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ORIGINAL ARTICLE

Heterologous production of a ginsenoside saponin (compound K) and its precursors in transgenic tobacco impairs the vegetative and reproductive growth

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Abstract

Main conclusion **Production of compound K (a ginsenoside saponin) and its precursors in transgenic tobacco resulted in stunted growth and seed set failure, which may be caused by strong autotoxicity of heterologously produced phytochemicals against the tobacco itself.**

Panax ginseng roots contain various saponins (ginsenosides), which are major bioactive compounds. A monoglucosylated saponin, compound K (20-*O*-(*β*-D-glucopyranosyl)-20(*S*)-protopanaxadiol), has high medicinal and cosmetic values but is present in undetectable amounts in naturally grown ginseng roots. The production of compound K (CK) requires complicated deglycosylation of ginsenosides using physicochemical and/or enzymatic degradation. In this work, we report the production of CK in transgenic tobacco by co-overexpressing three genes (*PgDDS*, *CYP716A47* and *UGT71A28*) isolated from *P. ginseng*. Introduction and expression of the transgenes in tobacco lines were confirmed by genomic PCR and RT-PCR. All the lines of transgenic tobacco produced CK including its precursors, protopanaxadiol and dammarenediol-II (DD). The concentrations of CK in the leaves ranged from 1.55 to 2.64 μg/g dry weight, depending on the transgenic line. Interestingly, production of CK in tobacco brought stunted plant growth and gave rise to seed set failure. This seed set failure was caused by both long-styled flowers and abnormal pollen development in transgenic tobacco. Both CK and DD treatments highly suppressed in vitro germination and tube growth in wild-type pollens. Based on these results, metabolic engineering for CK production in transgenic tobacco was successfully achieved, but the production of CK and its precursors in tobacco severely affects vegetative and reproductive growth due to the cytotoxicity of phytochemicals that are heterologously produced in transgenic tobacco.

Keywords Compound K · Ginsenoside · Metabolic engineering · Saponin · Transgenic tobacco

Abbreviations

PgDDS	*Panax ginseng* dammarenediol-II synthase
CYP716A47	Protopanaxadiol synthase
UGT71A28	Protopanaxadiol 20-glycosyltransferase
qPCR	Real-time polymerase chain reaction
CK	Compound K
DD	Dammarenediol-II
PPD	Protopanaxadiol

Introduction

Panax ginseng is one of the most famous medicinal plants (Shibata 2001). The major pharmacologically active components in ginseng roots are triterpenoid saponins known as ginsenosides. Ginsenosides are found exclusively in *Panax* species (Christensen 2008). The major saponin constituents in *Panax* species are dammarene-type ginsenosides, which are further divided into two groups based on their aglycone structures, protopanaxadiol (PPD) and protopanaxatriol (PPT) (Shibata 2001).

Ginseng roots and their processed products are typically administered orally. The absorption of intact ginsenosides

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by the gastrointestinal tract is extremely low because of the high molecular structure of the sugar groups attached to triterpene (Tawab et al. 2003). The deglycosylated or partially deglycosylated ginsenosides in the stomach (acid hydrolysis) and intestine (bacterial hydrolysis) are mainly absorbed into the bloodstream of the intestine (Liu et al. 2009; Karikura et al. 1991). Compound K (CK), a monoglucosylated saponin, is a major metabolite of ginsenosides detected in the urine and blood after oral administration of ginseng extract (Hasegawa et al. 1996). It is now generally accepted that monoglucosylated saponin (CK) or deglycosylated sapogenin (PPD) has greater biological effects than other natural forms of ginsenosides (Hasegawa et al. 1997; Jia et al. 2004; Popovich and Kitts 2004; Bae et al. 2004; Saklani and Kutty 2008; Du et al. 2011; Musende et al. 2012). CK exhibited multiple pharmacological properties such as anti-carcinogenic, anti-inflammation, anti-allergic, anti-diabetic, anti-angiogenic, anti-aging, neuroprotective and hepatoprotective effects (Yang et al. 2015b). CK exists in trace amounts in the naturally grown roots of *P. ginseng*. Due to its remarkable pharmaceutical properties, various methods have been devised to convert major ginsenosides into CK by heating (Kim et al. 2000), acid hydrolysis (Han et al. 1982), alkali treatment (Chen et al. 1987), microbial conversion (Tawab et al. 2003) and enzymatic conversion (Yang et al. 2015b).

Dammarenediol-II synthase (*PgDDS*) of *P. ginseng* catalyzes the production of triterpene (DD) from 2,3-oxidosqualene, as shown in Fig. 1 (Han et al. 2006). Subsequently, DD is converted to PPD sapogenin by hydroxylation of DD at C-12; the reaction is catalyzed by a cytochrome P450 (*CYP716A47*) (Han et al. 2011). CK is then produced by selective glycosylation of C-20*S*-OH of PPD by UDP-glycosyltransferase (*UGT71A27*) (Yan et al. 2014). The production of CK in plant cells or microbial hosts via genetic engineering may be a promising technology for cost-efficient production. Yan et al. (2014) attempted to produce CK in an engineered yeast expression system. Plant metabolic engineering is another useful technology for the production of secondary compounds (Verpoorte and Memelink 2002; Wu and Chappell 2008; Dudareva et al. 2013). To date, there has been no report of saponin (glucosylated triterpenes) production by heterologous expression of genes in plants.

Plants have evolved to confer an allopathic effect by releasing allelochemicals during adaptation to various environmental stresses (Rice 1995; Cheng and Cheng 2015). Ginsenosides have been accepted as active allelochemicals (Lei et al. 2010; Zhang et al. 2011). These compounds are released into the soil, thereby changing the soil microbial population structure and inhibiting the germination of seeds from the same or different species (Zhao et al. 2005; Li et al. 2008; He et al. 2009). Autotoxicity is a type of intraspecific allelopathy that occurs when plants release toxic chemicals into the environment. It directly or indirectly inhibits germination and growth in continuous cropping or short rotation (Huang et al. 2013; Singh et al. 1999). Autotoxicity is a major problem hindering successive cultivation of ginseng (Zhao et al. 2005; Li et al. 2008; He et al. 2009; Yang et al. 2015b). Production of unprecedented saponin in heterologous host plants using genetic engineering may cause autotoxicity. However, little is known about the autotoxic effects of saponin heterologously produced in transgenic plants.

In this work, we constructed transgenic tobacco co-overexpressing *P. ginseng PgDDS*, *CYP716A47* and *UGT71A27* genes for producing CK ginsenoside (Fig. 1). Enhanced CK production via callus culture of transgenic tobacco was achieved. Interestingly, transgenic tobacco showed stunted growth and male sterility. We discussed the autotoxicity of CK on the vegetative and reproductive growth of transgenic tobacco.

Materials and methods

Construction of transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47* and *UGT71A27*

The open reading frame (ORF) sequence of *PgDDS* was isolated with PCR using the primers 5′-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3′ and 5′-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3′ (Han et al. 2006). The ORF of *CYP716A47* was amplified using the PCR primers 5′-ATG GTG TTG TTT TTC TCC CTA TCT-3′ and 5′-TTA ATT GTG GGG ATG TAG ATG AAT-3′. The ORF of UGT71A28 was amplified using the PCR primers 5′-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3′ and 5′-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3′. Three genes were cloned via the GATEWAY vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA, USA) and transferred into the destination vector pZIP-Bar under the control of the double 35S CaMV promoter (Fig. 2a). Eventually, the overexpression construct harboring *PgDDS*, *CYP716A47* and *UGT71A28* was introduced into competent cells of the *Agrobacterium tumefaciens* GV3101 strain using the heat-shock method. The procedure for developing transgenic tobacco with the *PgDDS*, *CYP716A47*, and *UGT71A28* genes by *A. tumefaciens*-mediated transformation was essentially the same as described by Han et al. (2014), with the exception of the change in selection medium.

Transgenic plant screening and RT-PCR analysis

Genomic DNA (gDNA) was extracted from the leaves of putative transgenic plants regenerated in antibiotic (20 mg/

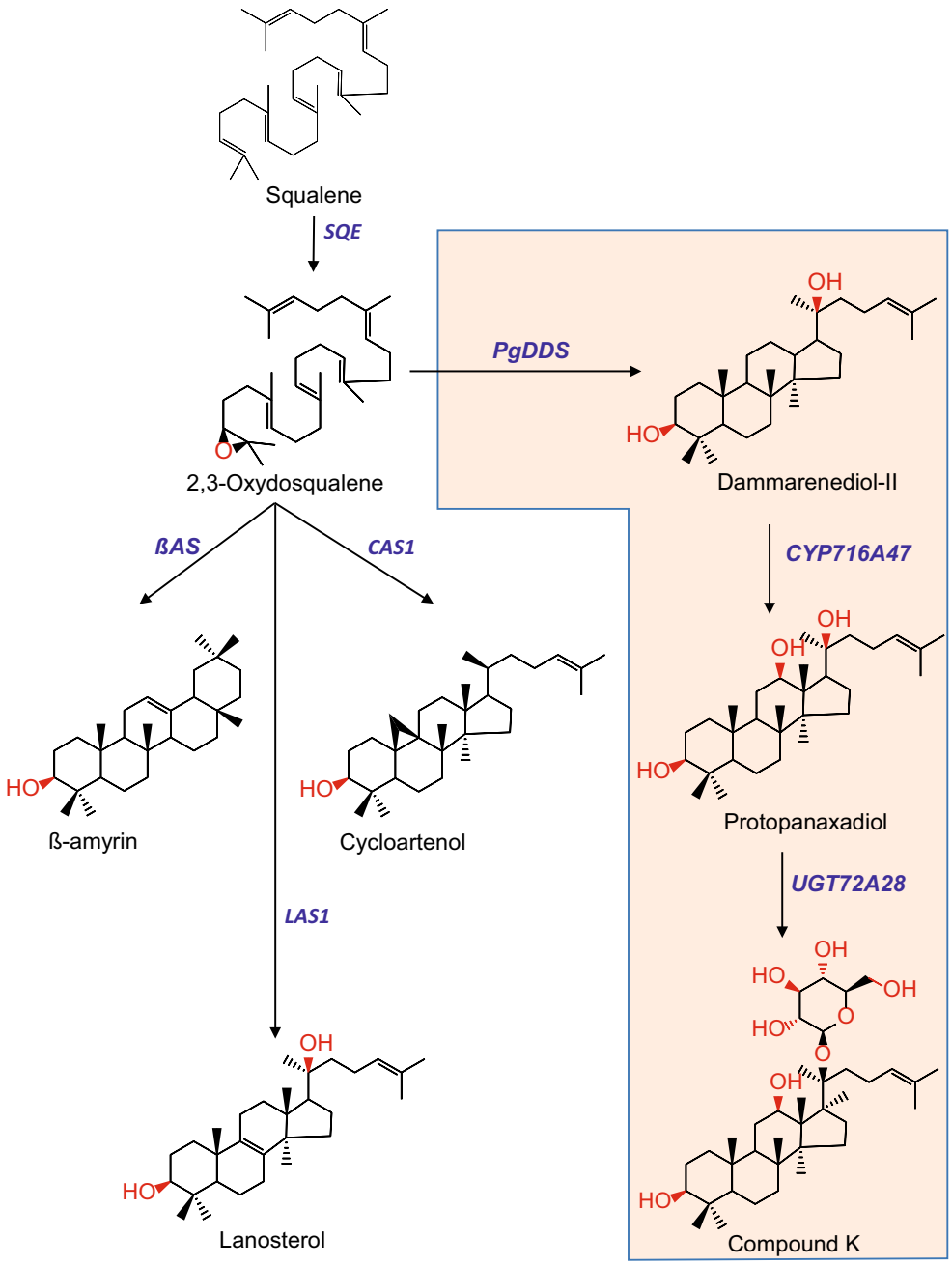

Fig. 1 General oxidosqualene metabolism in tobacco and pathway engineering for CK production. Three *P. ginseng*-derived genes (*PgDDS*, *CYP716A47*, and *UGT72A28*) were introduced in tobacco, among which *PgDDS* converts 2,3-oxidosqualene to a triterpene (dammarenediol-II). The triterpene subsequently undergoes oxidation by *CYP716A47* to produce protopanaxadiol (PPD). Glycosylation of PPD by *UGT71A28* finally produces CK. Heterologously co-expressed genes in this study, along with their products, are highlighted in the box. *SQE* squalene synthase, *PgDDS* dammarenediol synthase, *βAS* β-amyrin synthase, *CAS1* cycloartenol synthase, *LAS1* lanosterol synthase

L basta) supplemented media using RBC's genomic DNA extraction kit (RBC, YGP100) according to the manufacturer's instructions. Extracted gDNAs were used to screen the tobacco lines using primers that are bar specific (5′-AGG ACA GAG CCA CAA ACA CC-3′ and 5′-ATG CTT GTA TCC AGC TGC G-3′), *PgPPDs*-specific (5′-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3′ and 5′-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3′), *CYP716A47* specific (5′-ATG GTG TTG TTT TTC TCC CTA TCT-3′ and 5′-TTA ATT GTG GGG ATG TAG ATG AAT-3′), and *UGT71A28* specific (5′-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3′ and 5′-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3′). PCR was conducted with a DNA thermal cycler (Applied

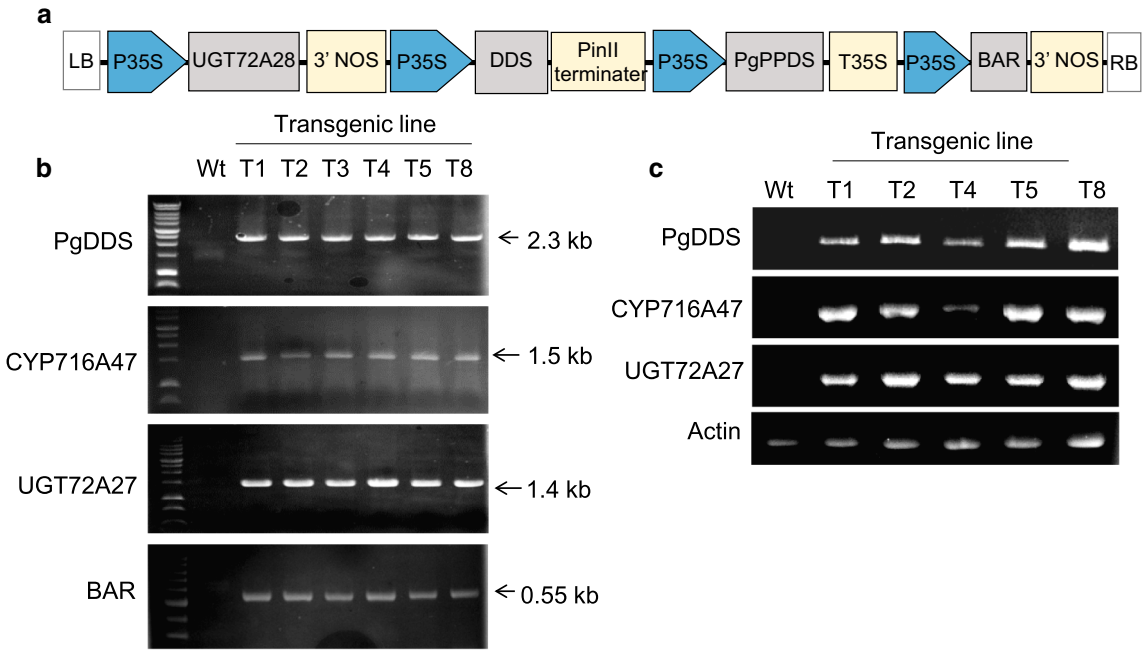

Fig. 2 Detection and expression analysis of introduced genes in transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47* and *UGT71A28*. **a** T-DNA region of plasmid for co-overexpression of the three genes. **b** Screening of transgenic tobacco lines (T1, T2, T3, T4, T5, and T8) by gDNA PCR via detection of introduced genes (*PgDDS, CYP716A47, UGT71A28*, and *BAR*) in their genomes. **c** Expression (RT-PCR) analysis of the introduced *PgDDS*, *CYP716A47* and *UGT71A28* genes in transgenic lines (T1, T2, T3, T14, T5, and T8); β-actin was used as an internal control. Wild-type (WT) was used as a negative control for the introduced genes in **b** and **c**

Biosystems, CA, USA) under the following conditions: one cycle of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; a final 10-min extension. The PCR products were loaded, and electrophoresis was carried out in 1% agarose gel (0.5% TBE). Only the positive lines showing amplicons for all genes tested were maintained and multiplied for subsequent analyses.

For RT-PCR, total RNA was isolated from WT and putative transgenic tobacco plants using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions and was reverse transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The first-strand cDNA was used as a template for RT-PCR analysis, which was performed as follows: 96 °C for 5 min; 30 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; a final 10-min extension at 72 °C. Aforementioned primers for *BAR*, *PgDDS*, *CYP716A47* and *UGT71A28* gene were used for RT-PCR analysis under the same PCR conditions mentioned above. RT-PCR analysis of *β-actin* (5′-CGT GAT CTT ACA GAT AGC TTC ATG A-3′ and 5′-AGA GAA GCT AAG ATT GAT CCT CC-3′) was used as an internal control to confirm RNA integrity and loading accuracy. Electrophoresis of the products was performed using 1% agar/0.5% TBA buffer. The RT-PCR analyses were repeated twice, and representative data are presented in Fig. 2c.

Callus induction

The leaves of transgenic tobacco plants were cut into segments and transferred onto MS (Murashige and Skoog 1962) medium containing various concentrations of 2,4-D (0. 0.1, 0.5, 1.0, and 2.0 mg/L), 3% sucrose, 20 mg/L of hygromycin and 0.27% Gelrite. All media were adjusted to pH 5.8 prior to autoclaving at 120 °C for 15 min. Approximately 15 segments were cultured on a Petri dish for 5 weeks. The culture room was maintained at 22 ± 1 °C under 24-μmol/m^2/s^2 white fluorescence illumination.

LCMS-IT-TOF analysis of transgenic tobacco

Milled powder (200 mg) from freeze-dried leaf samples of transgenic sources was soaked in 100% methanol and 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 filtered with a SepPak C-18 Cartridge (Waters, Milford, MA, USA).

LC analyses were conducted on a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC-20AD binary pump, DGU-20A degasser, SIL-20A autosampler, CTO-20AC column oven and SPD-M20A PDA detector. The mobile phase (delivered at 0.5 mL/min) consisted of solvent A (H_2O) and solvent B (CH_3CN). A binary gradient elution CK, PPD and DD was performed: initial 25% B for 5.0 min, 25–55% B from 5 to 10 min, 55–70% B from 10 to 15 min, 70–90% B from 15 to 20 min, 90–100% B from 20 to 30 min, 100–75% B from 30 to 35 min, returned to initial 25% B and maintained until 40 min for column balance. Chromatographic separation was achieved on a YMC-Pack Pro C18 RS column (150 × 2.0 mm D, S-5 μm, 8 nm, YMC Co., LTD., Japan) at 40 °C.

The mass spectrometer of LCMS–IT-TOF (Shimadzu, Kyoto, Japan) was equipped with an APCI source in the positive and negative ion modes. 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 region, 1.5×10^{-4} Pa; ion trap pressure, 1.7×10^{-2} Pa; ion accumulated 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 CID experiments, and the collision energy was set at 50% for MS2. Authentic DD, PPD and CK were directly subjected to the same conditions.

Ex vitro plant transfer for plant and floral morphology analysis

The confirmed transgenic tobacco lines were transferred (three plants per line) to soil along with three WT plants. For acclimatization, plants were covered with thin polyvinyl for 1 week and regularly inspected. Plants were grown under natural sunlight for 2 months after which their morphological differences (as compared to WT) were observed and plant heights were noted. The fresh biomass of the plants was measured after carefully removing soil around the roots and measuring the plant weight. To assess possible variation in floral morphology between transgenic and WT lines, plants were further grown until flowering.

Transgenic pollen germination test and effect of CK, PPD and DD on WT pollen

Pollen germination medium was prepared with ½ strength MS with 10% sucrose and 7% plant agar. Ten-milliliter aliquots of medium were poured on small Petri dishes (60 × 15 mm). After media solidification, pollen derived from NT was spread onto semi-solid medium, and 200 μL of liquid medium supplemented with different concentrations (0, 1, 5, 10, 20, and 50 mg/L) of CK, DD, PPD or a mixture of all three (total 24 treatments) was dropped onto the plate using a pipette. The pollen on semi-solid medium was left for 24 h in the dark at 25 °C, and the germination and tube growth of the pollen were observed using a light microscope. To study the effect of CK, PPD and DD on pollen germination and pollen tube growth, pollen from WT tobacco plants was cultured on semi-solid medium supplemented with various concentrations (0, 1, 5, 10, 20, and 50 mg/L) of CK, PPD and DD alone and in complete mixture (total 24 treatments). The frequency of the germination and growth of pollen were assessed after overnight incubation, and all treatments were carried out in triplicate.

Self- and cross-pollination between WT and transgenic tobacco plants

For self-pollination, the flowers were capped with a small paper cone to avoid possible cross-pollination. Both transgenic and WT flowers were hand-pollinated to minimize experimental error. For cross-pollination, the mature but unopened flowers were forced open with forceps, emasculated and covered with a paper cone. After 24 h, they were cross-pollinated with pollen of alternative origin (transgenic or WT) and kept covered with a cone at least until the styles degenerated. After maturity, sepals were removed from the seedpods and seed development status was observed.

Statistics

Values of all data represent the mean ± standard error (SE). The percentage data for pollen germination were arcsine transformed followed by ANOVA, while those for pollen tube length were analyzed with a nonparametric Kruskal–Wallis test using the statistical package 'R v3.3.2 in 'RStudio v1.0.1.36'. The significance level was set to $\alpha = 0.05$. Sample sizes are mentioned along with the corresponding data.

Results

Construction of transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47* and *UGT71A27*

Biosynthesis of CK from 2,3-oxidosqualene in *P. ginseng* requires a unique sequence of three steps with enzymes encoded by *PgDDS* (GenBank accession number, GU183405), *CYP716A47* (GenBank accession number,

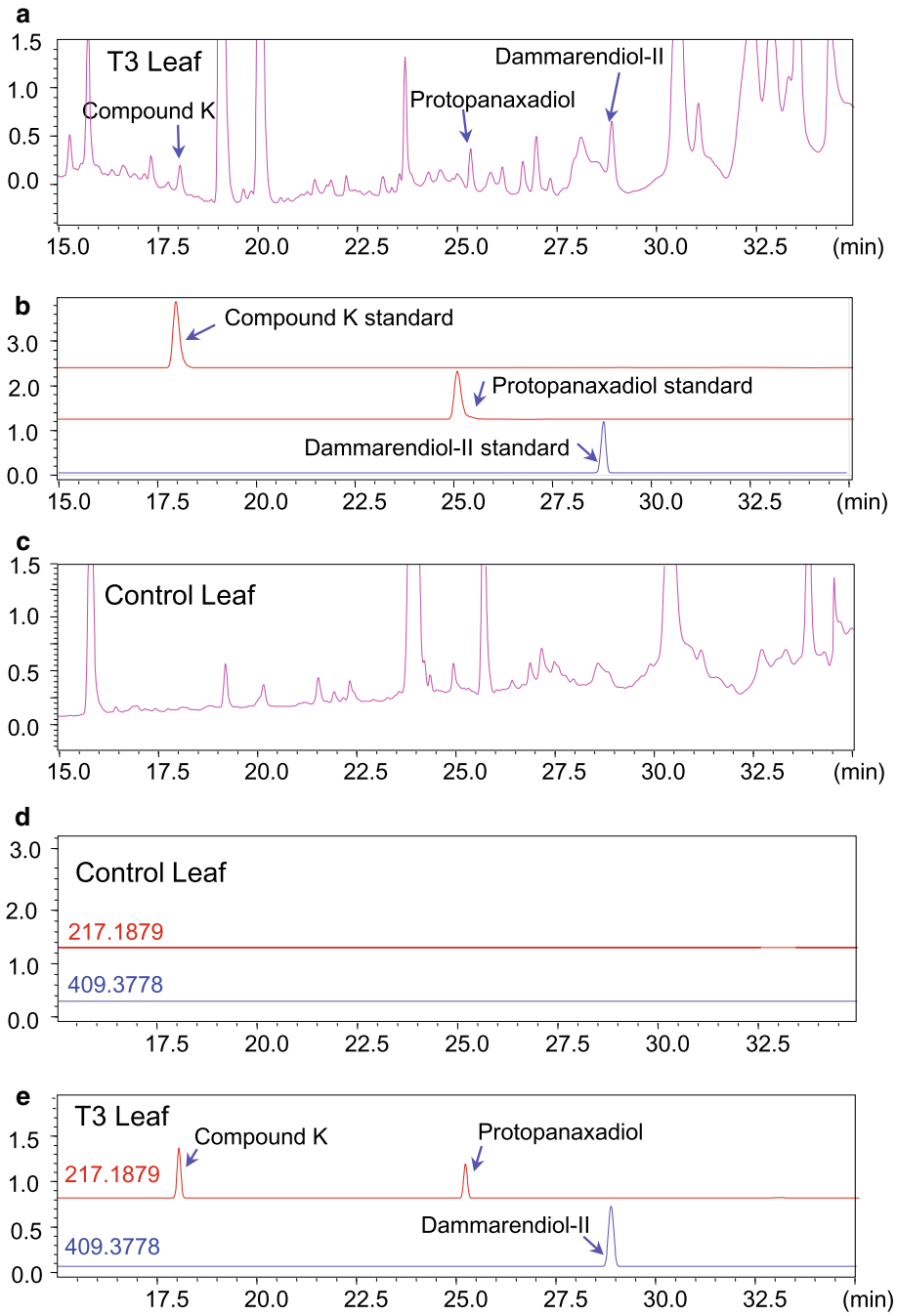

Fig. 3 LC-MSMS analyses of leaf extracts from transgenic tobacco (T3) co-overexpressing *PgDDS*, *CYP716A47* and *UGT71A28*. **a** TIC chromatogram of DD, PPD and CK in leaves of transgenic tobacco (T3). **b** Chromatogram of authentic standards DD, PPD and CK. **c** TIC chromatogram of WT tobacco leaf extracts. **d** Detection of DD, PPD and CK by single ion mode chromatogram of the WT tobacco. **e** Detection of DD, PPD and CK by single ion mode chromatogram of transgenic tobacco

JN604537) and *UGT71A27* (GenBank accession number, KM491309) (Fig. 1). A binary vector was constructed to enable co-overexpression of the three genes under the control of the CaMV35 promoter (Fig. 2a). Among more than 20 independent transgenic tobacco lines derived from *Agrobacterium*-mediated transformation, 7 lines were selected for further analysis (Fig. 2b). RT-PCR of cDNA revealed successful transcription of the introduced *BAR*, *PgDDS*, *CYP716A47* and *UGT71A27* genes in transgenic tobacco (Fig. 2b). As expected, no amplicon was detected in non-transgenic plants.

Analysis of introduced gene products in transgenic tobacco

The total ion chromatogram (TIC) from the liquid chromatograph–mass spectrometer-ion trap/time-of-flight (LCMS-IT-TOF) analysis showed that co-expression of *PgDDS*, *CYP716A47* and *UGT71A27* in tobacco showed three new peaks (Fig. 3a) at retention times of 18.2, 25.3 and 28.8 min, which were the same retention times for authentic CK and its precursors (PPD and DD), respectively (Fig. 3b). Furthermore, the peaks of authentic compounds were

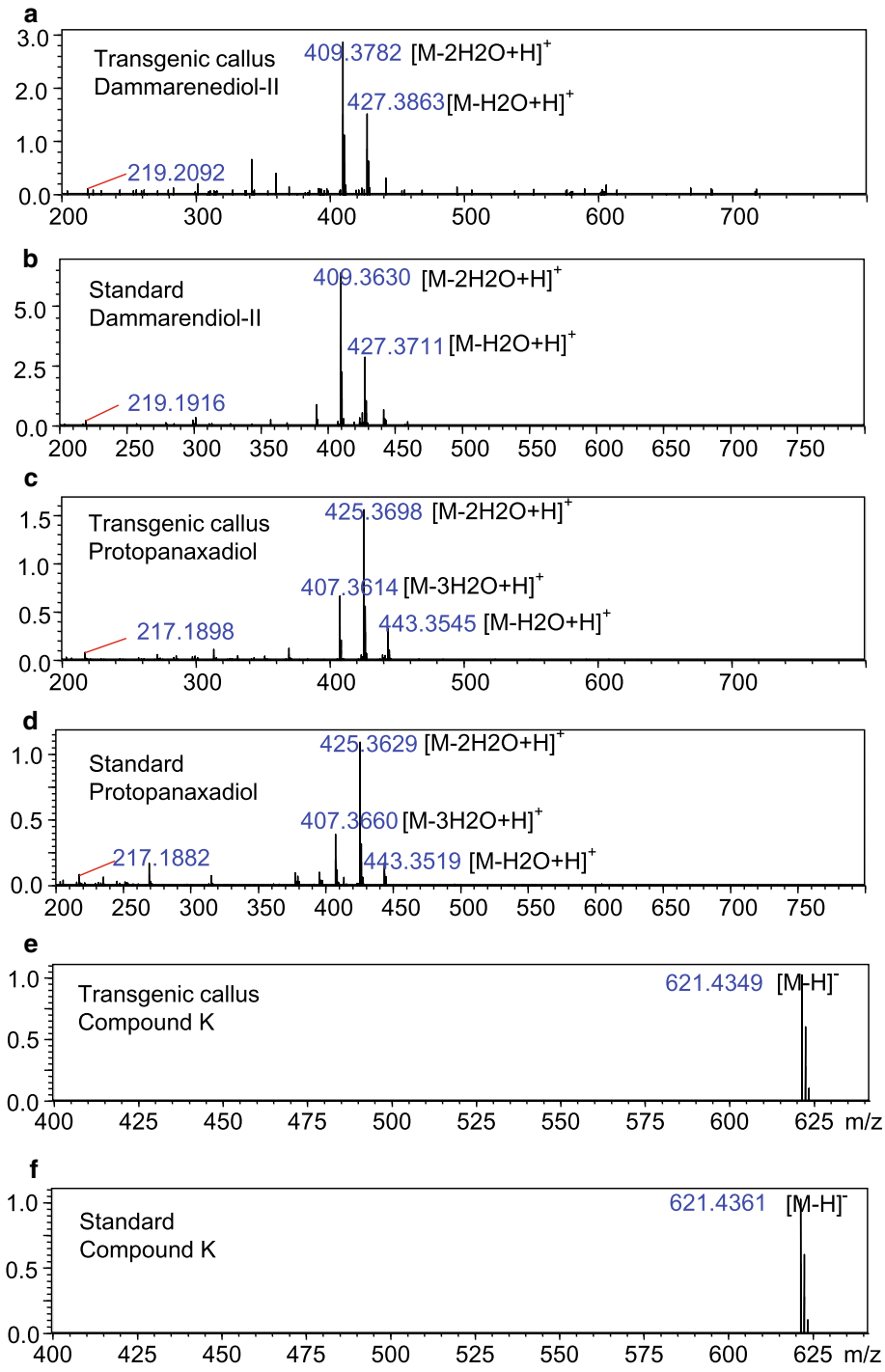

Fig. 4 APCI-MS_1 ionization spectra of DD, PPD and CK peaks in transgenic tobacco calli co-overexpressing *PgDDS*, *CYP716A47* and *UGT71A28*. Positive ionization of APCI MS^1 spectrum of the DD peak in sample (**a**) and DD standard (**b**). Positive ionization of the APCI MS^1 spectrum of a PPD peak in sample (**c**) and PPD standard (**d**). Negative ionization of the APCI MS^1 spectrum of a CK peak in sample (**e**) and CK standard (**f**)

exactly matched with those of tobacco extracts detected in selected ion monitoring (SIM) mode (Fig. 3e). None of these three signals were detected in non-transgenic plants (Fig. 3c, d). Additionally, atmospheric pressure chemical ionization (APCI) mass spectrometry (MS_1)-derived spectra of the putative peaks in the samples with 18.2, 25.3 and 28.8 min retention times were identical to those in authentic CK, PPD and DD compounds, respectively (Fig. 4).

The amount of CK ranged from 1.55 μg/g (T4 line) to 2.64 μg/g 65 (T8 line) on a dry weight (DW) basis among the transgenic lines. The proportion of CK including DD and PPD varied among the transgenic lines and organ types (Fig. 5). When the best transgenic line (T8) was checked for organ-specific CK accumulation, the root was found to contain the highest amount (4.65 μg/g), followed by the leaf (2.65 μg/g) and stem (1.70 μg/g). Interestingly, the

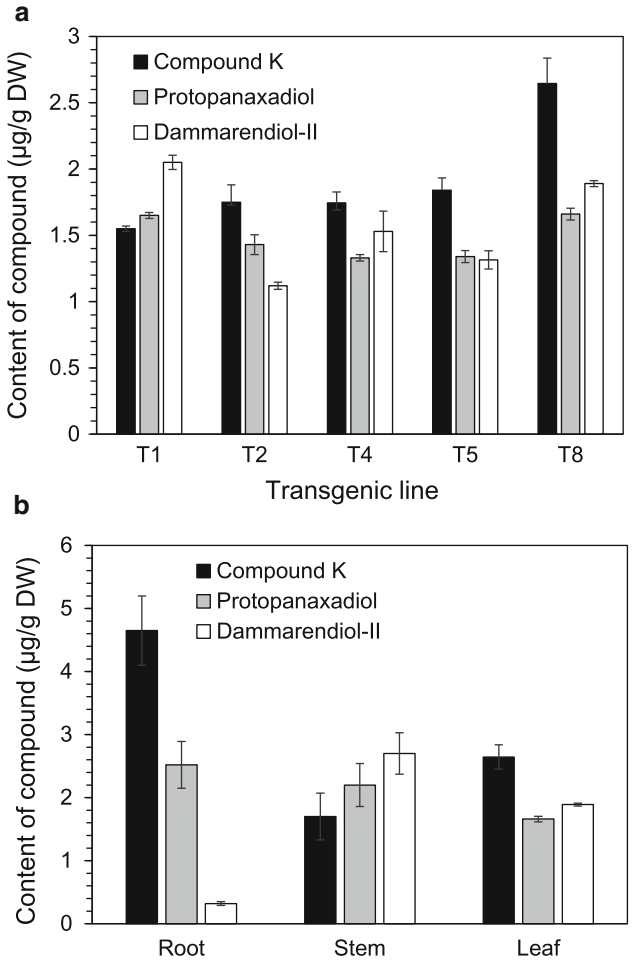

Fig. 5 DD, PPD and CK content in transgenic tobacco co-overexpressing *PgDDS*, *CYP716A47* and *UGT71A28*. **a** Comparative analysis of DD, PPD, and CK accumulation in leaves of various transgenic tobacco lines. **b** Organ-specific DD, PPD and CK content analysis in transgenic tobacco (line 8). Values represent the mean ± SE, $n = 3$

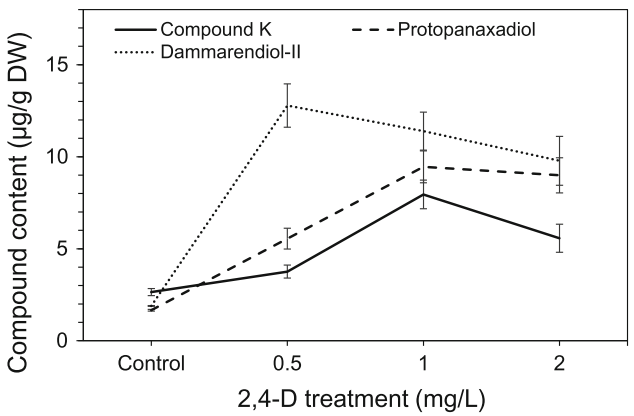

Fig. 6 Content of DD, PPD and CK in transgenic tobacco calli produced on medium supplemented with different concentrations of 2,4-D. Values represent the mean ± SE, $n = 3$

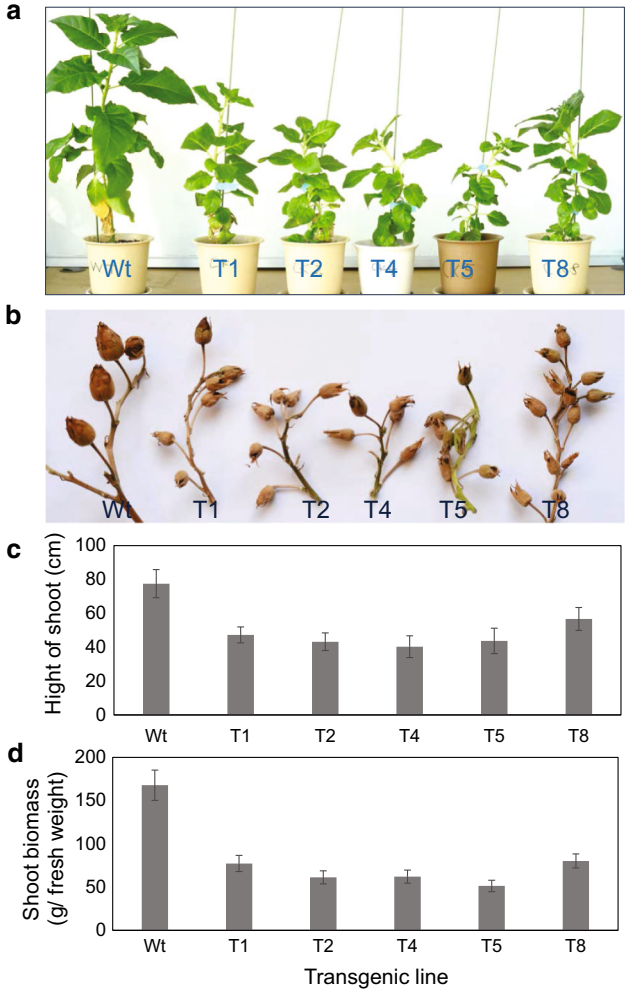

Fig. 7 Effect of co-overexpressed *PgDDS*, *CYP716A47* and *UGT71A28* on plant growth and biomass production. **a** WT and transgenic lines after 2 months of soil transfer and acclimatization. Compared to WT, transgenic lines showed a distinctive stunted growth pattern. **b** Comparative observation of seedpod morphology in WT and transgenic lines. Without assisted pollination, transgenic lines did not produce any seeds and the seedpods were smaller. **c** Comparative analysis of plant height among WT and transgenic lines. **d** Comparative analysis of biomass production among WT and transgenic lines. Values in **c** and **d** represent the mean ± SE, $n = 3$

highest DD content was found in the stem (2.70 μg/g), followed by the leaf (1.89 μg/g) and root (0.32 μg/g). The PPD content was found to be somewhat "balanced" in all three tissues tested (Fig. 5b).

Production of CK in callus and cell suspension culture induced by 2,4-D treatment

Leaf segments of the transgenic line (T8) were cultured on solid MS medium supplemented with various 2,4-D concentrations for callus induction. After 4 weeks of culture,

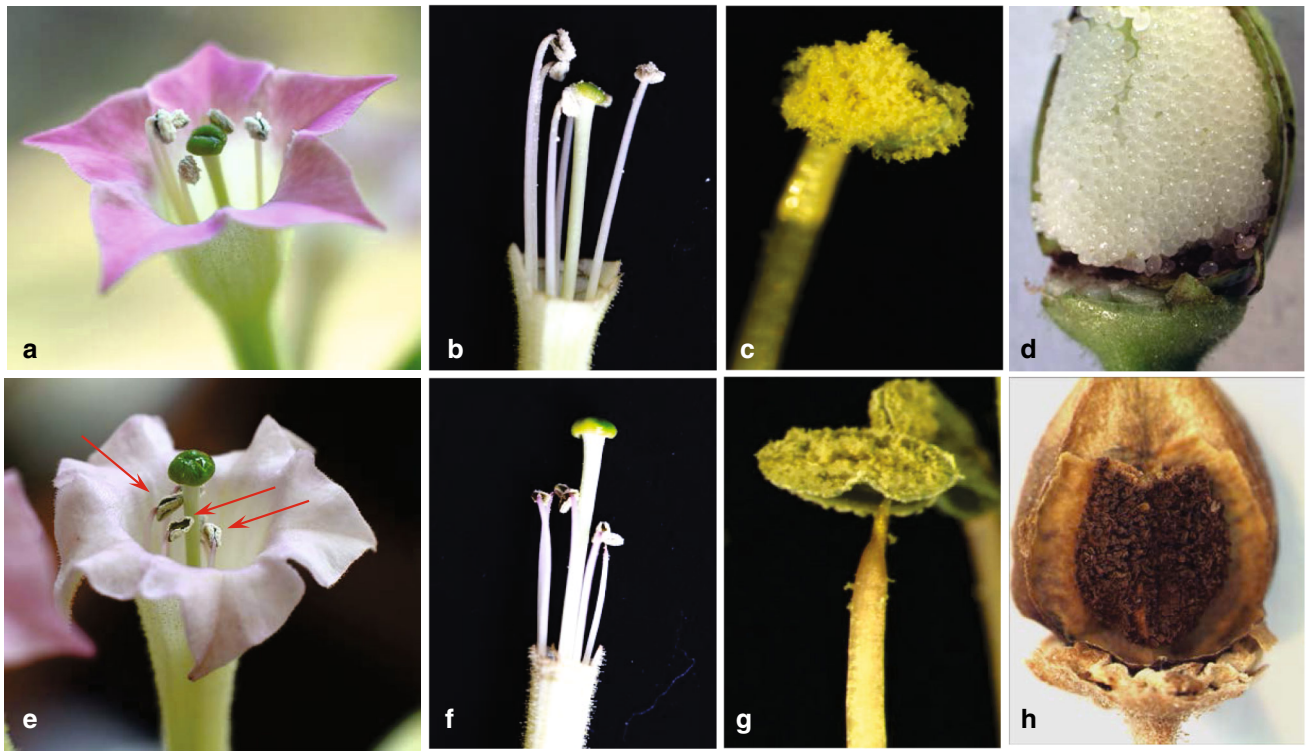

Fig. 8 Effect of co-overexpressed *PgDDS*, *CYP716A47* and *UGT71A28* in floral morphology and seed production. **a** WT tobacco flower with homostylous morphology. **b** Closed view of stamen and pistil of WT tobacco flower. **c** WT gave rise to anthers with profuse pollen. **d** Normal seed production in WT flower. **e** Flower of transgenic tobacco producing heterostylous (pin) flowers. **f** Closed view of stamen and pistil of transgenic tobacco. **g** Transgenic tobacco with anthers containing far less pollen. **h** No seed production without assisted pollination. *Arrows* in **e** point to shortened stamens

the contents of CK, PPD and DD in the calli were analyzed by LC/MS. The result revealed that CK, PPD and DD accumulation was greatly stimulated by 2,4-D treatment (Fig. 6). CK content was threefold higher than intact leaves with 1 mg/L 2,4-D treatment, and both PPD and DD production increased up to five- to sixfold. DD content was found to increase rapidly at 0.5 mg/L 2,4-D treatment (Fig. 6). For 2,4-D treatments, the amount of compounds was in the order of DD > PPD > CK.

Stunted plant growth and seed set failure in transgenic tobacco

All soil-transferred transgenic tobacco lines showed peculiar morphological differences with wild-type (WT) counterparts (Fig. 7). Compared to WT, transgenic plants showed retarded growth, short internode length and small leaves. The quantitative analysis of plant height and fresh biomass among WT and transgenic lines after 2 months of soil transfer clearly showed hindered growth and reduced plant biomass in transgenic lines (Fig. 7b, c). The plant heights of transgenic lines ranged from 40.3 cm (T4) to 66.7 cm (T8), while WT was 77.5 cm tall (Fig. 7b). Similarly, fresh biomass in transgenic lines ranged from 51.3 g/plant (T5) to 90.2 g/plant (T8), while WT contained 167.8 g/plant (Fig. 7c). Furthermore, all transgenic lines failed to set seeds under natural conditions (Fig. 8h). Upon close observation, transgenic tobacco plants were found to produce heterostylous flowers, while WT plants produced homostylous flowers (Fig. 8a, e). The stamen–style position in flowers from transgenic lines was 'pin' type (tall style and short stamens) (Fig. 8e, f). Morphologically, the stigma appeared normal in transgenic lines while the anthers produced far less pollen compared to their WT counterparts (Fig. 8c, g).

Abnormal pollen morphology in transgenic tobacco

Comparative observation of pollen between the WT and transgenic tobacco lines revealed their size differences. While the pollen morphology of WT was highly homogenous, that of transgenic tobacco was highly heterogeneous, with small and normal sizes. Moreover, negligible numbers of deformed pollen were present in WT, but large numbers of deformed pollen were observed in transgenic lines, indicating their developmental abnormalities (Fig. 9a, b). Further germination tests revealed a high germination rate (~70%) in WT pollen, while pollen from transgenic lines

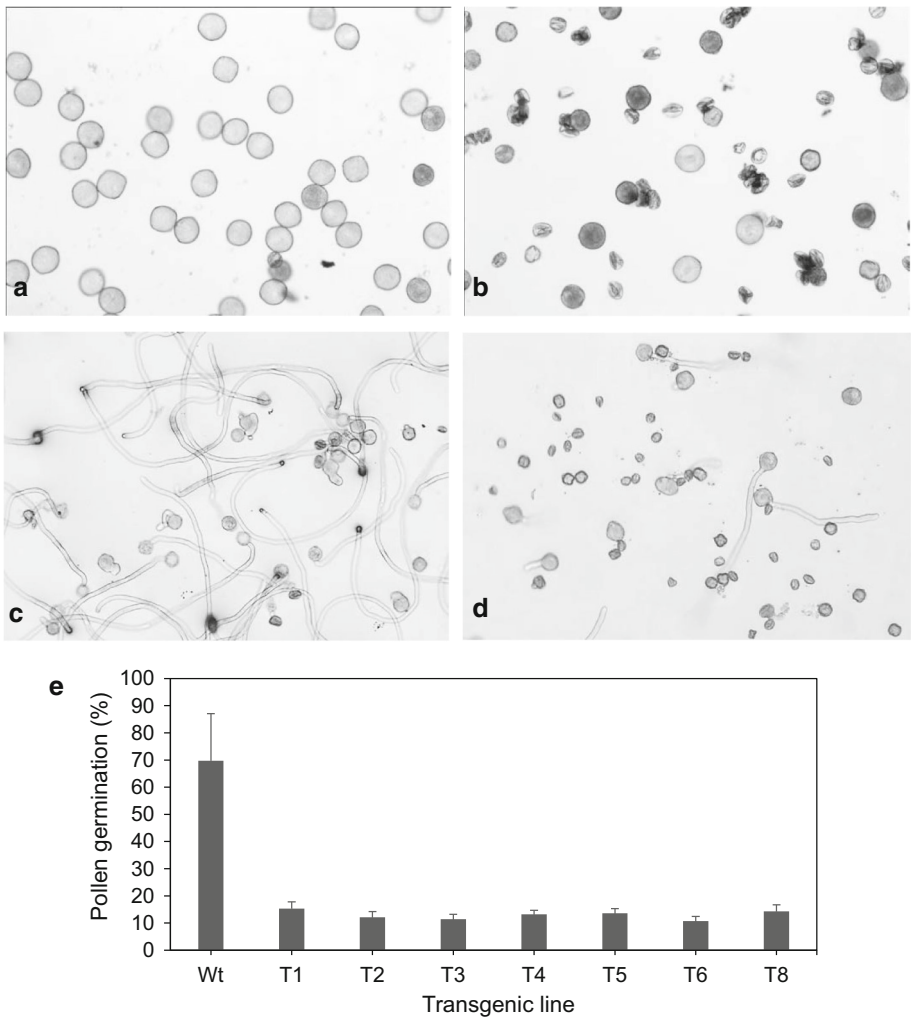

Fig. 9 Pollen germination test. **a** Pollen from WT plants with homogenous size. **b** Pollen from transgenic tobacco with heterogeneous sizes. **c** Germination of WT pollen. **d** Germination of transgenic tobacco pollen. **e** Frequency of pollen germination among WT and transgenic tobacco lines. Values in **e** are the mean ± SE, $n = 3$

showed a low germination rate (11–15%) (Fig. 9c–e). Transgenic pollen with equivalent size to WT pollen germinated, while smaller pollen showed no response (Fig. 9d).

Suppression of pollen germination by in vitro treatments of CK, PPD, and DD

To address the individual toxicity of CK, PPD and DD, WT pollen was treated with various concentrations of those compounds alone and in a mixture during in vitro pollen germination culture. The effect of CK, PPD and DD on in vitro pollen germination and pollen tube growth was assessed. The results clearly revealed the inverse relationship of compound mixture concentration and pollen germination (Fig. 10a) as well as pollen tube growth (Fig. 10b), indicating the toxicity posed by these chemicals to pollen. At a 1 mg/L concentration of a single-compound treatment, the reduction of the germination rate was not significantly different from the control, while a mixture of three compounds significantly reduced this rate ($p < 0.001$). The single-compound treatment obviously reduced the germination frequency of pollen at concentrations ≥5 mg/L (Fig. 11). Suppression of pollen germination and pollen tube growth by CK or DD treatment was more severe than by PPD at all concentrations. At a concentration of 50 mg/L, all treatments except for PPD inhibited germination completely (Figs. 11, 12). The inhibitory effect of the treatments on pollen germination frequency and pollen tube growth was mixture > DD > CK > PPD.

Self- and cross-pollination in planta for the viability test of gametes of transgenic origin

Manually assisted self- and cross-pollination of WT and transgenic tobacco flowers clearly indicated developmental abnormalities in both pollens and ovules (Fig. 12). Except for self-pollinated WT flowers, all other crosses produced smaller seedpods (Fig. 12a–d). While self-pollinated WT lines produced a profuse number of seeds, self-pollinated

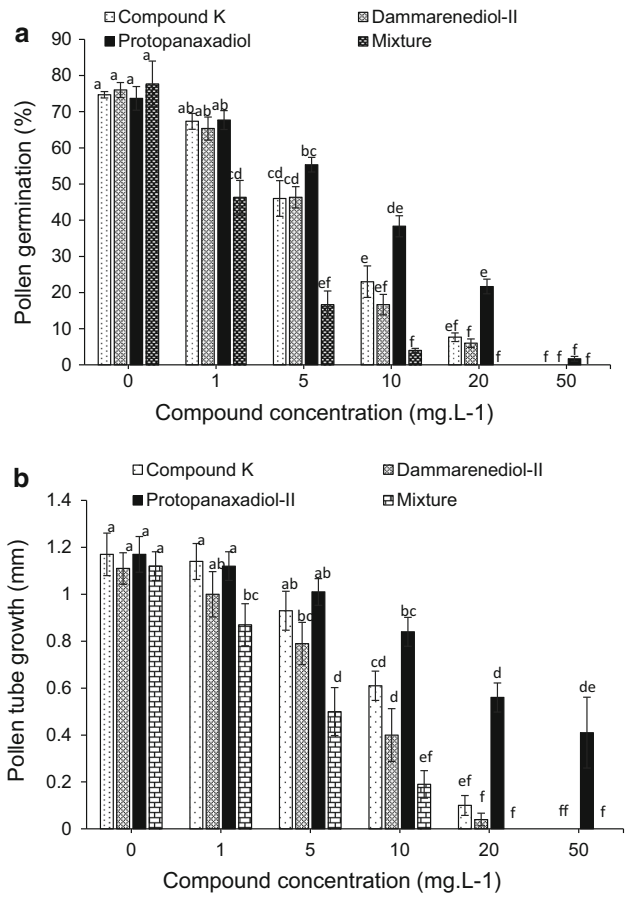

Fig. 10 Germination rate (**a**) and pollen tube growth (**b**) in WT pollen under various concentrations of CK, DD and PPD and their mixture. Values represent the mean ± SE. *Bars* with the same character are not significantly different (tested with DMRT for germination rate and Kruskal–Wallis test for pollen tube growth, $p \leq 0.05$)

transgenic lines and cross-pollinations [WT (♂) × Tr (♀) and Tr (♂) × WT (♀)] produced far fewer seeds. Moreover, both self- and cross-pollinated WT flowers produced seeds of more homogeneous size with a negligible number of deformed ones, as compared to those produced in transgenic flowers (Fig. 12).

Discussion

CK production and its tissue-specific accumulation

CKs have been reported to show much higher biological activities than natural ginsenosides (Hasegawa et al. 1997). However, all are present in undetectable amounts in naturally grown ginseng roots. To produce CK from ginseng plants, complicated enzymatic and chemo-physical treatments are required for deglycosylation of ginsenosides (Leung and Wong 2010; Lee et al. 2012). Thus, metabolic engineering of CK in transgenic tobacco may offer greater advantages for cost-effective production of the target compounds.

The first step of saponin (triterpene glycoside) production via metabolic engineering requires introduction of triterpene synthase to construct the triterpene skeleton. Furthermore, cytochrome P450 monooxygenase (for sapogenin production from triterpene) and uridine glucosyltransferase (for glucuronidation of the sapogenin) are necessary. To produce CK in tobacco, three *P. ginseng* genes (*PgDDS*, *CYP716A47*, and *UGT71A27*) should be introduced and expressed (Fig. 1). Transgenic tobacco lines were constructed via *Agrobacterium*-mediated transformation. Heterologous co-expression of three genes (*PgDDS*, *CYP716A47* and *UGT71A27*) in transgenic tobacco resulted in the production of CK together with its precursors. The CK content in transgenic tobacco leaf was 0.75–3.32 µg/g dry weight among different transgenic lines. However, the transgenic plants also showed the production of the intermediate precursors PPD and DD at high levels. Involvement of the electron donor counterpart, NADPH-cytochrome P450 reductase (CPR), has been known to be vital for efficient functioning of CYP enzymes (Jensen and Møller 2010). Moreover, plants use distinct CPR isoforms to meet high reductive demand of CYP-mediated reactions under stressed conditions (Ro et al. 2002). The suboptimal conversion of precursors into CK might be related to inadequate activity in tobacco CPR.

Tissue-specific LC–MS analysis revealed that the roots showed the highest CK content (4.65 µg/g DW). Ginsenosides are known to be synthesized in the leaves of ginseng plants followed by their long-distance transport to its roots (Kim et al. 2014). The disparity of CK and its precursors among organs of tobacco plants might be caused by tissue-specific accumulation and transportation systems.

The production of CK by callus and cell suspension culture compared to field-cultivated transgenic plants has great advantages because they are free from limitations such as regulation of pollen and seed dispersal of transgenic plants; they are also independent of the climate, season and geographical environment. Our study showed that leaf-derived calluses induced by 2,4-D treatments in transgenic tobacco had threefold higher CK content than intact tobacco leaves. In our earlier study, we reported that auxin treatment highly enhanced the accumulation of PPD in transgenic tobacco overexpressing *PgDDS* and *CYP716A47* (Chun et al. 2015). The auxin treatment can also enhance the expression of upstream genes such as HMGR (Aoyagi et al. 1993; Chun et al. 2015) and squalene epoxidase (Chun et al. 2015). These two are the rate-limiting enzymes for saponin and sterol biosynthesis (Ryder 1991; Chappell et al. 1995; Lee et al. 2004). Enhanced production of CK in 2,4-D-treated transgenic calli is likely

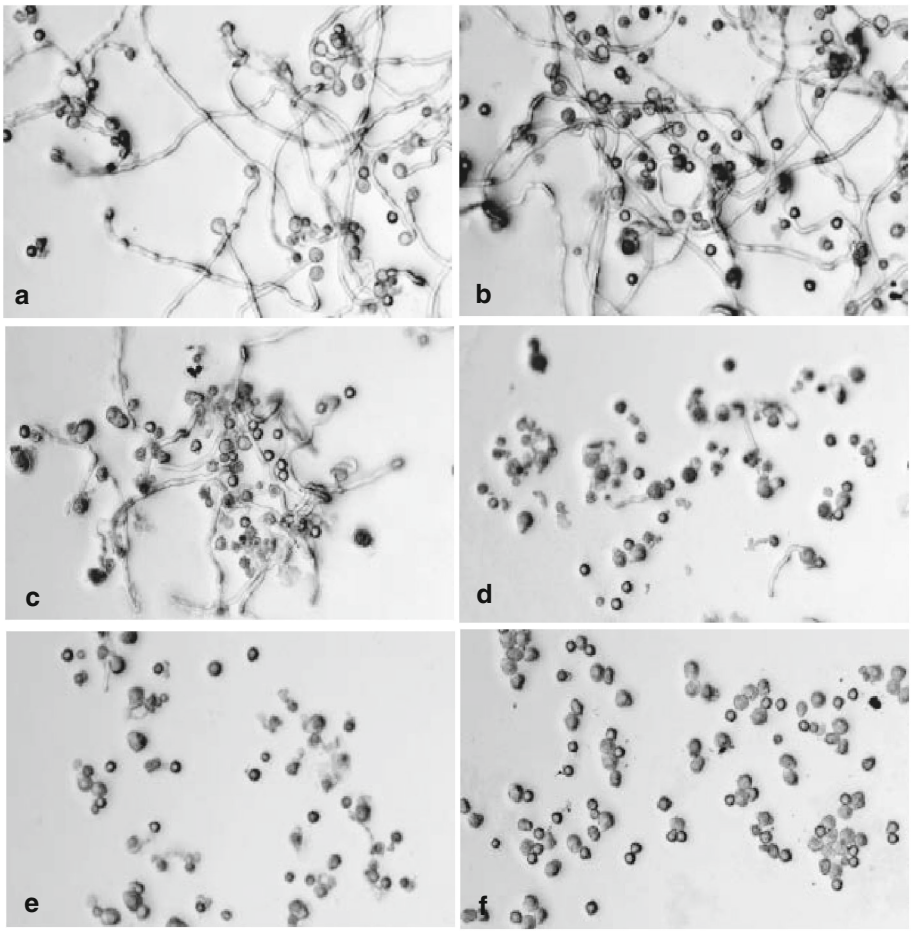

Fig. 11 Suppression of germination of WT pollen cultured in medium supplemented with 0 mg/L (**a**), 1 mg/L (**b**), 5 mg/L (**c**), 10 mg/L (**d**), 20 (**e**) and 50 mg/L (**f**) of CK, DD, and PPD mixture

due to the enhanced expression of upstream genes in the pathway of saponin biosynthesis.

Autotoxicity of CK, PPD, and DD in transgenic tobacco

Heterologous production of CK, PPD and DD in tobacco caused serious damage to plants such as stunted growth and seed set failure. This result indicates that the accumulation of CK, PPD and DD in transgenic tobacco has strong phytotoxic activities on tobacco itself. In our previous report (Han et al. 2014), transgenic tobacco lines producing moderate amounts of DD were acclimatized normally after exposure to open air conditions, which successfully set seeds. However, the transgenic line T14, which had higher DD content and showed normal growth in plastic culture bottles, could not survive upon soil transfer (Han et al. 2014). In our study, abnormal pollen morphology and seed-set failure in transgenic tobacco producing CK were unexpected. The suppression of filaments of stamen and impaired pollen development heterogeneous seed setting by self- and cross-pollinated transgenic flowers indicate that CK, PPD and DD toxicity is more severe to germ cells. In addition, an in vitro pollen germination test revealed that CK, PPD and DD treatments directly suppressed the germination and tube growth of WT pollen. Moreover, the effect of three-compound mixture treatments was more severe compared to single-compound treatments, which indicates strong phytotoxic properties for CK, PPD and DD. The toxic effect on overall plant morphology and floral development seemed to be the cumulative effect of the three compounds. Based on the above results, it is evident that CK, PPD and DD accumulation in transgenic tobacco resulted in severe impairment of both vegetative and reproductive growth, although the physiological mechanisms of autotoxic effects are yet to be elucidated.

The ginsenoside saponin has been accepted as an allelochemical (Lei et al. 2010; Zhang et al. 2011). Ginseng saponins inhibit germination of ginseng seeds (Yang et al. 2015a), stimulate or suppress the ginseng root pathogen (Nicol et al. 2003; Yousef and Bernards 2006), and cause antifeeding activity against insect larva (Zhang et al. 2015). Several ginsenosides (R1, Rg1, Re, Rg2, and Rd) cause autotoxicity against seedling emergence, growth and root cell vigor at a concentration of 1.0 μg/mL (Yang et al. 2015a). Although genetically engineered transgenic plants

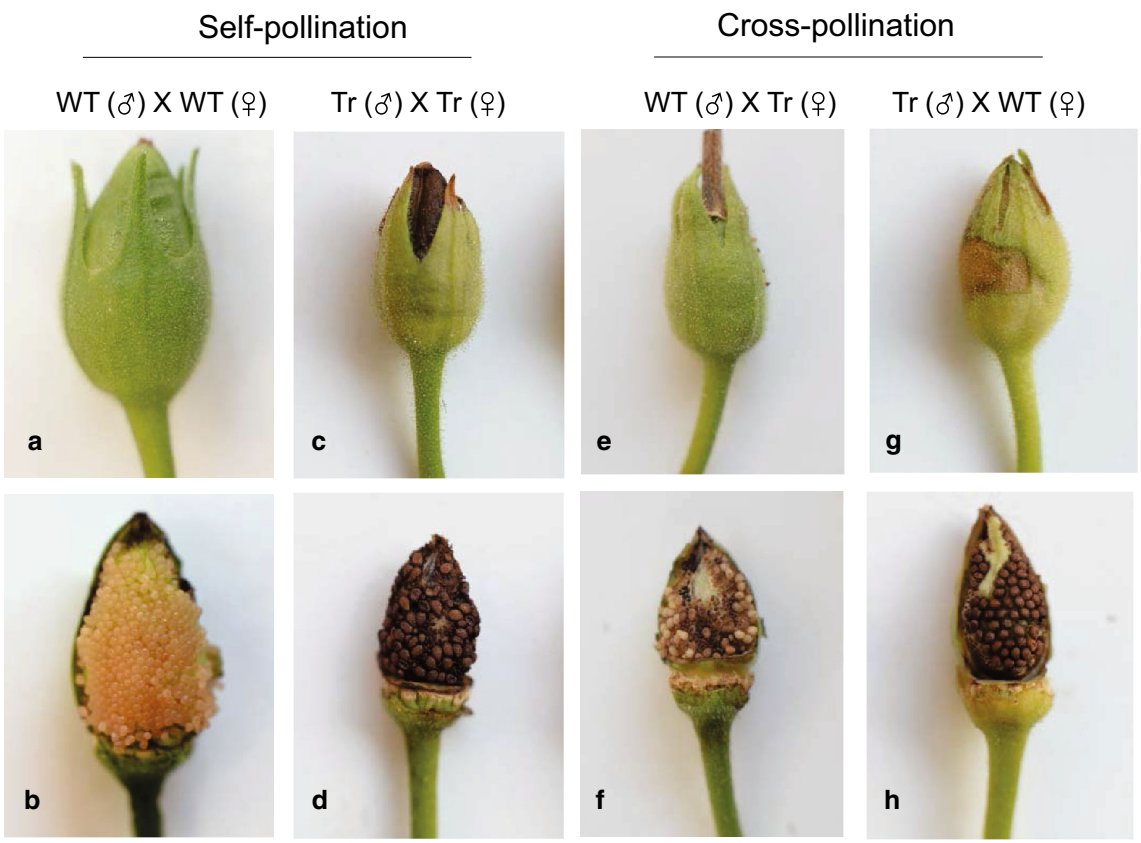

Fig. 12 Seed production by self- and cross-pollination between WT and transgenic tobacco (line 8). **a**, **b** Self-pollinated WT flower gave rise to plump seed pods and produced seeds of homogenous sizes in abundance. **c**, **d** Artificial self-pollinated seeds in transgenic plants gave smaller seed pods with a highly reduced number of seeds of heterogeneous size. **e**, **f** Cross-pollination between WT (♂) and transgenic tobacco (♀) produced small seed pods with fewer seeds than in WT seed pods. **g**, **h** Cross-pollination between WT (♀) and transgenic tobacco (♂) produced more seeds with more homogenous sizes compared to those from self-pollinated transgenic flowers (**f**) and from cross-pollinated flowers between WT (♂) and transgenic tobacco (♀) (**g**). All crossings were performed manually

producing a medicinally important ginsenoside saponin (CK) might be a promising alternative, we were confronted with the phytotoxic property of the compound in transgenic tobacco. The development of a mechanism to avoid such properties for CK, PPD and DD in transgenic tobacco must be considered.

CK toxicity in eukaryotes

Due to its highly valued medicinal properties, the effect of CK in animal models has been extensively studied. CK induces apoptosis in various types of cancer cells (Kim et al. 2009; Lee et al. 2010; Kim et al. 2010; Jeong et al. 2012; Wang et al. 2013; Law et al. 2014). Arrest of the cell cycle at the G_1 phase upon CK treatment has been reported in both normal and cancerous colon cells (Jeong et al. 2012). A similar effect for CK was reported in human monocytic leukemia cells (Kang et al. 2005). Furthermore, Kang et al. (2006) reported that CK treatment reduced telomerase activity and caused apoptosis in leukemia cells. Additionally, Lee et al. (2010) demonstrated CK-mediated ROS generation leading to apoptosis in human colon cancer cells by modulating a mitochondria-dependent apoptotic pathway and the mitogen-activated protein kinase pathway. Kim et al. (2010) also reported apoptosis in human breast cancer cells upon CK treatment due to ROS generation and activation of 5′ adenosine monophosphate-activated protein kinase (AMPK, a cellular energy sensor). Activation of AMPK has also been reported in human hepatoma cells upon CK treatment (Kim et al. 2009). AMPK regulates all aspects of cell function, particularly for energy homeostasis, by maintaining constant intracellular ATP concentrations (Hardie 2011, 2015). In addition, AMPK plays a crucial role in the reproductive aspects of gonad cell proliferation and germ cell maturation in numerous animal species (Hardie 2011, 2015; Bertoldo et al. 2015). Its plant ortholog, snf1-related protein kinase (*SnRK1*), is also closely related to energy metabolism, and both its overexpression and suppression bring about abnormalities in plant growth and development (Baena-Gonzalez et al. 2007; Nukarinen et al. 2016). Its overexpression in *Arabidopsis* brought about various vegetative

and reproductive abnormalities in the plant (Baena-Gonzalez et al. 2007), while its knockout exhibited overall hampered protein synthesis, leading to more severe effects in plants (Nukarinen et al. 2016). In our study, the cytotoxic effect of the CK in transgenic tobacco is comparable to CK-induced apoptosis in animal cells. Hence, in light of these reports and our observations, it can be inferred that the accumulated CK in transgenic tobacco may have played a negative role in energy metabolism to cause stunted growth and abnormal flower and pollen development. Further physiological and molecular study with a single CK treatment would shed more light on the subject.

Conclusively, we have shown the first case heterologous production of saponin in transgenic plants. The strong cytotoxic effects of CK, PPD and DD in transgenic tobacco are unexpected results. The expression of introduced genes driven by organ- or tissue-specific promoters may reduce the autotoxic effects of heterologously produced phytochemicals. Moreover, rapid sequestration or secretion of produced phytochemicals may increase the tolerance to toxic chemicals in transgenic tobacco plants.

Author contribution statement YEC designed the research and wrote the paper. JYH performed the genetic transformation of tobacco and analysis of phytochemicals. YSG analyzed the RT-PCR experiments with transgenic tobacco. CHA and PBA performed the analysis of the morphology of transgenic tobacco and pollen germination culture. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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