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Expression and RNA Interference-Induced Silencing of the Dammarenediol Synthase Gene in *Panax ginseng*

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Panax ginseng **is one of the most highly valued herbal medicines in the Orient, where it has gained an almost magical reputation for being able to maintain the quality of life. The root of ginseng contains noble tetracyclic triterpenenoid saponins (ginsenosides), which are thought to be the major effective ingredients in *P. ginseng*. The first committed step in ginsenoside synthesis is the cyclization of 2,3-oxidosqualene to dammarenediol II by oxidosqualene cyclase, dammarenediol synthase (DDS). The gene encoding DDS has been characterized. Here, we investigated the expression of the *DDS* gene together with the genes involved in ginsenoside biosynthesis (*SS*, *SE*, *PNX*, *PNY*, *PNY2* and *PNZ*). Expression of *DDS* mRNA was higher in flower buds compared with root, leaf and petiole of ginseng plants. Elicitor (methyl jasmonate) treatment up-regulated the expression of *DDS* mRNA. Ectopic expression of *DDS* in a yeast mutant (*erg7*) lacking lanosterol synthase resulted in the production of dammarenediol and hydroxydammarenone which were confirmed by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC/APCIMS). RNA interference (RNAi) of *DDS* in transgenic *P. ginseng* resulted in silencing of *DDS* expression which leads to a reduction of ginsenoside production to 84.5% in roots. These results indicate that expression of *DDS* played a vital role in the biosynthesis of ginsenosides in *P. ginseng*.**

Keywords: Ginsenoside — 2,3-Oxidosqualene cyclases — RNAi — Saponins — Triterpene.

Abbreviations: DDS, dammarenediol synthase; EST, expressed sequence tag; IBA, indole-3-butyric acid; LC/APCIMS, liquid chromatography-atmospheric pressure chemical ionization mass spectrometry; ORF, open reading frame; OSC, oxidosqualene cyclase; *PNA*, peptide nucleic acid; RNAi, RNA interference; RT–PCR, reverse transcripion–PCR; SE, squalene epoxidase; SS, squalene synthase; TLC, thin-layer chromatography.

The nucleotide sequence data reported in this paper appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB122080.

Introduction

Triterpenoid saponins are secondary metabolites of isoprenoidal natural products present in higher plants. They exhibit a wide range of both structural diversity and biological activity among plant species. These molecules also have considerable commercial value and are exploited as drugs and medicines (Hostettmann and Marston 1995, Vogler et al. 1999, Shibata 2001). The natural role of saponins in plants is likely to be in defense against attack by pathogens and pests (Osbourn 1996).

The root of *Panax ginseng* is one of the most famous and widely used medicinal plants (Shibata 2001). Its preparations have been taken orally as health products or natural drugs for a long time. It is generally believed that ginsenosides are mainly responsible for the pharmacological activities of ginseng (Vogler et al. 1999, Shibata 2001). The first committed step in ginsenoside synthesis is the cyclization of 2,3-oxidosqualene to dammarenediol II, catalyzed by the dammarenediol synthase (DDS) of the oxidosqualene cyclase (OSC) group (Kushiro et al. 1997). Ginsenosides are believed to be synthesized from dammarenediol II after hydroxylation by cytochrome P450 (Shibuya et al. 2006) and subsequently by glycosylation via glycosyltransferase (Kushiro et al. 1997, Kushiro et al. 1998, Haralampidis et al. 2001a, Choi et al. 2005). Seven dammarene-type tetracyclic triterpenes (ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1) are reported as major constituents, and only ginsenoside Ro is an oleanane-type pentacyclic triterpene, which is a minor component in *P. ginseng*. Each ginsenoside has been shown to have different pharmacological effects, including immune system modulation, antistress activities, antihyperglycemic activities, and anti-inflammatory, antioxidant and anticancer effects (Fig. 1) (Briskin 2000, Shibata 2001).

Triterpenoid saponins, like sterols, are synthesized from mevalonic acid via the isoprenoid pathway.

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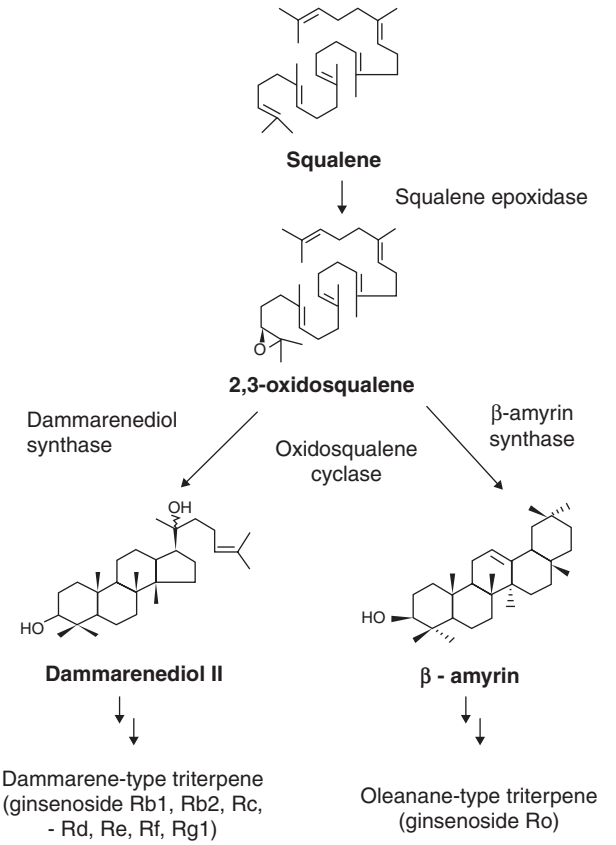

Fig. 1 Biosynthetic pathway of ginsenosides from squalene in *P. ginseng*. Triterpene undergoes oxidation, glycosylation and finally is converted into triterpene saponins (ginsenosides).

During triterpenoid saponin synthesis, 2,3-oxidosqualene is cyclized to one of a number of different potential products by OSCs. The primary components of triterpenoid saponins are oleanane (β-amyrin), ursane (α-amyrin), lupeol or dammarene-type triterpenoid skeletons. The former three compounds are pentacyclic triterpenes and the latter is a tetracyclic titerpene. Recently, a large number of pentacyclic triterpene synthases have been cloned and functionally characterized from various plant species, β-amyrin synthase from *P. ginseng* (Kushiro et al. 1998), *Medicago truncatula* (Iturbe-Ormaetxe et al. 2003), *Glycyrrhiza glabra* (Hayashi et al. 2001) and *Pisum sativum* (Morita et al. 2000), α-amyrin synthase from *Pisum sativum* (Morita et al. 2000), lupeol synthase from *Olea europaea* (Shibuya et al. 1999), *Taraxacum officinale* (Shibuya et al. 1999) and *Arabidopsis thaliana* (Herrera et al. 1998), and multifunctional triterpene synthase from *P. sativum* (Morita et al. 2000) and *Arabidopsis thaliana* (Kushiro et al. 2000). The gene encoding dammarenediol II synthase, a tetracyclic triterpene, has recently been characterized from *P. ginseng* by Tansakul et al. (2006).

There are many reports of the characterization of the gene for OSCs by ectopic expression in an *ERG7*-deficient yeast mutant. Mutation in an *OSC* gene in oat resulted in failure to synthesize triterpene saponins (Haralampidis et al. 2001b). RNA interference (RNAi) has been used for the identification or validation of biological functions of the targeted transcripts of individual genes or small groups of genes (Limpens et al. 2004, Ogita et al. 2003). However, there is no report to assess whether the *OSC* gene regulates the final product of triterpene saponins by RNAi or overexpression of the gene in transgenic plants.

Here, the expression of *DDS* together with that of other genes involved in ginsenoside biosynthesis in *P. ginseng* has been analyzed. Moreover, the response of those genes to elicitor (methyl jasmonate) treatments has been analyzed. It was demonstrated that the post-transcriptional gene silencing of *DDS* by RNAi strongly suppresses the expression of *DDS* mRNA and resulted in reduced ginsenoside accumulation in ginseng roots.

Results and Discussion

Isolation, sequence analysis and genomic organization of the DDS gene

A full-length cDNA coding DDS was isolated from expressed sequence tags (ESTs) in the flower cDNA library of *P. ginseng*. Recently, Tansakul et al. (2006) reported the peptide nucleic acid (*PNA*) cDNA (accession No. AB265170 in the DDBJ sequence database) determining DDS by functional analysis of heterologous expression in the *erg7* yeast mutant. The *PNA* cDNA has the same sequence as our *DDS* cDNA (accession No. AB122080 in the DDBJ/EMBL/GenBank sequence database). However, the detailed function of the *DDS* gene in *P. ginseng* is yet to be characterized.

The *DDS* cDNA was 2,562 bp long and carried a 2,309 bp full open reading frame (ORF) fragment (Fig. 2). The deduced amino acids of DDS (769 amino acids with a predicted molecular mass of 88.4 kDa) were found to be 56.2, 55.7, 50.5 and 47.9% identical to those of PNY2 (β-amyrin synthase in *P. ginseng*), PNY (β-amyrin synthase in *P. ginseng*), PNX (cycloartenol synthase in *P. ginseng*) and PNZ (lanosterol synthase in *P. ginseng*) (Suzuki et al. 2006). The relatively low identities of the DDS protein with other OSC proteins of *P. ginseng* suggest that this gene belongs to a new type of OSC gene family in *P. ginseng*. Comparison of the amino acid sequences of OSCs in *P. ginseng* revealed that six copies of the QW-motif, which occurs repeatedly in the sequences of all known OSCs (Poralla et al. 1994), are present in the DDS sequence, as well as a DCTAE motif implicated in substrate binding, that are characteristic for this family of enzymes (Abe and Prestwich 1994, Abe and Prestwich 1995).

A phylogenic tree constructed from the deduced amino acid sequences of plant OSCs is shown in Fig. 3.

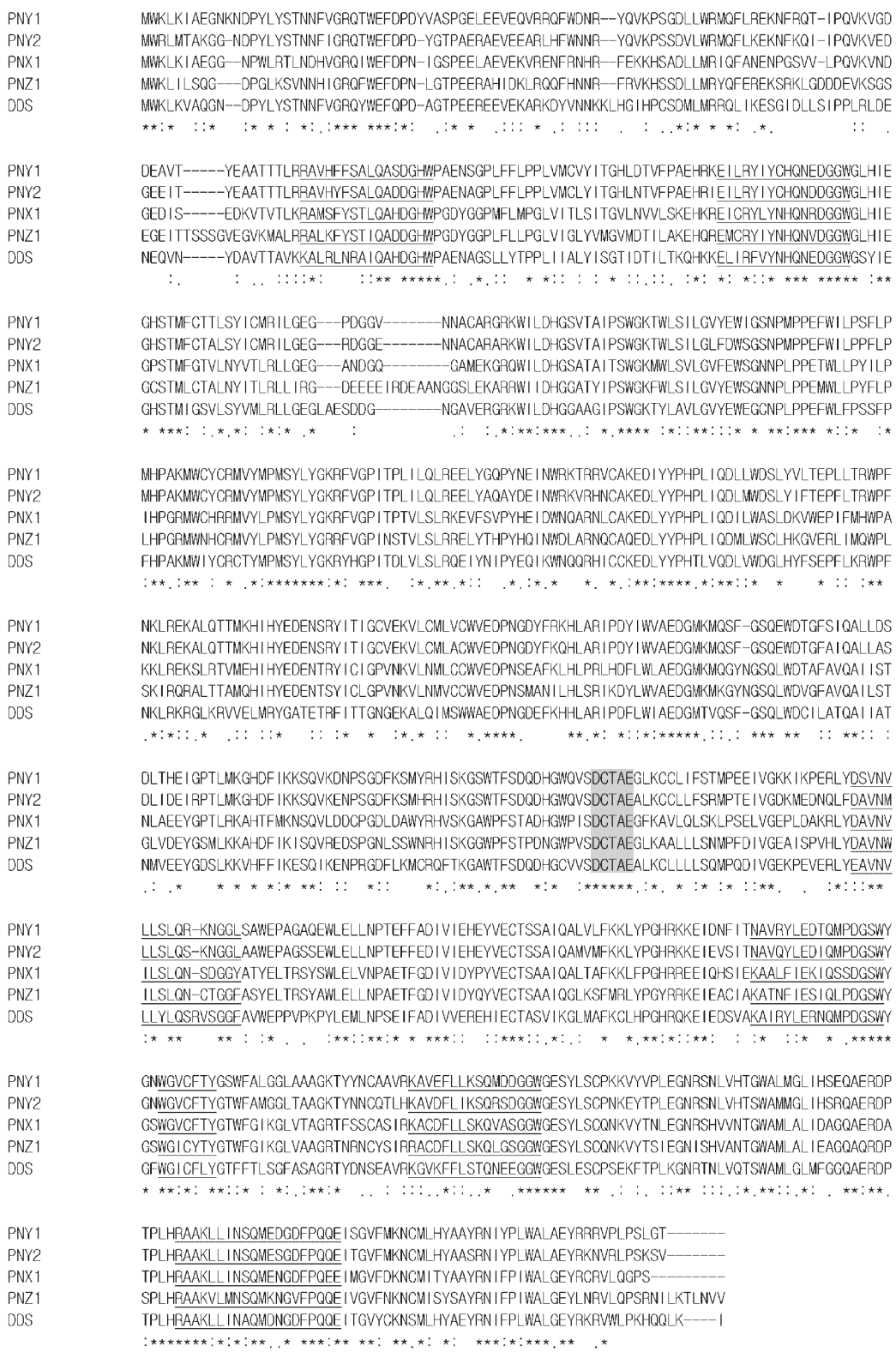

Fig. 2 Alignment of the deduced amino acid sequences of various types of OSCs in *P. ginseng*. The sequence identities between the DDS sequence and the other sequences are indicated in parentheses at the end of each sequence. The DDBJ/GenBank/EMBL accession numbers of the sequences are AB122080 (DDS, dammarenediol synthase), AB009030 (PNY, β-amyrin synthase), AB009029 (PNX, cycloartenol synthase), AB014057 (PNY2, β-amyrin synthase) and AB009031 (PNZ, lanosterol synthase).

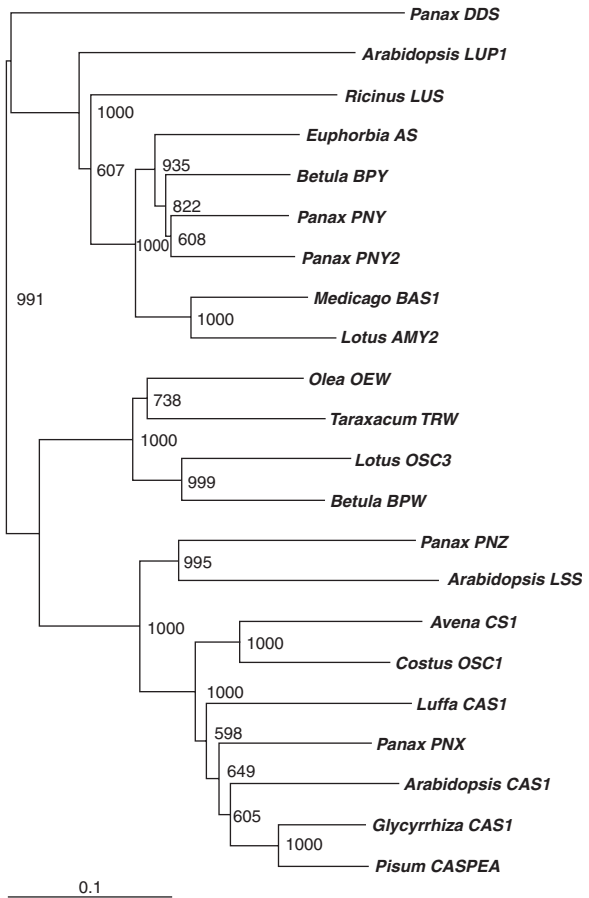

Fig. 3 Phylogenic tree of the deduced amino acid sequences of *DDS* and other plant OSCs. The DDBJ/GenBank/EMBL accession numbers of the sequences are AB122080 for dammarenediol synthase (*DDS*) in *P. ginseng*, AB055512 for β-amyrin synthase (*BPY*) in *Betula platyphylla*, AB009030 for β-amyrin synthase (*PNY*) in *P. ginseng*, AB206469 for β-amyrin synthase (*AS*) in *Euphorbia tirucalli*, AB014057 for β-amyrin synthase (*PNY2*) in *P. ginseng*, AJ430607 for β-amyrin synthase (*BAS1*) in *Medicago truncatula*, AF478455 for multifunctional β-amyrin synthase (*AMY2*) in *Lotus japonicus*, U49919 for lupeol synthase (*LUP1*) in *Arabidopsis thaliana*, DQ268869 for lupeol synthase (*LUS*) in *Ricinus communis*, AB025343 for lupeol synthase (*OEW*) in *Olea europaea*, AB025345 for lupeol synthase (*TRW*) in *Taraxacum officinale*, AB181245 for lupeol synthase (*OSC3*) in *Lotus japonicus*, AB055511 for lupeol synthase (*BPW*) in *Betula platyphylla*, AB009029 for cycloartenol synthase (*PNX*) in *P. ginseng*, AJ311790 for cycloartenol synthase (*CS1*) in *Avena strigosa*, AB058507 for cycloartenol synthase (*OSC1*) in *Costus speciosus*, AB033334 for cycloartenol synthase (*CAS1*) in *Luffa aegyptiaca*, AB025968 for cycloartenol synthase (*CAS1*) in *Glycyrrhiza glabra*, D89619 for cycloartenol synthase (*CASPEA*) in *Pisum sativum*, A49398 for cycloartenol synthase (*CAS1*) in *Arabidopsis thaliana*, AB009031 for lanosterol synthase (*PNZ*) in *P. ginseng*, and DQ508794 for lanosterol synthase (*LSS*) in *Arabidopsis thaliana*. Phylogenetic trees of plant OSC distances between each clone and group are calculated with the program CLUSTAL W. The distance between each clone was calculated using CLUSTAL W. Bootstrap analysis values are shown at the nodal branches. The indicated scale represents 0.1 amino acid substitution per site.

Phylogenetic analysis indicates that DDS shares greater amino acid sequence similarity with lupeol synthases than it does with β-amyrin synthases or cycloartenol synthases from higher plants. DDS might be a new class of plant triterpene synthase.

Expression of DDS and other OSCs in different parts of the plant

The expression of genes related to triterpene (ginsenoside) biosynthesis (*SS*, *SE*, *DDS*, *PNX*, *PNY1*, *PNY2* and *PNZ*) in different parts of the plant (flower bud, leaf, petiole and roots) was examined by reverse transcription–PCR (RT–PCR). *DDS* was expressed ubiquitously in various organs of *P. ginseng* such as the flower bud, leaf, petiole and root (Fig. 4). Significant accumulation of *DDS* mRNA occurred in flower buds and roots (Fig. 4). The high expression of *DDS* mRNA in the flower bud encouraged the isolation of a cDNA clone from flower ESTs of *P. ginseng*.

Response of ginseng OSCs to methyl jasmonate treatment

The expression of *DDS* in adventitious roots of *P. ginseng* was also examined by Nothern blot after 0, 3 and 24 h of treatment with 1 and 10 mM methyl jasmonate (Fig. 5). Methyl jasmonate treatment resulted in obvious accumulation of *DDS* mRNA in cultured roots compared with the control (Fig. 5A). In the control, *DDS* mRNA was faintly detected, but the expression was maintained at the same level during the treatment (Fig. 5A).

The expression of *SS*, *SE*, *PNX*, *PNY*, *PNY2* and *DDS* in adventitious roots of *P. ginseng* was examined by RT–PCR after 24 h of 0, 10, 20 and 30 μM methyl jasmonate treatments (Fig. 5B). Methyl jasmonate treatment resulted in the obvious accumulation of the mRNA of all OSCs (*DDS* and *PNY*) in adventitious roots as compared with the control (Fig. 5B). However, *PNY2* mRNA was constantly expressed by methyl jasmonate treatment. *DDS* accumulation was more obvious than the other *PNY*, *PNY2* coding for β-amyrin synthesase. In contrast, accumulation of *PNX* (cycloartenol synthase) and *PNZ* (lanosterol synthase) mRNA was relatively constant or decreased slightly (Fig. 5B). This result indicates that the *DDS* gene is highly responsive to the elicitor treatment.

It was reported that exogenously applied elicitor, methyl jasmonate, stimulates the biosynthesis of many secondary metabolites (Gundlach et al. 1992). Methyl jasmonate has also been used to enhance the ginsenoside content in cell suspension cultures (Lu et al. 2001) and root culture of *P. ginseng* (Han et al. 2006). The genes involved in triterpene biosynthesis were up-regulated by methyl jasmonate (Lee et al. 2004, Han et al. 2006).

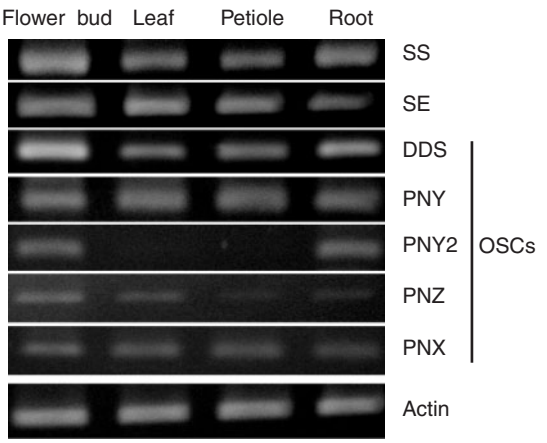

Fig. 4 Organ-specific expression of *DDS* in *P. ginseng*. The actin gene was used as a standard of RNA loading.

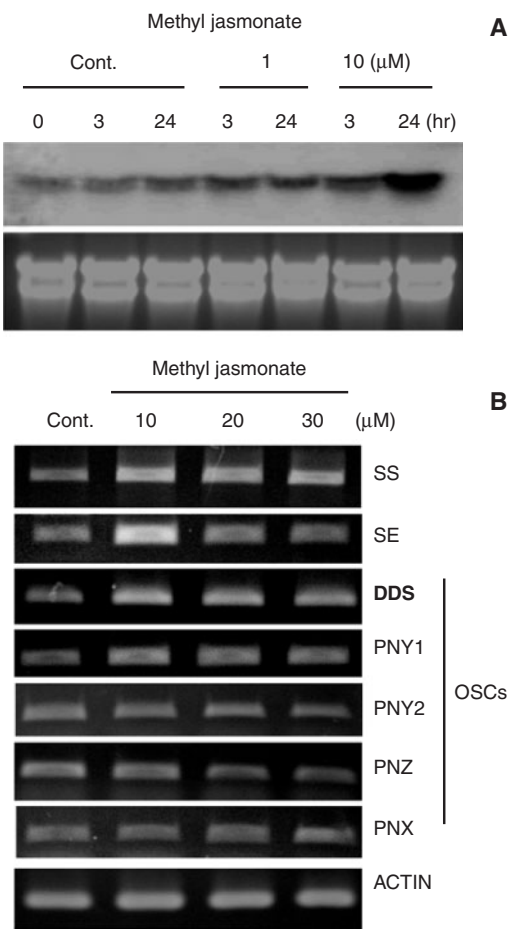

Fig. 5 Accumulation of mRNA involved in ginsenoside biosynthesis in adventitious roots of *P. ginseng* cultured on medium with methyl jasmonate. (A) Expression of *DDS* mRNA after 0, 3 and 24 h in 1 and 10 μM methyl jasmonate by Northern blot analysis. (B) Expression of *SS*, *SE*, *PNX*, *PNY*, *PNY2* and *DDS* genes after 24 h of 10, 20 and 30 μM methyl jasmonate treatment by RT–PCR. The actin gene was used as the standard for RNA loading.

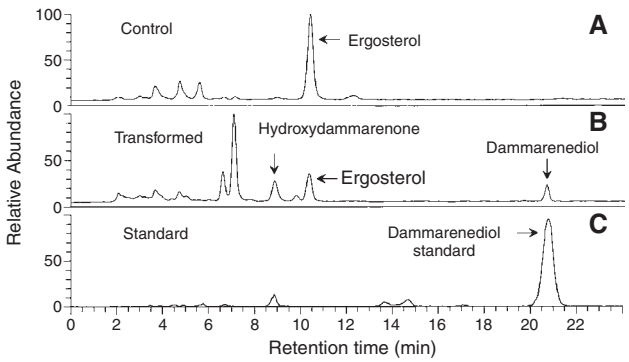

Fig. 6 Total ion chromatogram of LC/APCIMS analysis of the *DDS* product in yeast. (A) LC chromatogram of the cell extract from yeast with empty vector as a control. (B) LC chromatogram of the cell extract from yeast with pYES2-*DDS*. (C) LC chromatogram of a dammarenediol standard.

Ectopic expression of the DDS cDNA in the ERG7-deficient yeast mutant

A recent report of Tansakul et al. (2006) on *PNA* cDNA demonstrates that it has the same sequence as our *DDS* cDNA which encodes DDS. They have observed the production of dammaranediol II by the expression of *PNA* in an *ERG7*-deficient yeast mutant (GIL77). We inserted the ORF of *DDS* cDNA under the galactose-inducible *GAL* promoter in the pYES2.1 expression vector. The *DDS* gene was expressed in the *ERG7*-deficient yeast mutant (Karst and Lacroute 1977), which lacks a functional lanosterol synthase gene. The yeast extracts were analyzed by analysis of the total ion chromatogram of liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC/APCIMS). Dammarenediol and hydroxydammarenone were identified by the total ion chromatogram of LC/APCIMS and the retention times of peaks were 20.74 and 8.89 min, respectively (Fig. 6B). No signal of dammarenediol and hydroxydammarenone was detected in the control cells carrying the empty vector, although some peaks were detected (Fig. 6A). To analyze the clear fragmentation pattern, the extract of the pYES2.1-*DDS* transformant was loaded on a preparative silica gel thin-layer chromatography (TLC) plate and gave two clear spots, which were absent in the control pYES2.1 transformant with empty vector (data not shown). The two new spots were extracted from silica gel TLC and analyzed by LC/APCIMS (Fig. 7). Hydroxydammarenone and dammarenediol were identified by LC/APCIMS (Fig. 7). The LC/APCIMS fragmentation pattern was the same as that of authentic dammarenediol (*m*/*z* 409 $[M+H\text{-}2H_2O]^+$, 427 $[M+H\text{-}H_2O]^+$) (Fig. 7A). Tansakul et al. (2006) demonstrated that the expression of *PNA* in the *ERG7*-deficient yeast mutant (GIL77) resulted in dammarenediol II

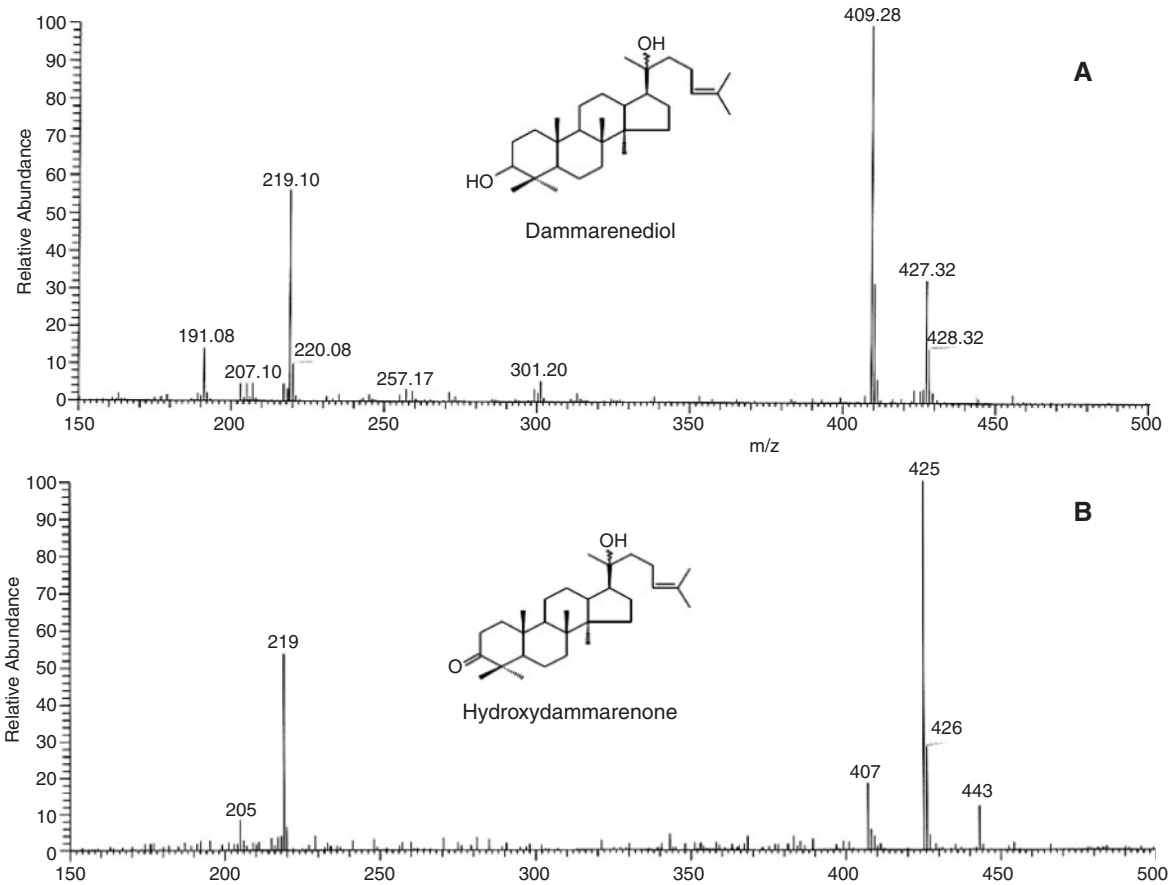

Fig. 7 LC/APCIMS analysis of the *DDS* product in yeast. (A) LC/APCIMS chromatogram of peaks detected in yeast with pYES2-*DDS*, indicating the same molecular weight as hydroxydammarenone (*m/z* 443 $[M+H]^+$, 425 $[M+H\text{-}H_2O]^+$, 407 $[M+H\text{-}2H^2O]^+$) and dammarenediol (*m/z* 409 $[M+H\text{-}2H_2O]^+$, 427 $[M+H\text{-}H_2O]^+$).

synthesis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR). Our LC/APCIMS fractions of the *DDS* gene product in the *ERG7*-deficient yeast mutant provided by Karst and Lacroute (1977) was interpreted as the same component demonstrated by Tansakul et al. (2006). We detected another component at 8.89 min of retention time which is hydroxydammarenone (*m/z* 443 $[M+H]^+$, 425 $[M+H\text{-}H_2O]^+$, 407 $[M+H\text{-}2H_2O]^+$). Hydroxydammarenone lacks H at the C-3 of the dammarenediol skeleton compared with dammarenediol (Fig. 7B). The chromatogram of the LC/APCIMS spectra is an exact match to the MS spectra of hydroxydammarenone extracted from dammar gum of Dipterocarpaceae trees (van der Doelen et al. 1998). The production of hydroxydammarenone might be derived from the oxidation of OH at the C-3 of the dammarenediol skeleton in in vivo conditions or during extraction from yeast, and not from multiple functions of the *DDS* gene.

RNAi-mediated silencing of DDS results in ginsenoside deficiency in P. ginseng plants

To analyze functionally the role of *DDS* in plants, transgenic *P. ginseng* plants constitutively expressing a *DDS*-RNAi were produced using the RNAi destination vector pK7GWIWG2(I) (Fig. 8A) by *Agrobacterium tumefaciens*-mediated genetic transformation of *P. ginseng* (Choi et al. 2001). Kanamycin-resistant *DDS*-RNAi somatic embryos were developed during the subculture on selection medium. Selected cotyledonary embryos were germinated on MS medium supplemented with 20 mM GA_3 and 50 mg l^{-1} kanamycin for 1 month, and the plants were maintained in culture bottles containing 1/2 MS medium with 2% sucrose (Fig. 8B). There is no obvious morphological change in *DDS*-RNAi transgenic plants compared with the wild type.

Since growth of *P. ginseng* is extremely slow in the field, the in vitro culture system of the roots was adopted to analyze the characteristics of transgenic plants.

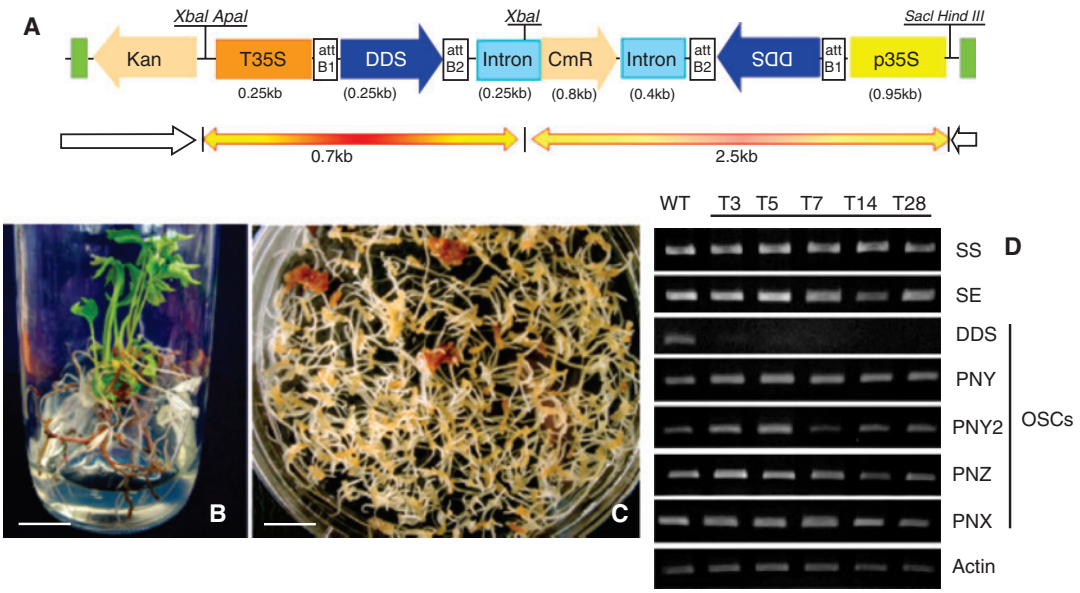

Fig. 8 RNA interference of *DDS* resulted in reduced ginsenoside production in *P. ginseng*. (A) Schematic representation of pK7GWIWG2(II)-*DDS* RNAi vector. (B) An RNAi transgenic plant (bar = 1.7 cm). (C) Adventitious root production from transgenic lines (bar = 1.2 cm). (D) RT–PCR analysis of wild-type and *DDS*-RNAi transgenic plants. The actin gene was used as the standard for RNA loading.

Induction and proliferations of roots were accomplished according to the protocol of Han et al. (2006). The adventitious roots derived from independent transgenic ginseng were regarded as an independent line (Fig. 8C). Thirty-seven kanamycin-resistant transformants were screened to analyze the *DDS* transcript level by RT–PCR analysis. Five lines designated as *DDS*-RNAi-3, -5, -7, -14 and -28, have shown strong silencing of *DDS* transcription (Fig. 8D). However, other *SS*, *SE* and OSCs transcripts were constantly expressed (Fig. 8D). As RNAi is a homology-dependent process, a specific gene or multiple members of a gene family can be silenced by targeting highly conserved sequence domains. *DDS*-RNAi construction using the 2,297–2,546 bp specific fragment of *DDS* cDNA resulted in specific silencing of the *DDS* transcript.

HPLC analysis revealed that the ginsenoside content in RNAi transgenic roots was markedly reduced in all transgenic lines, with a corresponding decrease in *DDS* transcript level (Figs. 8D, 9). Compared with a non-transformed line, the total ginsenoside content (ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2 and Rd) in lines 3 and 7 was reduced to 84.5 and 79.6%, respectively (Fig. 9B). The HPLC chromatogram revealed that all the ginsenosides were decreased in the T3 RNAi line (Fig. 9A). These results provide direct evidence for the expression of *DDS* in *P. ginseng*, and suggests its vital role in determining the biosynthesis of ginsenosides in *P. ginseng*. Overexpression of *DDS* will be useful for the enhanced production of pharmacologically important ginsenosides in *P. ginseng* or other plant species by genetic transformation.

Materials and Methods

Isolation and sequencing analysis of oxidosqualene cyclase (DDS) of P. ginseng

Total RNA was isolated from flowers of 4-year-old *P. ginseng* C.A. Meyer grown in the field. Poly(A)$^+$ RNA was isolated by using a Poly(A) quick mRNA isolation kit (Stratagene, La Jolla, CA, USA). The SMART cDNA library construction kit (Clonetech, Palo Alto, USA) was used to synthesize cDNA from poly(A)$^+$ RNA. Size-selected cDNA was ligated to λTriplEx2 vector arms and packaged into phage particles with Gigapack III Gold packaging extract (Stratagene, USA). ESTs were obtained from the ginseng flower cDNA library. Single-pass partial sequences were determined with an automated DNA sequencer (Applied Biosystems ABI Prism 3700, USA). A full-length cDNA clone of *DDS* was isolated from ESTs of the ginseng flower cDNA library. A full-length *DDS* gene and its predicted amino acid sequences were analyzed by using the DNASIS program (Hitachi, Japan).

Sequence and phylogenetic analysis

Multiple sequence alignments were generated using the CLUSTAL W program (Thompson et al. 1994). Phylogenetic analysis of deduced amino acid alignments was performed using the neighbor-joining method with the program and TreeView software (Page 1996). Bootstrap analysis with 1,000 replicates was used to assess the strength of nodes in the tree (Felsenstein 1985).

RT–PCR analysis

Total RNAs were isolated from flower buds, leafs, petioles and roots of field-grown *P. ginseng*. They were then reverse-transcribed by the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The first-strand DNAs were used as template for RT–PCR analysis, performed as follows: 96°C for 5 min; then 30 cycles of 96°C for 30 s, 60°C for 30 s, and 72°C

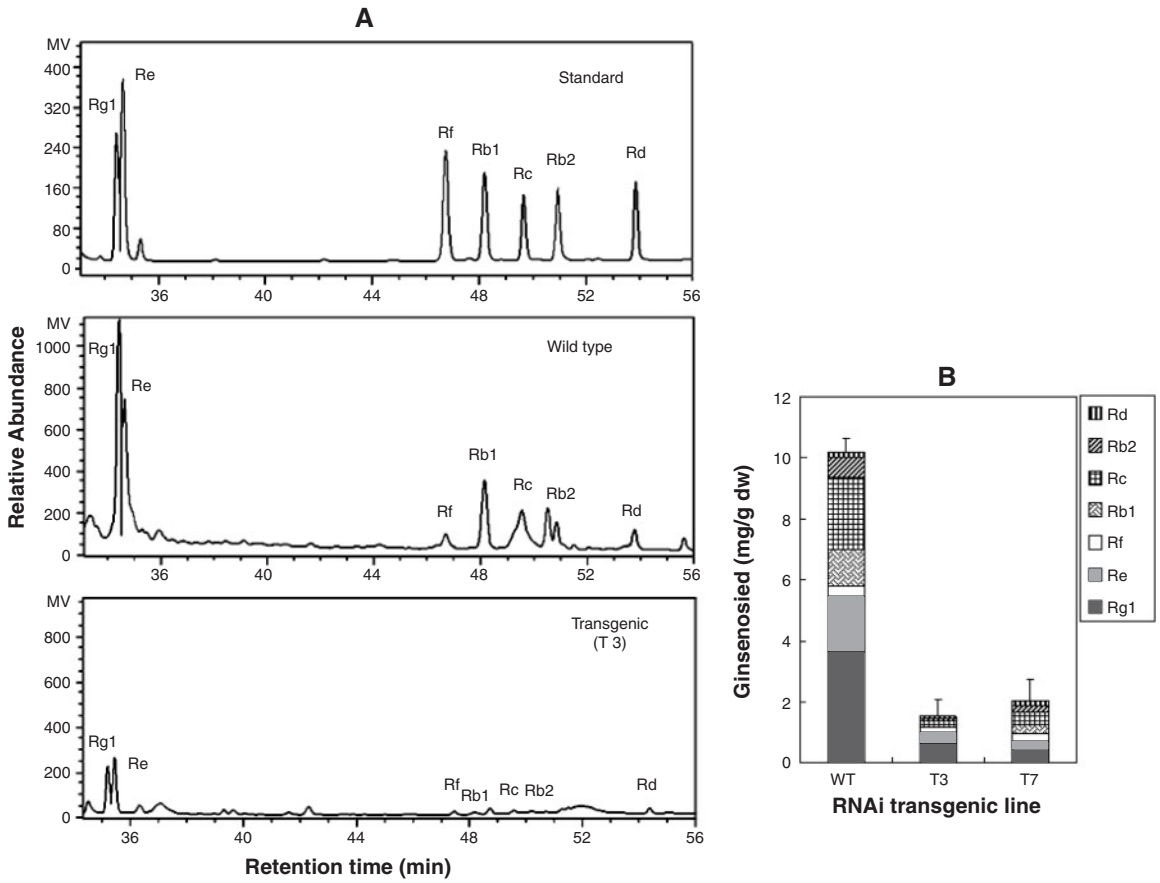

Fig. 9 HPLC analysis of ginsenosides of transgenic *DDS*-RNAi lines. (A) Authentic standard ginsenosides (top) and ginsenoside contents in the wild-type (middle) and *DDS*-RNAi transgenic roots (bottom). (B) Ginsenoside contents in T3 and T7 transgenic lines.

for 1 min; with a final 10 min extension at 72°C. Primers included 5′-ATGGGAAGTTTGGGGGCAATTCT-3′ and 5′-GTTCTC ACTGTTTGTTCAGTAGTAGGTT-3′ for *P. ginseng SS* (squalene synthase, AB115496); 5′-AGCAGCAGTTGACAAAGG-3′ and 5′-GCCACATTCGTTTTGGTGAAGG-3′ for *P. ginseng SE* (squalene epoxidase, AB003516); 5′-TCATCAGATGGCTCAT GGTACG-3′ and 5′-TCTCCTCCTGTGGGAAATCACC-3′ for *P. ginseng PNX* (cycloartenol synthase, AB009029); 5′-TATCC TGGACACCGAAAGAAGG-3′ and 5′-CTCCACTTATTTCC TGTTGGGG-3′ for *P. ginseng PNY* (β-amyrin synthase, AB 009030); 5′-GTGGATTTTCTAATAAAATCGCAACGCAG-3′ and 5′-TTTCATTTGAGTATTGGCAGGCCG-3′ for *P. ginseng PNY2* (β-amyrin synthase, AB014057); and 5′-ATGTGGAA GCTGAAGGTTGCTCAAGGA- 3′ and 5′-TTAAATTTTGA GCTGCTGGTGCTTAGGC-3′ for *P. ginseng DDS* (dammarenediol synthase, AB122080). RT–PCR analysis of β-actin (5′- CGTGATCTTACAGATAGCTTCATGA-3′ and 5′-AGAGA AGCTAAGATTGATCCTCC-3′) was used as the control to check for RNA integrity and accuracy of loading.

Expression of DDS by methyl jasmonate treatment

Flower buds, roots, petioles and leaves from 4-year-old ginseng plants that were grown in early spring were used for organ-specific expression of *DDS*, *SS*, *SE*, *PNX*, *PNY*, *PNY2* and *PNZ*.

Adventitious roots from ginseng were induced on 1/2 MS (Murashige and Skoog 1962) solid medium lacking NH_4NO_3 comprising 3.0 mg l^{-1} indole-3-butyric acid (IBA) and 3% sucrose. Then the roots were excised in 10 mm lengths (300 mg of fresh tissue) and were transferred to 100 ml Erlenmeyer flasks containing 30 ml of 1/2 MS liquid medium devoid of NH_4NO_3. To examine the expression of *DDS*, *SS*, *SE*, *PNX*, *PNY*, *PNY2* and *PNZ* by the influence of elicitors, adventitious root segments were cultured in a 1/2 MS liquid medium (devoid of NH_4NO_3 with 3.0 mg l^{-1} IBA and 3% sucrose) with 1, 10, 20 and 30 μM methyl jasmonate for 24 h. Flasks were agitated on a rotary shaker at 100 r.p.m. Each treatment consisted of three flasks, and the experiment was repeated three times. The culture room was maintained at 22 ± 1°C under 20 μmol m^{-2} s^{-1} from white fluorescent lamps.

Northern blot analysis

In order to confirm the expression of *DDS*, total RNA (30 μg) was separated on 1.2% (w/v) formaldehyde/agarose gels from the adventitious root segments cultured in a 1/2 MS liquid medium (devoid of NH_4NO_3 with 3.0 mg l^{-1} IBA and 3% sucrose) with (0, 1 and 10 μM) and without (control) methyl jasmonate for 0, 3 and 24 h. RNAs were blotted onto a Hybond-N^+ membrane (Amersham Pharmacia, Herdfordshire, UK). The full-length *DDS* gene was used for the hybridization probes. The fragments were labeled using the AlkPhos direct system (Amersham Pharmacia, UK). The membrane was pre-hybridized for 30 min and then hybridized with the probe overnight.

Functional expression in the yeast mutant ERG7 and analysis by TLC

To construct an expression plasmid for yeast, the ORF of *DDS* cDNA produced by PCR (25 cycles with 40 s at 94°C, 40 s at 50°C and 2 min at 72°C) with *Pfu* DNA polymerase (Stratagene) was cloned into pYES2.1 using the TOPO TA expression kit (Invitrogen, Groningen, The Netherlands). Two 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′, were used to isolate the cDNA. The PCR product was ligated into pYES2.1/V5-His-TOPO, and transformed the recombinant vector in *Escherichia coli*. The ORF of the cDNA was ligated to the *GAL1* promoter in the sense orientation. The nucleotide sequence of the inserted DNA was confirmed by sequencing. The *erg7* mutant (*MATa erg7 ura3-1 trp1-1*) was kindly provided by F. Karst, Colmar, France (Karst and Lacroute et al. 1977). Cells were grown to the early stationary phase at 30°C in YPD medium containing 1% yeast extract (Oxoid, Basingstoke, UK), 2% peptone (Oxoid), 2% glucose (Merck, Darmstadt, Germany) and 20 μg ml^{-1} ergosterol [ethanol : Tween-80 (1 : 1; v/v)]. Inocula (0.5 ml 100 ml^{-1} of culture medium) were made from pre-cultures grown for 18 h.

The *erg7*-deficient yeast was transformed with pYES2.1/V5-His-TOPO by an electroporation method. The culture conditions, induction by galactose and preparation of the triterpene monoalcohol fraction have been described previously (Kushiro et al. 1998). After galactose induction, cells were collected and refluxed with 2 ml of 20% KOH/50% EtOH for 5 min. After extraction with the same volume of hexane, the extracts were analyzed by ODS-TLC (Merck, solvent mix 5 : 95 water : acetonitrile).

LC/APCIMS analysis of yeast mutant

LC-APCIMS analysis was carried out on a surveyor liquid chromatography system (Thermo Finnigan Co., San Jose, CA, USA), consisting of four solvent pumps, a Rheodyne injector (5 μl loop) and HTP Pal autosampler (CTC Analytics, Zwingen, Switzerland). The analytical column was a Capcell pak C8 column (5 μm, 2.0 × 150 mm, Shiseido Co., Tokyo, Japan) maintained at 40°C, eluted with 90% actonitrile at a flow rate of 0.2 ml min^{-1}. A triple quadrupole mass spectrometer, Finnigan TSQ Quantum Ultra (Thermo Electron Co., San Jose, CA, USA), fitted with an atmospheric pressure chemical ionization (APCI) system was used for detection. Analysis was performed in the positive mode, with a discharge current of 5.0 μA, vaporizer temperature of 300°C and an ion-transfer capillary temperature of 350°C. Nitrogen was used as the sheath gas (40 bar) and as the auxiliary gas (30 bar). Authentic dammarenediol was directly subjected to the same conditions. The representative fragmentation ion values by LC/APCIMS of authentic dammarenediol exhibited *m/z* 409 $[M+H\text{-}2H_2O]^+$, 427 $[M+H\text{-}H_2O]^+$. Dammarenediol II used as standards for TLC and LC/APCIMS analysis was kindly provided by Dr. Sung JW of IL-Wha Co in Korea.

Generation of the RNAi silencing construct

Two primers, including gateway adaptors (Invitrogen Life Technologies, Carlsbad, CA, USA), were designed to amplify the region from nucleotide 2,297 to 2,546 of *DDS*: forward primer, 5′-AAAAAGCAGGCTcggaaacgtgtttggttgcc-3′; reverse primer, 5′-AGAAAGCTGGGTcaaacaacacctatttccgatt-3′ (the upper case letters represent the adaptors). The amplified PCR product was cloned in the pSB1 vector and transferred to the RNAi destination vector pK7GWIWG2(I) (whose *nptII* gene confers kanamycin resistance to plant cells) in *E. coli* DH5α, as described by the manufacturer (Invitrogen Life Technologies). The construct was sequenced and subsequently transferred to *A. tumefaciens* GV3101 cells harboring plasmid pMP90 using standard molecular biology techniques.

Construction of DDS-RNAi transgenic P. ginseng and adventitious root induction from transgenic lines

Genetic transformation of *P. ginseng* was carried out as described in our previous report (Choi et al. 2001). The surviving cotyledonary somatic embryos on the selection medium containing 50 mg l^{-1} kanamycin and 200 mg l^{-1} cefotaxime were detached and transferred to MS medium supplemented with 20 μM GA_3 and 50 mg l^{-1} kanamycin. Then, they were germinated and maintained on half-strength MS medium with 2% sucrose.

Root segments of plants collected from wild-type and the transgenic RNAi line were cultured on a 1/2 MS medium that lacked NH_4NO_3 but contained 3.0 mg l^{-1} IBA and 3% (w/v) sucrose, which was solidified with 0.27% gelrite. After being adjusted to pH 5.8, the medium was autoclaved at 120°C for 15 min. The Petri dishes were incubated for 5 weeks under darkness at 22 ± 1°C. Adventitious roots were excised from the maternal explants and subcultured on the medium with the same composition as that for the initial root induction.

DDS expression in RNAi transgenic ginseng plants

To analyze the post-transcriptional silencing of *DDS* expression in RNAi transgenic ginseng plants, total RNA was isolated from adventitious roots of RNAi transgenic and wild-type *P. ginseng* using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For RT–PCR analysis, total RNAs were isolated from cultured roots and were then reverse-transcribed by the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA).

Ginsenoside analyses by HPLC

Ginsenosides were extracted according to the method described by Samukawa et al. (1995). A 1 g aliquot of milled powder of freeze-dried roots was soaked in 80% MeOH at 60°C. After evaporation, the residue was dissolved in H_2O and washed twice, followed by extraction with H_2O-saturated *n*-butanol. The butanol layer was then evaporated to obtain the saponin fraction. Each sample was dissolved in EtOH, then filtrated with a SepPak C-18 Cartridge (Waters, Mitford, MC, USA). The HPLC separation was performed on a C18 column (5 μm, 4.6 × 250 mm, Agilent, Wilmington, USA) with water and acetonitrile as mobile phase. The time and ratios of water and acetonitrile followed the protocol of Samukawa et al. (1995). The flow rate of the mobile phase was 1.0 ml min^{-1}, and ginsenosides were monitored at a wavelength of 202 nm. Each ginsenoside was compared with the authentic ginsenoside samples purchased from ChromaDex Inc (California, USA). Quantitative analysis was performed with a one-point curve method using external standards of authentic ginsenosides.

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