation by transverse and longitudinal semi-thin sections of the siliques cuticles revealed that the cell length increased. Furthermore a chemical analysis showed that *PgCYP704B1*ox lines increased their cutin monomers contents in the siliques. Our results suggest that *PgCYP704B1* has a conserved role during male reproduction for fatty acid biosynthesis and its overexpression increases cutin monomers in siliques that eventually could be used for seed production.

**Keywords** *Panax ginseng* · Cytochrome P450 · Overexpressing plants · *PgCYP704B1* · Reproduction · Fatty acid · Seed yield · Biomass

## Introduction

Cytochrome P450s (CYPs) are conserved heme-thiolate monooxygenases throughout the plant kingdom that are associated in the synthesis of essential backbone structures characteristic of distinct primary and secondary metabolite groups [1–3]. There are 244 CYP genes in the *Arabidopsis* genome, divided into nine clans, covering 65 families, based on their phylogeny [4]. CYPs divide into three groups based on the biochemistry catalyzed [5]. The first group biosynthesizes organic compounds and comprises clans 51, 97, 710, and 711. The second group catalyze the biosynthesis of secondary metabolites, and comprises clan 71. The third group biosynthesizes plant hormones and fatty acids, and comprises clans 72, 74, 85, and 86 [4].

Regarding plant reproductive development, *CYP704B* family belongs to the clan CYP86, reported to be involved in the ω-hydroxylation of long-chain fatty acids essential for pollen wall's exine formation, and particularly expressed

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during young microspore stage of the developing anther. Catalysis of ω-hydroxylated long-chain fatty acids, lipids and their derivatives, including fatty acids, waxes [6, 7], and phospholipids [8, 9], are important components for development of pollen wall [10–12]. Altered exine of pollen wall was observed in mutants of *Arabidopsis CYP704B1* [11], *Brassica napus CYP704B1* [13] and rice *CYP704B2* [12]. However, rice *CYP704B2*, besides the altered pollen exine, it showed undeveloped anther cuticle and male sterile phenotype [12]. Further studies in other plants will help to elucidate diversified *CYP704B* function regarding plant reproductive development.

*Panax ginseng* is a slow-growing perennial herb that has been cultivated for its highly valued root for medicinal purposes since 5000 years ago [14, 15]. In the third year of growth, *P. ginseng* flowers and, usually, after four years of cultivation the roots are harvested [15, 16]. Attempts to increase ginseng yield and amounts of ginsenosides have been done by developing ginseng hybrids, and although they display heterosis, F1 hybrid plants showed male sterility derived from pollen defects at the young microspores stage [16]. Previously we studied and described the morphogenesis of anther and carpel at a cytological level to comprehend and specify *P. ginseng* reproductive developmental phases [17, 18]. Despite the importance of ginseng reproductive development, studies on functional gene analysis and molecular regulation remains scarce. In the research presented here, we isolated and cloned *CYP704B1* gene from *P. ginseng*, *PgCYP704B1*, expressed highly in flower bud, during anther reproductive development. Overexpression of *PgCYP704B1* in *Arabidopsis* showed improved plant biomass in terms of seed yield, potentially caused by the accumulation of saturated fatty acids and 2-hydroxy fatty acids in siliques. Our results suggest that *PgCYP704B1* has a conserved role during male reproduction and also boosts the accumulation of fatty acids helping to improve seed yield.

## Materials and methods

### Plant materials and growth conditions

Columbia ecotype (CS60000) of *Arabidopsis thaliana* was used as a system model. Seeds were surface sterilized with 70% ethanol for 1 min. Then with 50% sodium hypochlorite for 5 min. Then, washed thoroughly five times with distilled water. Sterilized-seeds were sown on half-strength Murashige and Skoog medium (Duchefa Biocheme) containing 1% sucrose, 0.8% (w/v) agar, and pH 5.7. Under long-day photoperiod of 16 h light and 8 h dark at 23 °C, 3-day-old cold-treated seeds were germinated. The ginseng (*Panax ginseng*) plant organs (root body, stem, leaf, flower bud, fruit) were acquired from the Ginseng Bank in South Korea. Transformants were screened on hygromycin (50 µg/mL)-selective medium plates. Under the same light/dark conditions 10-day-old seedlings were transplanted to soil and cultivated for 5 weeks.

### Identification of PgCYP704B1 gene and sequence analysis

To obtain a full-length coding sequence of *PgCYP704B1* gene, homologous sequences of *CYP704* EST were searched against the SNU Genome database (kindly provided by Prof. Tae-Jin Yang, Seoul National University) using a BLASTX algorithm. A pTriplEx phagemid for *CYP704* cDNA was excised from the λpTriplEx2 and used as a template for sequence analysis. DNASIS program was used to analyze nucleotide and amino acid sequence (Hitachi, Japan).

The amino acid sequence of *PgCYP704B1* was utilized to search for homologous proteins via BLAST network services at the NCBI. Sequence alignment was done using Clustal X and the subcellular localization for N-terminal was predicted by psortb (https://www.psort.org/psortb/) [19]. The subcellular target of the transit peptide was identified by Predotar v. 1.03 (https://urgi.versailles.inra.fr/predotar/predotar.html) [20]. A neighbor-joining tree was constructed with MEGA4 software, using the reliability of each node established by the bootstrap method. A 3D model was prepared on a SWISS-MODEL Workspace in automated mode [21] using *CYP704* as a template. The generated 3D structure was visualized using the UCSF Chimera package. The hydropathy value was calculated using the method [22] and the secondary structures were evaluated using Multiple Alignment (SOPMA) self-optimized prediction [23]. The estimation of protein characteristics was evaluated using ProtParam [24].

### Vector construction and *Arabidopsis* transformation

The *CYP704* gene was amplified from *P. ginseng* cDNA library and cloned into *Kpn*I and *Sma*I sites of the pCAMBIA1390 vector containing the Cauliflower Mosaic Virus 35S promoter and the yellow fluorescent protein (YFP). Before plant transformation, we verified nucleotide sequencing of the transgene construct. *Agrobacterium tumefaciens* C58C1 (pMP90) [25] transformants comprising pCAMBIA1390 plasmids with *CYP704B1* were tested on a 50 mg/L kanamycin solid YEP medium. The transformation of plasmids into *A. tumefaciens* C58C1 was confirmed by PCR using *PgCYP704B1*-specific primers after plasmids were isolated from kanamycin-resistant C58C1 colonies. The construct was transformed into *Arabidopsis* using *A. tumefaciens* C58C1. The insertion of transgenes into the transformants was confirmed by PCR. Homozygous plants with a 3:1 segregation ratio on antibiotic plates were selected for additional analyses. Among several T2 independent lines,

two selected lines were further analyzed for statistical and metabolite analysis.

## Subcellular localization

Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems (UK) Ltd) was used to observe fluorescence (YFP excitation collected at 520–600 nm). The images were processed using the Leica TCS SP5 software (Leica Microsystems (UK) Ltd).

## RNA isolation and semi-quantitative RT-PCR analysis

Total RNA extraction from frozen samples was performed using RNeasy mini kit (Qiagen, Valencia, CA, USA). 1 µg of total RNA was used for cDNA synthesis. For semi qRT-PCR, specific primers for *PgCYP704B1* (F-5′-CAG TCC CTC AGG ATC CAA AA-3′ and R-5′-CAA GGC AAA TCC TAG GAC CA-3′) and a constitutively expressed Ginseng *actin* gene (F-5′-CGT GAT CTT ACA GAT AGC TTG ATG A-3′and R-5′-AGA GAA GCT AAG ATT GAT CCT CC-3′) were used, with the following program: 4 min at 95 °C; followed 33 cycles of 95 °C for 30 s; 54 °C for 30 s, 72 °C for 20 s; and a final extension of 72 °C 7 min. The signal instensities were measured with ImageJ 1.50i [26].

## RNA in situ hybridization

Different developmental stages of flowers were collected and fixed in formalin-acetic acid alcohol (FAA, 50% ethanol, 5% glacial acetic, 3.7% formaldehyde in water) at 4 °C for 16 h. Then dehydrated on graded ethanol series (85, 90, 95, 100%), followed by xylene series, embedded in Paraplast Plus (Oxford Labware), and sectioned at 7 µm. The *PgCYP704B1* cDNA was prepared from RNA isolated from ginseng flowers at different developmental stages and then cloned to the pJET clone vector. In vitro transcription under T7 promoter with RNA polymerase using DIG RNA labeling kit (Roche) was made for DIG-labeled anti-sense (forward, 5′-TTC ATC TAT AGA TGG AGC-3′; reverse, 5′-GCC TGA GCA AAG CAA TTC-3′) and sense probes. RNA samples-DIG-labeled were made according to the manufacturer's guidelines using DIG RNA labeling kit (SP6/T7) (Roche, Mannheim, Germany).

A sense probe was used as a negative control. RNA hybridization and immunological detection of the hybridized probes were performed as described [27].

## Histology

Semi-thin section was performed utilizing siliques of 5-weeks old plants, fixed in FAA. Then dehydrated on graded ethanol sequence (70, 80, 90, and 100%) 30 min each step. Then embedded in KULZER's Technovit 7100 cold polymerizing resin (Heraeus Kulzer GmbH Philipp-Reis-Straβe 8/13, D-61273 Wehrheim/Ts) by three steps of preinfiltration, infiltration, and embedding at 45 ºC [28–30]. Then sectioned 4 µm thick in an Ultratome III ultramicrotome (LBK), and stained with 0.25% toluidine blue O (Chroma Gesellshaft Shaud). Bright-field photographs of the anther and siliques sections were observed in a Nikon ECLIPSE 80i microscope.

SEM was performed using anthers that were fixed and washed as described for semi-thin section, except dehydration, instead using 20, 30, 40, 50, 60, 70, 80, 90, and 100% ethanol 3 min for each step. Then the samples were dried at critical point temperature (Leica EM CPD300). Followed by 5 nm thick Aurum coating with a Leica EM SCD050 ion sputter. Then the Aurum-coated samples were observed in a Hitachi S3400N scanning electron microscope.

Microtome sections of resin-embedded flowers and siliques were used for lipid staining with Sudan IV as described [31, 32] with modifications. Exine observation was performed accordingly as described [11].

Pollen viability test was performed using anthers stained with Alexander staining as described [33]. Nail polished slides were sealed and observed in a Nikon ECLIPSE 80i microscope.

## Analysis of silique cutin monomers

Cutin from siliques of 5-week old plants were examined as described [7] with modifications. 10–20 mg of dried siliques were dipped in 2 mL of chloroform 1 min. The resulting chloroform extract was spiked with 10 µg of tetracosane (Fluka) as internal standard and transferred to a new glass vial. Then the solvent was evaporated under a light stream of nitrogen. Then the compounds containing free hydroxyl and carboxyl groups were transformed to trimethysilyl ethers and esters using 20 µL bis-(N, N-trimethysilyl)-tri-fluoro-acetamine (Sigma-Aldrich) in 20 µL pyridine for 40 min 70 °C. The monomers were identified from their electron ionization-mass spectrometry spectra (70 eV, m/z 50 to 700) after GC separation (column 30 m X 0.32 mm X 0.1 µm film thickness [DB-1; JandW Scientific].

The cutin monomer composition of the siliques polyesters was analyzed as described [34]. The siliques were extracted for 2 weeks in freshly added 1 mL of chloroform:methanol (1:1 v/v). This step was repeated four times before the siliques were finally dried at 30 ºC for 16 h. Then the delipidated siliques were depolymerized by transesterification in 1 mL of 1 N methanolic HCL at 80 ºC for 2 h. Then added 2 mL of saturated $NaCl/H_2O$ and 10 µg of drotriacontane (Fluka) as internal standard. Then hydrophobic monomers were extracted three times with 1 mL of hexane. Then the organic phases were mixed, the solvent was evaporated, and

the remaining sample was derivatized as described above. GC–MS (Agilent gas chromatograph coupled to an Agilent 5973 N quadrupole mass selective detector) and GC-FID (Agilent 6890 gas chromatograph) analysis were performed.

### Statistical analysis

Data were analyzed with GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). All data are expressed as means ± standard error of the mean for at least six replicates. The statistical significance of all the differences between the phenotypic traits of height, seeds-per silique, seed size, and siliques per plant, was determined using a one-way ANOVA and Kruskal–Wallis non-parametric post-hoc test. P value ≤ 0.05 was considered significant.

## Results

### *PgCYP704B1* encodes a putative cytochrome P450 hydroxylase in ginseng

Full genomic DNA sequence of PgCYP704B1 was analyzed using the genomic DNA sequence retrieved from the database of ginseng genome (http:/im-crop.snu.ac.kr/new/index.php). An NCBI Blast search (http:/www.ncbi.nlm.nih.gov/BLAST/) displayed the conserved superfamily P450, suggesting that *PgCYP704B1* belongs to the P450 family. The CYP704 family belongs to the CYP86 clan of fatty-acid hydroxylases, and since the CYP704 family underwent only one duplication, it divided the subfamilies CYP704A and CYP704B, predicting the differentiation between monocots and [2, 4, 11]. To gain information about the potential function of *PgCYP704B1*, its protein sequence and its relatives in higher plants, including Arabidopsis [11] and rice [12], were used to build a phylogenetic tree (Fig. 1). Based on our phylogenetic comparison, *PgCYP704B1* was placed in clan 86 subfamily CYP704B, separated from the closest associated clan 71 [4]. Several members in the clan 86 have been shown to catalyze fatty acid hydroxylation [12, 35–38], and the *CYP704B* subfamily members are expressed in the reproductive organs, among plants from bryophytes to angiosperms [12, 39], indicating an essential and conserved function that *PgCYP704B1* may have in fatty acid hydroxylation during plant male reproductive development. In addition, multiple sequence alignment (Fig. S1) showed that the CYP704 proteins and PgCYP704B1 contain the conserved domains such as AGRDT, TETLR; PERFW, and the FQAFPRICLG (F, G and C residues), as a common feature for CYPs functions [4, 11]. The predicted transit peptide of PgCYP704B1 (indicated arrow in Fig. S1) is shown to be positioned at its N-terminal with a cytoplasmic location [19] targeting endoplasmic reticulum (ER) and could function in the hydroxylation of fatty acids. Fatty acids are hydroxylated in plant cells in the ER through members of the CYP family [5, 40, 41]. To confirm the subcellular location of PgCYP704B1, we developed a translational fusion of the full-length PgCYP704B1 coding region and yellow fluorescent protein (YFP) controlled by the cauliflower mosaic virus 35S promoter (*35Spro*:*PgCYP704B1*:*YFP*). As we expected, the fluorescence of *35Spro*:*PgCYP704B1*:*YFP* was targeted to the ER organelle (Fig. 1b–d). Consistently, previous studies have shown that *CYP704B1* is targeted to ER in *Arabidopsis* and *B. napus* [13, 42]. CYPs can be divided into four classes depending on how electrons from NAD(P)H are delivered to the catalytic site, and a typical feature of class II is to be independently anchored on the outer face of the ER by amino-terminal hydrophobic anchors [43], as also confirmed in our results (Fig. S3b). The cDNA encoding *PgCYP704B1* has a length of 1,557 bp, encoding 518 amino acids; moreover, the *PgCYP704B1* gene contains six exons and five introns (Fig. S2), which is a common characteristic with *Arabidopsis* and *B. napus CYP704B1* genes [11, 13].

PgCYP704B1 hydrophobicity profile and its nearest homologs indicate that both the N and C terminals are highly conserved (Fig. S3b). The conserved domains of CYPs (AGRDT, TETLR; PERFW, and FQAFPRICLG) are hydrophobic, compatible with their place in the predicted 3D model, as they form part of the helixes (Fig. S3a). A similar number of alpha-helices, beta-turns, extended strands, and random coils (Table 1) was found on the secondary structure analysis (Fig. S3c) of PgCYP704B1 and the CYP704 proteins with close phylogenetic relationship.

### *PgCYP704B1* is highly expressed in flower buds

To verify the conserved function of *PgCYP704B1* in the anther as it has been described for *Arabidopsis* [11], rice [12], and *B. napus* [13], we conducted *PgCYP704B1* expression analysis by semi qRT-PCR utilizing ginseng tissues such as root, stem, leaf, flower buds and fruit. The transcript of *PgCYP704B1* was expressed highly in flower buds, although expression was identified at lower intensity in stem, leaves, and fruit (Fig. 2a, Fig. S2C).

To gain insight into the spatial and temporal patterns of *PgCYP704B1* expression in anthers, we performed in situ hybridization (Fig. 2b–d). The results showed that *PgCYP704B1* is expressed in the tapetal cell layer and meiocytes during the anther reproductive developmental stages 3 to 5 (classification made by Kim et al. [17]). In stage 3, when the microspore mother cells initiate meiotic division, the maximum amount of *PgCYP704B1* in the tapetum and dyads was observed. The hybridization weakened to tapetal cells, tetrads, and microspores after the development of tetrads at stage 4. For control hybridization, a sense probe was used (Fig. 2e).

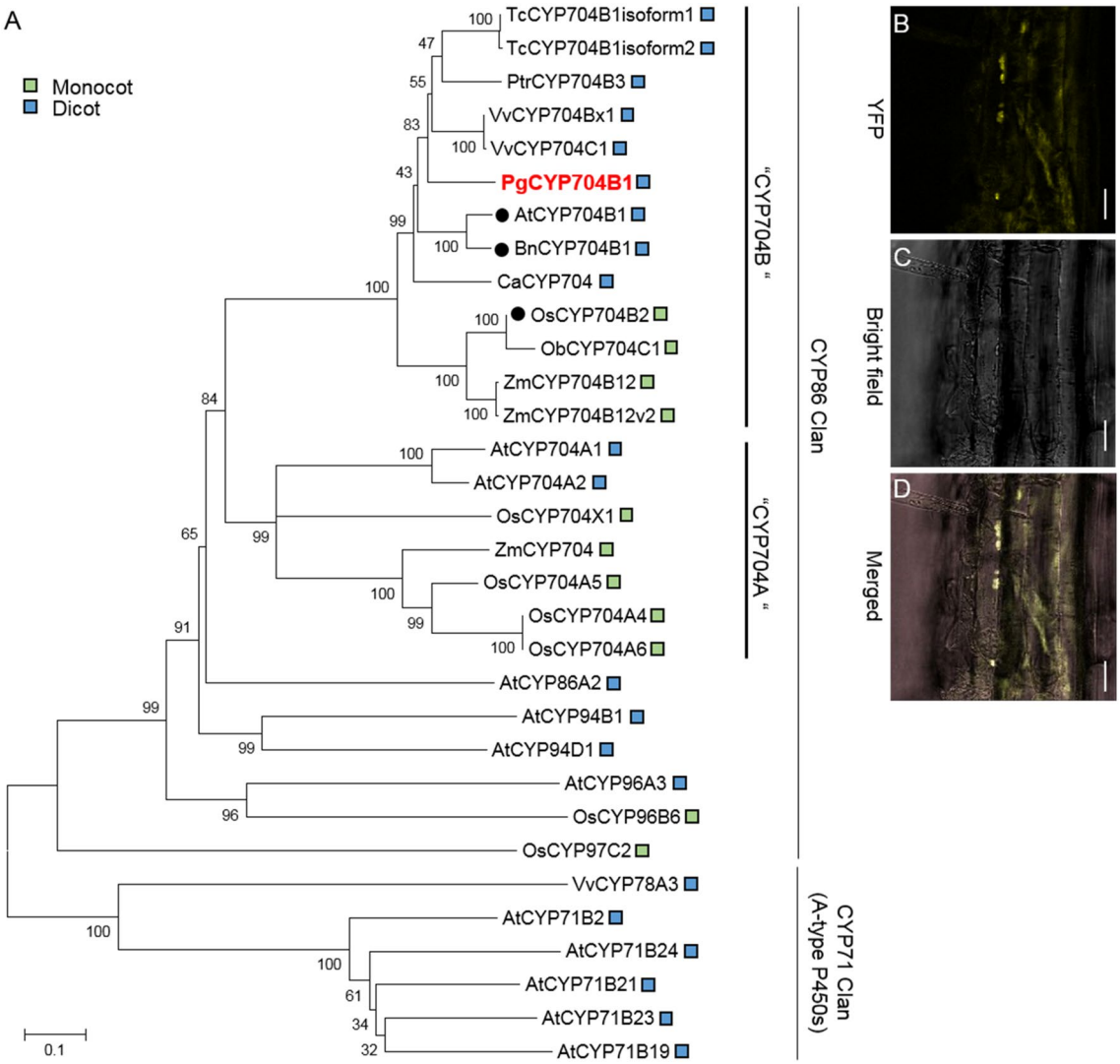


**Fig. 1** Phylogenic analysis of *PgCYP704B1*. **a** A neighbor-joining method analysis was performed using MEGA 4 based on the Table S1. Bootstrap values are percentage of 1000 replicates. The scale bar shows 0.1 amino acid substitutions per site. Black dots show reported *CYP704B* genes. Subcellular localization of *PgCYP704B1* by confocal laser scanning microscopy images of 7 d-old seedlings roots in transgenic lines expressing *35Spro*:*PgCYP704-YFP* in endoplasmic reticulum (ER), **b** YFP fluorescence, **c** bright field, and **d** merged images. Bars; **b–d** 100 mm

**Table 1** Secondary structure characteristics of ginseng *CYP704B1* and other plants with close phylogenetic relationship

| Protein | Alpha-helices | Beta-turns | Extended strands | Random coils |
|---|---|---|---|---|
| *PgCYP704B1* | 219 | 41 | 111 | 146 |
| *TcCYP704B1*isoform1 [EOY01870] | 250 | 41 | 95 | 149 |
| *TcCYP704B1*isoform2 [EOY01871] | 249 | 41 | 95 | 152 |
| *MnCYP704C1* [EXB92426] | 259 | 36 | 96 | 174 |
| *AtCYP704B1* [OAP12978] | 242 | 38 | 100 | 144 |
| *CcCYP704C1* [KYP54385] | 266 | 36 | 92 | 121 |

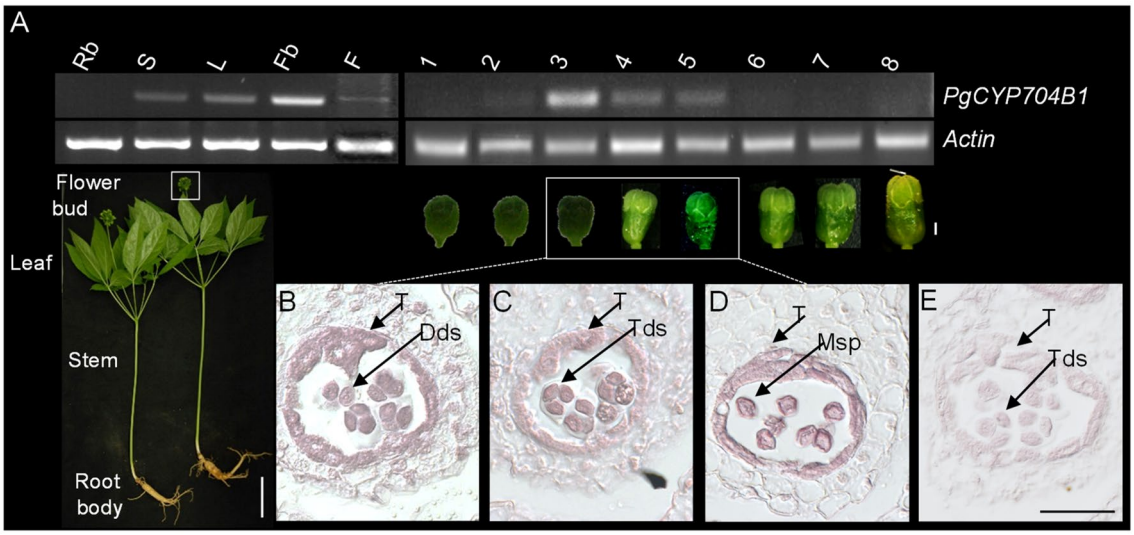


**Fig. 2** Expression pattern of *PgCYP704B1*. **a** Spatial and temporal expression analysis of *PgCYP704B1* transcript in selected tissues and anther development by semi qRT-PCR. Tissues from 5 year-old ginseng and flowers from 14 to 24 days after sprouting were analyzed for the presence of *PgCYP704B1*. Ginseng *actin* (*Pg Actin*) expression was used as a control. Rb, root body; S, stem; L, leaf; Fb, flower bud, and F, fruit; 1, stage 1; 2, stage 2; 3, stage 3; 4, stage 4; 5, stage 5; 6, stage 6; 7, stage 7; 8, stage 8. **b–d** RNA in situ analysis of *PgCYP704B1* in anthers. The anthers at stage 3 (**b**), 4 (**c**), and 5 (**d**) showing strong signal of *PgCYP704B1* in tapetal cells and microspores; **e** The sense probe produces no signal at stage 4 (control). *Dds*, dyads; *Msp*, microspore; *T*, tapetum; *Tds*, tetrads. Bars; **a** 10 cm and 1 mm, **b–d** 50 µm

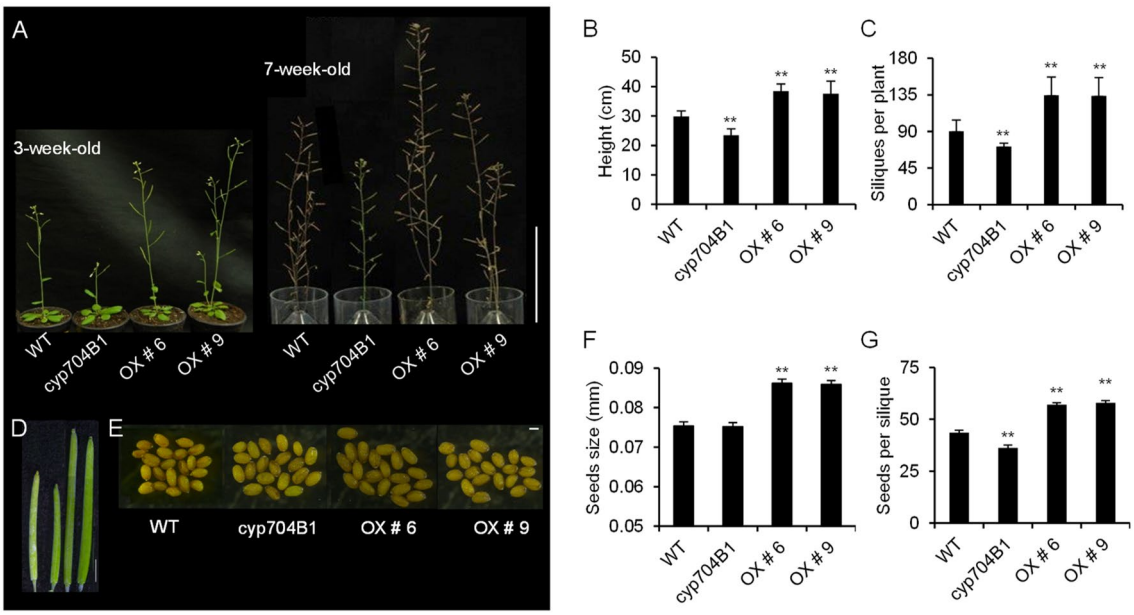


**Fig. 3** Overexpression of *PgCYP704B1* in *Arabidopsis* improves biomass. Greater growth in **a, b** height, **c** siliques per plant, **d** siliques size, **e, f** seed size, and **g** seeds per silique. Bars show mean expression levels ± SE. *WT,* Wild-type; *cyp704B1,* mutant; *OX # 6, PgCYP704B1*ox # 6; *OX # 9, PgCYP704B1*ox # 9. *P < 0.05. Bars; **a** 10 cm, **d** 5 mm **e–h** 500 µm

## Overexpression of *PgCYP704B1* improves plant productivity in transgenic *Arabidopsis*

Due to difficulties in obtaining transgenic regenerated *P. ginseng* plants, we observed the effect of overexpressing *PgCYP704B1* in transgenis *Arabidopsis* (*PgCYP704B1*ox). We examined plant biomass-related characteristics such as plant height, number and size of siliques and seeds. *PgCYP704B1*ox produced 21% taller plants compared with wild-type and mutant (Fig. 3a, b). As observed in

*PgCYP704B1*ox, the siliques increased 43.25% in number compared with wild-type and mutant (Fig. 3c, d). *PgCYP704B1*ox increased 10% in seed size and 45.25% in average yield compared with those of control (Fig. 3e–g).

In addition we stained mature anthers with lipophilic dye Sudan IV to observe the cuticular lipid deposition [31, 32]. Compared to wild-type, anthers of *cyp704B1* and *PgCYP704B1*ox showed increased lipidic compounds in the anther epidermal layer, and an opposite effect on the pollen wall (Fig. S4). As *cyp704B1* also shows high lipidic compounds, it confirms the conserved function of this *CYP704B* is the biosynthesis of lipidic precursors essential for anther reproductive development.

## *PgCYP704B1* can functionally complement the *Arabidopsis cyp704B1* mutant

The Arabidopsis *cyp704B1* was reported to show impaired pollen walls lacking a normal exine layer that displays a stripped surface called zebra phenotype [11]. To determine whether *PgCYP704B1* was able to complement the *Arabidopsis cyp704B1*, we performed a complementation assay by reciprocal hand pollination of *PgCYP704B1*ox with *cyp704B1*. The stable incorporation of the *PgCYP704B1* gene into *cyp704B1* was confirmed by PCR (Fig. S5). To observe the anthers and pollen phenotype, we used Alexander staining, semi-thin cross sections, Auramine O, and SEM. Pollen viability showed to not be affected, we assume that this recovery is due to PgCYP704B1 being involved in the sporopollenin biosynthesis and lipidic precursors, that could be stably co-transmitted to progeny plants and that it co-segregated with the introduced DNA. Although the anther of *PgCYP704B1*ox looks similar to the wild type and *cyp704B1*, the number of pollen grains increase and exhibits a compacted arrangement in *PgCYP704B1*ox (Fig. S6a–d). To characterize the alterations of the *PgCYP704B1*ox complementary lines we used Alexander staining showing that the anthers where reduced in size, in both height and width, but viable pollen (Fig. S7 a, b). Compared with wild-type, the anthers of *PgCYP704B1*ox and *cyp704B1* exhibited bigger pollen grains at stage 14 [44] (Fig. S6e–h). Laser scanning confocal microscopy (LSCM) of Auramine O-stained *PgCYP704B1*ox exhibits a more tightly organized exine architecture compared to wild-type, further we also confirmed that *cyp704B1* exhibits no exine membrane as Dobritsa et al. [11] reported (Fig. S6i–l). *PgCYP704B1* overexpression complemented the zebra pollen phenotype of *cyp704B1* checked by Auramine O (Fig. S7c, d), confirming the functional ortholog of *PgCYP704B1* to *CYP704B1* of Arabidopsis. Examination by SEM analysis showed that the outer surface of the pollen had a denser exine compared to wild-type and *cyp704B1* (Fig. S6m–p). Complementary-*PgCYP704B1*ox exhibits a normal pollen exine (Fig. S7g–h). Moreover, SEM analysis of anthers showed similar surface structure compared to wild-type (Fig. S6q–x). Complementary-*PgCYP704B1*ox exhibits no difference on the anther surface (Fig. S7g–j). Taken together, these results indicate that the overexpression of *PgCYP704B1* significantly affected pollen number and exine development.

## *PgCYP704B1* promotes significant silique cell elongation and affects fatty acids contents in transgenic *Arabidopsis*

We carried out light microscopic analysis of semi-thin sections to further observe the phenotype of the elongated siliques. The results revealed that *PgCYP704B1*ox exhibited longer exocarp cells observed by longitudinal sections and increased number of sclerenchyma cells observed by transverse sections in comparison with wild-type and *cyp704B1* (Fig. S8).

The cuticle is a hydrophobic layer, that coats the surface of the aerial organs such as leaves, stems, flowers, and fruits [12]. The cuticle layer functions not only in the interaction with the environment but also in plant development and growth [45]. All cuticles are biopolymers composed of two classes of lipophilic constituents, cutin and waxes [12, 46–50]. Because of the elongated siliques phenotype of *PgCYP704B1*ox and that the exocarp is made of cutin, we further performed gas chromatography-mass spectrometry (GC–MS) and gas chromatography-flame ionization detection (GC-FID). The levels of cutin monomers were found to increase up to 1.34 times of increment in *PgCYP704B1*ox in comparison with wild-type (Fig. 4a). The overexpression of *PgCYP704B1* significantly promoted the accumulation of saturated fatty acids such as C18:0 (1.5 times), C18:1 (1.8 times), C18:2 (1.6 times), C18:3 (1.8 times), C20:2 (2.1 times), and C24:0 (1.1 times) (Fig. 4b). The contents of 2-hydroxy fatty acids also significantly increased for C16:0 (1.2 times), C22:0 (1.5), C24:0 (1.2 times), C24:1 (1.2 times), C25:0 (1.9 times), and C26:0 (1.2 times) (Fig. 4c). Compared with the reported *AtCYP704*, *OsCYP704*, and *BnCYP704* the common effect with *PgCYP704B1* is the increment of long-chain fatty acids. However *PgCYP704B1* is also increasing very long-chain fatty acids, moreover *PgCYP704B1* did not show any increment of dicarboxylic fatty acids, terminal-hydroxy fatty acids, and alcohols (Fig. 4d–f). These data indicate that overexpression of *PgCYP704B1* in *Arabidopsis* synthesizes and promotes the accumulation of cutin in siliques.

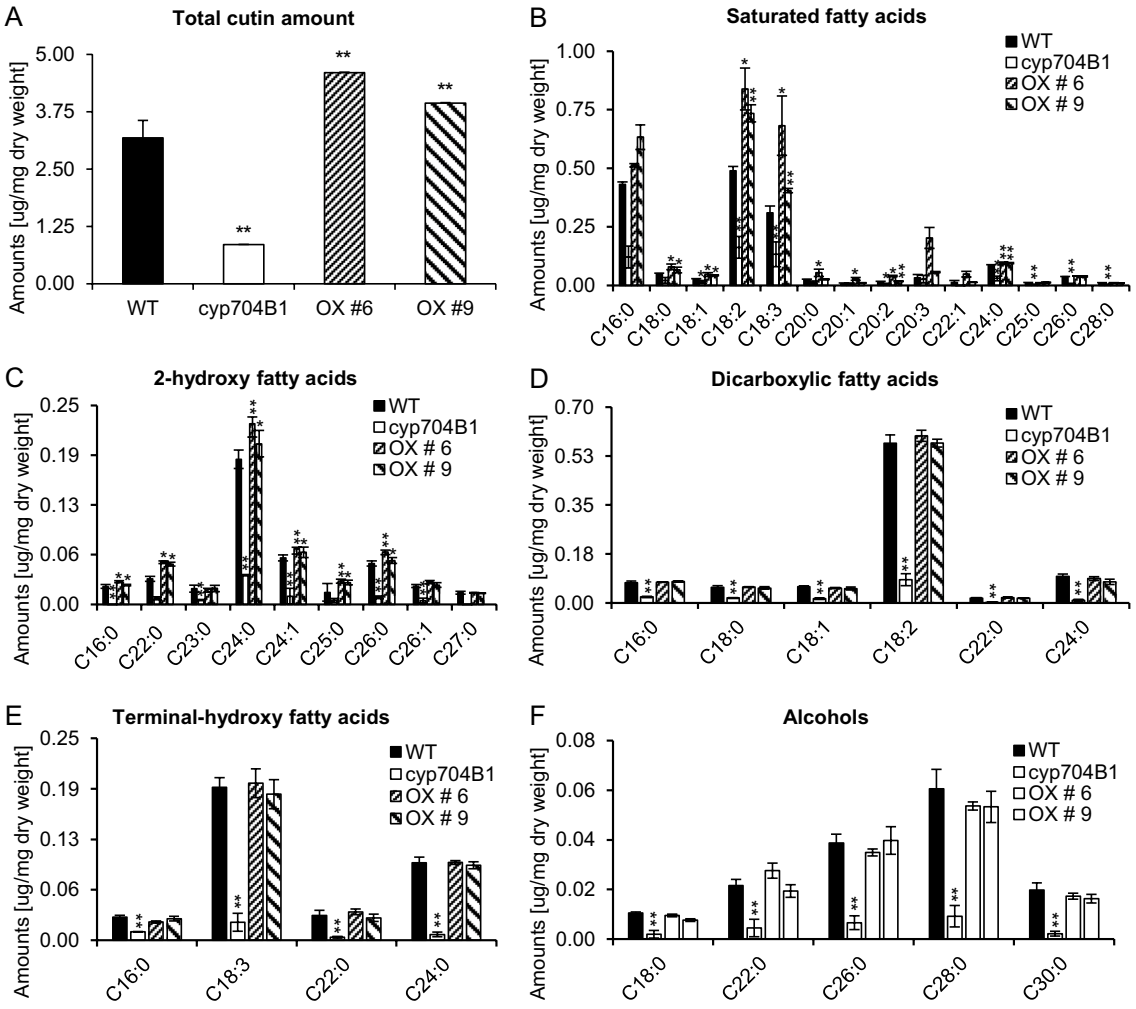


**Fig. 4** Chemical analysis of siliques cutin monomers in the wild-type, *cyp704B1*, and *PgCYP704ox* lines by GC–MS and GC-FID. **a** Total cutin amount per milligram of dry weight (μg/mg). **b** Saturated fatty acids per milligram of dry weight (μg/mg). **c** 2-hydroxy fatty acids per milligram of dry weight (μg/mg). **d** Dicarboxylic fatty acids per milligram of dry weight (μg/mg). **e** Terminal-hydroxy fatty acids per milligram of dry weight (μg/mg). **f** Alcohols per milligram of dry weight (μg/mg). The values indicate means of five biological replicates ± SD. *P < 0.05; **P < 0.01

## Discussion

### *PgCYP704B1* is ortholog of *AtCYP704B1* and it is highly expressed in the developing anther

*PgCYP704B1* is ortholog of *AtCYP704B1* (Fig. 1, S1) and it contains the four CYPs conserved domains (Fig. S1) indispensable for CYP functions [3, 11]. First, a Thr-containing binding pocket for molecular oxygen required in catalysis (AGRDT). Second, the E-R-R triade, using the consensus TETLR and PERW, generally thought to be involved in locking the heme pocket into position to assure stabilization of the conserved core structure. And lastly, the heme-binding domain (FQAGPRICLG) in the C terminus.

Both *PgCYP704B1* and *AtCYP704B1*, show a conserved function during anther reproductive development for sporopollenin biosynthesis [11] (Fig. 2). In accordance, *PgCYP704B1* expression in developing anther shows common tendencies with *Arabidopsis*, rice, and *B. napus* [11–13] at developing young microspores stage (Fig. 2). Although *CYP704B* expression in the developing anther slightly varies among *P. ginseng*, *Arabidopsis* rice, and *B. napus*. *AtCYP704B1* starts at the young microspore and it fades at vacuolation [11] and *OsCYP704B2* starts at the tetrad and it fades at mitosis I [12]. Whereas *BnCYP704* starts at early meiosis and it fades at pollen maturation [13]. *PgCYP704B1* starts at cell division (early meiosis) and it peaks its expression at meiosis fading at young microspore (stage 3, Fig. 2a). Suggesting that the tapetum might be

metabolically overactivated, thus increases meiotic division, and ultimately *PgCYP704B1*ox produces more number of pollen grains (Fig. S7).

*PgCYP704B1* complemented the zebra pollen phenotype of *cyp704B1* (Fig. S7c, d), confirming the functional ortholog of *PgCYP704B1* to *CYP704B1* of *Arabidopsis* and that *PgCYP704B1* is involved in fatty acid hydroxylation and is required for pollen exine formation.

### *PgCYP704B1* contributes to improve biomass of reproductive tissues and affects fatty acid contents

*PgCYP704B1* overexpression in transgenic *Arabidopsis* improved plant biomass as observed in plant height, size and number of siliques and seeds. The increases were 21% in plant height, 42.75% in siliques size, 43.25% in siliques number, 10% in seed size, and 45.25% in seed yield, compared to wild-type (Fig. 3). Consistently, *PgCYP704B1* expression was observed at lower intensity in the fruits (Fig. 2a), the phenotype of *PgCYP704B1*ox displayed longer siliques and longer exocarp cells in the siliques of the transgenic *Arabidopsis* (Fig. 3), and the long-chain and very long-chain fatty acids were also increased (Fig. 4). It is well known that sporopollenin is a highly cross-linked biopolymer of hydroxylated fatty acids, aliphatic compounds, and phenolics [5, 11, 51–62]. In rice, sporopollenin precursors are delivered from the tapetum in the form of Ubisch bodies [63]. The tapetum is responsible for the biosynthesis of sporopollenin building blocks in the post-tetrad stage [63]. According to Wang et al. [63], the synthesis of sporopollenin precursors involves eight vital enzymes in *Arabidopsis*, including ACYL-CoA SYNTHETASE5 (*ACOS5*) which catalyzes mid-/long-chain fatty acids into fatty acyl-CoA, then they are hydroxylated by *CYP703A2* and *CYP704B1*. POLYKETIDE SYNTHASE A (*PSKA*) and *PSKB* catalyze the hydroxylated products into triketides and tetraketides α-pyrones, conforming the substrates of TETRAKETIDE α-PYRONE REDUCTASE1 (*TKPR1*) and *TKPR2*. And MALE STERILE2 (*MS2*) catalyzes the protein of palmitoyl acyl carrier into a fatty alcohol as a fatty acyl reductase [5, 11, 53, 54, 57, 59, 64, 65]. The resulting precursors of sporopollenin are synthesized in the tapetum and transferred by the ATP-binding cassette transporter superfamily member (*ABCG26*) to the anther locule [63, 66]. *Arabidopsis ABCG26* has been reported to restore fertility in terms of seed yields [66] and is also predicted to transport synthesized sporopollenin precursors to the anther locule [63].

Taken together, this close association of *ABCG26* with *CYP704B1*, the fact that *ABCG26* regulates seed yield [66], and that *PgCYP704B1* overexpression showed increased lipidic compounds and increased plant biomass (Fig. 3), leads to hypothesize that *PgCYP704B1* overexpression might mediate plant biomass production in terms of fruits and seeds, synthesizing essential components of the anther and silique cuticle (Fig. 5), nevertheless this hypothesis requires further assessment by future studies. It will require further investigation to find out whether *PgCYP704B1* can directly interact with *ABCG* protein or whether this interaction depends on other molecular players.

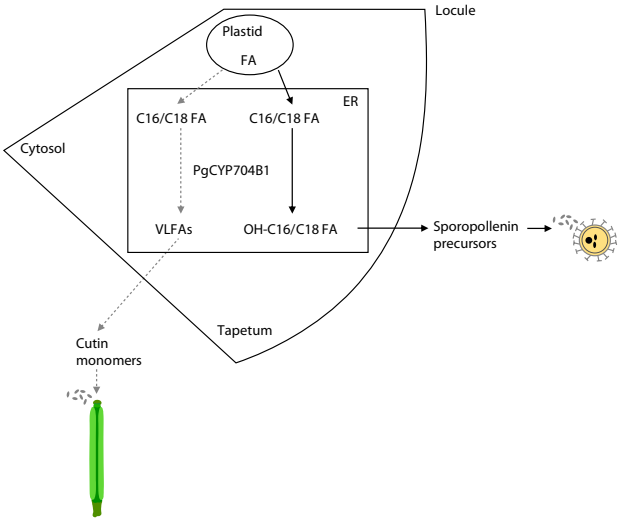


**Fig. 5** Hypothetical model of the role of *PgCYP704B1* in improving yield in reproductive tissues. Fatty acids produced in the plastid are transferred into the ER. Then they are synthesized to a maximum of C18 and C28 (VLFAs) in length. The resulting hydroxylated fatty acids are then transported to either use as precursors for sporopollenin in pollen, or as cutin monomers in siliques

## Conclusions

In this study, we have identified and characterized an unknown *P. ginseng* gene encoding a cytochrome P450 protein, which was designated *PgCYP704B1*. *PgCYP704B1* transcripts are low in the fruits and high in the flower buds, specially from the meiosis- to the young microspore-anther stages. Overexpression of *PgCYP704B1* in transgenic *Arabidopsis* improves plant biomass of reproductive tissues and enhances the accumulation of saturated fatty acids and 2-hydroxy fatty acids in siliques. It shows a conserved function in catalyzing ω-hydroxylation of long-chain fatty acids serving as building blocks of sporopollenin during anther reproductive development, suggesting its conserved role during male reproduction; furthermore, it exhibits a diversified function regarding reproductive tissues biomass improvement, particularly for seed production.

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**Author contributions** YJK, DCY, and DZ conceived and design the project and experiments. JS, JS, DM, PM conducted the experiments. JS, YJK and KHJ co-write the manuscript. JY contributed for cytological analysis, and JS performed the wax and cutin analysis. All authors read and approved the manuscript.

## Compliance with ethical standards

**Conflicts of interest** No conflict of interest was reported by the authors.

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