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Cytochrome P450 *CYP716A53v2* Catalyzes the Formation of Protopanaxatriol from Protopanaxadiol During Ginsenoside Biosynthesis in *Panax Ginseng*

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Rapid Paper

Ginseng (*Panax ginseng* C.A. Meyer) is one of the most popular medicinal herbs, and the root of this plant contains pharmacologically active components, called ginsenosides. Ginsenosides, a class of tetracyclic triterpene saponins, are synthesized from dammarenediol-II after hydroxylation by cytochrome P450 (CYP) and then glycosylation by a glycosyltransferase. Protopanaxadiol synthase, which is a CYP enzyme (*CYP716A47*) that catalyzes the hydroxylation of dammarenediol-II at the C-12 position to yield protopanaxadiol, was recently characterized. Here, we isolated two additional CYP716A subfamily genes (*CYP716A52v2* and *CYP716A53v2*) and determined that the gene product of *CYP716A53v2* is a protopanaxadiol 6-hydroxylase that catalyzes the formation of protopanaxatriol from protopanaxadiol during ginsenoside biosynthesis in *P. ginseng*. Both *CYP716A47* and *CYP716A53v2* mRNAs accumulated ubiquitously in all organs of ginseng plants. In contrast, *CYP716A52v2* mRNA accumulated only in the rhizome. Methyl jasmonate (MeJA) treatment resulted in the obvious accumulation of *CYP716A47* mRNA in adventitious roots. However, neither *CYP716A52v2* nor *CYP716A53v2* mRNA was affected by MeJA treatment during the entire culture period. The ectopic expression of *CYP716A53v2* in recombinant WAT21 yeast resulted in protopanaxatriol production after protopanaxadiol was added to the culture medium. In vitro enzymatic activity assays revealed that *CYP716A53v2* catalyzed the oxidation of protopanaxadiol to produce protopanaxatriol. The chemical structures of the protopanaxatriol products were confirmed using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC/APCIMS). Our results indicate that the gene product of *CYP716A53v2* is a protopanaxadiol 6-hydroxylase that produces protopanaxatriol from protopanaxadiol, which is an important step in the formation of dammarane-type triterpene aglycones in ginseng saponin biosynthesis.

Keywords: Cytochrome P450 • CYP716A53v2 • Ginsenoside • Protopanaxadiol • Protopanaxatriol.

Abbreviations: CYP, cytochrome P450; EST, expressed sequence tag; LC/APCIMS, liquid chromatography–atmospheric pressure chemical ionization mass spectrometry; MeJA, methyl jasmonate; ORF, open reading frame; RT–PCR, reverse transcription–PCR.

Introduction

Triterpenoid saponins are secondary metabolites of isoprenoid compounds that are present in higher plants. They exhibit great structural diversity and a wide range of biological activities among plant species. These compounds also have high commercial value, and many have been exploited as medicinal drugs (Hostettmann and Marston 1995, Vogler et al. 1999, Shibata 2001). The natural role of saponins in plants is probably in defense against attacks by pathogens and pests (Osbourn 1996). The primary components of triterpenoid saponins are dammarane, oleanane (μ-amyrin), ursane (α-amyrin) and lupeol-type triterpenoids.

Ginseng (*Panax ginseng* C.A. Meyer) is a perennial herbaceous species belonging to the family Araliaceae. This species has been used as an important traditional Asian medicine since ancient times. Ginseng root, one of the most famous and expensive crude drugs, has been commonly used to enhance quality of life (Ellis and Reddy 2002, Coleman et al. 2003). Immune system modulation, anti-stress activity, anti-cancer activity and anti-diabetic activity are the most notable features of ginseng in laboratory and clinical trials (Vogler et al. 1999, Shibata 2001, Yun 2001, Dey et al. 2003, Kiefer and Pantuso 2003). Approximately 12 ginseng (*Panax*) species are distributed around the world (Wen and Zimmer 1996, Zhu et al. 2004).

Ginsenosides are considered the primary components of the ginseng root that are responsible for its bioactivity. *Panax ginseng* roots contain at least 4% ginsenosides by dry weight (Shibata 2001). Seven dammarane-type tetracyclic triterpenes (ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1) are reported to be the major ginsenoside constituents, and only ginsenoside Ro is an oleanane-type pentacyclic triterpene. This compound is

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found only at low levels in *P. ginseng*. Dammarane-type ginsenosides are divided into two groups according to their aglycone structures: protopanaxadiols (Rb_1, Rb2, Rc and Rd) and the protopanaxatriols (Rg_1, Re, Rf and Rg_2). The ginsenoside Ro is thought to be synthesized from oleanolic acid, which is a product of β-amyrin (Shibata 1977).

The first step in the biosynthesis of dammarane-type ginsenosides is the cyclization of 2,3-oxidosqualene to form dammarenediol-II, a reaction that is catalyzed by dammarenediol synthase (**Fig. 1**). Two homologous dammarenediol synthases from *P. ginseng* (*DDS* and *PNA*) have been functionally characterized (Han et al. 2006, Tansakul et al. 2006). Dammarenediol-II is thought to be converted to a ginsenoside by hydroxylation by CYP enzymes (Shibuya et al. 2006) and subsequent glycosylation by glycosyltransferases (Choi et al. 2005, Shibuya et al. 2006).

CYPs constitute a supergene family in plant genomes. In plants, CYPs catalyze a wide variety of monooxygenation reactions in primary and secondary metabolism (Schuler 1996). Two CYP genes are thought to be involved in dammarane-type ginsenoside biosynthesis in ginseng plants. The product of one of these genes catalyzes the hydroxylation of dammarenediol at the C-12 position. Another enzyme, protopanaxatriol synthase, hydroxylates protopanaxadiol at the C-6 position to yield protopanaxatriol.

The first identification of a triterpene hydroxylase cDNA was *CYP93E1*, and this gene converts β-amyrin into the sophoradiol 24-hydroxylase gene in *Glycine max* (Shibuya et al. 2006). Another CYP is β-amyrin 11-oxidase (*CYP88D6*), which converts β-amyrin to 11-oxo-β-amyrin during biosynthesis of the triterpene sweetener glycyrrhizin (Seki et al. 2008). Seki et al. (2011) reported that CYP72A154 is capable of catalyzing three sequential oxidation steps at C-30 of 11-oxo-β-amyrin to produce glycyrrhetinic acid.

CYP716A subfamily genes are involved in triterpene saponin biosynthesis. *CYP716A12* in *Medicago truncatula* catalyzes the oxidation of β-amyrin and erythrodiol at the C-28 position, yielding oleanolic acid (Carelli et al. 2011, Fukushima et al. 2011). Han et al. (2011) reported that *CYP716A47* is involved in the hydroxylation of dammarenediol-II at the C-12 position to yield protopanaxadiol in *P. ginseng* (Han et al. 2011). The next step of protopanaxatriol production is postulated to be catalyzed by another CYP gene product (Han et al. 2011). However, the CYP gene encoding the protopanaxatriol synthase remains uncharacterized.

In the present study, we showed that *CYP716A53v2* functions in protopanaxatriol production by hydroxylating protopanaxadiol at the C-6 position. The ectopic expression of *CYP716A53v2* in yeast conferred the ability to produce protopanaxatriol after feeding with protopanaxadiol. The in vitro reaction of the *CYP716A53v2* protein with protopanaxadiol resulted in the production of protopanaxatriol. These results indicate that *CYP16A53v2* is a protopanaxatriol synthase (protopanaxadiol 6-hydroxylase) and catalyzes a critically important step in ginsenoside biosynthesis.

Results

Classification of *CYP716A53v2* genes

In *P. ginseng*, two CYP genes are proposed to participate in dammarane-type ginsenoside biosynthesis (**Fig. 1**). We previously reported that *CYP716A47* is involved in the hydroxylation of dammarenediol-II at the C-12 position, which is the first step in producing protopanaxadiol from dammarenediol-II (Han et al. 2011). In this work, we isolated additional *CYP716A* subfamily genes from the GenBank entries for *P. ginseng*. Two genes belonging to the CYP716A subfamily were found, and two full genes (*CYP716A52v2*, GenBank accession No. JX036032; and *CYP716A53v2*, GenBank accession No. JX036031) were obtained by full gene sequencing of expressed sequence tag (EST) clones obtained from an in vitro cultured adventitious root cDNA library (Han et al. 2011).

Although the *CYP716A52v2* and *CYP716A53v2* genes are assigned to the same CYP716A subfamily, the similarity of the deduced amino acid sequences was only 52% (**Supplementary Fig. S1**). The deduced amino acid sequence of CYP716A47 (protopanaxadiol synthase) shares 44 and 49% identity with CYP716A52v2 and CYP716A53v2, respectively. CYP716A52v2 shares 73% identity with CYP716A12 in *M. truncatula*, which is a multifunctional oxidase involved in oleanane-type triterpenoid biosynthesis (Carelli et al. 2011). CYP716A53v2 shows 53% identity to CYP716A12 (**Supplementary Fig. S1**). Phylogenetic analysis revealed that CYP716A52v2 is grouped with CYP716A12, CYP716A15 and CYP716A17, which are involved in oleanane-type triterpene biosynthesis. However, both CYP716A47 and CYP716A53v2 are somewhat separated from CYP716A12, CYP716A15 and CYP716A17 (**Supplementary Fig. S2**).

Panax notoginseng also contains genes that are highly similar to *P. ginseng CYP716A52v2* and *CYP716A53v2*. The deduced amino acid sequence of CYP716A52v2 has 97.71% identity and 97.92% similarity to a CYP716A52v1 (GenBank accession No. GU997666) in *P. notoginseng*. CYP716A53v2 has 96.8% identity and 97.86% similarity to a CYP716A53v1 (GenBank accession No. GU997670) in *P. notoginseng*.

Transcriptional activity of three CYP genes

Reverse transcription–PCR (RT–PCR) analysis of transcriptional activities of the three CYP genes in different parts of ginseng plants revealed that both *CYP716A47* and *CYP716A53v2* mRNAs accumulated in all organs of ginseng plants, including the flower buds (Fb), leaves (L), leaf petioles (Lp), sympodia (S), rhizomes (Rz) and roots (R), but the accumulation of *CYP716A53v2* mRNA in flower buds was less pronounced than that of *CYP716A47* mRNA (**Fig. 3**). In contrast, *CYP716A52v2* mRNA accumulated to high levels only in the rhizome, with very low levels in other organs (**Fig. 3**), indicating that *CYP716A52v2* has a unique expression pattern.

Methyl jasmonate (MeJA) treatment stimulates the biosynthesis of many secondary metabolites (Gundlach et al. 1992, Yendo et al. 2010, Lambert et al. 2011), including ginseng

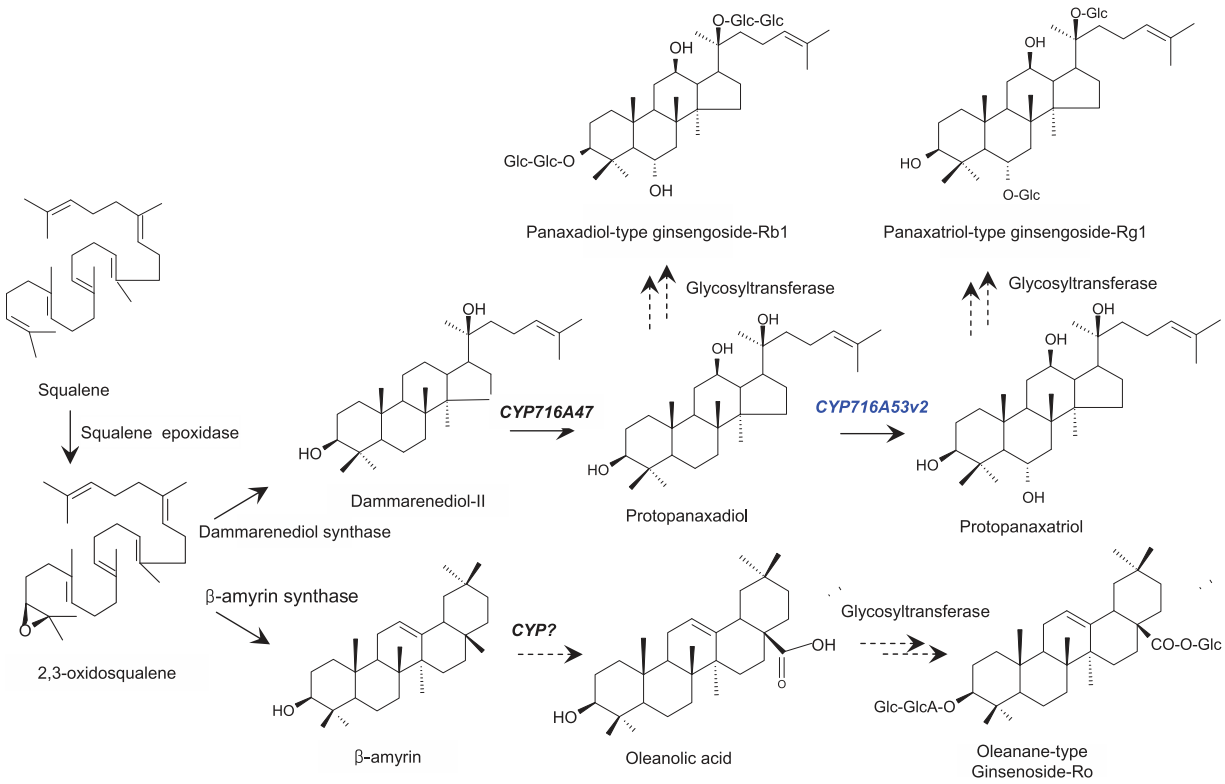

Fig. 1 Proposed biosynthetic pathway for ginsenosides in *P. ginseng*. Squalene epoxidase converts squalene into 2,3-oxidosqualene, which is then converted into a triterpene aglycone (dammarenediol-II or β-amyrin) by dammarenediol synthase or β-amyrin synthase. The triterpene aglycones subsequently undergo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

saponins in *P. ginseng* (Han et al. 2006). The transcription of *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* mRNAs in the adventitious roots of *P. ginseng* was monitored at 12, 24 and 48 h after MeJA (10 μM) was added to the culture medium. Without treatment, all three mRNAs were detected at low levels (**Fig. 4**). MeJA treatment resulted in the obvious accumulation of *CYP716A47* mRNA in the adventitious roots relative to the level in the control (**Fig. 4**). The transcription of *CYP716A47* increased to a maximum level after 24 h of MeJA treatment and then decreased slowly as the culture time proceeded (**Fig. 4**). However, neither *CYP716A52v2* mRNA nor *CYP716A53v2* mRNA responded to MeJA treatment during the entire culture period (**Fig. 4**).

Ectopic expression of *CYP716A53v2* cDNA in WAT21 yeast

The full-length cDNA clone of *CYP716A53v2* was 1,560 bp long [open reading frame (ORF) 1,410] with a 470 amino acid ORF, yielding a protein with a predicted molecular mass of 55.3. kDa The full-length cDNA clone of *CYP716A52v2* was 1,779 bp long (ORF 1,446) with a 481 amino acid ORF, yielding a protein with a predicted molecular mass of 54.0 kDa.

To examine the hydroxylation activity of *CYP716A52v2* and *CYP716A53v2* functionally, the ORFs of *CYP716A52v2* and *CYP716A53v2* cDNA were inserted into the pYES2.1 vector and expressed in WAT21 yeast expressing *Arabidopsis thaliana* NADPH-CYP reductase (Urban et al. 1997) under the control of a constitutive promoter (GAL1). The yeast extracts were analyzed using total ion chromatograms obtained using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC/APCIMS). Because protopanaxadiol was exogenously applied to the yeast, both protopanaxadiol and protopanaxatriol were analyzed in the total ion chromatogram from LC/APCIMS. The retention times of the protopanaxadiol and protopanaxatriol standards were 33.6 and 19.2 min, respectively (**Fig. 5D**, **E**). The protopanaxatriol signal was not detected in the control yeast harboring the empty vector, but there was a peak for the exogenously added protopanaxadiol (**Fig. 5**A). Interestingly, both protopanaxadiol (retention time, 33.6 min) and protopanaxatriol (retention time, 19.2 min) were identified in *CYP716A53v2*-expressing yeast (**Fig. 5C**). However, *CYP716A52v2*-expressing yeast did not produce protopanaxatriol, yielding the same chromatogram pattern as the control yeast (**Fig. 5B**).

The protopanaxatriol signal at 19.2 min from yeast expressing *CYP716A53v2* was analyzed using MS (**Fig. 6A**). The LC/APCIMS fragmentation pattern for yeast expressing *CYP716A53v2* included fragments with *m/z* ratios of 405 $[M\text{-}3H_2O + H]^+$, 423 $[M\text{-}2H_2O + H]^+$ and 441 $[M\text{-}H_2O + H]^+$, which are the same as those for pure protopanaxatriol (**Fig. 6B**). This result clearly indicates that *CYP716A53v2* is a protopanaxadiol 6-hydroxylase gene (protopanaxadiol synthase) and that

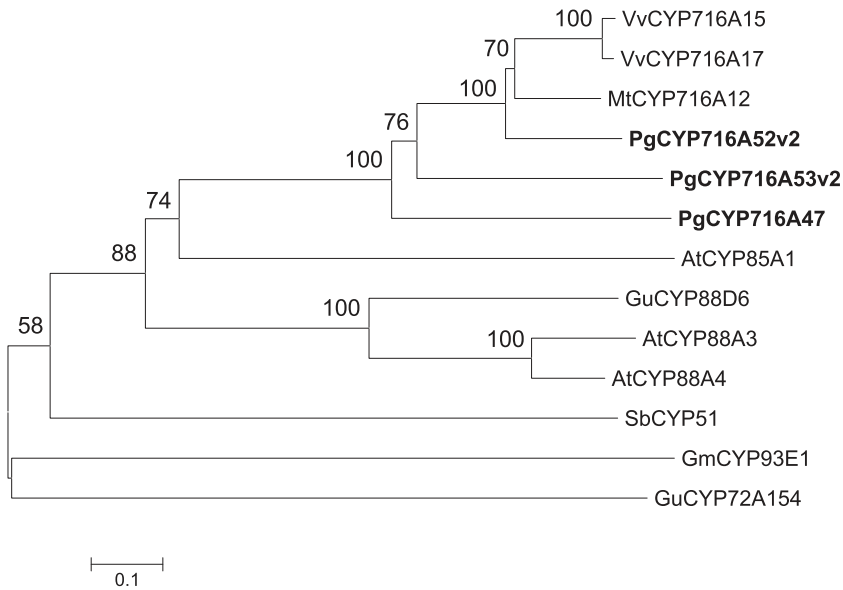

Fig. 2 Unrooted phylogenetic tree constructed based on the deduced amino acid sequences of the three *P. ginseng* CYPs (bold letters) and other plant CYPs. The tree was constructed by the Neighbor–Joining method with Poisson correction using the ClustalX program. Bootstrap values (percentage of 1,000 replicates) for each cluster are shown at the nodes. Bar = 0.1 amino acid substitutions per site. Two-letter codes are used for species: At, *Arabidopsis thaliana*; Gm, *Glycine max*; Gu, *Glycyrrhiza uralensis*; Mt, *Medicago truncatula*; Pg, *Panax ginseng*; Sb, *Sorghum bicolor*; Vv, *Vitis vinifera*. The following CYPs (with their GenBank accession numbers) were used in this analysis: AtCYP85A1 (AB035868), AtCYP88A3 (NM_100394), AtCYP88A4 (NM_001202728), GuCYP72A154 (AB558153), GuCYP88D6 (AB433179), GmCYP93E1 (AB231332), MtCYP716A12 (FN995112), PgCYP716A47 (JN604536), PgCYP716A53v2 (JX036031), PgCYP716A52v2 (JX036032), SbCYP51 (U74319), VvCYP716A15 (AB619802) and VvCYP716A17 (AB619803).

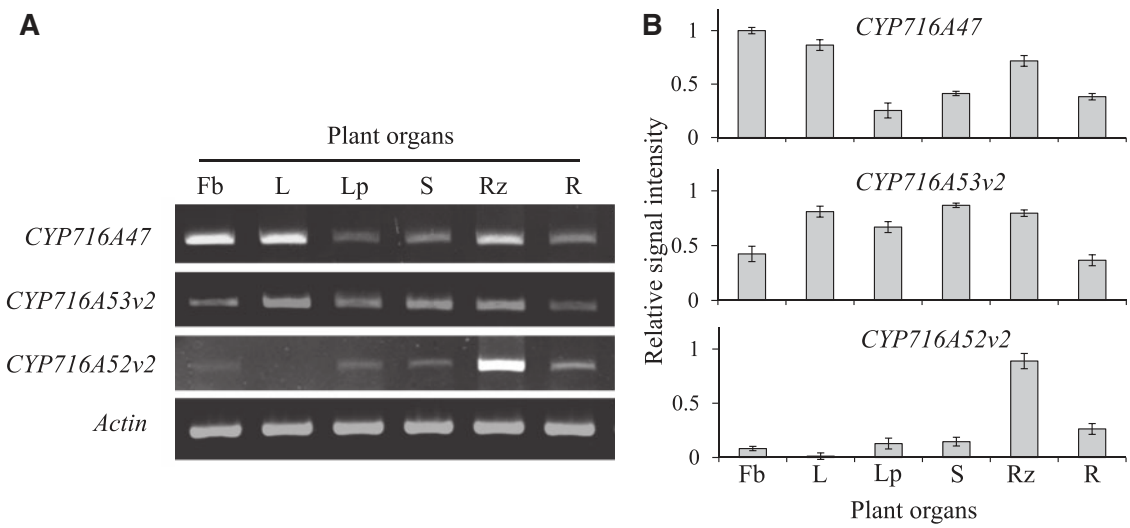

Fig. 3 RT–PCR analysis of the *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* mRNAs from different *P. ginseng* plant organs. (A) Accumulation of *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* mRNAs in different organs. (B) Densitometric analysis of *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* mRNAs in plant organs. Flower bud (Fb), leaf (L), leaf petiole (Lp), sympodium (S), rhizome (Rz) and root (R). Densitometric analysis represents the mean ± SE of three independent experiments, each performed in triplicate. The relative levels of target mRNA expression were determined by normalizing their individual band intensity to β-actin band intensity. The level of transcription was expressed as a level relative to CYP716A47 mRNA in flower bud.

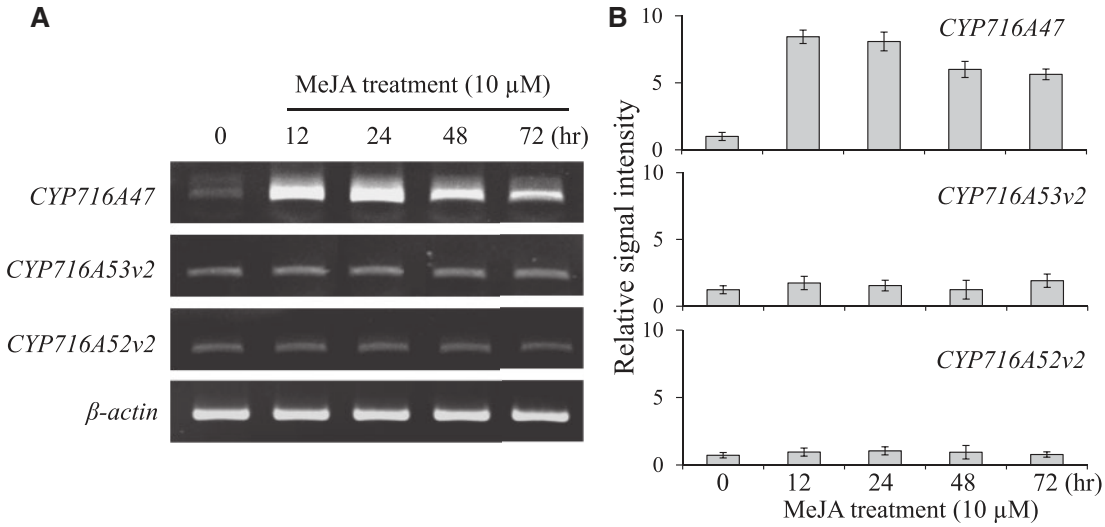

Fig. 4 RT–PCR analysis of *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* mRNAs in cultured adventitious roots after treatment with 10 μM MeJA for 12, 24, 48 and 72 h. Adventitious roots without MeJA treatment were used as a control. (A) Accumulation of *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* mRNAs in adventitious roots during various culture times after 10 μM MeJA treatment. (B) Densitometric analysis of *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* mRNAs in adventitious roots. Densitometric analysis represents the mean ± SE from three independent experiments, each performed in triplicate. The relative levels of target mRNA expression were determined by normalizing their individual band intensity to β-actin band intensity. The levels of transcription were expressed as a level relative to CYP716A47 mRNA in control roots.

its product converts protopanaxadiol into protopanaxatriol. These results also indicate that *CYP716A52v2* does not participate in protopanaxadiol synthesis.

In vitro CYP716A53v2 enzymatic activity assay

To verify the hydroxylation activity of the CYP716A53v2 enzyme in vitro, microsomal fractions from WAT21 yeast expressing *CYP716A53v2* were incubated with protopanaxadiol in the presence of NADPH for 2 h at 30°C. LC/APCIMS analysis of the reaction mixture revealed that *CYP716A53v2* converted protopanaxadiol (retention time, 33.6 min) into a new product (retention time, 19.2 min) with the same retention time as the pure protopanaxatriol standard (**Fig. 7A**). No activity was observed in boiled enzyme preparations of *CYP716A53v2* reacted with protopanaxadiol (**Fig. 7B**) or in yeast transformed with the empty vector (**Fig. 7C**). The LC/APCIMS fragmentation pattern of the new product (retention time, 19.2 min) included fragments with *m/z* ratios of 405 $[M\text{-}3H_2O + H]^+$, 423 $[M\text{-}2H_2O + H]^+$ and 441 $[M\text{-}1H_2O + H]^+$, which are the same as those of pure protopanaxatriol (**Fig. 6B**). This result also clearly demonstrates that *CYP716A53v2* catalyzes the conversion of protopanaxadiol into protopanaxatriol.

Discussion

CYP716A53v2 is a protopanaxadiol 6-hydroxylase that converts protopanaxadiol into protopanaxatriol

In *P. ginseng*, dammarane-type triterpenes are major ginsenosides whose triterpene aglycones are constituted of protopanaxadiols or protopanaxatriols. Protopanaxadiol is produced from dammarenediol-II by the hydroxylation of dammarenediol-II at the C-12 position. Protopanaxatriol is produced by the hydroxylation of protopanaxadiol at the C-6 position. Recently, we reported that the *CYP716A47* gene product is a dammarenediol 12-hydroxylase that produces protopanaxadiol from dammarenediol-II (Han et al. 2011).

We hypothesized that the next step of hydroxylation (protopanaxadiol 6-hydroxylase) for protopanaxatriol production from protopanaxadiol might be performed by an enzyme in the CYP716 family because both the *CYP716A47* and *CYP716A53v2* genes may be evolutionarily derived from a common ancestor and may act on a common dammarenediol skeleton. Thus, we focused on other *CYP716A* subfamily genes as candidate protopanaxatriol synthases. In addition to *CYP716A47*, two more genes (*CYP716A52v2* and *CYP716A53v2*) belonging to the CYP716A subfamily were found among the ESTs obtained from an in vitro cultured adventitious root cDNA library (Han et al. 2011).

The ORFs of *CYP716A52v2* and *CYP716A53v2* cDNA were heterologously expressed in yeast. Extracts of recombinant yeast expressing *CYP716A53v2* converted protopanaxadiol into protopanaxatriol, indicating that this enzyme has protopanaxadiol 6-hydroxylase activity. In contrast, *CYP716A52v2* did not exhibit this activity. The hydroxylation activity of *CYP716A53v2* was further confirmed by an in vitro enzymatic activity assay. LC/APCIMS clearly demonstrated the production of protopanaxatriol from protopanaxadiol, and the protopanaxatriol production was confirmed by the MS fragmentation pattern, which matched that of pure protopanaxatriol. Thus, these two CYP genes, *CYP716A47* and *CYP716A53v2*, were

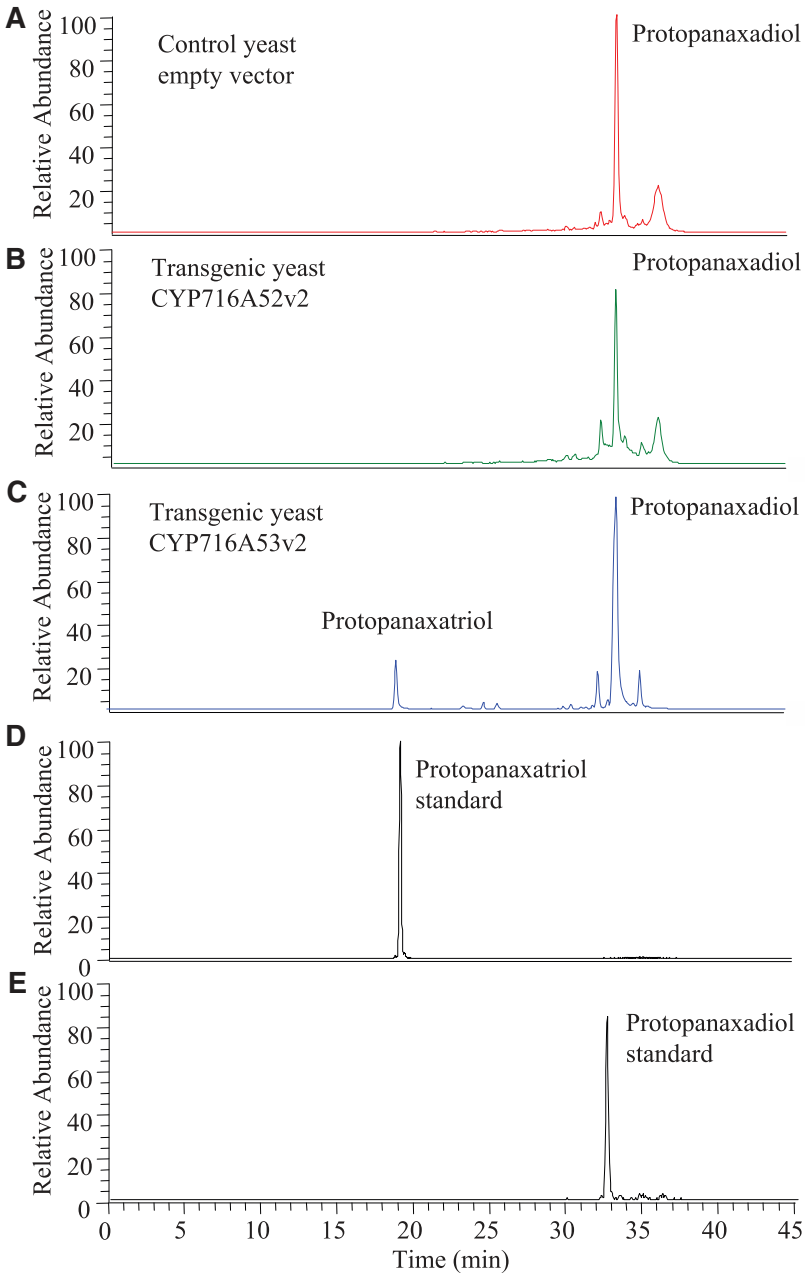

Fig. 5 The total ion chromatograms from the LC/APCIMS analysis of the products in yeast expressing *CYP716A52v2* or *CYP716A53v2*. (A) Only protopanaxadiol was detected in the cell extract of yeast harboring the empty vector as a control. (B) Only protopanaxadiol was detected in the cell extract of yeast harboring pYES2-*CYP716A52v2*. (C) A new peak at 19.2 min, which corresponds to the retention time of the protopanaxatriol standard, was detected in the cell extract of the yeast harboring pYES2-*CYP716A53v2*. (D) The LC chromatogram of the protopanaxatriol standard. (E) The LC chromatogram of the protopanaxadiol standard.

determined to be involved in the synthesis of two dammarenediol-type triterpene aglycones (protopanaxadiol and protopanaxatriol), and the products of these two genes are a dammarenediol 12-hydroxylase and a protopanaxadiol 6-hydroxylase, respectively. Although these two CYP genes are grouped together in the CYP716A subfamily, the deduced amino acid sequences of the two genes exhibit only 49% identity. Thus, there are considerable differences in the amino acid sequences of the protopanaxadiol synthase (*CYP716A47*) and the protopanaxatriol synthase (*CYP716A53v2*), even though they both participate in the same biosynthetic pathway, i.e. dammarenediol-type ginsenoside biosynthesis.

In our previous work, we demonstrated that *CYP716A47* had no hydroxylase activity other than protopanaxadiol 6-hydroxylase activity (Han et al. 2011). In the present study, we tested *CYP716A53v2* for additional activity to determine whether the gene product has protopanaxadiol synthase activity. Feeding dammarenediol to yeast expressing *CYP716A53v2*

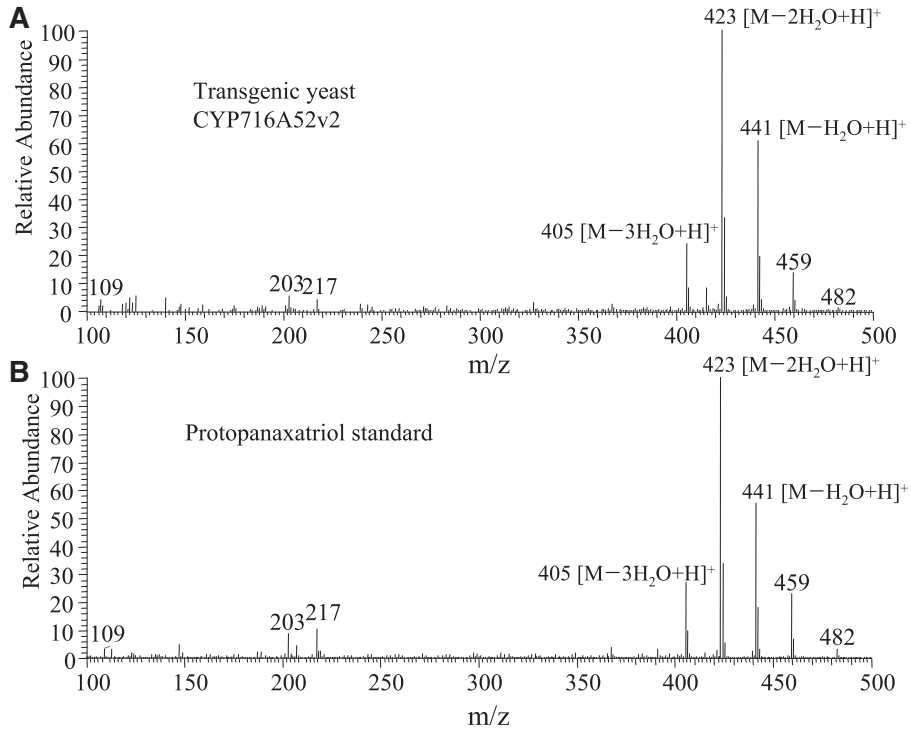

Fig. 6 LC/APCIMS fragmentation pattern of the new peak detected in the extract of yeast expressing *CYP716A53v2*. (A) The MS spectrum for the new peak at 19.2 min detected in yeast expressing *CYP716A53v2*. The LC/APCIMS fragmentation pattern included fragments with *m/z* ratios of 405 $[M\text{-}3H_2O + H]^+$, 423 $[M\text{-}2H_2O + H]^+$ and 441 $[M\text{-}H_2O + H]^+$, which are the same as those for pure protopanaxatriol. (B) The MS spectrum for the peak detected in the protopanaxatriol standard.

did not result in protopanaxadiol production (no dammarenediol 12-hydroxylase activity) (data not shown). These results indicate that neither *CYP716A47* nor *CYP716A53v2* is multifunctional. In contrast, multifunctional activity is frequently found in CYP enzymes involved in the biosynthesis of pentacyclic triterpenes in Fabaceae plants (Fukushima et al. 2011).

Panax ginseng roots contain one minor oleanane-type ginsenoside, ginsenoside Ro (Matsuda et al. 2010). The starting aglycone for the ginsenoside Ro is oleanolic acid, which is postulated to be derived from β-amyrin by CYP (Han et al. 2011). Recently, *CYP716A12* from *M. truncatula* was shown to catalyze β-amyrin and erythrodiol oxidation at the C-28 position, yielding oleanolic acid (Carelli et al. 2011, Fukushima et al. 2011). Interestingly, the amino acid sequences of *CYP716A52v2* show high identity (73%) with *CYP716A12*. Thus, *CYP716A52v2* in *P. ginseng* is suggested to have a function similar to that of *CYP716A12* in *M. truncatula* and to that of *CYP716A15* in *Vitis vinifera*, both of which participate in oleanolic saponin biosynthesis. Thus, *CYP716A52v2* might be postulated to participate in the biosynthesis of ginsenoside Ro in *P. ginseng*.

Transcriptional activity of the three ginseng CYP genes

The transcriptional activity of the three CYP genes was analyzed by RT–PCR in different parts of ginseng plants. The overall patterns of *CYP716A47* and *CYP716A53v2* mRNA accumulation in different parts of ginseng plants were similar, most probably because these two genes are involved in the same biosynthetic pathway. Interestingly, the roots showed less accumulation of *CYP716A47* and *CYP716A53v2* mRNA than did the leaves. The organ-specific mRNA accumulation patterns of *CYP716A47* and *CYP716A53v2* also corresponded to the pattern of ginsenoside accumulation in different parts of ginseng plants (Shi et al. 2007). The *P. ginseng* root is considered to be the main part of the plant used for medicinal purposes, and most studies on ginsenosides have focused on the *P. ginseng* root (Shi et al. 2007, Kim et al. 2009). However, the total ginsenoside content in ginseng plants is higher in the leaves than in the roots and is lower in the stem than in other parts (Shi et al. 2007). Kim et al. (2009) reported that berries produced more total ginsenosides than any other part of the plant, and the ginsenoside profile in the berries differed from that in the roots. A similar pattern of ginsenoside accumulation was reported for *Panax quinquefolius* plants (Wang et al. 2006).

Interestingly, the level of *CYP716A52v2* mRNA accumulation was much higher in the rhizome than in the other parts of plants, a pattern that is clearly different from the patterns of *CYP716A47* and *CYP716A53v2* mRNA accumulation. It was reported that the rhizome contains a high level of the oleanane-type ginsenoside Ro (Matsuda et al. 2010). Thus, the pattern of *CYP716A52v2* mRNA accumulation suggests that *CYP716A52v2* is a putative CYP gene whose product is involved in oleanane-type ginsenoside biosynthesis.

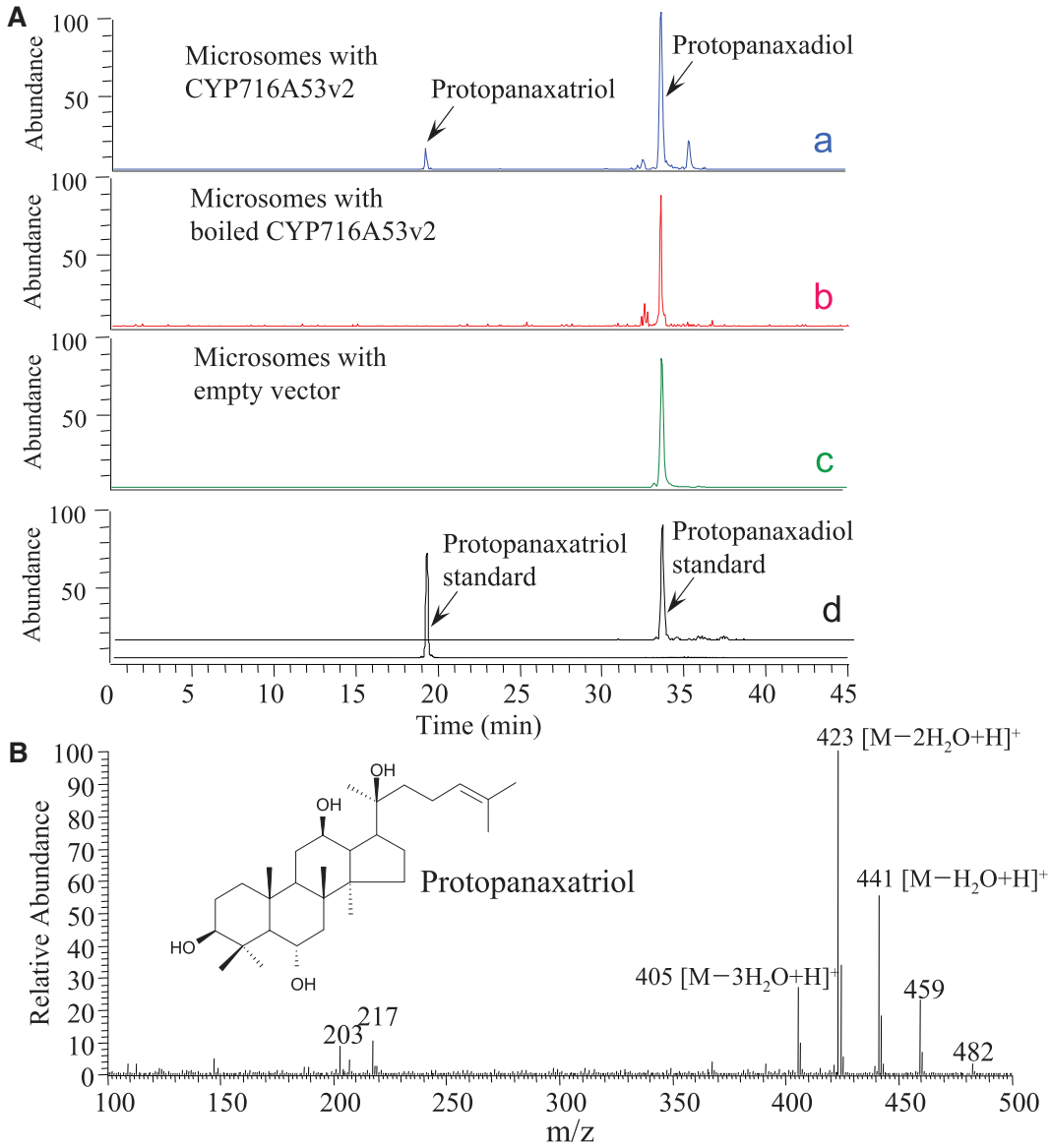

Fig. 7 In vitro conversion of protopanaxadiol into protopanaxatriol using microsomes from yeast expressing *CYP716A53v2*. (A) LC/APCIMS chromatograms of the products of the reaction of microsomes with protopanaxadiol. Line a shows the protopanaxatriol (19.2 min) produced by the reaction of microsomes from yeast harboring pYES2-*CYP716A53v2* and fed protopanaxadiol. Lines b and c show the products of the reaction of boiled microsomes with protopanaxadiol and the reaction of microsomes from yeast harboring the empty vector with protopanaxadiol, respectively. Line d shows the chromatograms for the protopanaxadiol and protopanaxatriol standards. (B) The MS spectrum of a peak (19.2 min) detected for the reaction products of microsomes from yeast harboring *CYP716A53v2* in line a of A. The LC/APCIMS fragmentation pattern included fragments with *m/z* ratios of 405 $[M\text{-}3H_2O + H]^+$, 423 $[M\text{-}2H_2O + H]^+$ and 441 $[M\text{-}H_2O + H]^+$, which are the same as those for pure protopanaxatriol (**Fig. 6B**).

The transcription of *CYP716A47*, *CYP716A53v2* and *CYP716A52v2* in adventitious roots was examined by RT–PCR at 12, 24, 48 and 72 h after treatment with 10 μM MeJA (**Fig. 5B**). MeJA treatment resulted in the obvious accumulation of *CYP716A47* mRNA relative to the level in the control. However, the *CYP716A53v2* mRNA level was not affected by MeJA treatment. This result indicates that the *CYP716A53v2* gene responds differently from *CYP716A47* after MeJA treatment even though the two genes participate in the same pathway of ginsenoside biosynthesis in *P. ginseng*. Yu et al. (2002) reported that MeJA treatment of ginseng adventitious roots resulted in >5-fold increases in the levels of protopanaxadiol-type ginsenosides (particularly Rb_1) after 7 d of MeJA treatment, but the content of protopanaxatriol-type ginsenosides did not change. These results suggest that protopanaxadiol- and protopanaxatriol-type ginsenosides might respond to different stimuli and have different functional roles in the biological defense mechanisms of ginseng plants.

Over 30 ginsenosides have been identified in *P. ginseng*, and they have been divided into two groups according to aglycone structure: the protopanaxadiols (Rb_1, Rb_2, Rc and Rd) and the protopanaxatriols (Rg_1, Re, Rf and Rg_2). Each ginsenoside has been shown to have different pharmacological effects, including immune system modulation, anti-stress, anti-hyperglycemic, anti-inflammatory, anti-oxidant and anti-cancer effects (Briskin 2000, Shibata 2001). Recent studies have demonstrated that ginsenoside aglycones, including both protopanaxadiol and protopanaxatriol, have greater biological effects than ginsenosides (triterpene glycosides) with larger molecular structures (Jia et al. 2004, Popovich and Kitts 2004). Protopanaxadiol has been shown to exhibit apoptotic effects on cancer cells through various signaling pathways and has been reported as cytotoxic to multidrug-resistant tumors (Jia et al. 2004, Popovich and Kitts 2004, Li et al. 2006). The identified protopanaxadiol synthase (*CYP716A47*) and protopanaxatriol (*CYP716A53v2*) can be used in metabolic engineering studies to produce these compounds directly in microorganisms and plants or to enhance the production of ginsenosides by the genetic transformation of *P. ginseng*.

Materials and Methods

Isolation of cDNA clones for *CYP716A52v2* and *CYP716A53v2*

Two full-length cDNA clones (CYP716A52v2, GenBank accession No. JX036032; and CYP716A53v2, GenBank accession No. JX036031) were obtained by full gene sequencing of ESTs obtained from an in vitro cultured adventitious root cDNA library (Han et al. 2011).

Comparison of CYP protein sequences

To analyze the phylogenic relationships among these gene sequences, amino acid sequences were obtained from EMBL, GenBank and DDBJ. Multiple sequence alignments were generated using the CLUSTALW program (Thompson et al. 1994). Phylogenetic analysis of the deduced amino acid alignments was performed using the Neighbor–Joining method with TreeView software (Page 1996). Bootstrap analysis with 1,000 replicates was used to assess the strength of the nodes in the tree (Felsenstein 1985).

RT–PCR analysis

Total RNA was isolated from the MeJA-treated adventitious roots or from different organs of ginseng plants and reverse-transcribed using the ImProm-II Reverse Transcription System (Promega). First-strand cDNA 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 cDNA for β-actin was used as a control for RNA integrity and loading accuracy. The products were electrophoresed on a 1% agarose gel in 0.5× TBA buffer. The RT–PCR analyses were repeated twice, and representative data are shown in the figures. The primers used were 5′-ATG GTG TTG TTT TTC TCC CTA TCT CTT CTT CT-3′ and 5′-TTA ATT GTG GGG ATG TAG ATG AAT-3′ for *P. ginseng CYP716A47*; 5′-ATG GAA CTC TTC TAT GTC CCT CT-3′ and 5′-TTA GGC TTT GTG TGG AAA TAG GC-3′ for *P. ginseng CYP716A52v2*; and 5′-ATG GAT CTC TTT ATC TCA TCT CAA-3′ and 5′-TTA AAG CGT ACA AGG TGA TAG ACG-3′ for *P. ginseng CYP716A53v2*. The β-actin cDNA (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 a control for RNA integrity and loading accuracy. The band intensity was measured using a scanning densitometer, and quantified using MULTI GAUGE 3.0 (Fujifilm, http://www.fujifilm.com). These experiments were performed in triplicate.

Ectopic expression of *CYP716A52v2* and *CYP716A53v2* in yeast

To construct an expression plasmid vector for yeast, the ORFs of *CYP716A52v2* and *CYP716A53v2* were amplified from cDNA using PCR (25 cycles of 40 s at 94°C, 40 s at 55°C and 2 min at 72°C) with *Pfu* DNA polymerase (Invitrogen) and cloned into pYES2.1 using the TOPO TA expression kit (Invitrogen). The primer pairs used to isolate the cDNAs were 5′-ATG GAA CTC TTC TAT GTC CCT CT-3′ and 5′-TTA GGC TTT GTG TGG AAA TAG GC-3′ for *CYP716A52v2* synthase and 5′-ATG GAT CTC TTT ATC TCA TCT CAA-3′ and 5′-TTA AAG CGT ACA AGG TGA TAG ACG-3′ for *CYP716A53v2* synthase. The cloned pYES2.1/V5-His-TOPO vector was transformed into *Escherichia coli*. The ORFs were then ligated to the GAL1 promoter in the sense orientation. The nucleotide sequence of the inserted DNA was confirmed by sequencing. Expression vectors for *CYP716A52v2* and *CYP716A53v2* and an empty vector were used to transform the *Saccharomyces cerevisiae* strain WAT21, which expresses the *A. thaliana* NADPH-CYP reductase (Urban et al. 1997).

WAT21 yeast cells were transformed using a modified lithium acetate procedure, as described previously (Gietz et al. 1992). Transformed cells were selected by SC-U (SC minimal medium lacking uracil) and subcultured on YPG medium after 3 d of growth (Kribii et al. 1997). The culture conditions and methods for galactose induction and the preparation of the triterpene monoalcohol fraction have been described previously (Kushiro et al. 1998), with the exception that protopanaxadiol (10 mg l^{-1}) was added to the medium as a substrate. After galactose induction for 1 d, the cells were collected by centrifugation at 500×*g* for 5 min and refluxed with 2 ml of 20% KOH/50% EtOH for 5 min. After extraction with the same volume of hexane, the extracts were dissolved in 80% acetonitrile and 20% water to remove sterol, and analyzed by LC/APCIMS.

In vitro enzymatic activity assay. Extraction of proteins from yeast *CYP716A52v2* and *CYP716A53v2* was done as described by Olsen et al. (2010) with some modifications. The microsomal fractions were collected by ultracentrifugation at 100,000×*g* for 60 min. The enzymatic activity of *CYP716A53v2* was tested in a

total volume of 500 μl of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM NADPH, 20 μg of substrate and 1 mg of microsomal fraction protein. The reaction mixture was incubated for 2 h at 30°C, and the reaction was extracted twice with the same volume of hexane. The hexane extracts were evaporated and analyzed using LC/APCIMS.

LC/APCIMS analysis of the recombinant yeast

LC/APCIMS analysis was performed on a surveyor LC system (Thermo Finnigan Co.) consisting of four solvent pumps, a Rheodyne injector (5 ml loop) and an HTP Pal autosampler (CTC Analytics). The analytical column was a YMC pack-pro C18 RS (5 mm, 2.0 × 150 mm, YMC Co. Ltd. Japan) maintained at 40°C. The durations of steps in the water and acetonitrile gradient and the ratios of these constituents were as follows: 0 min, 20% acetonitrile and 80% water; 30 min, 90% acetonitrile and 10% water; 32 min, 95% acetonitrile and 5% water; 34 min, 95% acetonitrile and 5% water; 36 min, 20% acetonitrile and 80% water; and 45 min, 20% acetonitrile and 80% water at a flow rate of 0.2 ml min^{-1}. A triple quadrupole Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Electron Co.) was fitted with an atmospheric pressure chemical ionization (APCI) system, which was used for detection. The analysis was performed in positive mode with a 5.0 μA discharge current, a 320°C vaporizer temperature and a 320°C ion-transfer capillary temperature. Nitrogen was used as the sheath (15 psi) and auxiliary gas (10 psi). Pure protopanaxadiol and protopanaxatriol were analyzed under the same conditions. The protopanaxadiol and protopanaxatriol used as the standards for the LC/APCIMS analysis were purchased from Sigma-Aldrich Co.

Supplementary data

Supplementary data are available at PCP online.

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