CYP716A Subfamily Members are Multifunctional Oxidases in Triterpenoid Biosynthesis

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Triterpenoids are a diverse group of secondary metabolites that are associated with a variety of biological activities. Oleanolic acid, ursolic acid and betulinic acid are common triterpenoids in plants with diverse biological activities, including antifungal, antibacterial, anti-human immunodeficiency virus (HIV) and/or antitumor activities. In the present study, using the gene co-expression analysis tool of Medicago truncatula, we found a strong correlation between CYP716A12 and β-amyrin synthase (βAS), which encodes the enzyme responsible for the initial cyclization of 2,3-oxidosqualene to β-amyrin (the basic structural backbone of most triterpenoid saponins). Through an in vitro assay, we identified CYP716A12 as a β-amyrin 28-oxidase able to modify β-amyrin to oleanolic acid (through erythrodiol and, possibly, oleanolic aldehyde). We also confirmed its activity in vivo, by expressing CYP716A12 in transgenic yeast that endogenously produce β-amyrin. In addition, CYP716A12 was evaluated for its potential β-amyrin- and lupeol-oxidizing activities. Interestingly, CYP716A12 was able to generate ursolic acid (through uvaol and, possibly, ursolic aldehyde) and betulinic acid (through betulin).

Hence, CYP716A12 was characterized as a multifunctional enzyme with β-amyrin 28-oxidase, β-amyrin 28-oxidase and lupeol 28-oxidase activities. We also identified homologs of CYP716A12 in grape (CYP716A15 and CYP716A17) that are involved in triterpenoid biosynthesis, which indicates the highly conserved functionality of the CYP716A subfamily among plants. These findings will be useful in the heterologous production of pharmacologically and industrially important triterpenoids, including oleanolic acid, ursolic acid and betulinic acid.

Keywords: Cyt P450 monooxygenase, CYP716A, Medicago truncatula, Triterpenoids, Vitis vinifera.

Abbreviations: ADH, alcohol dehydrogenase; aAS, α-amyrin synthase; bAS, β-amyrin synthase; CYP, Cyt P450 monooxygenase; CPR, Cyt P450 reductase; EIC, extracted ion chromatogram; EST, expressed sequence tag; GC-MS, gas chromatography–mass spectrometry; HIV, human immunodeficiency virus; LUS, lupeol synthase; OSC, oxidosqualene cyclase; Rt, retention time; RT–PCR, reverse transcription–PCR; Sf9, Spodoptera frugiperda 9; TIC, total ion chromatogram; UGT, uridine diphosphate-dependent glycosyltransferase.

The nucleotide sequences reported in this paper have been submitted to the GenBank database under the accession numbers Glycyrrhiza uralensis lupeol synthase (LUS), AB663343; Vitis vinifera CYP716A15, AB619802; Vitis vinifera CYP716A17, AB619803.

Introduction

Triterpenoid saponins are a diverse group of secondary metabolites produced by many plant species. Examples include medicinal plants that are exploited as drug sources such as licorice and ginseng, as well as crop plants, such as legumes and oats (Haralampidis et al. 2002). Interest in triterpenoid saponins has increased recently because of data showing their diverse biological activities and beneficial properties, which include antifungal, antibacterial, antiviral, antitumor, molluscicidal, insecticidal and antifeedant activities (Suzuki et al. 2002,
Sparg et al. 2004, Huhman et al. 2005). Hence, elucidation of the pathways underlying the biosynthesis of triterpenoid saponins at the molecular level will help increase production levels within native hosts and/or facilitate engineering of these pathways in heterologous hosts (Julsing et al. 2006).

The first committed step in triterpenoid saponin biosynthesis is the cyclization of 2,3-oxidosqualene. This reaction is catalyzed by specific oxidosqualene cyclases (OSCs), including β-amyrin synthase (bAS), and has been functionally characterized in several plants (Herrera et al. 1998, Kushiro et al. 1998, Morita et al. 2000, Suzuki et al. 2002, Iturbe-Ormaetxe et al. 2003) (Supplementary Fig. S1). Subsequent modifications that impart functional properties and diversify the basic triterpenoid backbone include the addition of small functional groups, including hydroxyl, keto, aldehyde and carboxyl moieties, generally followed by glycosylation reactions (Augustin et al. 2011). Cyt P450 monooxygenases (CYPs) perform many of these prior-to-glycosylation modifications (Croteau et al. 2000). To date, a number of CYPs that use β-amyrin as a substrate have been identified in dicotyledonous plants, whereas just one (CYP51H10) has been identified in monocots (Supplementary Fig. S1). CYP88D6 catalyzes the C-11 oxidation of β-amyrin in licorice (Glycyrrhiza uralensis, Fabaceae) glycyrrhizin biosynthesis (Seki et al. 2008). Both soybean (Glycine max) CYP93E1 (Shibuya et al. 2006) and licorice CYP93E3 (Seki et al. 2008) catalyze the C-24 hydroxylation of β-amyrin in soyasaponin biosynthesis. Although biochemical characterization is still needed, oat (Avena strigosa) CYP51H10 (Sad2) may catalyze an early modification of β-amyrin in avennacin biosynthesis (Qi et al. 2006).

Identifying the specific CYP enzyme responsible for the production of a particular metabolite is a difficult task due to the large number of members and significant diversity within the CYP multigene family (Augustin et al. 2011). A comprehensive approach that includes genomic and expressed sequence tag (EST) sequence data, transcript and metabolite profiling, and heterologous expression to assess enzymatic activity is necessary to annotate new biosynthetic gene functions correctly (Marasco and Schmidt-Dannert 2007). Such a combined approach has been used successfully to identify several glycosyltransferase genes involved in the biosynthesis of triterpenoid saponins in Medicago truncatula (Achnine et al. 2005).

Various saponins have been isolated from the genus Medicago (Bialy et al. 1999, Bialy et al. 2004, Broeckling et al. 2005, Kapusta et al. 2005a, Kapusta et al. 2005b, Bialy et al. 2006, Schliemann et al. 2008, Tava et al. 2009). In fact, > 30 saponins have been identified in the model legume, M. truncatula (Huhman and Sumner 2002). The core of this diversity is centralized in a few aglycones (sapogenins). According to the position of decorations, the aglycones could be differentiated into two groups: those that are hydroxylated at the C-24 position (soyasapogenols), and those that are hydroxylated at the C-23 and carboxylated at the C-28 position (hemolytic sapogenins) (Carelli et al. 2011) (see Fig. 1).

Gene co-expression analysis has emerged as a powerful tool for predicting gene function, because genes whose expression...
patterns are strongly correlated with each other are expected to be involved in the same biological processes. Furthermore, large amounts of high-quality microarray data in public primary databases have facilitated the calculation of gene co-expression scores across thousands of samples (Usadel et al. 2009). In M. truncatula, Naoumkina et al. (2010) developed a database of co-expressed genes based on comprehensive clustering of methyl jasmonate-induced transcript expression patterns along with chromosomal location analysis.

Here, we report the identification of CYP716A12 as a multi-functional oxidase in triterpenoid saponin biosynthesis through bioinformatics analysis (e.g. M. truncatula gene atlas co-expression analysis and biochemical pathway database data) and heterologous expression in Spodoptera frugiperda 9 (Sf9) insect cells and transgenic yeast. Large amounts of oleanolic acid accumulate in grape skin (Zhang et al. 2004), and both oleanane and lupane saponins have been isolated from seedless raisins (Rivero-Cruz et al. 2008). We also identified homologs of CYP716A12 in grape (CYP716A15 and CYP716A17) that are involved in triterpenoid biosynthesis, showing the highly conserved functionality of the CYP716A subfamily beyond plant lineages.

Results

Selection of candidate CYPs

We used the gene co-expression analysis tool of M. truncatula (http://mtgea.noble.org/v2/correlation_search_form.php) to find CYP genes co-expressed with bAS (Supplementary Table S1, Supplementary Fig. S2). Genes encoding CYPs belonging to the CYP72A (three genes), CYP716A (one gene) and CYP93E (one gene) subfamilies had correlation coefficients to bAS that were >0.7 (Table 1). We tested the β-amin oxidizing activity of those CYPs in vitro and/or in vivo. CYP93E2 (R = 0.791) oxidized β-amin at C-24, producing 24-OH-β-amin (Fig. 1) and probably β-amin-24-oic acid (Supplementary Fig. S3). Consistent with this activity, we found 79.8 and 86.4% amino acid sequence identities between CYP93E2 and the previously reported β-amin 24-oxidase CYP93E1 (Shibuya et al. 2006) and CYP93E3 (Seki et al. 2008), respectively.

All of the remaining genes had correlation coefficient values greater than that for CYP93E2 (Table 1); however, they did not show β-amin oxidizing activity, except for CYP716A12.

β-amin 28-oxidase activity of M. truncatula CYP716A12

Microsomes containing CYP716A12 were obtained from Sf9 insect cells and assayed in vitro with β-amin (compound 1 in Fig. 2) as the substrate. The obtained samples were extracted with ethyl acetate and analyzed by gas chromatography–mass spectrometry (GC-MS). The total ion chromatogram (TIC) showed a new peak [4, retention time (Rt) = 19.74 min, Fig. 3] in the CYP716A12-containing reaction, with respect to the empty vector (control) and boiled microsome reactions. The Rt and mass spectrum of this new peak matched with those of authentic oleanolic acid (compound 4 in Figs. 2, 3).

To confirm these results, we evaluated the activity of CYP716A12 with the engineered yeast system used to characterize CYP88D6 (Seki et al. 2008). This system is based on the co-expression of OSC, CYP and CPR (Cyt P450 reductase, a redox partner of CYP) in yeast cells (Supplementary Fig. S4). The TIC of an ethyl acetate extract of bAS/CPR/CYP716A12-expressing yeast showed three new peaks compared with the control sample (carrying bAS and CPR alone) (Fig. 4A). Peak 2 (Rt = 19.02 min) was identified as erythrodiol (compound 2 in Fig. 2) and peak 4 (Rt = 19.52 min) was identified as oleanolic acid (4), based on a comparison with the Rts and mass spectra of authentic standards (Supplementary Fig. S5). Additionally, a peak (Rt = 19.45 min) presumably corresponding to oleanaldehyde (compound 3 in Figs. 2, 4A, Supplementary Fig. S5) was detected, although we were unable to confirm that result using an authentic standard.

In this way, CYP716A12 was identified as a β-amin 28-oxidase that catalyzes three sequential oxidation reactions at C-28 of the oleanane backbone.

Activity of M. truncatula CYP716A12 toward other triterpenes

To assess further the substrate specificity of CYP716A12, we tested its activity using α-amin (compound 5 in Fig. 2) and lupeol (compound 9 in Fig. 2) as potential substrates. Ursolic acid (compound 8 in Fig. 2) and betulinic acid (compound 12 in Fig. 2) are triterpenoids with many reported biological properties, including antitumor and anti-human immunodeficiency virus (HIV) activities (Fulda and Kroemer 2009). Interestingly, these triterpenoids have the same molecular weight and share the C-28 carboxyl group with oleanolic acid (4) (see Fig. 2). Consequently, it is thought that CYP716A12 may use α-amin (5) and lupeol (9) as substrates to produce ursolic acid (8) and betulinic acid (12), respectively. To validate this assumption, CYP716A12 was tested in α-amin synthase (aAS)/CPR and
Fig. 3  In vitro activity of *M. truncatula* CYP716A12, and of *Vitis vinifera* CYP716A15 and CYP716A17, toward β-amyrin. The TICs of our in vitro assay reaction products (produced by GC-MS) are shown. Oleanolic acid (1) was detected in CYP716A12- and CYP716A15- and CYP716A17-expressing Sf9 microsomes, but not in boiled (denatured) microsomes. Mass spectra showing the observed peaks compared with those produced for the oleanolic acid standard.
lupeol synthase (LUS)/CPR backgrounds as described in the analysis of β-amyрин 28-oxidase activity. Peaks corresponding to uvaol (compound 6 in Fig. 2; peak 6 in Fig. 4B; Rt = 19.11 min) and ursolic acid (peak 8 in Fig. 4B; Rt = 20.12 min) were found in an ethyl acetate extract of aAS/CPR/CYP716A12-expressing yeast (Fig. 4B, Supplementary Fig. S6). In addition, a peak (7; Rt = 20.03 min) that presumably corresponds to ursolic aldehyde (compound 7 in Fig. 2) was observed, though it was not possible to make a comparison with the corresponding standard. We also detected erythrodiol (2) and oleanolic acid (4) as minor products (Supplementary Fig. S6). There is no monofunctional aAS; all reported OSCs that include β-amyрин (5) as an enzymatic product have been shown to be multifunctional enzymes (Saimaru et al. 2007). In this work, we used an OSC from olive that mainly produces β-amyрин (5); however, it has also been reported to produce β-amyрин (1) as one of its minor products (Saimaru et al. 2007). Thus, it is reasonable that β-amyрин (1)-oxidized derivatives (erythrodiol and oleanolic acid) were observed in addition to α-amyрин (5)-oxidized derivatives.

**Fig. 4** In vivo production of oleanolic acid, ursolic acid and betulinic acid in transgenic yeast co-expressing bAS, aAS or LUS; CPR; and CYP716A12. The TICs of ethyl acetate extracts (produced by GC-MS) from each yeast culture are shown. (A) Erythrodiol (2) and oleanolic acid (4) were detected in bAS/CPR/CYP716A12-expressing yeast. (B) Uvaol (6), probable ursolic aldehyde (7) and ursolic acid (8) were detected in aAS/CPR/CYP716A12-expressing yeast. Erythrodiol (2) and oleanolic acid (4) were also observed. An enlarged chromatogram corresponding to Rt = 18–21 min is shown as an inset. (C) Betulin (10) and betulinic acid (12) were detected in LUS/CPR/CYP716A12-expressing yeast. All yeast expression assays were done in duplicate. Insets, enlarged chromatographs at 19–20 min. Yeast cultures expressing bAS, aAS or LUS and CPR were used as controls, respectively.
On the other hand, peaks corresponding to betulin (compound 10 in Fig. 2; peak 10 in Fig. 4C; Rt = 19.26 min) and betulinic acid (peak 12 in Fig. 4C; Rt = 19.46 min) were found in an ethyl acetate extract of LUS/CPR/CYP716A12-expressing yeast (Fig. 4C, Supplementary Fig. S7). None of these peaks was observed in the control samples.

Together, these results suggest that CYP716A12 is a multifunctional oxidase that modifies β-amyrin (1) to produce oleanolic acid (4), and that it also modifies α-amyrin (5) and lupeol (9) to produce ursolic acid (8) and betulinic acid (12), respectively.

Cloning and functional characterization of grape CYP716As

Among the CYP716A family members listed on the Cytochrome P450 Homepage (Nelson 2009), 13 full-length sequences and seven partial sequences were found in grape (Fig. 5, Supplementary Table S2). From these sequences, we obtained the full-length cDNAs of CYP716A15 and CYP716A17, which showed 75.3% and 75.1% amino acid identities, respectively, to CYP716A12 (Supplementary Fig. S8). CYP716A15 and CYP716A17 showed similar expression patterns in two cultivars (cv. Cabernet Sauvignon and cv. Pinot Noir). They were highly expressed in stem and fruit skin, especially at the young and intermediate stages (Supplementary Fig. S9).

When we tested the in vitro activity of CYP716A15 and CYP716A17 with β-amyrin as the substrate, both proteins catalyzed the production of oleanolic acid (4) compared with control reaction samples, as described for CYP716A12 (Fig. 3). Therefore, we suggest that CYP716A15 and CYP716A17 are involved in the production of oleanolic acid (4) in grape skin.

Further we tested the in vivo activity of CYP716A15 in three different transgenic yeast backgrounds: bAS/CPR, aAS/CPR and LUS/CPR. CYP716A15 modified each of these substrates. Peaks corresponding to erythrodiol (2), possibly oleanolic aldehyde (3) and oleanolic acid (4) were detected in bAS/CPR/CYP716A15-expressing yeast (Fig. 6A, Supplementary Fig. S10).

Similiar to results described previously for CYP716A12, we detected peaks identified as uvaol (6), ursolic acid (8) and possibly ursolic aldehyde (7), along with other minor products (2, 4), in aAS/CPR/CYP716A15-expressing yeast (Fig. 6B, Supplementary Fig. S11).

Finally, betulin (10) and betulinic acid (12) were detected in LUS/CPR/CYP716A15-expressing yeast (Fig. 6C, Supplementary Fig. S12). None of these peaks was detected in the control samples (carrying the LUS and CPR genes only). These results might implicate CYP716A15 in lupeane saponin biosynthesis in grape.

Discussion

Oleanolic acid, ursolic acid and betulinic acid are widespread triterpenoids in plants and show diverse biological activities, including antifungal, antibacterial, anti-HIV and/or antitumor activities (Sasazuka et al. 1995, Fulda and Kroemer 2009, Wu 2009). In the present study, we identified three multifunctional enzymes belonging to the CYP716A subfamily (a CYP85 clan member) that catalyze three sequential oxidation reactions at C-28 of β-amyrin, producing oleanolic acid. In addition, when CYP716A12 and CYP716A15 were expressed in yeast, they were able to modify α-amyrin and lupeol, generating ursolic acid and betulinic acid, respectively (Fig. 2).

CYP clans are deep gene clades observable on phylogenetic trees. Land plants comprise 11 clans. Due to the great diversity of CYPs that belong to the CYP71, CYP72 and CYP85 clans, the prediction of substrate preferences based on sequence data is difficult (Nelson and Werck-Reichhart 2011); however, the majority of the enzymes reported to be involved in triterpenoid biosynthesis belong to these clans (Supplementary Fig. S1). Moreover, these enzymes catalyze modifications of β-amyrin (C-11 oxidation and C-24 hydroxylation) that are relatively rare among saponins (Augustin et al. 2011). More common modifications, including C-23 hydroxylation and C-28 carboxylation of the oleanane backbone, were implied to be catalyzed by more conserved yet uncharacterized CYPs (Augustin et al. 2011). CYP716 family members are widespread among plants (Fig. 5, Supplementary Table S2); in fact, oleanolic acid, ursolic acid and betulinic acid have been isolated from numerous plant species. Indeed, oleanolic acid has been found in >1,620 species corresponding to >146 families, including monocots and gymnosperms (Fai and Tao 2009). The present study revealed the function of CYP716A subfamily members that are highly conserved among plants and appear to share the same C-28 oxidizing activity, suggesting their biosynthetic role in plants containing oleanolic acid, ursolic acid and/or betulinic acid.

The phylogenetic tree in Fig. 5 shows that the enzymes identified in this work are closely related, suggesting that oleanolic acid-producing CYPs form a functional cluster beyond plant lineages.

Interestingly, CYP716 family members are apparently restricted to dicotyledonous plants (Nelson 2009); however, as mentioned above, oleanolic acid has been isolated from many species, some of which belong to the monocot families Cyperaceae, Poaceae, Iridaceae and Orchidaceae (Fai and Tao 2009). Thus, additional studies are needed to elucidate whether CYP716 family members are also present in (at least) these plant families.

Conventionally, CYPs are classified into A-type and non-A-type. A-type CYPs are thought to be primarily involved in plant-specific pathways, while non-A-type CYPs are primarily involved in metabolic housekeeping functions (Werck-Reichhart et al. 2002, Schuler and Werck-Reichhart 2003). Here, we identified CYPs belonging to the CYP716A subfamily, which belongs to the non-A-type CYP85 clan, and which is involved in the biosynthesis of secondary metabolites. This is not the first time a non-A-type CYP has been implicated in a plant-specific pathway; previously, we implicated a non-A-type CYP85 clan member, CYP88D6, in glycyrrhizin biosynthesis.
Although it has been proposed that CYP85 along with the clans CYP51 and CYP710 evolved from a sterol-metabolizing CYP51 ancestor (Nelson and Werck-Reichhart 2011), CYP88D6 belongs to a legume-specific CYP subfamily and is involved in the biosynthesis of glycyrrhizin, a genus-specific compound. In contrast, the CYP716A subfamily and its main product (oleanolic acid) are broadly distributed among plants. Therefore, CYP716A subfamily members could have an important role in plant development or signaling along with their function in the...
oleanolic acid pathway. Recently, mutants defective in CYP716A12 expression were found to be unable to produce hemolytic saponins (sapogenins and glycosides, derived from oleanolic acid; Fig. 1). Moreover, they showed a strong decrease in growth, reaffirming the possible dual role of CYP716A12 in plant defense and development (Carelli et al. 2011).

Gene clusters involved in secondary metabolism, which are common in bacteria, have recently been reported in plants. Examples include genes involved in the biosynthesis of thalianol in Arabidopsis, benzoxazinoids in maize and momilactone in rice (Mizutani and Ohta 2010). Although the CYP716A12 locus is not clear in the M. truncatula genome, a unique cluster of CYP716A genes (CYP716A21–CYP716A31) was found in the

![Fig. 6](https://academic.oup.com/pcp/article-abstract/52/12/2050/1821099)
grapevine genome. All of these genes, except CYP716A29, are clustered in chromosome 18 (Supplementary Fig. S13); this suggests that they arose via lineage-specific gene duplication and might be involved in the biosynthesis of lineage-specific metabolites. Furthermore, CYP716A15 and CYP716A17 are located in tandem (within 320 kb) in the same transcriptional direction on chromosome 11 (Supplementary Fig. S13). Interestingly, CYP716A17 is located adjacent to a bAS-like gene, similar to the operon-like gene clusters observed in Arabidopsis, oat and rice (Shimura et al. 2007, Field and Osbourn 2008).

Semi-quantitative reverse transcription–PCR (RT–PCR) revealed that CYP716A15 and CYP716A17 are coordinately expressed in aerial organs, especially in stems and fruit skins (Supplementary Fig. S9). Taking into account their structural similarity (95% amino acid sequence identity), genomic synteny and biochemical activity, they are most probably functionally redundant genes in plants. CYP716A19 and CYP716A20 are also located adjacent to bAS-like genes on chromosomes 4 and 11, respectively (Supplementary Fig. S13). Further functional characterization of these bAS-like and CYP716A genes is needed to clarify the biological significance of these proposed operon-like gene clusters in grapevine.

Here, we successfully identified CYPs that produce oleanolic acid, ursolic acid and betulinic acid, using a combined approach of co-expression analysis to identify possible candidate genes followed by heterologous expression in SF9 cells (in vitro) and yeast (in vivo). We redirected the endogenous sterol pathway by introducing different OSC genes into wild-type yeast and subsequently co-expressed CPR and CYP716A genes. Although oleanolic acid, ursolic acid and betulinic acid are widespread among plants, obtaining large amounts for commercial/industrial purposes is laborious. In addition, it is common to find them simultaneously in natural sources, which complicates their isolation for practical applications. The use of engineered yeast might offer increased product yields, as a feasible alternative for the production of useful compounds.

Materials and Methods

Chemicals (sapogenin authentic standards)

All sapogenins purchased were at least of analytical grade. β-Amyrin (>98.5% purity), α-amyrin (>98.5% purity), oysteriodiol (>97% purity), uvaol (>95% purity), oleanolic acid (>97% purity) and ursolic acid (>98.5% purity) were purchased from Extra syntche. Lupeol (>94% purity), betulin (>98% purity) and betulinic acid (>98% purity) were purchased from Sigma-Aldrich.

Full-length cDNA amplification

Medicago truncatula CYP716A12 and CYP93E2. Gene-specific primers (shown in Supplementary Table S3) were designed for each sequence. To obtain full-length CYP716A12 and CYP93E2 cDNAs (GenBank: ABC59076.1), RT–PCR was conducted using KOD Plus DNA Polymerase (Toyobo) and RNA isolated from the stems, leaves and roots of 4-week-old M. truncatula (R-108) plants with RNAwiz™ (Ambion). First-strand cDNA synthesis was performed using a Smart RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s instructions. Primers 1–2 (for in vitro assay) and 3–4 (for in vivo assay) were used to amplify CYP716A12, and primers 5–6 were used to amplify CYP93E2.

Grape CYP716A15 and CYP716A17. cDNAs for RT–PCR were prepared from various grapevine tissues as described previously (Ono et al. 2010). PCR was performed using primers 7–8 for CYP716A15/CYP716A17 and 9–10 for ubiquitin. To distinguish the two genes (CYP716A15/17), the amplified fragments were digested with SpeI, which cuts CYP716A15 specifically. The digested PCR products were separated by 1% agarose gel electrophoresis and detected by ethidium bromide staining. After digestion, the CYP716A15 PCR product (1,443 bp) was divided into two fragments (by SpeI) at 1,315 bp, unlike CYP716A17 (1,443 bp).

aAS. To test the activity of the isolated CYPs using α-amyrin as the substrate, we expressed an OSC from olive that mainly produces α-amyrin, named OEA (Saimaru et al. 2007); for clari ty, in this paper, we refer to OEA as aAS. Total RNA was prepared from leaves of Olea europaea (cv. Nevadillo Blanco) plants using a SuperScript™ III first-strand synthesis system (Invitrogen). Primers 11–12 were used to amplify the full-length sequence. The sequence was then transferred to pENTR™/D-Topo® and subsequently transformed into pYES3/CT(AUR) (Seki et al. 2008) to produce pYES-ADH-aAS.

LUS. The LUS gene from G. uralensis was isolated using primers 13–14, which were designed based on a previously reported LUS gene from G. glabra (Hayashi et al. 2004), and total RNA prepared from stolons of G. uralensis plants with a SMART RACE cDNA Amplification Kit (Clontech). We produced pYES-ADH-LUS as described before for pYES-ADH-aAS.

In vitro enzyme assays

CYP716A12. The amplified fragment was initially cloned into pMD19 (TAKARA). The insert was then digested with BamHI and Sall and introduced into pFastBac1 (Invitrogen). The pFastBac1-CYP716A12 construct was then used to prepare recombinant bacmid DNA by transformation of the Escherichia coli DH10Bac strain (Invitrogen) according to the manufacturer’s instructions. The expression of CYP716A12 in SF9 cells and in vivo enzyme assays were performed as described previously (Ohnishi et al. 2006, Seki et al. 2008).

CYP716A15 and CYP716A17. The amplified fragments were cloned into pDEST™8 via pENTR-D TOPO (Invitrogen). The obtained insect cell baculovirus expression clones were used to generate recombinant bacmid DNAs by transformation of the E. coli DH10Bac strain. CYP716A15 and CYP716A17 expression in SF9 cells and in vitro enzyme assays were performed as described previously (Ohnishi et al. 2006, Seki et al. 2008).
Constructures for the yeast in vivo assays

The pYES3-ADH-OSC1 plasmid (for the constitutive expression of *Lotus japonicus* bAS; under the control of the ADH1 promoter) and pELC (for the galactose-inducible expression of *L. japonicus* CPR alone) were constructed as described previously (Seki et al. 2008). Similarly, to create pDEST52-CYP716A12 (for the galactose-inducible expression of CYP716A12), full-length CYP716A12 cDNA was cloned into pENTR™/D-Topo™ to produce pENTR-CYP716A12. Next, the cDNA was transferred to pYES-DEST52 (Invitrogen) using Gateway LR Clonase Enzyme Mix (Invitrogen). The same strategy was used for CYP716A15.

Yeast in vivo assays

*Saccharomyces cerevisiae* INVSc1 (MATα his3D1 leu2 trp1-289 ura3-52; Invitrogen) harboring pYES3-ADH-aAS, pYES3-ADH-OSC1 or pYES3-ADH-LUS was co-transformed with pDEST52-CYP716A12 or pELC-CYP716A12 using Frozen-EX Yeast Transformation ITTM (Zymo Research). A sample harboring pYES3-ADH-aAS, pYES3-ADH-OSC1 or pYES3-ADH-LUS alone was used as a control. Recombinant yeast cells were cultured in synthetic complete medium (5 ml) containing 2% glucose without tryptophan and leucine (SC-W-L) at 30°C for 1 d at 170 r.p.m. The cells were then collected and resuspended in SC-W-L medium (10 ml) containing 13 μM hemin and 2% galactose instead of glucose, and cultured at 30°C for 2 d at 170 r.p.m. The obtained cultured samples were distributed into two 10 ml Falcon tubes and stored at −30°C. All assays were performed in duplicate (two independent assays obtained from different clones) to confirm our results.

Extraction and GC-MS analysis

In vitro assay. The standard reaction mixture (200 μl) consisted of 10 μM substrate, 5 mM NADPH, 25 mM potassium phosphate buffer (pH 7.5), microsomes expressing the CYP716A gene and Arabidopsis NADPH-cytochrome P450 reductase (Mizutani and Ohta 1998), and then incubated at 30°C for 2 h. The same volume (200 μl) of ethyl acetate was added to the reaction mixture, and mixed for extraction of reacted compounds. The supernatant (ethyl acetate upper layer) collected by centrifugation was dried, and trimethylsilylated with 50 μl of NO-bis(trimethylsilylamide) (Nacalai tesque Inc.) for 30 min. This procedure was repeated three times. The remaining powder was transferred to a silica-gel column (6 ml; Waters Corp.) and washed with 10 ml of ethyl acetate and 10 ml of chloroform–methanol (1:1 dilution). The samples were then placed into an evaporator for 60 min. After resuspending the obtained pellet in 300 μl of chloroform–methanol, 100 μl was transferred to a vial and placed in an evaporator for 30 min. Finally, the pellet was trimethylsilylated with 50 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich) for 30 min at 80°C. The evaporated samples were stored at 4°C until needed.

GC-MS was performed using a JMS-AMSUN200 mass spectrometer (JEOL Ltd.) connected to a gas chromatograph (6890A; Agilent Technologies) with a DB-1 (30 m × 0.25 mm, 0.25 μm film thickness; J&W Scientific) or HP-5 (30 m × 0.32 mm, 0.25 μm film thickness; J&W Scientific) capillary column. The injection temperature was 250°C. The column temperature program was as follows: 80°C for 1 min, followed by an increase to 300°C at a rate of 20°C min⁻¹, and a hold at 300°C for 20 or 28 min. The carrier gas was He, and the flow rate was 1.2 or 1.0 ml min⁻¹, respectively; the interface temperature was 300°C with a splitless injection (Seki et al. 2008). Peaks were identified by comparing the Rts and mass spectra with those of the authentic standards.

Supplementary data

Supplementary data are available at PCP online.

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