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ORIGINAL PAPER

The isolation and characterization of dammarenediol synthase gene from *Panax quinquefolius* and its heterologous co-expression with cytochrome P450 gene *PqD12H* in yeast

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Abstract *Panax quinquefolius* is one of perennial herbs and well known for its outstanding pharmacological activity. Ginsenosides are thought to be the main active ingredients in *Panax quinquefolius* and exist in many kinds of plant genus *Panax* (ginseng). Dammarenediol synthase, which is considered as a key enzyme in ginsenoside biosynthesis pathway can convert 2, 3-oxidosqualene into dammarenediol-II. However, the dammarenediol synthase gene in *Panax quinquefolius* has not been identified. Here, we cloned and identified a dammarenediol synthase gene from *Panax quinquefolius* (*PqDS,* GenBank accession No. KC316048) at the first time, and reverse transcription-PCR (RT-PCR) analysis also showed an obvious transcription increase of *PqDS* in the methyl jasmonate (MeJA)-induced hairy roots. Ectopic expression of *PqDS* in yeast resulted in the production of dammarenediol-II was confirmed by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC/APCIMS). Moreover, overexpression of *PqDS* in transgenic hairy roots could increase the transcription of gene *PqDS* and another P450 gene *PqD12H* (encoding protopanaxadiol synthase in *Panax quinquefolius*), the accumulation of ginsenosides also increased at the same time. In addition, both *PqDS* and *PqD12H* gene co-expressed in recombinant yeast result in the production of protopanaxadiol was detected by LC/APCIMS; this result also provides a new strategy for the abundant production of protopanaxadiol in vitro.

Keywords *Panax quinquefolius* · Dammarenediol synthase · Overexpression · Heterologous co-expression · Protopanaxadiol

Introduction

Panax quinquefolius (P. quinquefolius) as well as *Panax ginseng (P. ginseng)* are commended for its pharmacological effects and widely used from ancient times (Assinewe et al. 2003). Ginsenosides, the secondary metabolites of *P. quinquefolius*, are famous for its pharmacological activity in enhancing the quality of life (Coleman et al. 2003). It was confirmed that ginsenosides have function of immune system modulation, anti-stress activity, anti-cancer activity, and anti-diabetic activity (Vogler et al. 1999; Dey et al. 2003; Kiefer and Pantuso 2003). In addition, the triterpene glycosides, triterpene aglycones also have biological activities, such as soyasapogenol B has hepatoprotective activity, oleanolic acid has anti-inflammatory and anti-tumor activities (Sasaki et al. 1997; Banno et al. 2004). At present, more than 40 ginsenoside compounds have been identified in *Panax* and divided into three groups according to their aglycone structures: the Rb group (protopanaxadiols, including Ra_1, Ra_2, Rb_1, Rb_2, Rb_3, Rc, Rd, Rg_3, Rh_2, and others), the Rg group (protopanaxatriols, including Rg_1, Rg_2, Re, Rf, Rh_1, and others), and the Ro group (oleanolic acid) (Attele et al. 1999; Kima et al. 2007). Generally, seven dammarane-type ginsenoside (Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1) are regarded as the major pharmaceutical ginsenoside constituents in *P. quinquefolius*.

Hao-Jie Cao and Yao Sun assisted to this work.

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Several studies have demonstrated the biosynthesis of ginsenoside in *Panax*; squalene is synthesized via mevalonic acid (MVA) pathway which is located in the cytoplasm and almost present in all organisms in nature, then converted into 2, 3-oxidosqualene by the function of squalene epoxidase (SE) (Liang and Zhao 2008). As shown in Fig. 1, the cyclization of 2, 3-oxidosqualene was performed by different oxidosqualene cyclases (OSCs) as following steps: cycloartenol synthase (CAS) converts 2, 3-oxidosqualene into cycloartenol and leads to the formation of sterols; β-amyrin synthase (β-AS) converts 2, 3-oxidosqualene into β-amyrin and leads to the production of oleanane; lupeol synthase (LS) converts 2, 3-oxidosqualene into lupeol. Furthermore, there is another branched pathway which leads to the biosynthesis of dammarane-type ginsenosides: after the conversion of 2, 3-oxidosqualene into dammarenediol-II by dammarenediol synthase (DS), dammarenediol-II is converted into protopanaxadiol under the hydroxylation of a cytochrome P450 enzyme (D12H) at C-12 position, follow with another cytochrome P450 enzyme (P6H) involves in hydroxylation at C-6 position, then protopanaxadiol could be converted into protopanaxatriol. Subsequently, a variety of ginsenosides is synthesized by adding one or several monosaccharides to these triterpene aglycones under the function of glycosyltransferase (GT) (Cai et al. 2007).

In previous studies, we have comprehended that dammarenediol-II possess enormous value in medical field (Usami et al. 2008), and dammarenediol-II isolated from dammar resin or oil showed potent inhibitory effects against viruses in the animal system (Poehland et al. 1987; Akihisa et al. 2004). In dammarane-type ginsenoside biosynthesis of *Panax*, dammarenediol-II is an important precursor, and the complementary DNA (cDNA) of dammarenediol synthase has been obtained from many plants except for *P. quinquefolius* (Han et al. 2006; Tansakul et al. 2006).

In our study, a dammarenediol synthase gene (*PqDS*) (GenBank accession No. KC316048) was cloned and identified successfully from *P. quinquefolius* hairy roots for the first time; we also analyzed the transcription of hairy root with the treatment of methyl jasmonate (MeJA). The ectopic expression of *PqDS* in yeast analyzed by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry

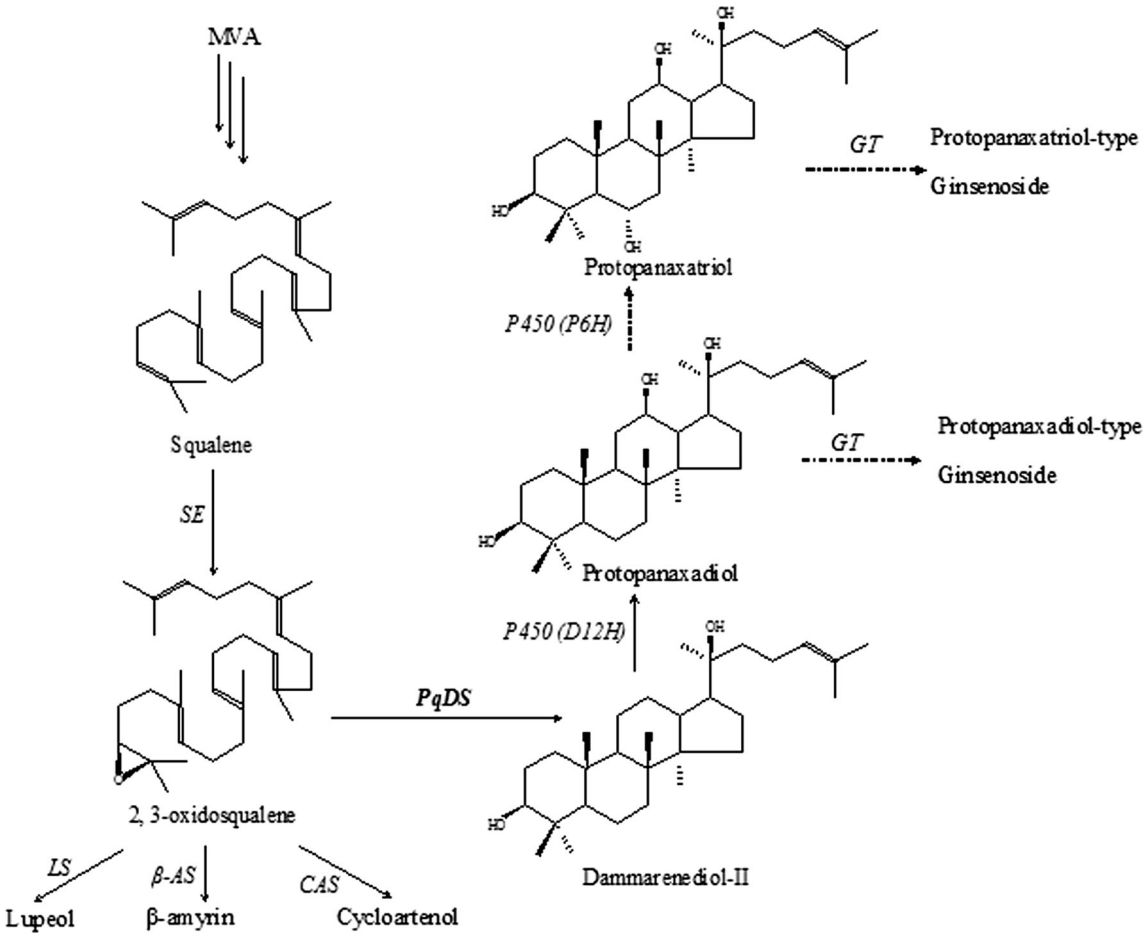

Fig. 1 Proposed biosynthetic pathway of ginsenosides in *P. quinquefolius*. MVA mevalonate, *SE* squalene epoxidase, *CAS* cycloartenol synthase, *LS* lupeol synthase, *β-AS* beta-amyrin synthase, *DS* dammarenediol synthase, *GT* glucosyltransferase. *Dotted lines* indicate uncharacterized region of pathway

(LC/APCIMS) indicates that *PqDS* is a dammarenediol synthase. Moreover, we investigated the content change of ginsenosides with treatment of overexpression gene *PqDS* in hairy roots. At the same time, we constructed recombinant yeast with both vector pAUR123-*PqDS* and pAUR123-*PqD12H* inside to research the heterologous expression of protopanaxadiol in yeast cell.

Materials and methods

Cloning and sequence analysis of *PqDS* by bioinformatics method

Total RNA was extracted by the RNA Reagent kit (TaKaRa) from hairy roots of *P. quinquefolius* which is cultured in B5 medium at 25 °C and the first-strand cDNA as template for PCR was obtained with PrimeScript™ RT-PCR Kit (TaKaRa). Degenerate primers were designed after comparing the conserved sequences between *P. ginseng DDS* (GenBank accession No. GU183405.1) and *Panax notoginseng DS* (GenBank accession No. KC953035) by BLAST P, then the full-length gene was amplified by 5′ and 3′ RACE. The PCR product was analyzed by 1 % agarose electrophoresis. After sequencing, using CLUSTAL–X to perform the sequence analysis, and the phylogenetic tree was constructed based on the deduced amino acid sequences of *P. quinquefolius PqDS* and other OSCs from plants.

RT–PCR analysis of *PqDS* gene in the MeJA-treated hairy roots

Total RNA was isolated from the MeJA-treated *P. quinquefolius* hairy roots and then reverse-transcribed into first-strand cDNA for RT-PCR, the protocols as follows: primers: *PqDS*-F: 5′-GTTTTCCTTTTCATCCAG-3′ and *PqDS*-R: 5′-ATCCTCAGCAATCCATAA-3′; The PCR program 94 °C for 5 min, 30 cycles of 94 °C for 40s, 50 °C for 30s, 72 °C for 1 min, and then 8 min extension at 72 °C, hold at 4 °C. Actin gene was utilized as the standard for RNA loading, production was analyzed by electrophoresis with 1 % agarose.

Ectopic expression of *PqDS* in yeast

In order to achieve the expression of *PqDS* in yeast, shuttle plasmid pAUR-123 (TaKaRa) was used to construct the expression vector. We designed primers with restriction sites SmaI and SacI to insert the ORF of *PqDS* into multiple clone site of pAUR-123, primers were as follows: SmaI-*PqDS*-F: 5′-GAGCTCATGGCAGCAGCAATGGTGTT-3′ and SacI-*PqDS*-R: 5′-CCCGGGGTGGGGATGTAGATGAATGGGA-3′; The PCR conditions 94 °C for 5 min, 35 cycles of 94 °C for 50s, 62 °C for 40s, 72 °C for 1 min, and then 8 min extension at 72 °C, hold at 4 °C, product was analyzed by electrophoresis with 1 % agarose. Then recombinant vector named pAUR123-*PqDS* after sequencing.

Recombinant vector pAUR123-*PqDS* transformed into competent WAT21 by a modified lithium acetate procedure as described previously (Gietz et al. 1992), and the transformed cells were selected by solid medium of YPD with antibiotics AbA (Aureobasidin A) for 3 days at 28 °C, followed by subcultured in YPD liquid medium.

Overexpression of *PqDS* in transgenic hairy roots and analyses ginsenoside by HPLC

To further study the expression of *PqDS* in *P. quinquefolius*, we constructed an overexpression vector which could be transformed by *Agrobacterium rhizogenes* A4. We designed primers with restriction sites HindIII and PstI to insert the ORF of *PqDS* into multiple clone site of the plant expression vector pBI121, the primer sequences are as follows: HindIII-*PqDS*-F: 5′-AAGCTTATGGCAGCAGCAATGGTGTT-3′ and PstI-*PqDS*-R: 5′-CTGCAGGTGGGGATGTAGATGA ATGGGA-3′. Previous cDNA was used as the template, and PCR amplification was performed as follows: 94 °C for 5 min, 30 cycles of 94 °C for 40s, 47 °C for 30s, 72 °C for 1 min, and then 8 min extension at 72 °C, hold at 4 °C.

The overexpression component and vector pBI121 were double digested (HindIII and PstI) and ligated together. Then, the recombinant vector pBI121-*PqDS* was transformed into competent *E. coli* cell by heat shock. After sequencing, the recombinant plasmid was isolated and transformed into competent *A. rhizogenes* A4, and then the pBI121-*PqDS* overexpression engineering bacteria was constructed.

P. quinquefolius hairy roots with treatment of pBI121-*PqDS* overexpression component was harvested according to the method described in our previous study (Sun et al. 2013). The 4-year-old *P. quinquefolius* plant roots in good growth condition were cut into 1-cm-thick pieces after sterilized with mercury, then pre-cultured on B5 solid medium with 3 % sucrose at 25 °C; after 2 days of growing, immersed root segments in *A. rhizogenes* A4 (containing pBI121-*PqDS*) for 8–10 min at room temperature, placed on sterilized filter paper for 10 min, then root segments cultured on B5 solid medium supplemented with 20 mg/l kanamycin. When hairy roots appeared around the edges of root segments with 4–5 weeks culture, we picked them up and transferred to new B5 medium without kanamycin for subculture and HPLC analysis.

Ginsenosides of *P. quinquefolius* hairy roots with pBI121-*PqDS* overexpression were extracted according to the method described by Samukawa et al. (1995), and analysis of ginsenoside by HPLC was performed according to the method

described by Han et al. (2006). HPLC performed with water and acetonitrile as mobile phase. The flow rate of the mobile phase was 1.0 ml min^{-1}, ginsenoside were monitored at a wavelength of 203 nm, and column was maintained at 30 °C. Each ginsenoside was compared with the authentic ginsenoside samples. Quantitative analysis was performed with a one-point curve method using external standards of authentic ginsenoside.

Heterologous expression of protopanaxadiol in yeast

The ORF of *PqD12H* (GenBank accession No. JX569336) was amplified by PCR using primers as follows: SmaI-*PqD12H*-F: 5′-GAGCTCATGGTGTTGTTT TTCTCCCT-3′ and SacI-*PqD12H*-R: 5′-CCCGGGTTAA TTGTGGGGATGTAGA-3′, then the ORF was inserted into pAUR123 to get recombinant vector pAUR123-*PqD12H* followed by transformed pAUR123-*PqD12H* into competent WAT21 yeast which containing pAUR123-*PqDS* already. In that way, we obtained the WAT21 yeast including both pAUR123-*PqDS* and pAUR123-*PqD12H* in a single cell.

Analysis of the recombinant yeast by LC/APCIMS

Both recombinant yeast WAT21 and the control yeast were cultured in YPD liquid medium for 2 days; then, we collected the cells by centrifuging at 500×*g* for 5 min and refluxed the cell pellets with 2 ml of 20 % KOH/50 % EtOH for 5 min. After extraction with the equal volume of hexane, the extracts were analyzed by LC/APCIMS. The LC/APCIMS analysis was performed on a surveyor LC system, and the analytical column was Shim-pack VP-ODS C18 (5 μm, 4.6×250 mm, Shimadzu, Japan) maintained at 30 °C and monitor wavelength at 203 nm. The gradient applications of mobile phases (water and acetonitrile) and the ratio of the constituents were as follows: 0 min, 45 % acetonitrile and 55 % water; 10 min, 50 % acetonitrile and 50 % water; 20 min, 52 % acetonitrile and 48 % water; 40 min, 80 % acetonitrile and 20 % water; 55 min, 90 % acetonitrile and 10 % water; 60 min, 100 % acetonitrile and 0 % water; and 65 min, 45 % acetonitrile and 55 % water at a flow rate of 0.8 ml.min^{-1}. A triple quadrupole mass spectrometer, Finnigan TSQ Quantum Ultra which fitted with an atmospheric pressure chemical ionization (APCI) system was used for detection. As Han et al. (2011) described, the

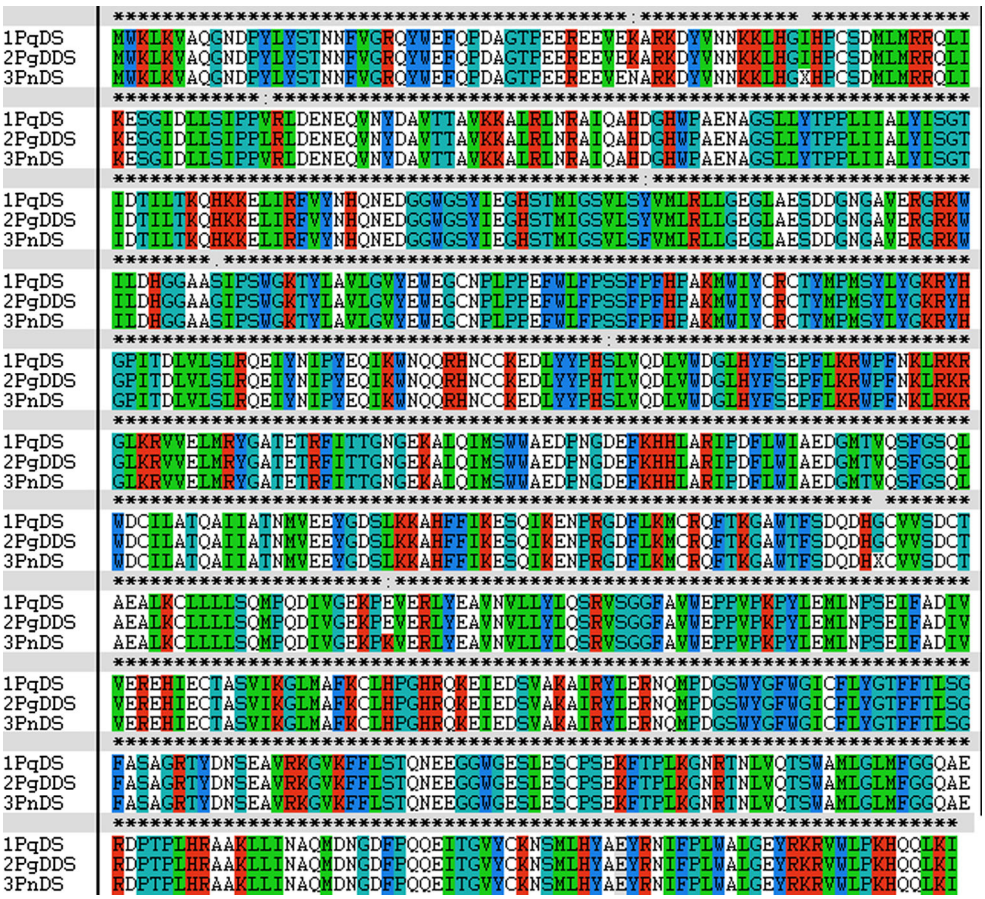

Fig. 2 Alignment results of deduced amino acid sequences of *PqDS* (KC316048) with *PgDDS* (GU183405.1) and *PnDS* (KC953035)

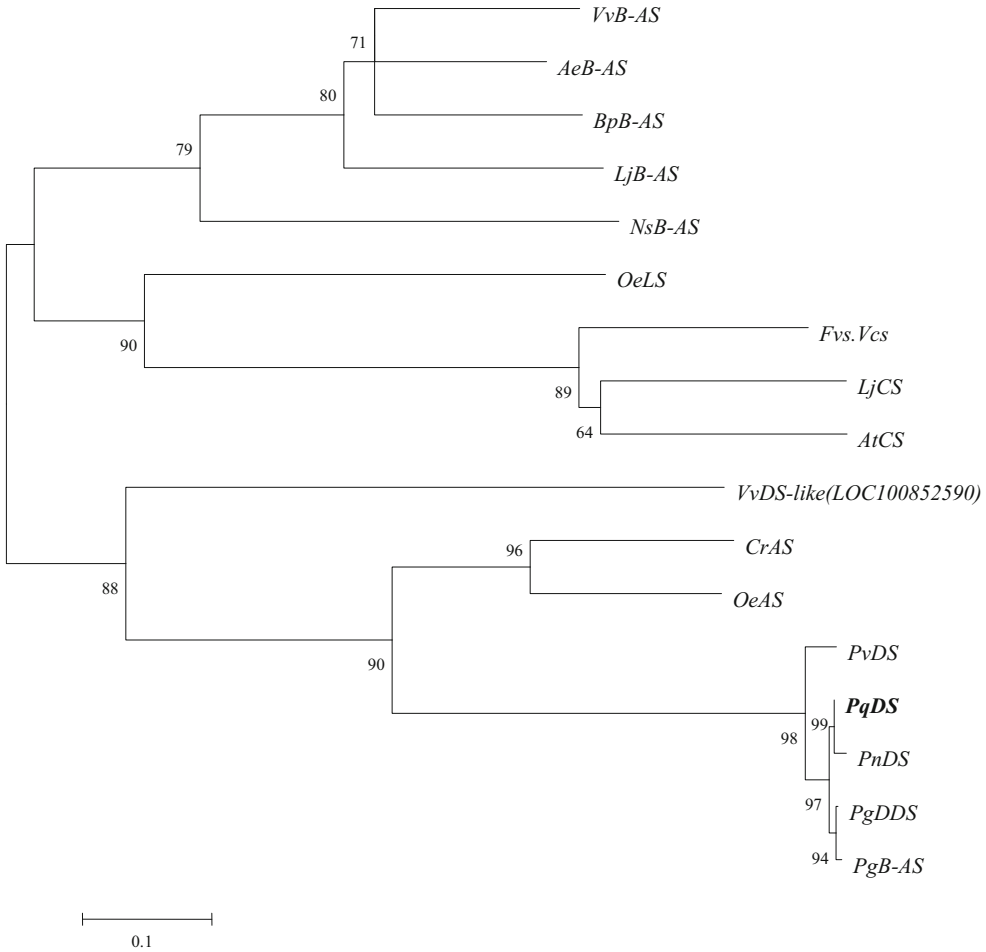

Fig. 3 Phylogenetic tree constructed between *PqDS* and other OSCs from plants. It was constructed by the Neighbor–Joining method using the Clustal X program. Bar=0.1 amino acid substitutions/site. *PqDS*, *Panax quinquefolius* dammarenediol synthase (KC316048); *PgDDS*, *Panax ginseng* dammarenediol synthase (GU183405.1); *PnDS*, *Panax notoginseng* dammarenediol synthase (GU997680.1); *PvDS*, *Panax vietnamensis* dammarenediol synthase (KF306328.1); *VvDS*-like (LOC100852590), *Vitis vinifera* dammarenediol synthase (XM_003632941.1); *PgB-AS*, *Panax ginseng* beta-amyrin synthase (AB122080); *CrAS*, *Catharanthus roseus* amyrin synthase (JQ027033.1); *OeAS*, *Olea europaea* mixed amyrin synthase (AB291240); *AtCS*, *Arabidopsis thaliana* cycloartenol synthase (AAC04931); *Fvs. VCS*, *Fragaria vesca subsp. vesca* cycloartenol synthase-like (XP_004299756.1); *VvB-AS*, *Vitis vinifera* beta-amyrin synthase (XM_002270898.1); *LjB*-AS, *Lotus japonicas* beta-amyrin synthase (BAE53429); *LjCS*, *Lotus japonicas* cycloartenol synthase (BAE53431); *AeB-AS*, *Aralia elata* beta-amyrin synthase (ADK12003); *OeLS*, *Olea europaea* lupeol synthase (AB025343); *BpB-AS*, *Betula platyphylla* beta-amyrin synthase (Q8W3Z1); *NsB-AS*, *Nigella sativa* beta-amyrin synthase (FJ013228)

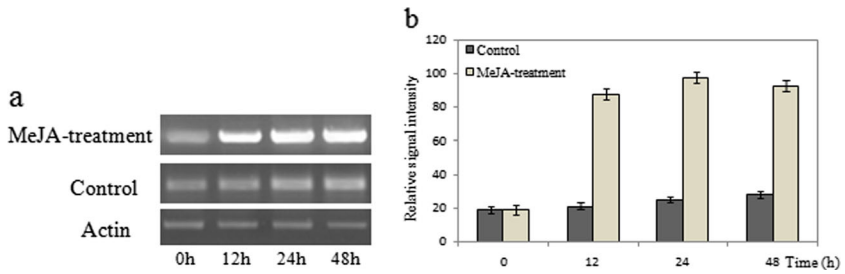

Fig. 4 RT–PCR analysis of *PqDS* mRNA from *P. quinquefolius* hairy roots with MeJA-treated. **a** RT–PCR analysis of *PqDS* gene expression with MeJA-treated at 0, 12, 24, and 48 h, respectively; whereas the control was the hairy roots treated with distilled water and actin gene was utilized as the standard for RNA loading. **b** Densitometric analysis of *PqDS* with MeJA-treatment, *graphs* show quantification for *PqDS* gene amplified PCR products as a percent of the maximum band intensity recorded with MeJA-treated. *Error bars* represent±SE from three independent samples

analysis was performed in the positive mode with a 5.0-mA discharge current, 320 °C vaporizer temperature, and 320 °C ion-transfer capillary temperature. Meanwhile, authentic samples of dammarenediol-II and protopanaxadiol were detected under the identical conditions.

Results

Isolation and sequence analysis of *PqDS*

In our study, using the degenerate primers and RACE (rapid-amplification of cDNA ends), we obtained a gene of full

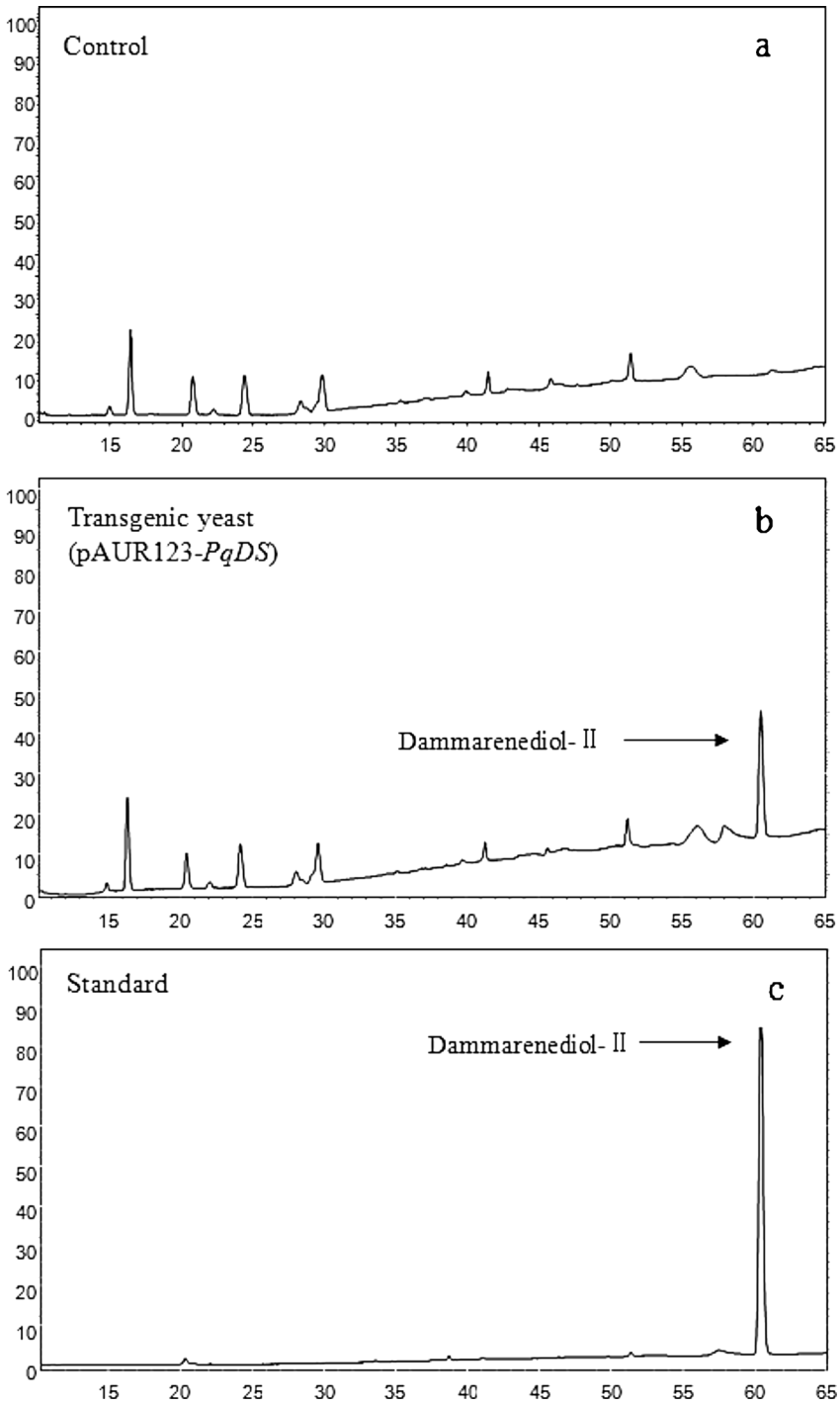

Fig. 5 The total ion chromatogram from LC/APCIMS analysis of the *PqDS* product in yeast. **a** The LC chromatogram of yeast extracts with an empty vector as control. **b** The LC chromatogram of yeast extracts with pAUR123-*PqDS*. (c) The LC chromatogram of dammarenediol-II standard

length is 2,319 bp with an open reading frame (ORF) of 2,310 bp encoding a protein of 769 amino acids with a calculated molecular mass of 88.4 kDa. For further analysis, this gene was named *PqDS* (GenBank accession No. KC316048).

The BLAST P search revealed that the deduced amino acid sequence of *PqDS* shared extremely high similarity of 99.61 and 99.59 % with that of *P. ginseng* dammarenediol synthase *(PgDDS)* and *Panax notoginseng (PnDS)*, respectively (Fig. 2). The phylogenetic tree also showed that gene *PgDDS* and *PnDS* have the highest homology to *PqDS* (Fig. 3).

Transcript analysis of *PqDS* gene in the MeJA-treated hairy roots

RT-PCR analysis on transcriptional activity of *PqDS* gene in *P. quinquefolius* hairy roots after exposed to 10 μm MeJA for 0, 12, 24, and 48 h, hairy roots without treatment were used as control. As reported before, MeJA could greatly improve the expression of squalene synthase, squalene epoxidase, and dammarenediol synthase in ginsenoside biosynthesis (Han et al. 2006). Compared with control, the transcription of *PqDS* after treatment with MeJA increased significantly except for at 0 h, the transcription were almost similar (Fig. 4a), and it reached the maximum level at 24 h then decreased slowly over time (Fig. 4b).

Ectopic expression of *PqDS* gene in WAT21 yeast

The open reading frame (ORF) region of *PqDS* was inserted into the yeast expression vector pAUR-123 to construct recombinant vector pAUR123-*PqDS* and then transformed into WAT21 yeast. After cultured with antibiotic Aureobasidin A (AbA) for 2 days, collected the yeast extract and detected by LC/APCIMS. The total ion chromatogram from LC/APCIMS indicated there was a peak at the retention time of 59.87 min (Fig. 5b), which is the same as the authentic sample of dammarenediol-II (Fig. 5c), and it was not detected in the control yeast with empty vector (Fig. 5a). Using MS fragmentation pattern analysis the dammarenediol-II signal at the retention time of 59.87 min from yeast with recombinant vector pAUR123-*PqDS*, the LC/APCIMS fragmentation pattern includes the m/z ratios of 409 $[M\text{-}2H_2O+H]^+$ and 427 $[M\text{-}H_2O+H]^+$ (Fig. 6a), which was the same as that of authentic dammarenediol (Fig. 6b). This result indicates that ectopic

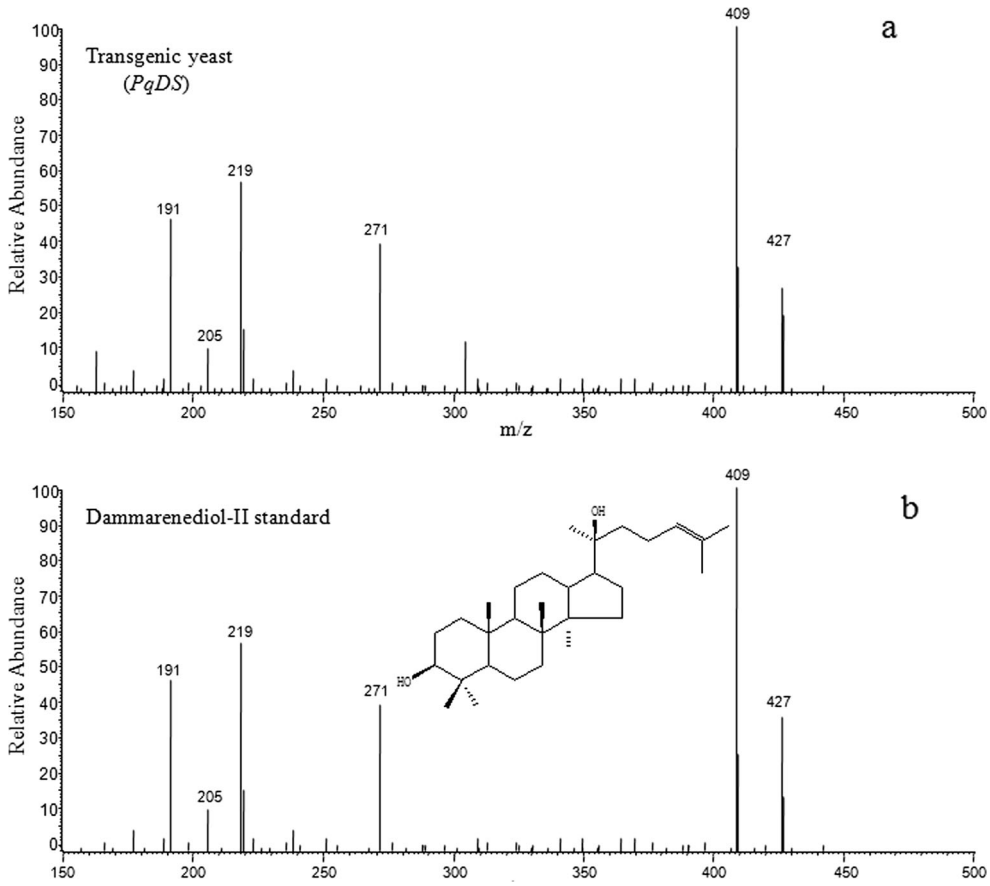

Fig. 6 LC/APCIMS spectra of peaks detected in yeast with *PqDS*. **a** The MS spectrum for a peak detected in yeast with *PqDS* at a retention time of 59.87 min. **b** The MS spectrum for the peak detected in the dammarenediol-II standard include the m/z ratios of 409 $[M\text{-}2H_2O+H]^+$ and 427 $[M\text{-}H_2O+H]^+$.

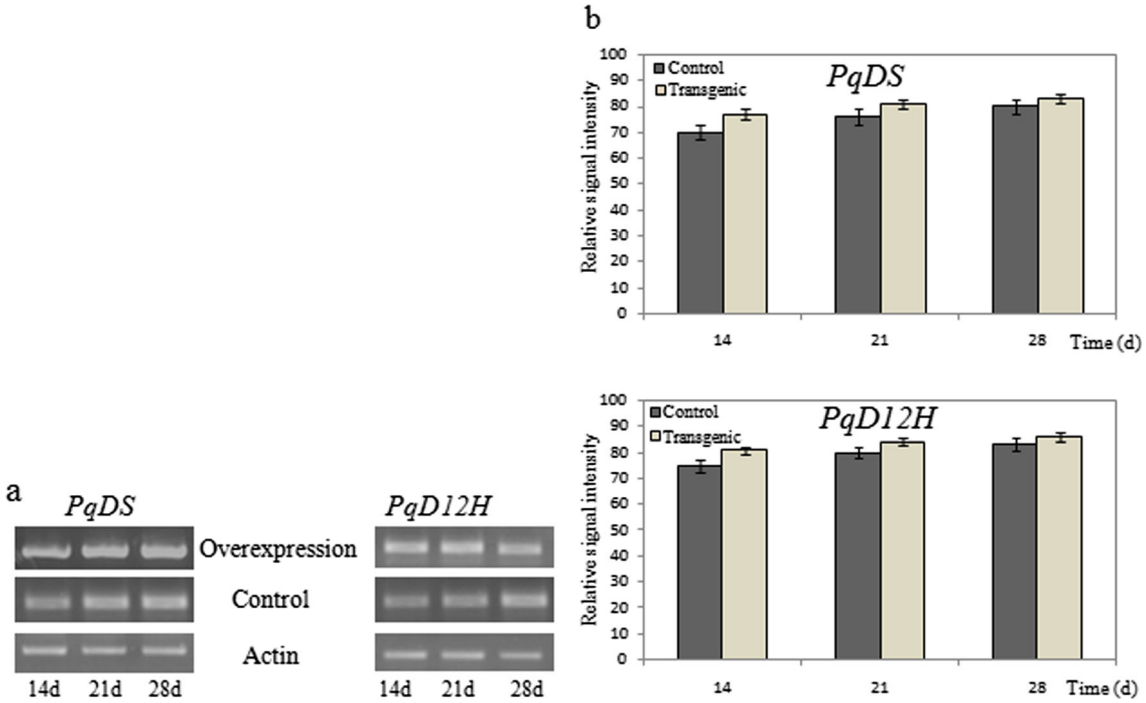

Fig. 7 RT–PCR analysis of *PqDS* and *PqD12H* mRNAs from *P. quinquefolius* hairy roots within overexpression *PqDS* treated. **a** RT–PCR analysis the transcription of *PqDS* and *PqD12H* gene with overexpression *PqDS* treated after 14, 21 and 28 days, respectively. The transcription of gene *PqDS* and *PqD12H* from *P. quinquefolius* hairy root without overexpression *PqDS* treated as control respectively. The actin gene was utilized as the standard for RNA loading. **b** Densitometric analysis for *PqDS* and *PqD12H* with overexpression *PqDS* treated respectively. *Graphs* show quantification for *PqDS* and *PqD12H* gene amplified PCR products as a percent of the maximum band intensity recorded within the overexpression *PqDS* treated. *Error bars* represent± SE from three independent samples

expression of *PqDS* in yeast contributes to the production of dammarenediol-II and *PqDS* is the authentic gene of dammarenediol synthase in *P. quinquefolius*.

Response to gene *PqDS* overexpression in *P. quinquefolius* hairy roots

In order to detect whether gene *PqDS* could regulate the expression of *PqD12H in P. quinquefolius hairy roots*, we selected 14, 21, and 28 days hairy roots with pBI121-*PqDS* overexpression treated, then the messenger RNA (mRNA) of *PqDS* and *PqD12H* was isolated and analyzed by RT-PCR; compared with control, both *PqDS* and *PqD12H* have higher accumulation of mRNA in the transgenic hairy roots and the increase ratio decreased slightly over culture time (Fig. 7).

Ginsenosides were measured by HPLC to analyze the effects of gene *PqDS* overexpression on ginsenoside contents in *P. quinquefolius* hairy root. The result indicated that the accumulation of ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1) were enhanced significantly in transgenic hairy roots compared to these of wild-type (Fig. 8a–c). Detailed data was shown in Table 1. Densitometric analysis also demonstrated that treatment of gene *PqDS* overexpression could increase the expression of ginsenosides (Fig. 8d).

Heterologous expression analysis of protopanaxadiol in yeast

We use LC/APCIMS to analyze the heterologous expression of protopanaxadiol in WAT21 yeast and choose yeast with empty vector as control. As shown in Fig. 9, the LC chromatogram of transformed yeast only contain pAUR123-*PqD12H* (Fig. 9b) was as the same as control (Fig. 9a), and no protopanaxadiol was detected. Then we added exogenous dammarenediol-II (50 mg l^{-1}) dissolved with ethanol and Tween-80 (1:1, *v*/*v*) into the medium as substrate for 1 day (Kushiro et al. 1998), the LC chromatogram from the extract identified two peak at a retention time of 59.87 and 49.46 min (Fig. 9c), which had the same retention time with the authentic samples of dammarenediol-II and protopanaxadiol respectively (Fig. 9e). In addition, both dammarenediol-II and protopanaxadiol were identified from the LC chromatogram

Fig. 8 HPLC and densitometric analysis of ginsenosides in transgenic ▶ overexpression *PqDS* hairy roots **a** Ginsenosides chromatograms in the wild-type (WT) of roots. **b** Ginsenosides chromatograms in the transgenic (overexpression PqDS) of roots. **c** Ginsenosides chromatograms of authentic ginsenosides. **d** Densitometric analysis of ginsenosides contents of transgenic overexpression *PqDS* hairy roots. *Error bars* represent±SE from three independent samples

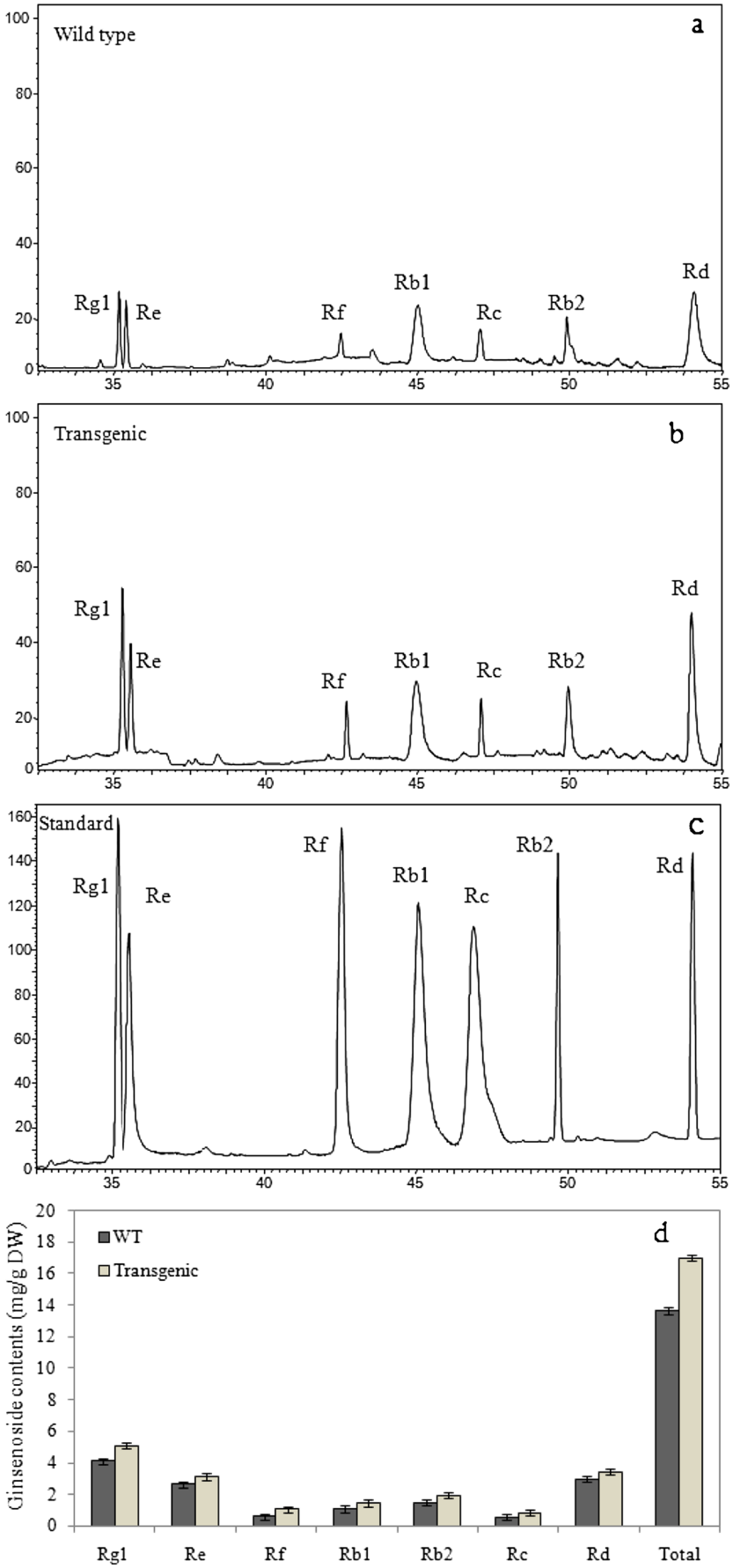
Wild type
a
Rg1
Re
Rf
Rb1
Rc
Rb2
Rd
Transgenic
b
Standard
c
WT
Transgenic
d
Ginsenoside contents (mg/g DW)
Rg1
Re
Rf
Rb1
Rb2
Rc
Rd
Total

Table 1 Ginsenoside contents in the extracts from in vitro cultured hairy roots of the wild-type and transgenic of *PqDS* overexpression of *P. quinquefolius*

Explant	Ginsenoside (mg/g DW)[a]							Total
	Protopanaxatriol			Protopanaxadiol				
	Rg1	Re	Rf	Rb1	Rb2	Rc	Rd	
WT	4.21±0.18	2.63±0.21	0.61±0.10	1.11±0.15	1.52±0.14	0.57±0.09	2.99±0.16	13.64±0.67
Transgenic	5.08±0.23	3.12±0.17	1.06±0.09	1.46±0.19	1.98±0.16	0.85±0.11	3.44±0.21	16.99±0.77
Increased ratio[b]	1.21	1.19	1.74	1.32	1.30	1.49	1.15	1.25

Rg1, Re, Rf, Rb1, Rb2, Rc, and Rd indicate ginsenoside Rg1, -Re, -Rf, -Rb1, -Rb2, and -Rc, respectively

[a] Data represent the mean value±SE of three independent experiments

[b] Increased ratio values represent the fold increase by transgenic hairy roots as compared to wild-type roots; values >1.0 represent the increase of the ginsenoside, whereas those <1.0 represent a decrease

of yeast with both pAUR123-*PqDS* and pAUR123-*PqD12H* (Fig. 9d).

The protopanaxadiol signal at the retention time of 49.46 min was analyzed using the MS fragmentation patterns (Fig. 10a). The LC/APCIMS fragmentation included the m/z ratios of 407 $[M\text{-}3H_2O+H]^+$, 425 $[M\text{-}2H_2O+H]^+$, and 443 $[M\text{-}H_2O+H]^+$, which are the same as for authentic protopanaxadiol (Fig. 10b). This result indicates that heterologous expression of both genes *PqDS* and *PqD12H* in yeast could be used for the production of protopanaxadiol, and protopanaxadiol in extract of WAT21 yeast with both pAUR123-*PqDS* and pAUR123-*PqD12H* was approximately 18.09 μg g^{-1} FW with 2 days of culture while still trace amounts dammarenediol-II in extract. Moreover, the retention time of the protopanaxatriol standard (Fig. 9e) was at 21.15 min, and it was not detected in LC/APCIMS chromatograms, so there was no protopanaxatriol produced in all extracts.

Discussion

Previous studies reported there were two stereoisomers (S and R configuration at C-20) of dammarenediol as natural products, and dammarenediol synthase of *Panax* yielded dammarenediol-II with (20S)-isomer as its sole product (Kushiro et al. 1997; Tansakul et al. 2006). In recent years, many researchers have isolated cDNA of dammarenediol synthase from *P. ginseng* with degenerate primers (Tansakul et al. 2006) and expressed sequence tags (ESTs) in flower cDNA library (Han et al. 2006). Whereas, the similar research of dammarenediol synthase in *P. quinquefolius* was few. In our study, we cloned a dammarenediol synthase gene with degenerate primers designed from the most conserved regions of the known dammarenediol synthase *P. ginseng (PgDDS)* and *Panax notoginseng (PnDS)*. Deduced amino acid sequence analysis of *PqDS* indicated the extreme similarity with the dammarenediol synthase in *P. ginseng (PgDDS)* and *Panax notoginseng (PnDS)*. Phylogenetic analysis has shown that *PqDS* also shared greater similarity with *Panax vietnamensis* dammarenediol synthase (*PvDS*) and *Panax ginseng* beta-amyrin synthase (*PgB-AS*) than other OSCs. These demonstrated that *PqDS* is the most probable gene that could encodes dammarenediol synthase in *P. quinquefolius*.

Many researches have shown that the treatment of MeJA could induce the upregulation of ginsenoside biosynthesis in hairy roots (Lee et al. 2004) and the content of protopanaxadiol-type ginsenosides could increased fivefold after 7 days MeJA-treated (Yu et al. 2002). In this study, the expression of *PqDS* in hairy root with MeJA-treatment was increased obviously compared with the control, the transcription increased to the highest level after 24 h treatment and then decreased slowly over time. This indicated that *PqDS* gene is highly responsive to the elicitor treatment by MeJA.

Our previous work have cloned and characterized that *PqD12H* was a protopanaxadiol synthase with hydroxylase activity involved in the dammarenediol-II hydroxylation at the C-12 position in *P. quinquefolius* (Sun et al. 2013). In this work, both *PqDS* and *PqD12H* have remarkable increase of transcription with the treatment of overexpression *PqDS* in transgenic *P. quinquefolius*, the results suggested that *PqDS* is an upstream gene of *PqD12H* during the ginsenoside biosynthesis in *P. quinquefolius* and the overexpression of *PqDS* gene could greatly upregulate the expression of *PqD12H*. This is consistent with the conclusion of Hey et al. (2006), that the transcription level of downstream genes could increased obviously with the treatment of overexpression upstream gene in transgenic *P. ginseng*. In particular, all of ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1) increased significantly with the treatment of overexpression *PqDS,* protopanaxadiol-type ginsenosides (Rb1, Rb2, Rc, and Rd)

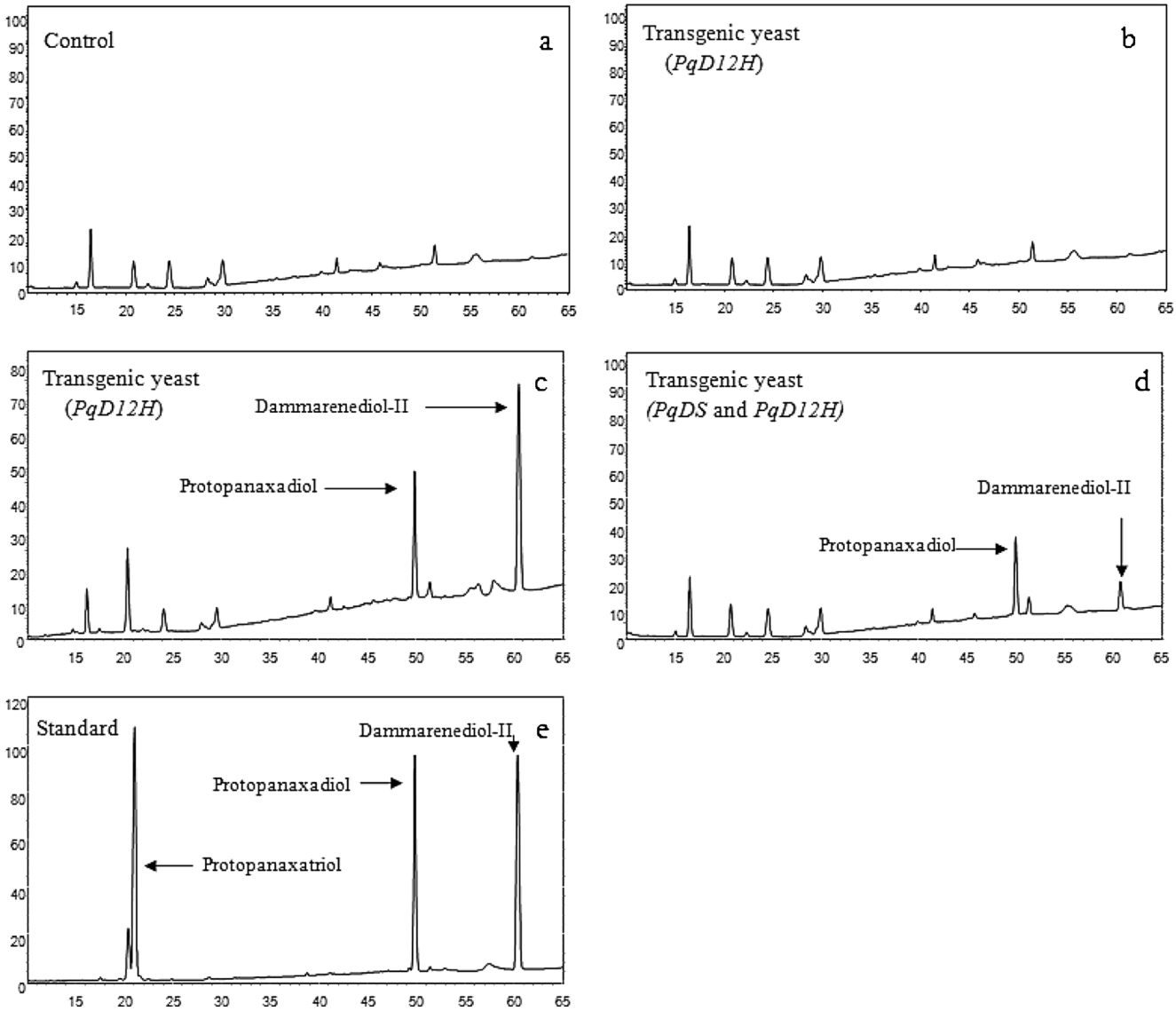

Fig. 9 The total ion chromatogram from LC/APCIMS analysis of protopanaxadiol production in WAT21 yeast with both *PqDS* and *PqD12H*. **a** The LC chromatogram of the control yeast extracts. **b** The LC chromatogram of the yeast extract with pAUR123-*PqD12H*. **c** The LC chromatogram of the yeast cell extract with pAUR123-*PqD12H* after addition of dammarenediol-II in medium. **d** The LC chromatogram of cell extract from the yeast with both pAUR123-*PqDS* and pAUR123-*PqD12H*. **e** The LC chromatogram of dammarenediol-II, protopanaxadiol and protopanaxatriol authentic samples, respectively

increased by 1.15–1.49 times that of the control, whereas protopanaxatriol-type ginsenosides (Rg1, Re and Rf) increased by 1.17–1.74 times and total ginsenosides increased by 1.25 times. This result suggested that overexpression of *PqDS* could be useful for the enhanced production of important ginsenosides in *P. quinquefolius.*

Yeast WAT21 is a kind of unicellular fungi; many researchers treated it as a eukaryotic expression system for yielding multiple sterols and intermediate metabolites which also involved in the metabolic synthesis in higher plants (Landl et al. 1996). Han et al. used it to perform the ectopic expression of *PgDDS* and *CYP716A47* at 2006 and 2011, respectively; Kim et al. (2009) also used it for the expression of *CabAS*. In our study, we chose it for the expression identification of *PqDS* and heterologous expression of protopanaxadiol. Dammarenediol-II was detected in extracts of yeast with recombinant vector pAUR123-*PqDS* by LC/APCIMS; the result demonstrated that expression of dammarenediol-II in yeast is successful and also proved that *PqDS* we isolated from *P. quinquefolius* is authentic gene for encoding dammarenediol synthase. No protopanaxadiol signal was detected in extracts of yeast with pAUR123-*PqD12H* while protopanaxadiol was detected after exogenous

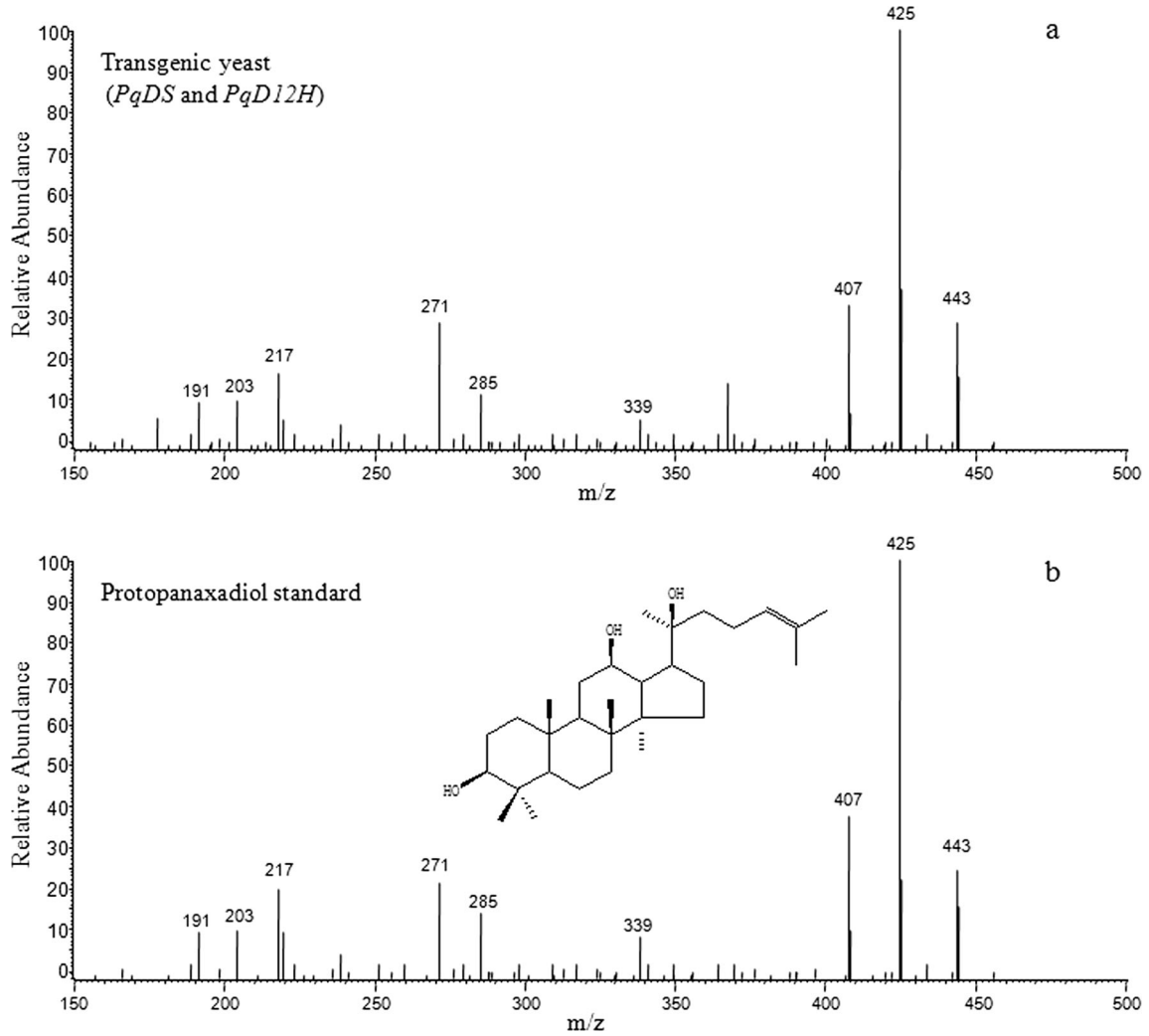

Fig. 10 LC/APCIMS spectra of peaks detected protopanaxadiol signal at the retention time of 49.46 **a** The MS spectrum for a peak detected in yeast with both genes *PqDS* and *PqD12H* at a retention time of 49.46 min. **b** The MS spectrum for the peak detected in the protopanaxadiol standard include the m/z ratios of 407 $[M\text{-}3H_2O+H]^+$, 425 $[M\text{-}2H_2O+H]^+$ and 443 $[M\text{-}H_2O+H]^+$

dammarenediol-II added into medium demonstrated that yeast with single recombinant vector pAUR123-*PqD12H* is unable to express protopanaxadiol without exogenous dammarenediol-II; In addition, protopanaxadiol signal was detected in extracts of yeast with both pAUR123-*PqDS* and pAUR123-*PqD12H* by LC/APCIMS; this result demonstrated that heterologous expression of protopanaxadiol in yeast is successful and the yield was considerable.

In *P. quinquefolius*, the content of triterpenoid saponin is about 5 % in dried roots (Wang et al. 2006), while it is only 4 % in *P. ginseng* (Shibata 2001). As is known to all, the growth cycle of *Panax* plant is extremely slow, and the procedure to extract ginsenoside in vivo is quite complicated, an available method for harvesting abundant ginsenoside is imperative. So, our research provides a new mode to produce protopanaxadiol through heterologous expression in yeast.

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