



Research Article

Production of ginsenoside aglycone (protopanaxatriol) and male sterility of transgenic tobacco co-overexpressing three *Panax ginseng* genes: *PgDDS*, *CYP716A47*, and *CYP716A53v2*

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ARTICLE INFO

Article history:

Received 11 October 2017

Received in Revised form

5 January 2018

Accepted 8 February 2018

Available online 15 February 2018

Keywords:

Male sterility

Metabolic engineering

Protopanaxatriol

Sapogenin

Transgenic tobacco

ABSTRACT

Background: Protopanaxatriol (PPT) is an aglycone of ginsenosides, which has high medicinal values. Production of PPT from natural ginseng plants requires artificial deglycosylation procedures of ginsenosides via enzymatic or physicochemical treatments. Metabolic engineering could be an efficient technology for production of ginsenoside sapogenin. For PPT biosynthesis in *Panax ginseng*, damarenediol-II synthase (*PgDDS*) and two cytochrome P450 enzymes (*CYP716A47* and *CYP716A53v2*) are essentially required.

Methods: Transgenic tobacco co-overexpressing *P. ginseng* *PgDDS*, *CYP716A47*, and *CYP716A53v2* was constructed via *Agrobacterium*-mediated transformation.

Results: Expression of the three introduced genes in transgenic tobacco lines was confirmed by Reverse transcription-polymerase chain reaction (RT-PCR). Analysis of liquid chromatography showed three new peaks, dammarenediol-II (DD), protopanaxadiol (PPD), and PPT, in leaves of transgenic tobacco. Transgenic tobacco (line 6) contained 2.8 µg/g dry weight (DW), 7.3 µg/g DW, and 11.6 µg/g DW of PPT, PPD, and DD in leaves, respectively. Production of PPT was achieved via cell suspension culture and was highly affected by auxin treatment. The content of PPT in cell suspension was increased 37.25-fold compared with that of leaves of the transgenic tobacco. Transgenic tobacco was not able to set seeds because of microspore degeneration in anthers. Transmission electron microscopy analysis revealed that cells of phloem tissue situated in the center of the anther showed an abnormally condensed nuclei and degenerated mitochondria.

Conclusion: We successfully achieved the production of PPT in transgenic tobacco. The possible factors deriving male sterility in transgenic tobacco are discussed.

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1. Introduction

Panax ginseng is a well-known medicinal plant in Asia and has long been used to cure various diseases and improve physical performance [1]. Recently, various clinical effects of ginseng have been reported [2–4]. Ginsenosides, a group of triterpenoid saponins, are the main pharmacological constituents of ginseng. The major components of ginsenosides are reported as dammarane-type ginsenosides [5]. Dammarane-type ginsenosides are divided into two groups according to their aglycone structure. One is protopanaxadiol (PPD), and the other is protopanaxatriol (PPT). The difference between them is further hydroxylation of PPD at the C-6 position in PPT.

Biosynthetic pathways of dammarane-type ginsenosides in *P. ginseng* are shown in Fig. 1. 2,3-Oxidosqualene is cyclized to dammarenediol-II (DD) by dammarenediol-II synthase [6,7]. Then, DD is hydroxylated to PPD by dammarenediol-II 12-hydroxylase (*CYP716A47*) [8], and then PPD is converted to PPT by protopanaxadiol 6-hydroxylase (*CYP716A53v2*) [9]. Final ginsenosides are produced from glycosylation of the aglycones of PPD or PPT by glycosyltransferase [10].

Ginseng roots and their products are typically administered orally. The absorption of glycosylated ginsenosides by the gastrointestinal tract is extremely low [11]. Oral administration of ginseng preparations exposes ginsenosides to acid hydrolysis in the stomach, which are further cleaved by intestinal microflora stepwise

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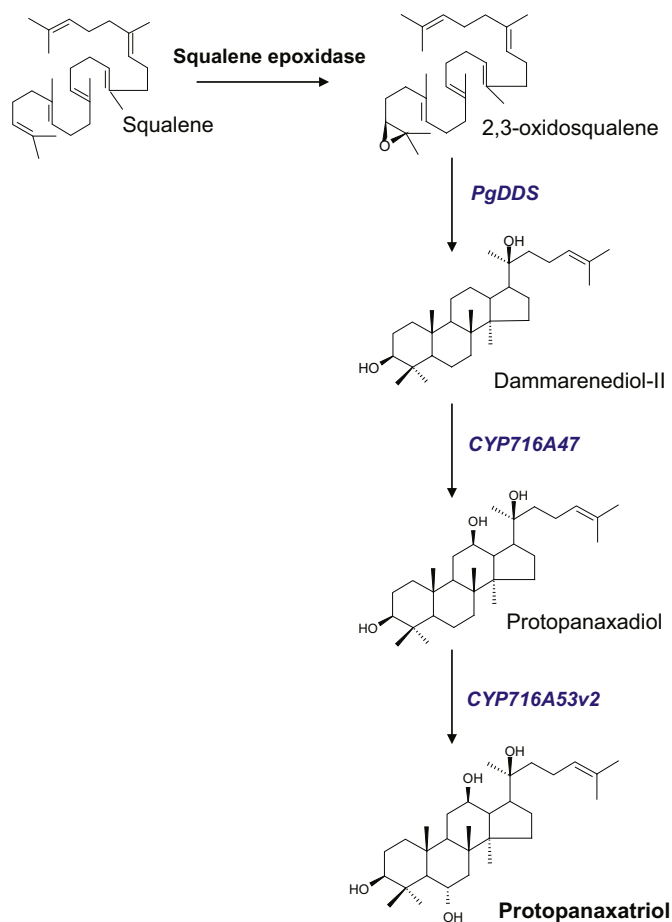


Fig. 1. Biosynthetic pathway engineering for PPT production from 2,3-oxidosqualene. Three *P. ginseng*-derived genes (*PgDDS*, *CYP716A47*, and *CYP716A53v2*) are necessary for production of PPT in tobacco. *PgDDS* converts 2,3-oxidosqualene to a triterpene (DD). The triterpene subsequently undergoes hydroxylation by *CYP716A47* to produce PPD, and PPD undergoes further hydroxylation into PPT by *CYP716A53v2*. DD, dammarenediol-II; PPD, protopanaxadiol; PPT, protopanaxatriol.

from the terminal sugar at C-3 or C-20 positions [12–15]. Recently, metabolized ginsenosides have gained more attention because completely deglycosylated aglycones or partially hydrolyzed ginsenosides possess more beneficial biological effects than naturally produced ginsenosides [16–19]. In addition, deglycosylated ginsenosides are readily absorbed into the bloodstream [13]. The conversion of major ginsenosides into deglycosylated forms had been attempted using physicochemical transformation methods, such as heating [20], acid treatment [19], or alkaline cleavage [21].

PPT, a saponin of the PPT group of ginsenosides, shows anti-cancer activity [16,22,23] and has beneficial effects on the cardiovascular system [24–26]. Antidiabetic activity of PPT also has been reported [27,28]. Ginseng plants contain very low and undetectable amounts of PPT because this is an intermediate product. To produce PPT from ginseng plants, enzymatic and physicochemical treatments are required for complete deglycosylation of PPT-type ginsenosides [3,29]. Alternatively, metabolic engineering technology could be a suitable method to produce the saponin via heterologous expression of genes in other plants.

Tobacco plants have few saponins (tobacco saponin A and B), including furostanol-type aglycones only in seeds but not in mature plant organs [30]. The first step in ginsenoside synthesis is the cyclization of 2,3-oxidosqualene to DD, catalyzed by the *PgDDS* of the oxidosqualene cyclase group [6]. This *PgDDS* enzyme is

somewhat unique in *Panax* species but probably exists in a few other plant species producing dammar resin [31,32]. To produce PPT in transgenic tobacco, three ginseng genes (*PgDDS*, *CYP716A47*, and *CYP716A53v2*) should be introduced into tobacco.

In this study, three ginseng genes (*PgDDS*, *CYP716A47*, and *CYP716A53v2*) were heterologously expressed in transgenic tobacco. PPT production was confirmed in both leaves of transgenic tobacco lines and cell suspension cultures. The culture of cells in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) was effective for enhanced production of PPT. Interestingly, male sterility caused from abnormal microsporogenesis in transgenic tobacco was demonstrated.

2. Materials and methods

2.1. Construction of overexpression vector and transgenic tobacco

The full lengths of *PgDDS* (GenBank accession number AB122080), *CYP716A47* (GenBank accession number JN604536), and *CYP716A53v2* (GenBank accession number JX036031) used in this study were isolated by PCR. The primers used for isolation of the open reading frame sequences of *PgDDS*, *CYP716A47*, and *CYP716A53v2* are follows. The forward primer to amplify the *PgDDS* gene was 5'-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3', and the reverse primer used was 5'-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3'. The forward primer used for the *CYP716A47* gene was 5'-ATG GTG TTG TTT TTC TCC CTA TCT-3', and the reverse primer was 5'-TTA ATT GTG GGG ATG TAG ATG AAT-3'. The forward primer used for *CYP716A53v2* was 5'-ATG GAT CTC TTT ATC TCA TCT CAA-3', and the reverse primer was 5'-TTA AAG CGT ACA AGG TGA TAG ACG-3'. To generate overexpression, the open reading frame sequences of these genes were cloned into GATEWAY vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA) and then transferred to the pZIP-Bar destination vector, in which *PgDDS*, *CYP716A47*, and *CYP716A53v2* were driven by the 35S Cauliflower mosaic virus (CaMV) promoter. The vector was inserted into competent cells of *Agrobacterium tumefaciens* GV3101 and transformed into tobacco plants by using the same methods as in the study by Han et al [33].

2.2. DNA analysis

Genomic DNA was isolated from the leaf tissue of putative transgenic tobacco plants and wild-type (WT) tobacco plants using the RBC Genomic DNA mini kit (Real biotech, Taiwan) according to the manufacturer's protocols. To amplify the *PgDDS* gene, the forward primer 5'-CTG CAG ATG TGG AAG CTG AA-3' and the reverse primer 5'-CCC GGG TTA AAT TTT GAG CT-3' were used. The forward primer used for the *CYP716A47* gene was 5'-ATG GTG TTG TTT TTC TCC CTA TCT-3', and the reverse primer was 5'-TTA ATT GTG GGG ATG TAG ATG AAT-3'. The forward primer used for *CYP716A53v2* was 5'-CTG CAG ATG GAT CTC TTT AT-3', and the reverse primer was 5'-GGA TCC TTA AAG CGT ACA AG-3'. The primers used for the *Bar* gene were 5'-CGG ACA TGC CGG TCT GC-3' and 5'-GCT GCC AGA AAC CCA CGT CA-3'. PCR was performed in a DNA thermal cycler (Applied Biosystems, CA, USA). The PCR conditions were 5 min at 94°C; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension of 7 min at 72°C.

2.3. RT-PCR analysis

The total RNA was extracted using an RNeasy plant mini kit (Qiagen, Germany) from tobacco plants following the manufacturer's instructions. To produce the first-strand cDNA, the RNA was reverse transcribed using the ImProm-2 Reverse Transcription System (Promega Corporation, Madison, WI, USA). Then, the cDNA

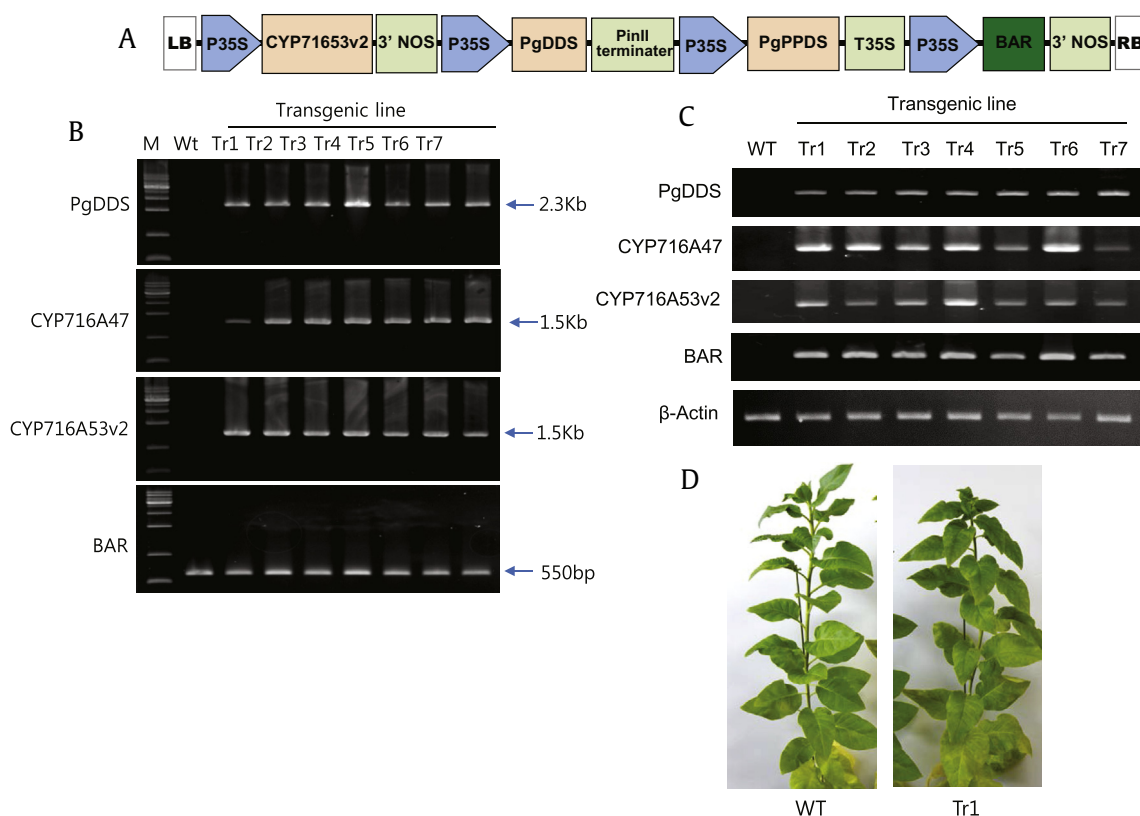


Fig. 2. Detection and expression analysis of introduced genes in transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2*. (A) T-DNA region of plasmid for co-overexpression of the three genes. (B) Genomic DNA PCR for screening of transgenic tobacco lines via detection of introduced genes. (C) RT-PCR analysis of the introduced *PgDDS*, *CYP716A47*, *CYP716A53v2*, and *BAR* genes in transgenic lines (Tr1, Tr2, Tr3, Tr4, Tr5, Tr6, and Tr7). β -Actin was used as an internal control for the introduced genes. (D) Photograph of WT (left) and transgenic tobacco (right). WT, wild-type.

was used for RT-PCR, which was performed in these conditions: 5 min at 96°C; 30 cycles of 96°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension of 10 min at 72°C. The forward primer used for the *PgDDS* gene was 5'-CTG CAG ATG TGG AAG CTG AA-3', and the reverse primer was 5'-CCC GGG TTA AAT TTT GAG CT-3'. The forward primer used for the *CYP716A47* gene was 5'-ATG GTG TTG TTT TTC TCC CTA TCT-3', and the reverse primer was 5'-TTA ATT GTG GGG ATG TAG ATG AAT-3'. The forward primer used for *CYP716A53v2* was 5'-CTG CAG ATG GAT CTC TTT AT-3', and the reverse primer was 5'-GGA TCC TTA AAG CGT ACA AG-3'. The tobacco β -actin gene was amplified as the control to identify the integrity of RNA and check the loading accuracy. The primers 5'-ACA GGT ATT GTG TTG GAT TC-3' and 5'-TGT TGG AAG GTG CTG AGA G-3' were used for the tobacco β -actin gene. The RT-PCR products were analyzed by performing gel electrophoresis using 1% agarose gel and 0.5× Tris-borate-EDTA (TBE) buffer.

2.4. Callus induction and cell suspension culture

Leaves from *in vitro* transgenic tobacco plants were cut into small pieces (approximately 0.5–1.0 cm) and were placed on Murashige and Skoog MS medium that contained 3% sucrose, 0.27% Gelrite, and 1.0 mg/L 2,4-D. The medium pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. The culture room was maintained at 22 ± 1°C under 24 μ mol/m² white fluorescence illumination. After 5 weeks of culture, the callus was observed and was transferred to MS liquid medium for cell suspension culture. The MS liquid medium contained 3% sucrose with different types of auxins (1 mg/L 2,4-D, 1 mg/L naphthaleneacetic acid (NAA), 1 mg/L indole-3-butyric acid (IBA), and 1 mg/L indole-3-acetic acid (IAA))

in a 250-ml Erlenmeyer flask. After 3 weeks, cells were harvested for analysis of PPT contents.

2.5. Liquid chromatography mass spectrometry ion trap/time of flight analysis of transgenic tobacco

Milled powder (200 mg) from freeze-dried leaf samples of transgenic sources was soaked in 100% methanol and was sonicated at a constant frequency of 20 kHz for a range of processing temperatures (10–30°C). The supernatant obtained by centrifugation was collected. After evaporation, the residue was dissolved in 100% methanol and then filtered with a Sep-Pak C-18 Cartridge (Waters, Milford, MA, USA).

Liquid chromatography (LC) analyses were conducted on a HPLC system (Shimadzu, Kyoto, Japan) consisting of an LC-20AD binary pump, DGU-20A degasser, SIL-20A autosampler, CTO-20AC column oven, and SPD-M20A photodiode array (PDA) detector. The mobile phase (delivered at 0.5 mL/min) consisted of solvent A (H₂O) and solvent B (CH₃CN). A binary gradient elution was performed: 25–35% B for 5 min, 35–55% B for 10 min, 55–70% B for 15 min, 70–90% B for 20 min, 90–100% B for 30 min, 100–75% B for 35 min, 75–25% B for 40 min, and 25% B for 50 min. Chromatographic separation was achieved on a YMC-Pack Pro c-18 RS column (150 × 2.0 mm, D, S-5 μ m, 8 nm; YMC Co., Ltd, Japan) at 40°C.

A liquid chromatography mass spectrometry ion trap/time of flight (LCMS-IT-TOF) mass spectrometer (Shimadzu) was equipped with an Atmospheric pressure chemical ionization (APCI) source in positive and negative ion mode. The optimized analytical methods were as follows: detector voltage, 1.60 kV; nebulizing gas (N₂) flow, 1.5 L/min; dry gas (N₂) flow, 50 kPa; pressure of TOF

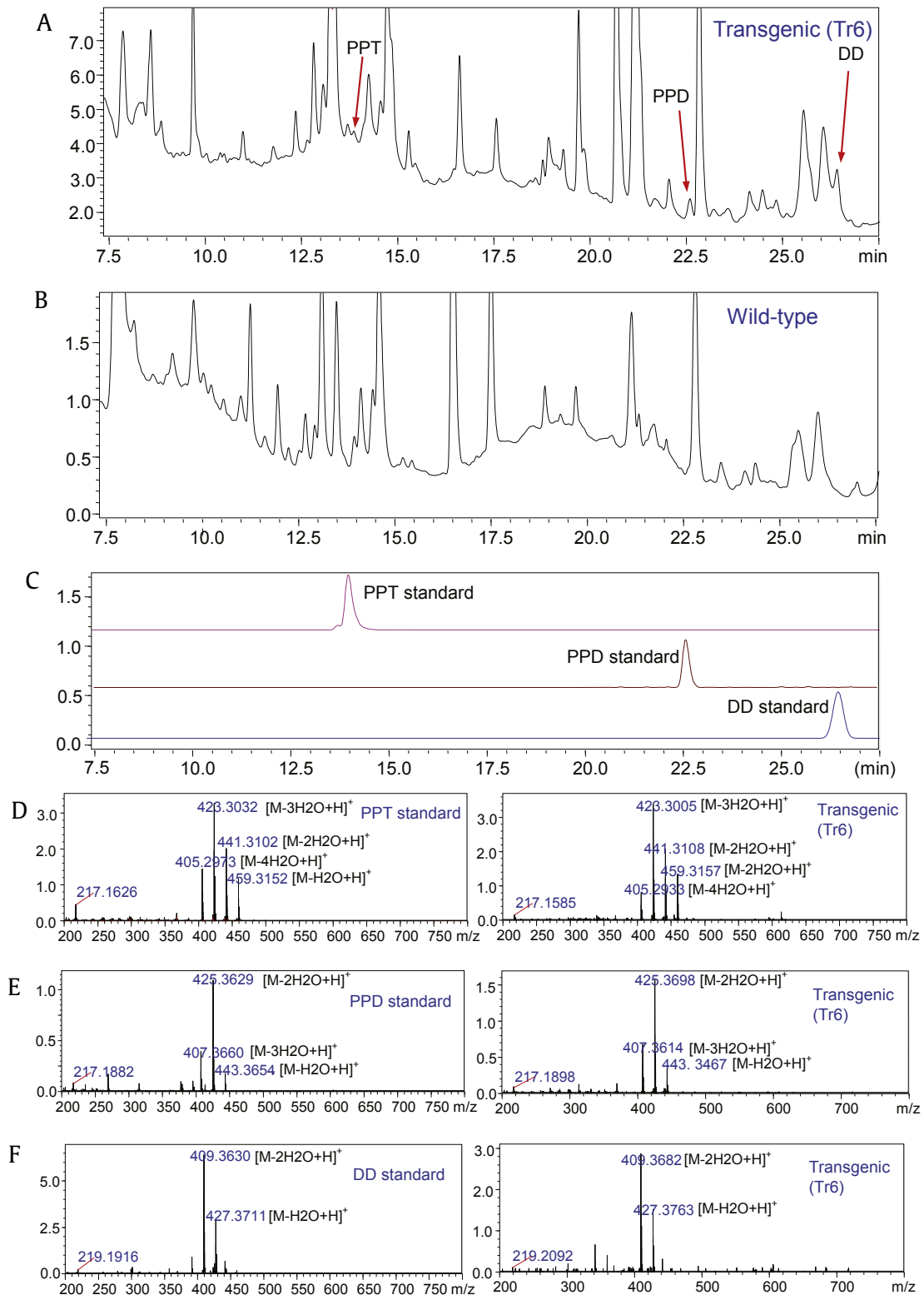


Fig. 3. LC-MS/MS analyses of leaf extracts from transgenic tobacco (line Tr6) co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2*. (A) TIC chromatogram of DD, PPD, and PPT in leaves of transgenic tobacco. (B) TIC chromatogram of WT tobacco leaf extracts. (C) Chromatogram of authentic standards DD, PPD, and PPT. (D–F) MS spectrum of DD, PPD, and PPT peaks from transformed tobacco leaves and those of authentic standards. DD, dammarenediol-II; LC, liquid chromatography; MS, mass spectrometry; PPD, protopanaxadiol; PPT, protopanaxatriol; TIC, total ion chromatogram; WT, wild-type.

region, 1.5×10^{-4} Pa; ion trap pressure, 1.7×10^{-2} Pa; ion accumulation time, 30 ms; precursor ion selected width, 3.0 amu. For qualitative analysis, scan ranges were set at m/z 100–1000 for MS1 and 100–500 for MS2; ultra-high-purity Ar was used as the cooling gas and the collision gas for Collision-induced dissociation (CID) experiments, and the collision energy was set at 50% for MS2. Authentic DD, PPD, and PPT were handled under the same conditions.

2.6. Transmission electron microscopy analysis of anther

Different stages of anthers were fixed with 3% glutaraldehyde immediately after sampling. Samples were dehydrated in an ethanol series and then treated with propylene oxide before embedding in Spurr's epoxy resin. Semithin sections were mounted on glass slides and stained with toluidine blue O. Ultrathin sections (approx. 40 nm) were placed on carbon-coated 200-mesh copper grids and stained with 1% aqueous uranyl acetate for 30 min. Sectioned samples were analyzed with an electron microscope LEO 912AB Omega (Leo Ltd., Germany). Images were captured by a ProScan slow-scan charge-coupled-device camera controlled by electron spectroscopic imaging (ESI) Vision 3.2 Software (SIS, Soft Imaging System, Münster, Germany).

3. Results

3.1. Heterologous expression of *PgDDS*, *CYP716A47*, and *CYP716A53v2* in transgenic tobacco

Tobacco plants do not synthesize any dammarane-type triterpene compounds. Three ginseng genes (*PgDDS*, *CYP716A47*, and *CYP716A53v2*) are essentially required for PPT aglycone formation from 2,3-oxidosqualene. Transgenic tobacco plants co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2* driven by CaMV35 promoters were constructed by *Agrobacterium*-mediated transformation (Fig. 2A). Seven independent transgenic lines were selected for analysis. Insertion of *PgDDS*, *CYP716A47*, *CYP716A53v2*, and *BAR* genes in transgenic tobacco plants was confirmed by performing PCR of genomic DNA (Fig. 2B).

Leaves of seven different lines of transgenic and WT tobacco plants were used in RT-PCR analysis to confirm the expression of introduced *PgDDS*, *CYP716A47*, and *CYP716A53v2* genes. All transgenic lines successfully transcribed these three genes and showed various patterns of expression among transgenic lines (Fig. 2C). No PCR amplification of *PgDDS*, *CYP716A47*, and *CYP716A53v2* mRNA was detected in WT tobacco (Fig. 2C). There is no clear phenotypical difference between WT and transgenic tobacco (Fig. 2D).

3.2. LCMS–IT–TOF analysis of transgenic tobacco

To analyze PPT in transgenic tobacco plants, leaf extracts of soil-transferred tobacco were analyzed by LCMS–IT–TOF. LC chromatogram revealed that transgenic tobacco clearly showed the production of three new compounds (PPT, PPD, and DD). These new peaks in leaf extracts of all transgenic lines were detected at retention times of 13.8 min, 22.9 min, and 27.1 min, which corresponded to authentic PPT, PPD, and DD, respectively (Fig. 3A). The MS fragmentation patterns of those three peaks were equal to those of standards (Fig. 3C). The control (WT) tobacco did not show any signal for PPT, PPD, or DD, even by single ion mode of analysis (Fig. 3B).

The MS fragmentation pattern with peak retention times of 13.8 min in transgenic tobacco included fragments with m/z ratios of 405 [M-4H₂O + H]⁺, 423 [M-3H₂O + H]⁺, 441 [M-2H₂O + H]⁺, and 460 [M-H₂O + H]⁺, which were the same as those for the pure

PPT standard (Fig. 3D). The MS fragmentation pattern with peak retention times of 22.9 min included fragments with m/z ratios of 407 [M-3H₂O + H]⁺, 425 [M-2H₂O + H]⁺, and 443 [M-H₂O + H]⁺, which were the same as those for the PPD standard (Fig. 3E). The MS fragmentation pattern with peak retention times of 27.1 min included fragments with m/z ratios of 409 [M-2H₂O + H]⁺ and 427 [M-H₂O + H]⁺, which were the same as those for the DD standard (Fig. 3F).

The content of PPT in leaves of transgenic tobacco was different among the transgenic lines (Fig. 4). Transgenic line 6 showed the highest PPT content [2.8 µg/g dry weight (DW)] in leaves. However, the contents of PPD and DD, which are the precursors of PPT, were also high in all transgenic lines (Fig. 4).

3.3. Production of PPT by cell suspension culture of transgenic line

After induction of callus of transgenic (Tr1, Tr6, and Tr7) and WT tobacco on solid medium with 1.0 mg/L 2,4-D, the calluses were cultured in liquid medium with different auxins (1.0 mg/L 2,4-D, IBA, IAA, and NAA). After 3 weeks of culture, the production of PPT, PPD, and DD in cell suspension was measured by LCMS–IT–TOF. An obvious new peak with a 13.8-min retention time, representing PPT, was detected in the callus (grown on 1.0 mg/L 2,4-D) of transgenic lines (Fig. 5A). The retention time of the new peak corresponded to that of authentic PPT (Fig. 5C). However, this peak was not detected in WT callus (Fig. 5B). The MS fragmentation patterns (405 [M-4H₂O + H]⁺, 423 [M-3H₂O + H]⁺, 441 [M-2H₂O + H]⁺, and 460 [M-H₂O + H]⁺) of the PPT peak (Fig. 5D) in cells of transgenic lines were the same as those of standards (Fig. 5E). However, the production of PPD and DD was not detectable, probably due to the full conversion of the PPD and DD precursors into PPT, as demonstrated by single ion mode analysis (Fig. 5F–H).

The effect of plant growth regulator (2,4-D, IBA, NAA, and IAA) treatment on the growth of cells and production of PPT was monitored. Cell growth was optimal in medium with NAA, and the quantitative order of cell growth was NAA > IBA > 2,4-D > IAA (Fig. 6A). However, the production of PPT was highly activated in the medium with 2,4-D, and the production rate of PPT in cells by hormonal treatments was ranked 2,4-D > IBA > NAA > IAA (Fig. 6B). The content of PPT in Tr6 cells grown in medium with 1.0 mg/L 2,4-D reached 104.3 µg/g DW, which was a 37.25-fold increase compared with the content of PPT in leaves of pot-grown transgenic tobacco. The PPT production order among different lines was Tr6 > Tr7 > Tr1, which showed the same pattern of PPT concentration in leaves among transgenic tobacco lines.

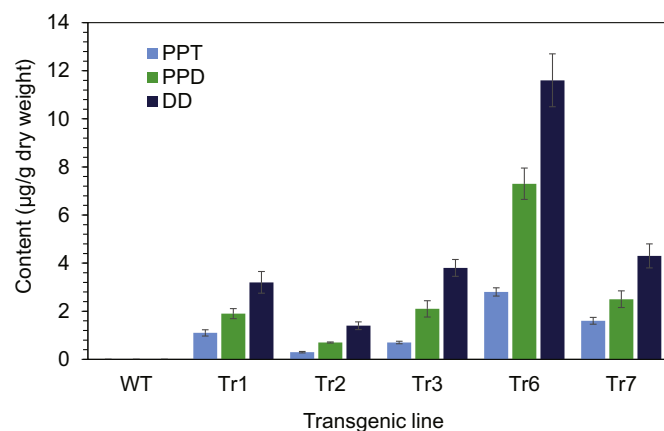


Fig. 4. DD, PPD, and PPT content in leaves among the lines of transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2*. CK, compound K; DD, dammareniol-II; PPD, protopanaxadiol; PPT, protopanaxatriol.

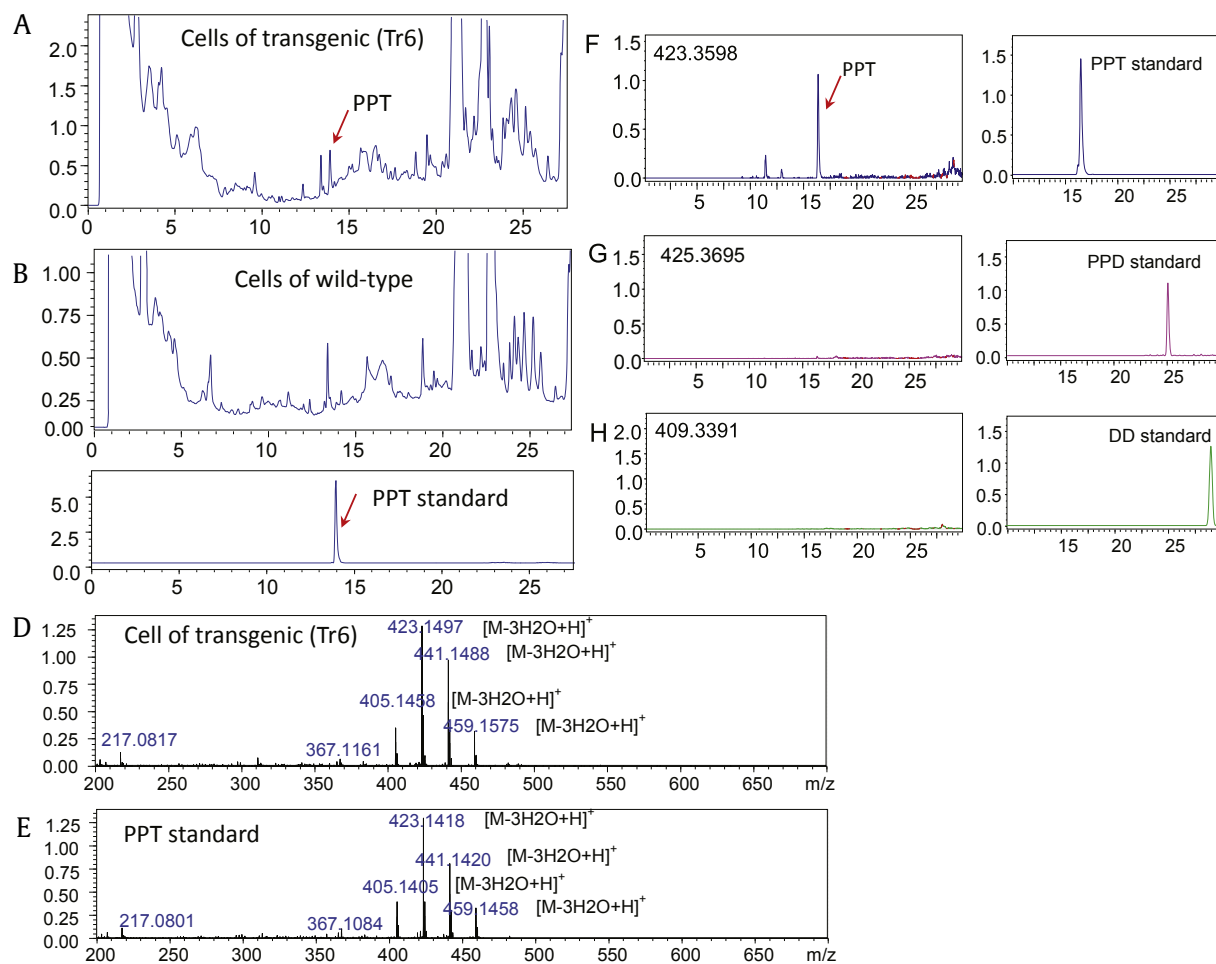


Fig. 5. LC-MS/MS analyses of extracts of transgenic tobacco cells co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2*. (A) TIC chromatogram of transgenic tobacco cells extracts, indicating obvious PPT peak (arrow). (B) TIC chromatogram of cell extracts of WT tobacco. (C) Chromatogram of authentic standard PPT. (D) MS spectrum of PPT peak from transgenic cell extracts co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2* (left panels) and PPT, PPD, and DD authentic standards (right panels). (E) MS spectrum of authentic PPT standard. (F–H) Detection of PPT (F), PPD (G), and DD (H) at retention times using single ion chromatography of the transgenic cell extracts co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2* (left panels) and PPT, PPD, and DD authentic standards (right panels). DD, dammarenediol-II; LC, liquid chromatography; MS, mass spectrometry; PPD, protopanaxadiol; PPT, protopanaxatriol; WT, wild-type.

3.4. Seed set failure in transgenic tobacco

All the lines of transgenic tobacco showed no obvious morphological differences (plant height and leaf shape) compared with the WT counterparts (data not shown). However, there were clear morphological differences in the size of the stamens and pollen production in flowers between transgenic tobacco and WT (Fig. 7). Transgenic tobacco plants were found to produce heterostylous flowers with long style and short stamens (Fig. 7F–H), whereas WT tobacco produced flowers that either were homostylous or had anther on the top of filament that was positioned slightly higher than the stigma of style (Fig. 7A–C). WT tobacco had numerous pollinated pollens on the stigma after blooming (Fig. 7D), but there was no pollen on the stigma of transgenic tobacco (Fig. 7I). The dehiscent anthers in flowers of transgenic tobacco did not contain any discernible pollen grains (Fig. 7I), but those of WT tobacco had numerous pollen grains in that position (Fig. 7D). Eventually all transgenic tobacco lines failed to set seeds under natural conditions (Fig. 7J), but WT tobacco had normally grown seed capsules (Fig. 7E).

Because transgenic tobacco did not produce fertile pollen grains, seeds were not produced by both self-pollination and artificial pollination. Although normally matured pollens of WT tobacco were manually pollinated on the stigma in transgenic tobacco, there were no fully developed normal seeds in the ovary of

transgenic tobacco because of empty embryos in ovules (Fig. S1A, SB). In contrast, self-pollinated WT tobacco set normal seeds in all ovules (Fig. S1C, SD).

3.5. Microspore degeneration in anthers of transgenic tobacco

Because dehiscent anthers in flowers of transgenic tobacco did not contain any mature pollen grains, microsporogenesis was observed in the anthers of both transgenic and WT tobacco. In WT tobacco, microspore tetrads in immature flower buds (at stage 1 in Fig. 8A) of WT tobacco were clearly observed (Fig. 8B) and developed into normal pollen grains in the anthers of mature flowers (at stage 5) (Fig. 8C). There were no obvious tetrad clusters of microspores in the immature anthers of transgenic tobacco, probably caused from impaired meiosis of microspore mother cells (Fig. 8D). These abnormally developed cells were rapidly degenerated during maturation of flowers, and no normal pollen grains were observed at stage 5 (just before flower opening) (Fig. 8E).

3.6. Premature necrosis in cells of vascular bundles of anthers in transgenic tobacco

Anthers of WT and transgenic tobacco were observed after semithin and ultrathin sections under light microscope and

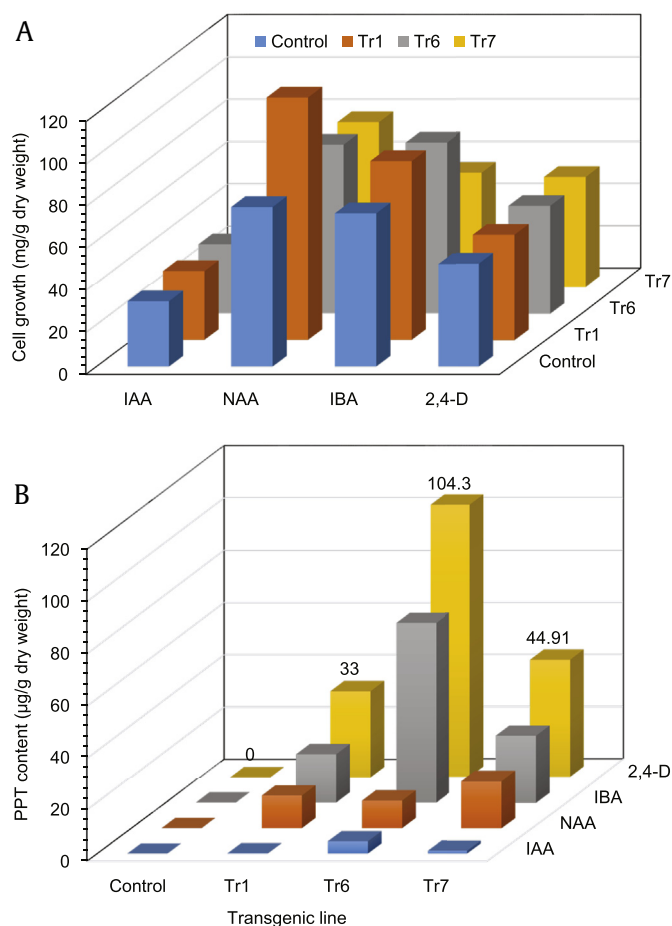


Fig. 6. Cell growth and contents of DD, PPD, and PPT in transgenic tobacco cells produced on media supplemented with different types of auxins (IAA, IBA, NAA, and 2,4-D). (A) Dry weight of cells of transgenic tobacco lines grown on media with different types of auxins. (B) Concentration of PPT, PPD, and DD in transgenic tobacco cells on media with various types of auxins. Values represent mean \pm SE, $n = 3$. DD, dammarenediol-II; PPD, protopanaxadiol; PPT, protopanaxatriol.

transmission electron microscope. Anther sacs of transgenic tobacco showed empty pollen grains (Fig. 9A), but those of WT contained well-developed pollen grains (Fig. 9B). There were striking differences between WT and transgenic tobacco in the cellular morphology in vascular bundles of connective tissues situated in the center of four-lobed anther sacs. In transgenic tobacco, densely stained nuclei in vascular bundles were detected by toluidine blue O (Fig. 9C), which were not detected in those of WT (Fig. 9D). Ultrathin sections of this portion revealed that the phloem parenchymatous cells of surrounding xylem tissues showed irregularly condensed chromatin in nuclei in transgenic tobacco (Figs. 10D, 10E) but evenly distributed chromatin in nuclei of WT (Fig. 10A, 10B). Mitochondria of WT tobacco cells showed inner-membrane folded structures (cristae) (Fig. 10C), but there were no conspicuous cristae in mitochondria of transgenic tobacco (Fig. 10F). Many mitochondria lost their internal constituents and showed damaged and/or broken cell membranes (Fig. 10F).

4. Discussion

4.1. PPT production in transgenic tobacco

Transgenic lines of tobacco co-expressing *PgDDS*, *CYP716A47*, and *CYP716A53v2* were constructed. The content of PPT in leaves

of tobacco transgenic lines ranged from 0.1 to 2.8 $\mu\text{g/g}$ DW. Two other compounds (PPD and DD) were also produced, which are the direct precursors in PPT biosynthesis. In naturally grown ginseng plants, all of these three triterpenes, intermediates of ginsenoside, are hardly detectable in roots, probably because of rapid conversion of these triterpenes into glycosylated ginsenosides. Interestingly, the two PPT precursors (PPD and DD) accumulated high amounts in leaves of all transgenic lines compared with those of WT. This result indicated that the conversion from triterpene molecules into PPT was not efficiently achieved in tobacco plants. For conversion from DD to PPT, two CYP enzymes (*CYP716A47* and *CYP716A53v2*) are necessary. For the catalytic activities of all CYP enzymes, NADPH:cytochrome P450 reductase (CPR) as the electron donor counterpart is required [34]. The incomplete conversion from DD to PPT in tobacco may be related to the compatibility between the tobacco CPR enzyme and heterologously expressing two ginseng cytochrome P450 enzymes (*CYP716A47* and *CYP716A53v2*).

4.2. PPT production in cell suspension culture

The heterologous yeast expression system has been used not only for functional characterization of triterpene synthase genes but also for large-scale production of triterpenes. Metabolic engineering technology has been extensively achieved via yeast microorganisms [35]. The production of DD has been achieved in engineered yeast (*Pichia pastoris*) [36]. PPD production was achieved through introducing *PgDDS*, *CYP716A47*, and *A. thaliana* CPR genes into yeast [37]. It is known that the role of triterpenes and glycosylated triterpenes (saponins) in plants provide protection against pathogens and pests [38]. DD production in transgenic tobacco overexpressing the *PgDDS* gene confers tobacco mosaic virus tolerance [39]. The plant genetic engineering system for production of triterpene has additional advantages because transgenic plants have new biological characteristics, such as disease and pest resistance.

Cell suspension cultures of transgenic plants have some advantages over field-grown transgenic plants because the production of triterpene in an engineered cell culture is free from climate, seasonal, and environmental variations and requires short culture time (2–4 weeks) for cell harvest. Moreover, in both microorganisms and field-cultivated transgenic plants, unintentional release of microorganisms and plant seeds and pollens in the wild could occur; however, it is not problematic in cultured cells because they cannot survive in the natural environment.

We attempted to produce PPT via cell suspension cultures of transgenic tobacco. After induction of callus from leaf segments of transgenic tobacco on medium containing 2,4-D, these calluses were transferred to liquid media containing different auxins (1.0 mg/l 2,4-D, IBA, IAA, and NAA). Interestingly, DD, PPD, and PPT production was clearly elevated in cell suspension cultures compared with that in leaves of transgenic tobacco. The same results were gained in transgenic tobacco producing DD [9] and compound K (CK) [40]. Auxin treatment clearly increased the production of PPT compared with the auxin-free condition, and the treatment of 2,4-D showed the strongest effect for the production of PPT in cells than other auxin types. Interestingly, the production of PPT precursors such as DD and PPD was highly reduced in cell culture because these two compounds were detected only at trace amounts. Generally, *in vitro*-cultured cells and organs produce lower quantities of secondary compounds than naturally grown plants [41]. It is still unclear why the content of PPT in cultured cells was higher than that in leaves of transgenic tobacco plants. It is known that 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and squalene epoxidase are rate-limiting enzymes of saponin

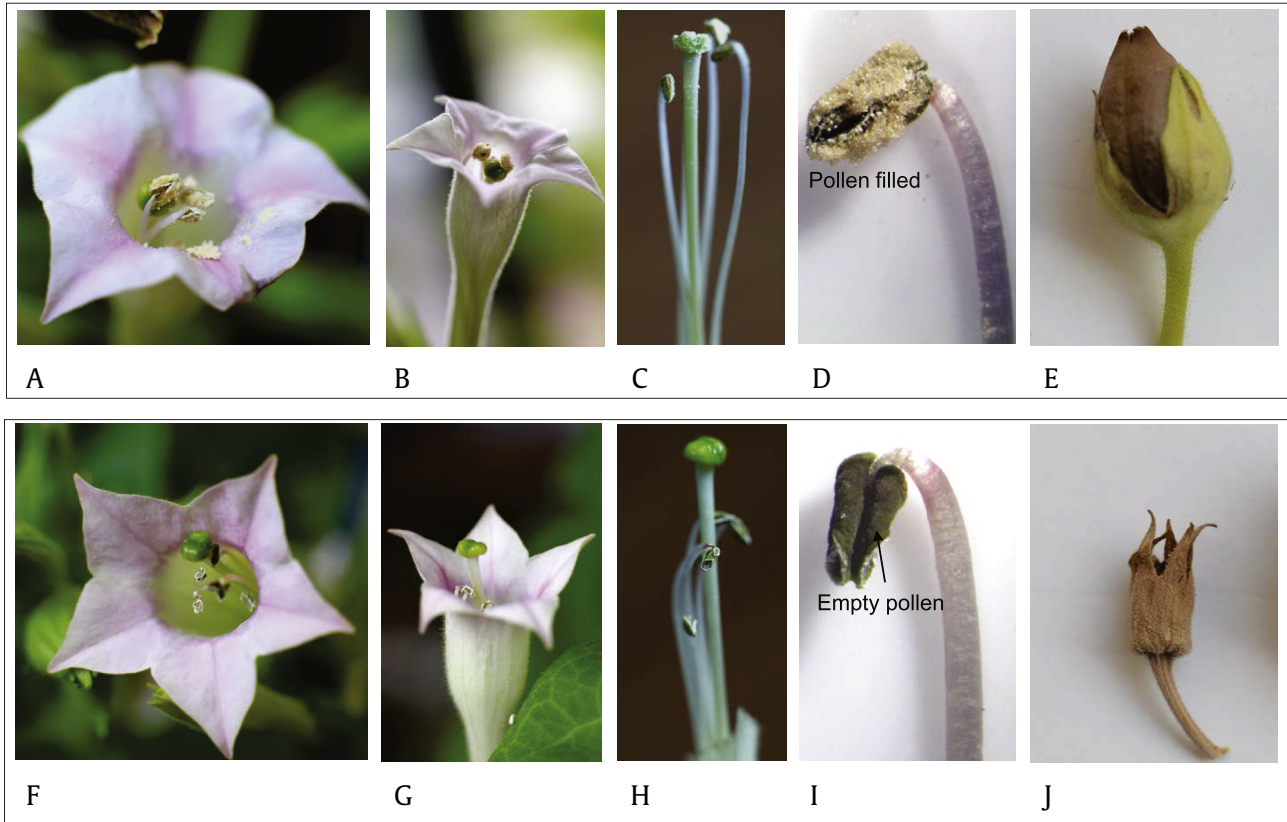


Fig. 7. Floral morphology and seed production in transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2*. (A–E) Flower structure and seed set in WT tobacco. (A, B) Homostylous morphology in WT tobacco flower. (C) Enlarged view of stamen and pistil of WT tobacco flower. (D) Dehiscent anthers with profuse pollens. (E) Normal seed set in WT flower. (F–J) Flower structure and seed set in transgenic tobacco. (F, G) Heterostylous flowers with elongated pistil in transgenic tobacco. (H) Enlarged closed view of short stamen and long pistil of transgenic tobacco. (I) No pollen grains on dehiscent anthers in transgenic tobacco. (J) Seed set failure in transgenic tobacco. Arrow in **i** points to empty pollen grain.

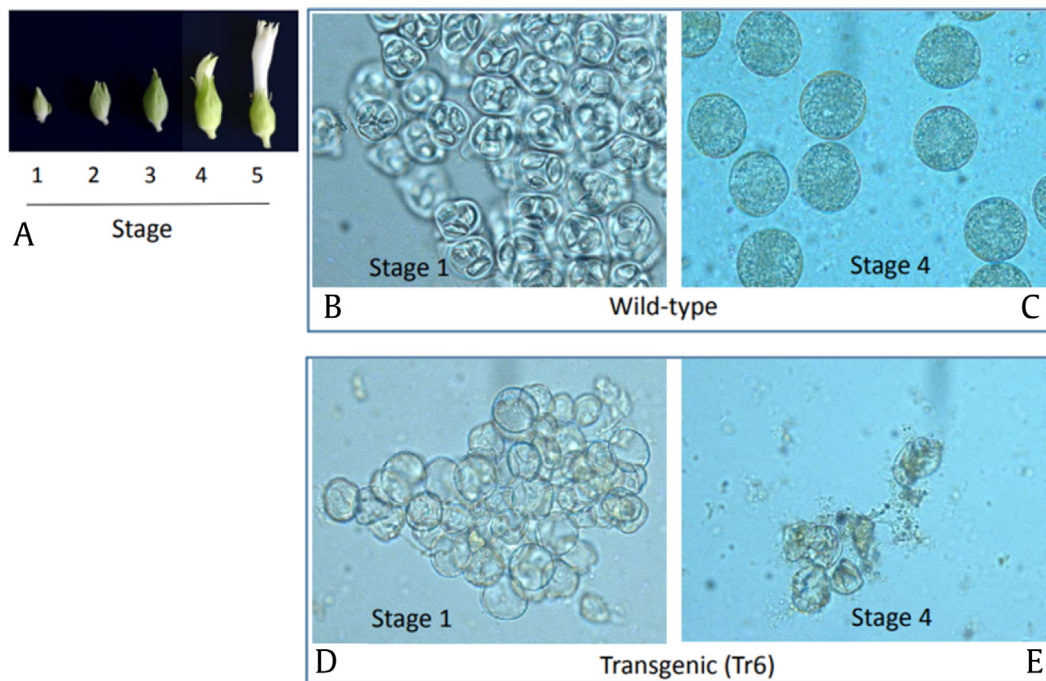


Fig. 8. Observation of microspores in anthers of WT and transgenic tobacco (Tr6). (A) Samples of immature flower buds at five different stages in WT tobacco for investigation of microspore in anthers. (B) Microspore tetrads in immature flower buds (stage 1) of WT tobacco. (C) Mature microspores in anthers before blooming of flowers (stage 5) in WT tobacco. (D) Abnormal microspore showing no obvious tetrads in immature flower buds (stage 1) of transgenic tobacco. (E) Degenerated microspores in anthers before blooming of flowers (stage 5) in transgenic tobacco.

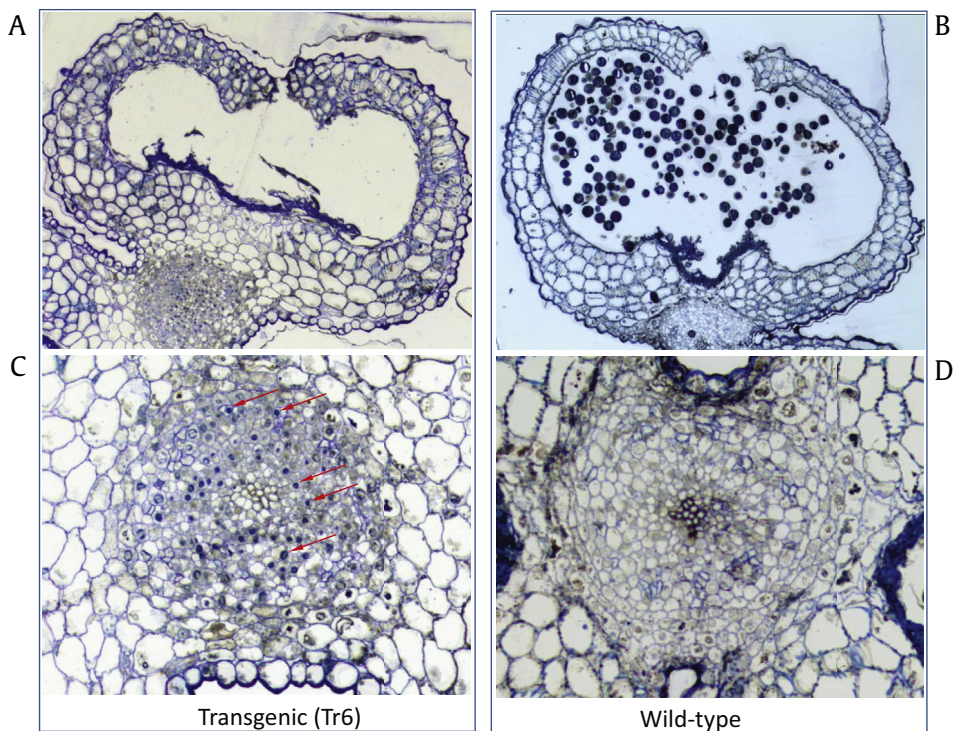


Fig. 9. Semithin sections of anthers in transgenic (Tr6) and WT tobacco. (A) Empty microspore in the anther sac of transgenic tobacco. (B) Semithin section of WT tobacco anthers revealed well-developed microspores within the anther sac. (C) Darkly stained nuclei (arrows) of phloem cells in connective tissue of anthers of transgenic tobacco. (D) Darkly stained nuclei of phloem cells in connective tissue not in anthers of WT tobacco.

and sterol biosynthesis [42–44]. Auxin treatment could enhance the expression of genes encoding HMG-COA reductase and squalene epoxidase in cell suspension cultures of tobacco [45]. Although 2,4-D is classified as an auxin, this chemical is usually used as a

pesticide because this auxin induces uncontrolled cell division. Most likely, metabolic flow for triterpene (PPT) biosynthesis in cells of transgenic tobacco might be strongly accelerated by 2,4-D treatment.

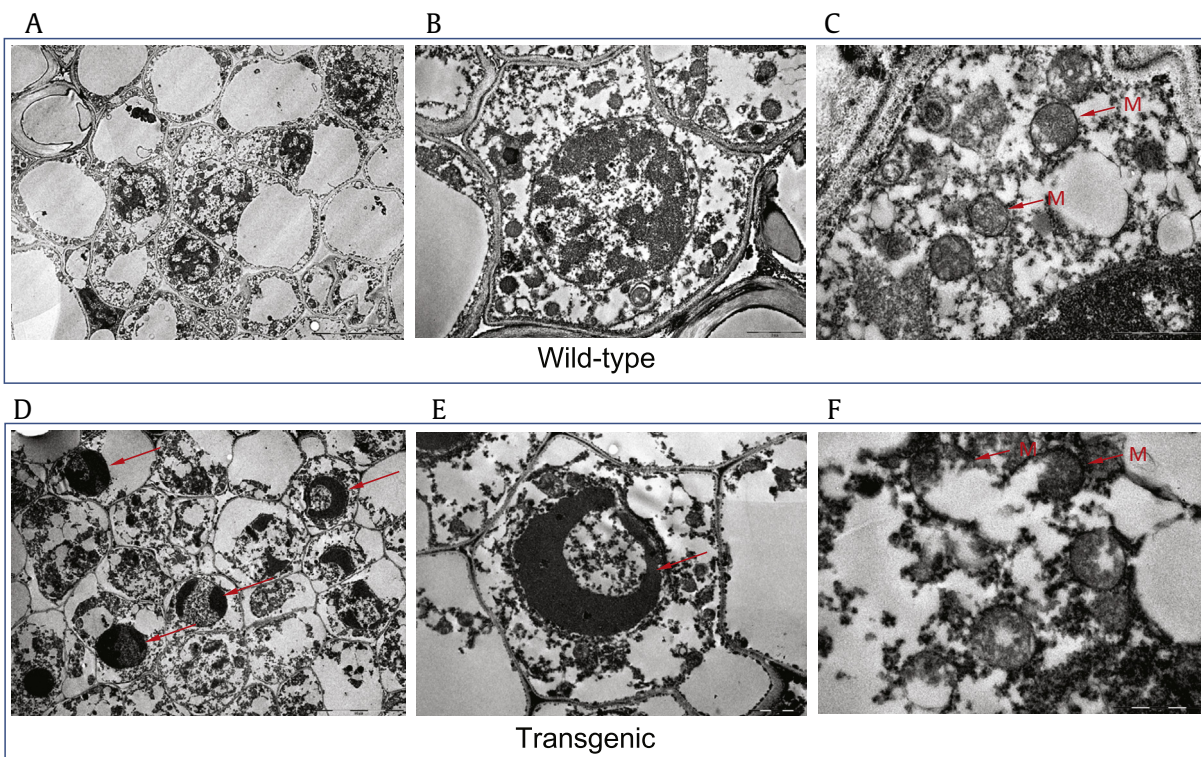


Fig. 10. Ultrathin section of connective tissue of anthers in transgenic and WT tobacco. (A, B) The phloem cells in connective tissue of anthers in WT tobacco revealed that chromatin in nuclei was evenly distributed. (C) Mitochondria had well-developed membranes and internal structures (cristae). (D, E) The phloem cells in connective tissue of anthers in transgenic tobacco contained compacted chromatin (arrow in D, E). (F) Mitochondria had no conspicuous internal structures (cristae) and membranes (arrows in F).

4.3. Male sterility in transgenic tobacco

Surprisingly, transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47*, and *CYP716A53v2* did not contain any mature pollen grains in anthers and eventually failed to set seeds. Cytological observation revealed that no obvious microspore tetrad cells were observed in young anthers of transgenic tobacco and that these abnormal microspores rapidly degenerated during the development of flowers. The occurrence of male sterility was also reported in transgenic tobacco producing CK (protopanaxadiol 20-O-glucoside) by co-overexpression of *PgDDS*, *CYP716A47*, and *UGT71A28* [40]. In an *in vitro* pollen germination test, CK, PPD, and DD treatments directly suppressed the germination and tube growth of tobacco pollen, indicating strong phytotoxic properties of CK, PPD, and DD [40]. However, this transgenic tobacco producing CK contained normally matured pollens at some proportions and was able to set seeds [40]. In contrast, transgenic tobacco producing PPT did not set seeds in any lines, indicating more severe damage of microsporogenesis.

Because microsporogenesis in male organs was damaged in transgenic tobacco, it is still questionable whether megasporogenesis in female organs is also affected in transgenic tobacco. To address this question, normal pollens from WT tobacco were pollinated onto the stigma of transgenic tobacco. This cross hybridization also resulted in failure of seed set because of empty embryos in ovules in transgenic tobacco. The seed set failure can be caused from abortion of megasporogenesis in ovules, as seen in microsporogenesis in transgenic tobacco. In our previous report, DD, PPD, and CK in transgenic tobacco can directly affect the germination of pollen [40]. The seed set failure can also be caused from the suppression of pollen tube growth along the downward of style of transgenic tobacco affected by the heterologously produced toxic DD, PPD, and PPT in transgenic tobacco. Further physiological and molecular studies are necessary to verify the mechanism of female sterility in transgenic tobacco.

The factors inducing microsporogenesis (not clear in megasporogenesis) failure in transgenic tobacco producing ginsenoside aglycone (PPT) are still largely unknown. In nature, saponins are described as toxic defensive chemicals (allelopathic agents) although their detailed mechanism is poorly understood. The toxic effect of ginseng aglycones in tobacco can be partly attributed to the auto-toxic phenomenon of heterologously produced allelochemicals. However, it is interesting why the toxic effect of ginsenoside aglycones is particularly severe in microsporogenesis.

Semithin and ultrathin sections of tobacco anthers revealed that the condensed chromatin in nuclei and degenerated mitochondria were conspicuous in phloem cells of connective tissue of anthers in transgenic tobacco but not in the cells of WT tobacco. These ultrastructural features of nuclei and mitochondria are common in programmed cell death due to the senescence of leaves, pathogen attack, wounding, and exposure to toxins [46,47]. In our previous reports, the two key enzymes (squalene synthase and squalene epoxidase) in triterpene biosynthesis were preferentially expressed in phloem cells in vascular bundles [48,49]. We interpreted that phloem cells are metabolically active sites for sterol and triterpene biosynthesis [49].

CYP enzymes are involved in the oxidative metabolism of a variety of lipophilic compounds. CPR transfers electrons from Nicotinamide adenine dinucleotide phosphate (NADPH) to CYPs. A tight coupling between electron transfer of CPR and CYP substrate oxidation is required for efficient catalysis. Poor coupling of the CYP catalytic cycle results in continuous production of reactive oxygen species, which can induce oxidative stress [50,51]. For production of PPT in transgenic tobacco, two *P. ginseng* CYP enzymes were introduced into tobacco. In general, tobacco plants are not saponin-

rich plants. Therefore, tobacco plants evolutionally may not be a good system for saponin aglycon production, accumulation, and transportation. Most likely, the overexpression of two ginseng CYP enzymes in tobacco might have resulted in incomplete coupling between *P. ginseng* CYPs and tobacco CPR and resulted in oxidative stress. Further physiological and molecular studies are necessary to verify the mechanism of male sterility in transgenic tobacco.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the Rural Development Administration, Republic of Korea [Next-Generation Bio-Green 21 Program (PJ01344401)].

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jgr.2018.02.005>.

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