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ORIGINAL PAPER

Production of dammarenediol-II triterpene in a cell suspension culture of transgenic tobacco

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Abstract

Key message **Dammarenediol-II is biologically active tetracyclic triterpenoid, which is basic compound of ginsenoside saponin. Here, we established the dammarenediol-II production via a cell suspension culture of transgenic tobacco overexpressing *PgDDS*.**

Abstract Dammarenediol-II synthase catalyzes the cyclization of 2,3-oxidosqualene to dammarenediol-II, which is the basic triterpene skeleton in dammarene-type saponin (ginsenosides) in *Panax ginseng*. Dammarenediol-II is a useful candidate both for pharmacologically active triterpenes and as a defense compound in plants. Dammarenediol-II is present in the roots of *P. ginseng* in trace amounts because it is an intermediate product in triterpene biosynthesis. In this work, we established the production of dammarenediol-II via cell suspension culture of transgenic tobacco. The dammarenediol-II synthase gene (*PgDDS*) isolated from *P. ginseng* was introduced into the *Nicotiana tobacum* genome under the control of 35S promoter by Agrobacterium-mediated transformation. Accumulation of dammarenediol-II in transgenic tobacco plants occurred in an organ-specific manner (roots > stems > leaves > flower buds), and transgenic line 14 (T14) exhibited a high amount (157.8 μg g^{-1} DW) of dammarenediol-II in the roots. Dammarenediol-II production in transgenic tobacco plants resulted in reduced phytosterol (β-sitosterol, campesterol, and stigmasterol) contents. A cell suspension culture was established as a shake flask culture of a callus derived from root segments of transgenic (T14) plants. The amount of dammarenediol-II production in the cell suspension reached 573 μg g^{-1} dry weight after 3 weeks of culture, which is equivalent to a culture volume of 5.2 mg dammarenediol-II per liter. Conclusively, the production of dammarenediol-II in a cell suspension culture of transgenic tobacco can be applied to the large-scale production of this compound and utilized as a source of pharmacologically active medicinal materials.

Keywords Dammarenediol-II synthase · Ginsenoside · Transgenic tobacco · Metabolic engineering

Abbreviations

HPT	Hygromycin phosphotransferase
DDS	Dammarenediol-II synthase
PgDDS	*Panax ginseng* dammarenediol-II synthase
BA	6-Benzylaminopurine
MS	Murashige and Skoog
MeJA	Methyl jasmonate
PCR	Polymerase chain reaction

Introduction

The root of *Panax ginseng* is one of the most famous and widely used medicinal plants (Shibata 2001). It is generally believed that dammarene-type ginsenosides, which are reported as major constituents of ginsenoside saponin in *Panax* species (Shibata 2001), are primarily responsible for the pharmacological activities of ginseng.

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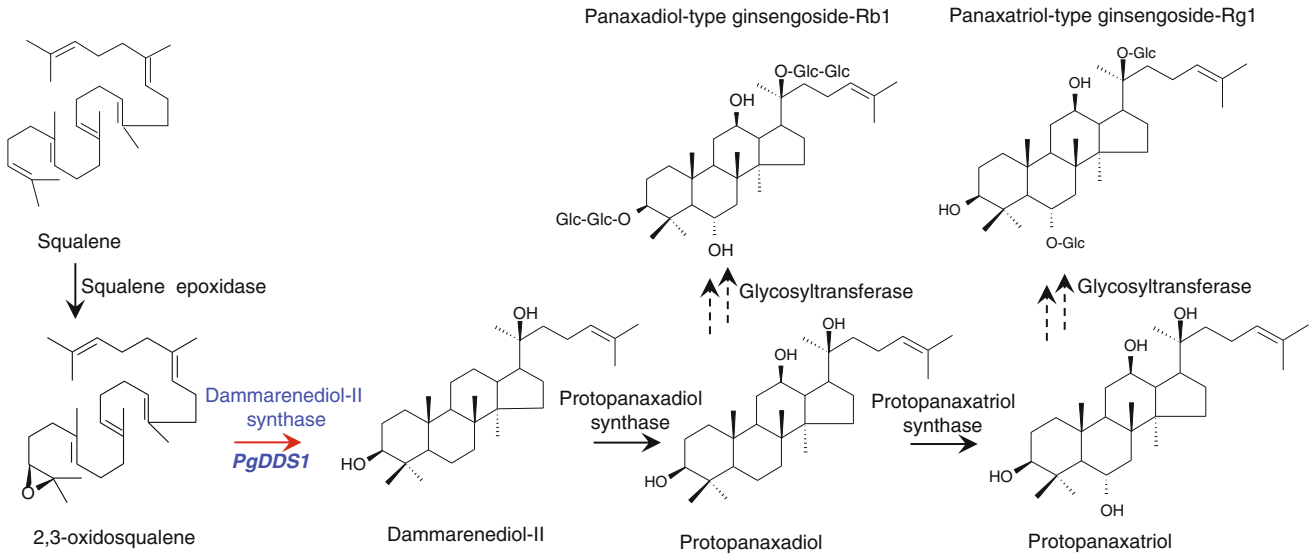

Fig. 1 Biosynthetic pathway for ginsenosides in *P. ginseng*. Squalene epoxidase converts squalene into 2,3-oxidosqualene, which is then converted into a triterpene aglycone (dammarenediol-II) by dammarenediol-II synthase (*PgDDS*). The triterpene aglycones subsequently undergo oxidation by CYP716A47 and CYP716A53v2 and glycosylation and are finally converted into triterpene saponins (ginsenosides)

The first committed step in dammarene-type ginsenoside synthesis is the cyclization of 2,3-oxidosqualene to dammarenediol-II (Fig. 1), catalyzed by dammarenediol-II synthase (DDS) (Han et al. 2006; Tansakul et al. 2006). Dammarenediol-II is converted to protopanaxadiol and protopanaxatriol by two cytochrome P450 genes (Han et al. 2011, 2012) and subsequently to ginsenosides by glycosylation via glycosyltransferase (Choi et al. 2005).

Recent studies have demonstrated that hydrolyzed ginsenosides or triterpene aglycones have greater biological effects than natural ginsenosides (Hasegawa et al. 1997; Jia et al. 2004; Popovich and Kitts 2004; Bae et al. 2004). Dammarenediol-II is the basic triterpenoid structure of ginsenoside saponin and is a useful candidate with potentially biologically active triterpenes. Dammarenediol-II isolated from dammar resin of trees in the *Dipterocarpaceae* family exhibits inhibitory effects against Herpes simplex virus types I and II and the Epstein-Barr virus (Poehland et al. 1987; Akihisa et al. 2004). Previously, we reported that transgenic tobacco expressing the *P. ginseng* dammarenediol-II synthase (*PgDDS*) produced 20–30 μg g^{-1} dry weight dammarenediol-II in leaves, conferring resistance to the tobacco mosaic virus (TMV) (Lee et al. 2012).

Dammarenediol-II is likely to be present in trace or undetectable amounts in *P. ginseng* plants because of the rapid flux of intermediates toward the final production of ginsenosides. Therefore, the natural production of dammarenediol-II in cultivated ginseng roots is nearly impossible to harness to afford commercially attractive yields of dammarenediol-II. Production of dammarenediol-II from other plant species or microbial hosts via genetic engineering might be a promising technology for efficient production. Heterologous expression of the *PgDDS* gene in ergosterol-deficient mutant yeast for characterization of this gene resulted in the production of dammarenediol-II (Tansakul et al. 2006; Han et al. 2006). Liang et al. (2012) attempted the production of dammarenediol-II in the engineered yeast expressing DDS and could produce dammarenediol-II up to 250 μg l^{-1}.

Plant cell suspension culture systems have been used for the production of useful secondary compounds by increasing the content of secondary metabolites or by producing the desired compounds via heterologous gene expression using an easily culturable host plant (Verpoorte and Memelink 2002; Wu and Chappell 2008; Dudareva et al. 2013). In this work, we focused on the establishment of dammarenediol-II production via a cell suspension culture of transgenic tobacco overexpressing *PgDDS*.

Materials and methods

Construction of overexpression vector harboring *PgDDS*

The full-length sequence of *PgDDS* (GenBank accession number, AB122080) was isolated by PCR using 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 open reading frame of *PgDDS* was cloned via GATEWAY vector pCR8/GW/TOPO

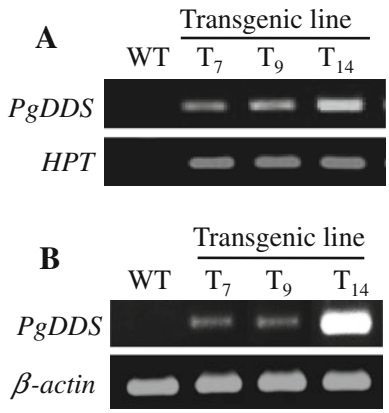

Fig. 2 Detection of introduced genes in transgenic tobacco overexpressing *PgDDS*. **a** Confirmation of the introduced genes (*PgDDS* and *HPT*) by genomic DNA PCR. **b** Expression of *PgDDS* in leaves of wild-type (WT) plants and the indicated transgenic lines (T7, T9 and T14) by RT-PCR. *β-actin* was used as a loading control. The wild-type line was not transformed with Agrobacterium

(Invitrogen) and transferred into binary destination vector pH7WG2, placing it under the control of the double 35S CaMV promoter (Fig. 2a). Eventually, the overexpression construct harboring *PgDDS* was introduced into *Agrobacterium tumefaciens* LBA4404 competent cells by the heat shock method.

Construction of transgenic tobacco with the *PgDDS* gene by *A. tumefaciens*-mediated transformation

A single colony of *A. tumefaciens* LBA4404 harboring the overexpression vector was cultured for 48 h at 28 °C in a yeast extract broth medium (YEB; peptone 10 g/l, yeast extract 10 g/l, NaCl 5 g/l, pH 7.0) with 30 mg/l hygromycin in a gyratory shaker at 200 rpm. The bacterial suspension (absorbance of ~0.6 at 600 nm) was pelleted by centrifugation for 5 min at 5,000 rpm and resuspended in hormone-free MS (Murashige and Skoog 1962) liquid medium.

Leaves from the in vitro cultured tobacco plants (*Nicotiana tabacum,* cv. Xanthi) were cut into 0.5–1 cm^2 squares and then immersed in the bacterial suspension for 20 min. The leaf pieces were then blotted on sterile filter paper to remove excess bacterial solution before transfer to a co-culture medium (MS medium with 2 mg/l BA) and incubated in darkness for 3 days. After 3 days of co-culture, leaf pieces were washed five times with sterile water containing 500 mg/l cefotaxime to remove excess bacteria, blotted on sterile filter paper, transferred to a first stage selection medium with 400 mg/l cefotaxime, and kept in a culture room maintained at 22 ± 2 °C with a 16-h photoperiod of 50 μmol m^{-2} s^{-1} light by cool white fluorescent tubes. After ~4 weeks of culture, adventitious shoots began to appear near the edges of leaf pieces. Those leaf pieces containing adventitious shoots were transferred to a second stage selection medium containing both 400 mg/l cefotaxime and 20 mg/l hygromycin. After a further 3 weeks of selection, hygromycin-resistant shoots had grown to ~2 cm and were transferred and maintained by consecutive subculture on 1/2 MS medium containing 40 mg/l hygromycin. Transgenic plantlets were gently rinsed in distilled water to remove agar from the roots and then transferred into 10 × 10 × 10 cm plastic soil pots. After 3 weeks of acclimatization, plantlets were moved to the greenhouse.

PCR analysis

Genomic DNA from the leaf tissue of putative transformed plants and wild-type plants was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The primers used for amplifying the *PgDDS* gene were 5′-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3′ and 5′-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3′, and the primers used for the *HPT* gene were 5′-GCG TGA CCT ATT GCA TCT CC-3′ and 5′-TTC TAC ACA GCC ATC GGT CC-3′. The PCR mixture was incubated in a DNA thermal cycler (Applied Biosystems, C.A. USA.) under the following conditions: one cycle of 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; with a final 10 min extension.

RT-PCR analysis

Total RNA was isolated from tobacco plants using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions and reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The first-strand DNA was used as the 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; and a final 10 min extension at 72 °C. The primers used for the *PgDDS* gene were 5′-ATG TGG AAG CTG AAG GTT GCT CAA GGA-3′ and 5′-TTA AAT TTT GAG CTG CTG GTG CTT AGG C-3′. 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 the control to check for RNA integrity and accuracy of loading. 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 the figures.

Cell suspension culture of transgenic tobacco cells

Newly formed roots of transgenic tobacco were cut into segments 10 mm in length; transferred onto MS medium

with 1.0 mg/l 2,4-D, 3 % sucrose, 20 mg/l hygromycin, and 0.27 % gelrite. All media were adjusted to pH 5.8 prior to being autoclaved at 120 °C for 15 min. Approximately 15 root segments were cultured in a Petri plate for 5 weeks. The culture room was maintained at 22 ± 1 °C under 24 μmol m^{-2} s^{-2} white florescent lamp illumination.

For the cell suspension culture, calluses derived from the root segments were transferred into MS liquid medium with 1 mg/L 2,4-D, 3 % sucrose, and 20 mg/l hygromycin in a 250-ml Erlenmyer flask and subcultured for a 3-week interval. When fine free floating cells were established, these cells were used for next experiment. To estimate the growth of cell mass during the culture periods, five grams of fresh cell mass (packed cell volume) was transferred to 400-ml MS liquid medium in a 1,000-ml Erlenmeyer flask. Cell growth and the amount of dammarenediol-II were assessed at 3-day intervals for 21 days of culture.

MeJA treatment

To investigate the effect of MeJA, cell suspensions at the vigorous growth phase after 15 days of culture were elicited by adding various concentrations of MeJA (0, 5, 10, 20, and 30 μM) and harvested after 21 days of culture. The culture room was maintained at 24 ± 2 °C under a 16/8-h (light/dark) photoperiod with light supplied by white fluorescent tubes at an intensity of 24 μmol m^{-2} s^{-2}. They were agitated by a gyratory shaker at 100 rpm. At least three flasks were cultured for each treatment, and this experiment was repeated three times.

GC–MS analysis of dammarenediol-II and phytosterols

Five hundred mg of milled powder from freeze-dried samples (leaves, flower buds, stems, roots, and suspension-cultured cells) was soaked in 100 % hexane. The supernatant collected by centrifugation was dried. After evaporation, the residue was dissolved in 100 % methanol, and then filtered with a SepPak C-18 Cartridge (Waters, Milford, MA, USA). A 10-ml aliquot of solution was analyzed by GC (Agilent 7890A) linked to an inert MSD system (Agilent 5975C) with a Triple-Axis detector and equipped with a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 mm). The injection temperature was 250 °C, and the column temperature program was as follows: 150 °C for 5 min, followed by a rise to 300 °C at a rate of 5 °C min^{-1} and a hold at 300 °C for 20 min. The carrier gas was He and the flow rate was 1.2 ml min^{-1}. The interface temperature was 300 °C with a split injection (10:1). The temperature of the ionization chamber was 250 °C, and ionization was by electron impact at 70 eV. The dammarenediol-II used as the standard for GC/MS analysis was provided by Professor Yong Soo Kwon of the Kangwon National University in Korea. The representative fragmentation ion values by GC–MS of authentic dammarenediol-II exhibited m/z 426.

Results

Production of transgenic tobacco

Transgenic tobacco plants overexpressing *PgDDS* (AB122080) under the control of the CaMV35 promoter were constructed. Of the 27 independent transgenic lines selected from different explants, three lines were finally selected for analysis based on high transcription of *PgDDS* gene by RT-PCR. *HPT* and *PgDDS* gene integration into the tobacco genome was confirmed by PCR of genomic DNA. All three transgenic lines indicated the presence of the expected PCR products for the *HPT* and *PgDDS* genes (Fig. 2a). However, no PCR signals were detected in those of wild-type tobacco (Fig. 2a).

Leaves from transgenic and wild-type plants were subjected to RT-PCR analysis to detect *PgDDS* transcription. All three selected transgenic lines showed clear accumulation of *PgDDS* mRNA. In particular, T14 exhibited exceptionally strong transcription of *PgDDS* as compared to the T7 and T9 lines. No PCR signal of *PgDDS* mRNA was found in wild-type plants (Fig. 2b). The accumulation of *PgDDS* mRNA in different parts of transgenic plant (flower bud, leaf, petiole and roots) was examined by RT-PCR. *PgDDS* mRNA was accumulated ubiquitously in all organs of tobacco. However, highest *PgDDS* mRNA accumulation occurred in leaves, the order of *PgDDS* mRNA accumulation was leaves > roots > stems > flower buds (data not shown).

GC/MS analysis of transgenic tobacco

To analyze the content of dammarenediol-II in transgenic tobacco, root extracts were analyzed by gas chromatography-mass spectrometry (GC/MS). All transgenic lines (T7, T9, and T14) showed a new product (dammarenediol-II) at a retention time of 37.6 min (Fig. 3a) as compared to the wild-type control, which is identical to the retention time of authentic dammarenediol-II (Fig. 3a). Furthermore, the MS spectra revealed that the fragmentation pattern of dammarenediol-II products with an ion at m/z 426 completely matched the MS spectra of authentic dammarenediol-II (Fig. 3b). Accumulation of dammarenediol-II in transgenic tobacco occurred in an organ-specific manner (roots > stems > leaves > flower buds) (Fig. 3c). In T14 plants, the amount of dammarenediol-II in the roots was at least threefold higher (157.8 μg g^{-1} DW) than in the other two lines (T7 and T9). Accumulation of dammarenediol-II in

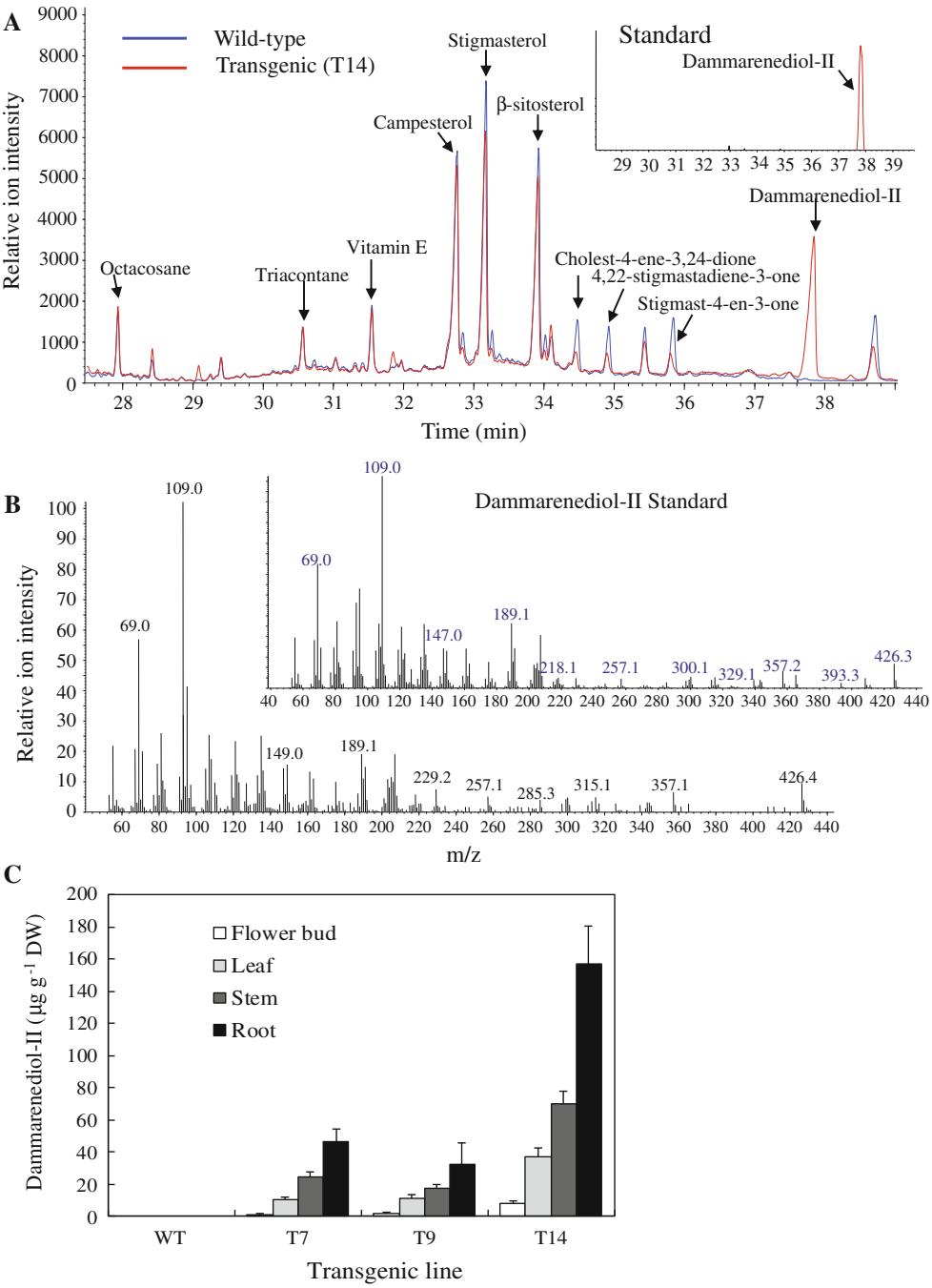

Fig. 3 GC–MS analyses of the extracts from wild-type and transgenic tobacco overexpressing *PgDDS*. **a** GC chromatogram of root extracts of transgenic (T14) and wild-type tobacco. The *blue line* represents wild-type tobacco (control); the *red line* represents transgenic tobacco. *Inset* represent GC chromatogram of standard dammarenediol-II. **b** MS spectrum of the identified dammarenediol-II peak obtained from a transgenic line (T14). *Inset* represents MS spectrum of standard dammarenediol-II. **c** Contents of dammarenediol-II in the leaves, stems, roots, and flower buds of transgenic tobacco overexpressing *PgDDS*. Samples from wild-type and transgenic lines (T7, T9, and T14) were processed to measure dammarenediol-II by GC–MS. Data are mean values with the standard deviation obtained from three independent plants

the roots in the T7 and T9 transgenic lines was 46.8 and 32.0 μg g^{-1} DW, respectively (Fig. 3c). In the wild-type plants, no traceable dammarenediol-II signal was detected (Fig. 3c).

Our result clearly demonstrated that the production of dammarenediol-II in transgenic tobacco is negatively related to phytosterol accumulation. In the T14 line, phytosterol (β-sitosterol, campesterol, and stigmasterol) and phytosterol derivatives (cholest-4-ene-3,24-dione, 4,22-stigmastadiene-3-one, and stigmast-4-en-3-one) were clearly reduced as compared to those of wild-type plants (Fig. 3a). The reduction of phytosterols in the T7 and T9 lines was also similar to that of the T14 line but less prominent (data not shown). Octacosane, triacontane, and vitamin E contents did not differ between transgenic and wild-type plants (Fig. 3a).

There was no obvious phenotypic difference between wild-type and transgenic plants when the plants were grown in plastic culture bottles. When in vitro cultured plantlets of transgenic tobaccos were transferred to soil,

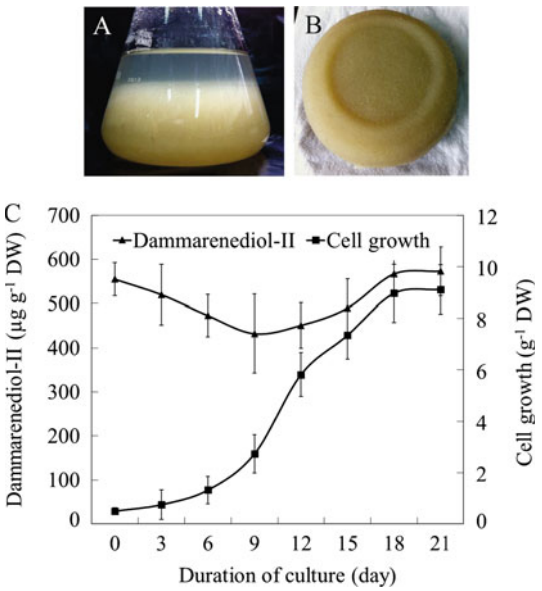

Fig. 4 Production of dammarenediol-II via cell suspension culture of transgenic tobacco. **a** Cell suspension in a 500-ml flask containing MS liquid medium with 1.0 mg/l 2,4-D after 21 day of culture. **b** Harvested cells after 21 day of culture for analysis. **c** Growth of cells (*filled square*) and production of dammarenediol-II (*filled triangle*) during 3 weeks of cell suspension culture of the T14 line. *Vertical error bars* indicate mean values with the standard deviation obtained from three independent plants

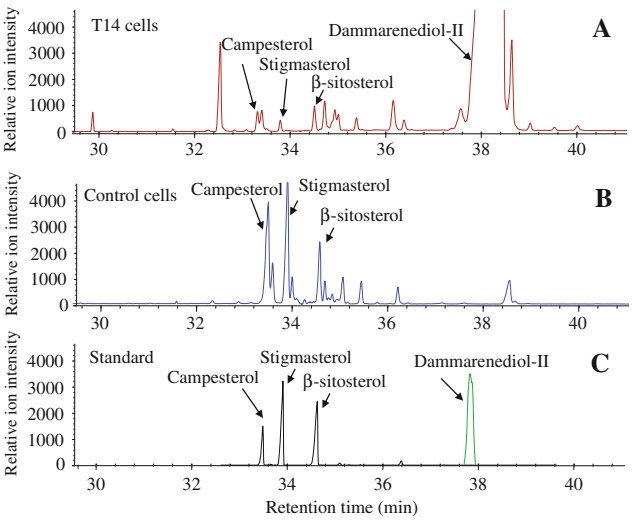

Fig. 5 GC–MS analysis of phytosterols (campesterol, β-sitosterol, and stigmasterol) and dammarenediol-II in cell suspensions of wild-type and transgenic tobacco overexpressing *PgDDS*. **a** GC chromatogram extracted from the T14 transgenic cell line. **b** GC chromatogram extracted from a wild-type cell line. **c** GC chromatogram of authentic phytosterols (campesterol, β-sitosterol, and stigmasterol) and dammarenediol-II

wild-type plants and transgenic lines T7 and T9 were acclimatized after exposure to open air conditions, and all successfully set seeds. However, the T14 line, which contains high dammarenediol-II, wilted soon after transfer to open air exposure, within several hours (data not shown).

Production of dammarenediol-II by cell suspension culture of transgenic lines

The production of plant materials by cell suspension culture is more advantageous because they are free from natural limitations such as seasonal climate and geographical environment. To establish the cell suspension culture, a callus was induced from root segments of transgenic line T14, transferred into liquid medium in a 250-ml Erlenmyer flask and maintained for 3-week subculture intervals. When free floating cell suspension was established (Fig. 4a, b), 5 g of packed cell volume was inoculated in 1,000-ml flask containing 400-ml MS liquid medium. The growth and dammarenediol-II production of cells were assessed at 3-day intervals during 21 days of culture. Fresh weight actively increased during days 9–18 of the culture (Fig. 4c). Approximately an 18.2-fold increase in fresh weight was attained after 21 days of culture (Fig. 4c). The amount of dammarenediol-II production reached 573 μg g^{-1} dry weight after 3 weeks of culture, which is 3.6-fold higher than that of root extracts of T14 plants. The total amount of dammarenediol-II production reached 5.2 mg l^{-1}. Production of dammarenediol-II was slightly lowered when cell growth was active (Fig. 4c). In the cell suspension culture, phytosterol amounts (β-sitosterol, campesterol, and stigmasterol) were strongly reduced in the T14 line (Fig. 5a) as compared to non-transgenic control cells (Fig. 5b). Fig. 5c revealed the total ion chromatogram of standard phytosterols (β-sitosterol, campesterol, and stigmasterol) and dammarenediol-II.

Effect of an elicitor on the production of dammarenediol-II in transgenic cells

Various concentrations (5, 10, 20, and 30 μm) of MeJA were added during the later stage (at 15 days of culture) of cell suspension culture of the transgenic T14 line. MeJA treatment resulted in reduced cell growth (Fig. 6) in a dose-dependent manner. However, this treatment did not significantly enhance the production of dammarenediol-II, although the data in Fig. 6 indicate a slight increase in dammarenediol-II production.

Discussion

Dammarenediol-II production in transgenic tobacco

Dammarenediol-II is the basic triterpenoid structure of ginsenoside saponin and is a useful candidate with potentially biologically active triterpenes. DDS catalyzes the

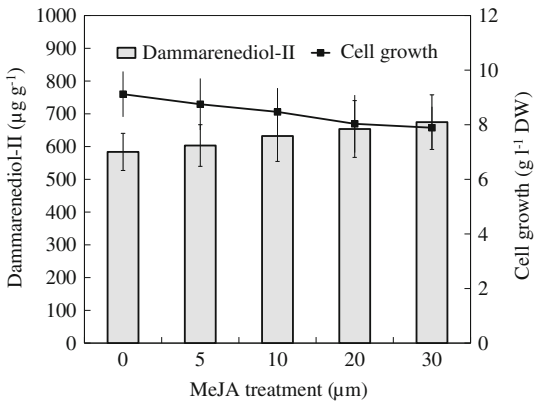

Fig. 6 Growth of cells (*filled square*) and production of dammarenediol-II (*vertical bar*) after MeJA treatment, at various concentrations, of a cell suspension culture of the T14 line. *Vertical error bars* indicate mean values with the standard deviation obtained from three independent plants

cyclization of 2,3-oxidosqualene into dammarene-type triterpenoid skeletons. Dammarenediol-II is exclusively present in some plant species, including *Panax* species, some trees in the *Dipterocarpaceae* family (Scalarone et al. 2005), and *Gynostemma* (Hu et al. 1996) and *Cacalia* species (Spencer 1981). However, dammarenediol-II's precursor, 2,3-oxidosqualene, is prevalent in all eukaryotes. Thus, the heterologous expression of single DDS alone can make it possible to produce dammarenediol-II in any eukaryote. In our previous work, we reported that transgenic tobacco expressing the *PgDDS* gene exhibited resistance to the TMV (Lee et al. 2012). In the present work, we established a production system for dammarenediol-II using a transgenic cell suspension culture.

Dammarenediol-II accumulation in transgenic tobacco overexpressing *PgDDS* occurred in an organ-specific manner, although expression of *PgDDS* was driven by the strong constitutive 35S promoter. Roots exhibited an at least twofold higher content of dammarenediol-II as compared to the leaves of transgenic tobacco plants. However, RT-PCR analysis revealed that leaf showed highest transcription of *PgDDS* among the other organs. The different amounts of dammarenediol-II in different parts of tobacco plants might not result from the transcription of *PgDDS* but from different availability of the 2,3-oxidosqualene precursor in different organs and/or specific accumulation affinity of dammarenediol-II in roots.

Reduced phytosterols in transgenic tobacco producing dammarenediol-II

In this work, dammarenediol-II production in transgenic tobacco plants resulted in reduced phytosterol (β-sitosterol, campesterol, and stigmasterol) contents. However, octacosane, triacontane, and vitamin E contents did not differ between transgenic and wild-type plants. Octacosane and triacontane are the main components of epicuticular wax in tobacco (Cameron et al. 2006). Vitamin E is synthesized in the plastids of plants from precursors derived from the shikimate and methylerythritol phosphate pathways (Hunter and Cahoon 2007). The reduced phytosterol accumulation in transgenic tobacco might be caused by competition for precursors, as both phytosterols and triterpenes in plants are synthesized from the product of cyclization of their common precursor, 2,3-oxidosqualene. Thus, consumption of 2,3-oxidosqualene for the production of dammarenediol-II in transgenic tobacco overexpressing *PgDDS* can restrain the metabolic flow for the biosynthesis of phytosterols, which might result in the reduced sterol accumulation observed in transgenic tobacco.

Transgenic tobacco (T14) producing a high amount of dammarenediol-II failed to survive after soil transfer. It is unclear why this line did not survive in open air. In the transgenic tobacco, sterol amounts were clearly reduced. Sterols are integral components of the membrane lipid bilayer, where, in conjunction with phospholipids, they regulate membrane permeability and fluidity (Mouritsen and Zuckermann 2004; Quartacci et al. 2002). Plant cell membranes are usually composed of β-sitosterol, campesterol, stigmasterol, and stanol esters (Hartmann 1998). The reduced phytosterol content in transgenic tobacco overexpressing DDS can have negative effects on the normal physiological functions in plants. Based on the above reports, the highly reduced phytosterol content in T14 transgenic tobacco plants might result in impaired cell membrane fluidity, which in turn resulted in transpiration disorder under open air exposure. However, this should be verified via further research. There have been some reports that altered terpenoid biosynthesis in transgenic plants results in retarded growth. Aharoni et al. (2003) suggest that the growth retardation in transgenic plants is a result of the depletion of isoprenoid precursors in the plants.

Active dammarenediol-II production via a cell suspension culture

In transgenic tobacco plant line T14, the root extracts contained 157.8 μg g^{-1} DW of dammarenediol-II. The cell suspension culture derived from the roots of the T14 line resulted in a threefold higher amount of dammarenediol-II production (573 μg g^{-1} DW). It is not clear why dammarenediol-II production was strongly increased in the cell culture as compared to the roots of the transgenic plants. In transgenic plants, biosynthesis of terpenoids is strictly controlled because terpenoid accumulation occurs in a tissue- and organ-specific manner (Tholl 2006; Nagegowda 2010). In this work, dammarenediol-II content in tobacco transgenic plants exhibited organ-specific accumulation,

although the expression of dammarenediol-II is driven by a strong 35S promoter. The enhanced dammarenediol-II accumulation in suspended cells as compared to transgenic plants is most likely caused by either the system in plants controlling the regulation of triterpenoids or differences in the precursor supply.

MeJA treatment for dammarenediol-II production in a cell suspension culture

MeJA is distributed throughout higher plants, in which it is derived from jasmonic acid via the octadecanoid pathway and the reaction is catalyzed by jasmonic acid carboxyl methyltransferase (Creelman and Mullet 1997). There have been many approaches to increasing the yield of ginsenosides from ginseng cell or organ cultures via treatment with MeJA (Wu and Zhong 1999; Lee et al. 2004; Kim et al. 2004, 2009). MeJA treatment resulted in the increased accumulation of all mRNAs involved in saponin biosynthesis in adventitious roots of *P. ginseng* as compared with the control (Han et al. 2006). However, MeJA treatment of transgenic tobacco cells overexpressing *PgDDS* increased dammarenediol-II production only slightly. A similar result was obtained in transgenic *Bupleurum falcatum* overexpressing *B. falcatum* squalene synthase, in which MeJA treatment failed to stimulate the mRNA accumulation of triterpene (β-amyrin) synthase (Kim et al. 2011). By contrast, MeJA treatment of wild-type roots strongly stimulated β-amyrin synthase mRNA accumulation (Kim et al. 2011). Kim et al. (2011) suggest that overexpression of squalene synthase in *B. falcatum* regulates the downstream genes for triterpene and phytosterol biosynthesis more powerfully than an elicitor treatment. The small increase in dammarenediol-II content in transgenic tobacco cells in response to MeJA treatment might be due to the limited flux of precursor because a large amount of precursor was already consumed for dammarenediol-II production. Conclusively, in transgenic tobacco cells overexpressing *PgDDS,* MeJA treatment is not effective for further enhancing dammarenediol-II production.

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