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Comparative functional analysis of CYP71AV1 natural variants reveals an important residue for the successive oxidation of amorpha-4,11-diene

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ABSTRACT

Artemisinin is an antimalarial sesquiterpenoid isolated from the aerial parts of the plant *Artemisia annua*. CYP71AV1, a cytochrome P450 monooxygenase was identified in the artemisinin biosynthetic pathway. CYP71AV1 catalyzes three successive oxidation steps at the C12 position of amorpha-4,11-diene to produce artemisinic acid. In this study, we isolated putative *CYP71AV1* orthologs in different species of *Artemisia*. Comparative functional analysis of CYP71AV1 and its putative orthologs, together with homology modeling, enabled us to identify an amino acid residue (Ser479) critical for the second oxidation reaction catalyzed by CYP71AV1. Our results clearly show that a comparative study of natural variants is useful to investigate the structure–function relationships of CYP71AV1.

1. Introduction

Artemisinin, a sesquiterpene endoperoxide lactone, was identified from *Artemisia annua* L. (Asteraceae) as a compound that is highly effective against *Plasmodium* spp., the parasite that causes malaria [1]. Because artemisinin and its analogs derivatives are highly poisonous, they are effective against multidrug-resistant *Plasmodium falciparum*, and since 2001, the World Health Organization has recommended artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated *P. falciparum* malaria. Consequently, the demand for artemisinin derivatives for use in ACTs currently overwhelms their supply. Many valuable contributions to the total synthesis of artemisinin have been reported [2–4], but the industrialization of total chemical synthesis

has not yet been achieved due to the high cost of the multistep synthesis process. The increasing demand for ACTs, along with limited supplies of plant-derived artemisinin, has led scientists to study its biosynthesis in *A. annua*.

In the artemisinin pathway, the first committed step is mediated by amorpha-4,11-diene synthase (ADS), which converts the common sesquiterpene precursor farnesyl diphosphate into amorpha-4,11-diene [5,6]. Subsequently, a cytochrome P450 monooxygenase (CYP71AV1) oxidizes amorpha-4,11-diene at the C12 position to artemisinic acid in three successive steps [7,8]. In an earlier study, artemisinic acid was proposed as a precursor of artemisinin [9]. However, recent data suggest that the primary route is through the artemisinic aldehyde intermediate resulting from the second oxidation step of CYP71AV1 [10,11]. Subsequently, artemisinic aldehyde is converted into artemisinin by several other enzymatic and non-enzymatic reactions (Supplementary Fig. 1) [12].

In addition to *A. annua*, the extracts of several other species, including *Artemisia afra* and *Artemisia absinthium* in the same genus, have been reported to show antiplasmodial activity [13–15]. However, our extensive chemical analysis failed to detect any traces of

artemisinin and its specific precursors (amorpha-4,11-diene and artemsinic acid) from these species (Suzuki et al., unpublished data). Hence, we investigated whether these *Artemisia* species express genes potentially encoding the artemisinin biosynthetic enzyme.

As a result, we isolated putative *CYP71AV1* orthologs, which encoded proteins with an identity of more than 94% amino acid with *A. annua* CYP71AV1 from both *A. afra* and *A. absinthium*. Moreover, a comparative structural and functional analysis of CYP71AV1 and putative orthologous proteins, combined with homology modeling and subsequent mutational analysis, successfully identified an amino acid residue (Ser479) that was important for the multiple oxidations of amorpha-4,11-diene, which are catalyzed by CYP71AV1.

2. Materials and methods

2.1. Plant materials and chemical standards

A. annua was obtained from the Research Center for Medicinal Plant Resources. *A. afra* was obtained from University of Pretoria. *A. absinthium* was purchased at a specialty herb store (e-tisanes) in Shizuoka, Japan.

Artemisinic acid was isolated from *A. annua* [16]. Artemisinic acid was esterified with CH_2N_2 and then reduced to the corresponding artemisinic alcohol with diisobutyl aluminum hydride. Artemisinic alcohol was mesylated with methanesulfonyl chloride and then reduced with lithium aluminum hydride to yield amorpha-4,11-diene. The product was purified using a column of silver nitrate-coated silica gel to give amorpha-4,11-diene as a colorless oil. Artemisinic alcohol was oxidized with MnO_2 to yield the corresponding artemisinic aldehyde. All compounds were identified by a comparison of proton nuclear magnetic resonance spectroscopy (¹H NMR), carbon nuclear magnetic resonance spectroscopy (¹³C NMR), and gas chromatography–mass spectrometry (GC–MS) with reference data [11,17,18].

2.2. RT-PCR and isolation of cDNAs

Total RNA was extracted from the leaves of *A. annua*, *A. afra*, and *A. absinthium* using RNAwiz (Ambion, Carlsbad, CA, USA) and treated with RNase-free DNase (Takara Bio Inc., Shiga, Japan). It was further purified using an RNeasy Plant Mini Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's specifications. First-strand cDNA synthesis was carried out using a SMART RACE cDNA Amplification Kit (Clontech/Takara Bio Inc.) with 1 μg total RNA. PCR was performed using primers 1–2 for *ADS*, 3–4 for *CYP71AV1*, and 5–6 for *ubiquitin* (for primer sequences see Supplementary Table 1).

To obtain the cDNAs encoding *afCYP71AV1* (GenBank ID: AB706289) and *abCYP71AV1* (GenBank ID: AB706290), RT-PCR was conducted using KOD Plus Polymerase (Toyobo, Osaka, Japan) with primers 3–4. The amplified fragment was cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced. Three individual clones were fully sequenced and showed no sequence variation.

2.3. Construction of chimeric cDNAs

To generate chimeric constructs consisting of cDNA fragments from *CYP71AV1* (GenBank ID: AB706288) and *abCYP71AV1*, the fragments to be fused were produced in separate PCR reactions. Primers 3–8, 3–10, 4–7, and 4–9 were used to amplify fragments from regions A, A–B, B–C, and C, respectively (Fig. 3A). The obtained fragments were mixed at a ratio of 1:1 and subsequently fused and amplified using primers 3–4.

2.4. Site-directed mutagenesis

CYP71AV1 S307A and CYP71AV1 L369M were constructed via two PCR steps using one forward and one reverse mutagenic primer for each site-directed mutant. The primer pairs incorporating point mutation were primers 11–12 (CYP71AV1 S307A) and 13–14 (CYP71AV1 L369M). CYP71AV1 M483V and CYP71AV1 S479F were constructed via one PCR with the following reverse mutagenic primers: 15 (CYP71AV1 M483V) and 16 (CYP71AV1 S479F). Coding regions were fully sequenced to verify that no undesired mutations had been introduced by PCR.

2.5. Yeast in vivo assays

The plasmids used in our study, pELC-GAL-P450 (galactose-inducible expression of *Lotus japonicus CPR* and *Artemisia* species *P450*, driven by the promoters *GAL10* and *GAL1*, respectively) and pYES3-ADH-ADS (constitutive expression of *ADS* driven by an *ADH1* promoter) were constructed as following sentences.

To obtain pELC-GAL-P450, the full-length cDNA was cloned via the gateway entry vector pENTR/D-TOPO (Invitrogen) into the Gateway-adopted version of the pELC vector [19]. The expression of *P450* and *CPR* was under the control of the galactose-inducible promoters *GAL1* and *GAL10*, respectively. pYES3-ADH-ADS was constructed using the pYES3/CT (AUR)-Gateway-1 vector (Akashi et al., unpublished data). The pYES3/CT (AUR)-Gateway-1 vector was constructed by replacing PGAL1 in the CYC1TT region of pYES3/CT (Invitrogen) with the fragment containing the *ADH1* promoter-terminator (PADH1-TADH1) of pAUR123 (Takara Bio Inc.), subsequent digestion with SmaI, and ligation to the GATEWAY conversion cassette frame B (Invitrogen).

The two vectors were introduced to *Saccharomyces cerevisiae* strain INVSc1 (*MATa his3D1 leu2 trp1-289 ura3-52*; Invitrogen) using Frozen-EZ Yeast Transformation II TM (Zymo Research, Irvine, CA, USA). A sample harboring pYES3-ADH-ADS and pELC-GAL-empty was used as a control. Recombinant yeast cells in synthetic complete medium (10 ml) containing 2% glucose without tryptophan and leucine (SC-W-L) were cultured at 30 °C for 1 day at 150 rpm. The cells were collected and resuspended in SC-W-L medium (10 ml) containing 13 μg/ml hemin and 2% galactose (instead of glucose) and cultured at 30 °C for 1 day at 150 rpm. The cultured samples obtained (5 ml) were disrupted by glass beads, acid-washed (425–600 μm; Sigma–Aldrich, St. Louis, MO, USA) and then extracted three times with ethyl acetate (5 ml). The extract was filtered through a sodium sulfate column and then concentrated by evaporation under a stream of nitrogen gas to minimum volume. The residue was dissolved with ethyl acetate (500 μl) to prepare the sample for gas chromatography–mass spectrometry (GC–MS) analysis.

2.6. In vitro enzyme assays

Recombinant yeast harboring pELC-GAL-P450 alone was used to obtain microsomal fractions. Yeast microsomal fractions were prepared according to a previously described technique [20] except for conditioning of the buffer. Briefly, cells were washed with buffer A (80 mM Hepes-NaOH (pH 7.2), 5 mM EGTA, 5 mM EDTA, 10 mM KCl, and 320 mM sucrose) [21] and resuspended and incubated in buffer B (buffer A supplemented with 10 mM β-Me). Cells were broken using glass beads in buffer C (buffer A supplemented with 2 mM β-Me and 1 mM PMSF). Microsomal fractions were prepared by centrifugation at $1500 \times g$ for 10 min at 4 °C to remove cellular debris followed by centrifugation at $100,000 \times g$ for 60 min at 4 °C. The final microsomal pellets were resuspended in a storage buffer (50 mM potassium phosphate buffer (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1.5 mM β-Me, and 20% glycerol).

Enzyme reactions were initiated by adding substrate to a final concentration of 20 μM to 2 ml McIlvaine buffer (pH 6) containing 2 mM dithiothreitol, 5 μM FAD, 5 μM FMN, 1 mM ascorbic acid, 5 mM NADPH, and 60–80 μg microsomal pellets. The reaction mixtures were incubated overnight and extracted with ethyl acetate (1 ml × 1, 500 μl × 2) and concentrated by evaporation under a stream of nitrogen gas to minimum volume. The residue was dissolved with ethyl acetate (400 μl) to prepare the sample for GC–MS analysis. GC–MS samples for artemisinic acid analysis were esterified with an excess of diazomethane. The conversion efficiency of a substrate to reaction products was calculated from the peak area of the total ion chromatogram as follows. Diene, alcohol, aldehyde and acid indicates amorpha-4,11-diene, artemisinic alcohol, artemisinic aldehyde and artemisinic acid, respectively.

$$\text{diene to alcohol} = \frac{\text{alcohol} + \text{aldehyde} + \text{acid}}{\text{diene} + \text{alcohol} + \text{aldehyde} + \text{acid}} \times 100 \ (\%)$$

$$\text{alcohol to aldehyde} = \frac{\text{aldehyde} + \text{acid}}{\text{alcohol} + \text{aldehyde} + \text{acid}} \times 100 \ (\%)$$

2.7. Homology modeling

A BLAST [22] search was used to find a suitable template for modeling CYP71AV1. On the basis of the highest identity (27%), human microsomal P450, CYP1A2 (PDB ID: 2HI4) [23] was selected. Homology modeling was performed with T-coffee [24] and Modeler [25] by threading the CYP71AV1 onto the structural coordinates for CYP1A2. Docking calculations were conducted with the PatchDock web server [26,27].

2.8. GC–MS analysis

GC–MS data for sesquiterpenoid analysis were corrected with a JMS-AM SUN200 quadrupole mass spectrometer (JEOL, Tokyo, Japan) interfaced to a model 6890 gas chromatograph (Agilent, Santa Clara, CA, USA) operated in the electron impact mode (70 eV). A capillary column was used under the following conditions: HP-5MS (30 m, 0.25 mm i.d., 0.25 μm film thickness, J&W; Agilent) with fused silica capillary tube (1 m, 0.25 mm i.d; GL Sciences, Tokyo, Japan); injection port temperature, 250 °C; interface temperature, 280 °C; ion source temperature, 250 °C; oven temperature program: 60 °C rising at 3 °C/min to 280 °C, held for 6 min, 40 s; split ratio, 1:10; carrier gas, helium; flow rate, 0.7 ml/min. The scanning speed of the mass spectrometer was 2 scans/sec from m/z 40 to m/z 350. The peaks were identified by a comparison of GC retention time and mass spectra to those of authentic compounds. Limit of detection was 2 ng/ml culture and reaction mixtures. Relative ion intensity is shown as a percentage relative to the most intense ion (100% intensity).

3. Results

3.1. Isolation of putative CYP71AV1 orthologs

To investigate whether the *Artemisia* species that were reported to have antimalarial activity expressed genes for artemisinin biosynthesis, RT-PCR was performed using RNA isolated from leaves of *A. afra* and *A. absinthium* with primers designed for both start and stop codons of *ADS* and *CYP71AV1*.
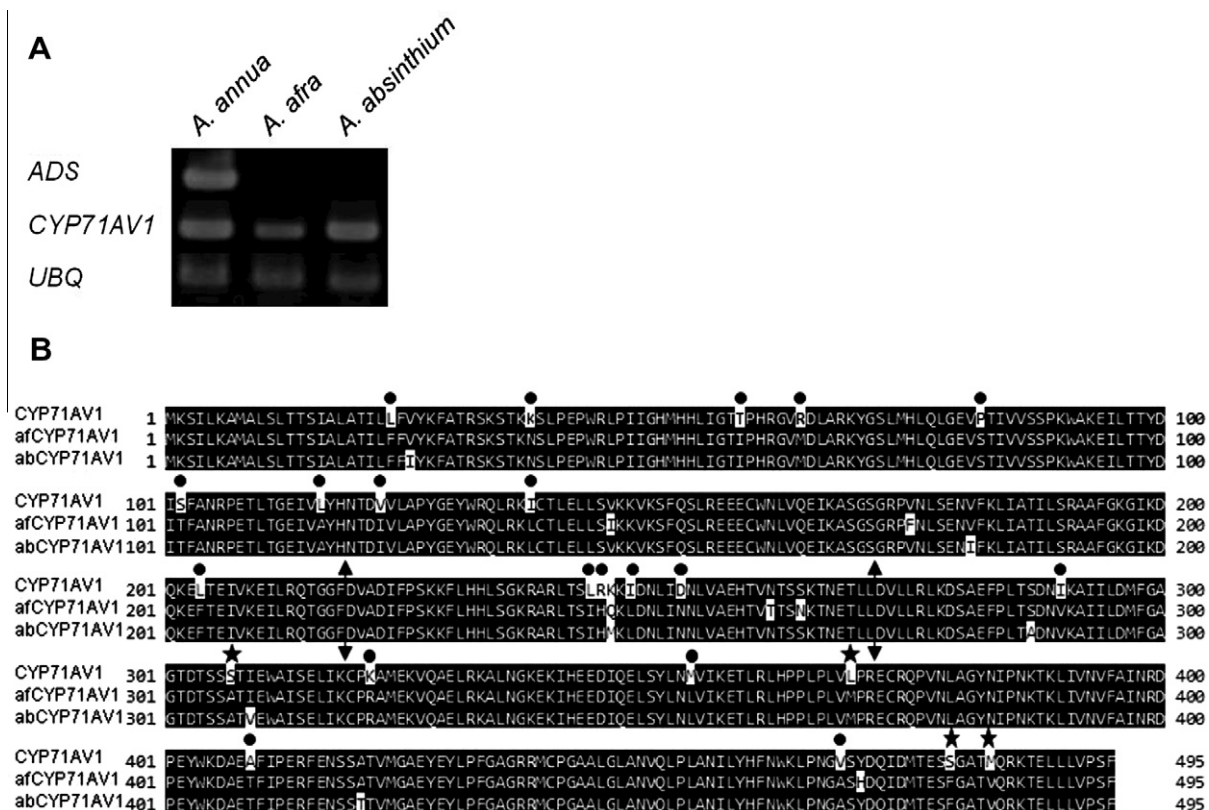

Fig. 1. Isolated putative *CYP71AV1* orthologs from *A. afra* and *A. absinthium*. (A) RT-PCR analysis of amorpha-4,11-diene synthase (*ADS*) and *CYP71AV1* mRNA levels in leaves. Ubiquitin (*UBQ*) was included as an internal control. (B) Alignment of deduced amino acid sequences of CYP71AV1 and putative CYP71AV1 orthologs (GENETYX Version 8; GENETYX Corp., Tokyo, Japan). Identical residues are indicated by dark shading. The large double arrows indicate the three regions of chimeric proteins. Circled marks and stars denote the conserved regions in afCYP71AV1 and abCYP71AV1 that differ in CYP71AV1. Stars show the positions of site-directed mutations.

The RT-PCR products of *ADS*, the gene of the first enzyme in the artemisinin-specific pathway, were not detectable in these *Artemisia* species (Fig. 1A). The same results were obtained when RT-PCR was performed with the other 14 different primer sets. The absence of the *ADS* transcript relates to the fact that no arte-misinin and its specific intermediates were detected from these *Artemisia* species.

PCR products were detected in both *A. afra* and *A. absinthium* with the primer set for *CYP71AV1*. The putative full length cDNAs encoded a polypeptide of 495 residues, which had a 94% identity to CYP71AV1. According to the deduced amino acid sequences from these putative *CYP71AV1* orthologous cDNAs, the P450 nomenclature committee (c/o Dr. D.R. Nelson, University of Ten-nessee, Memphis, TN) named them CYP71AV1. In this study, *A. afra* CYP71AV1 and *A. absinthium* CYP71AV1 were named *afCYP71AV1* and *abCYP71AV1*, respectively. These putative CYP71AV1 orthologs share a high amino acid identity (98%) and have 23 common amino acid residues that are different from CYP71AV1 (Fig. 1B, circled marks and stars).

3.2. Amorpha-4,11-diene oxidizing activity of putative CYP71AV1 orthologs

The potential amorpha-4,11-diene oxidizing activity of the CY-P71AV1 orthologs was examined in an engineered yeast strain that produces amorpha-4,11-diene endogenously. For this purpose *ADS* from *A. annua* was expressed in yeast cells, and subsequently, each of *afCYP71AV1* or *abCYP71AV1* was co-expressed with CPR (cyto-chrome P450 reductase, a redox partner of CYP) in the *ADS*-transformed yeast. Following the culture, ethyl acetate extracts were analyzed by GC–MS.

In the ethyl acetate extract of the *ADS/CPR/CYP71AV1*-expressing yeast culture, artemisinic acid was detected as the sole reaction product of the enzymatic oxidation (Fig. 2A), indicating that CY-P71AV1 is capable of catalyzing the three-step oxidation of amor-pha-4,11-diene to generate artemisinic acid, as reported previously [7,8]. Although both afCYP71AV1 and abCYP71AV1 catalyzed the initial oxidation step at the C12 position of amoprha-4,11-diene into artemisinic alcohol, the oxidation reaction of artemisinic
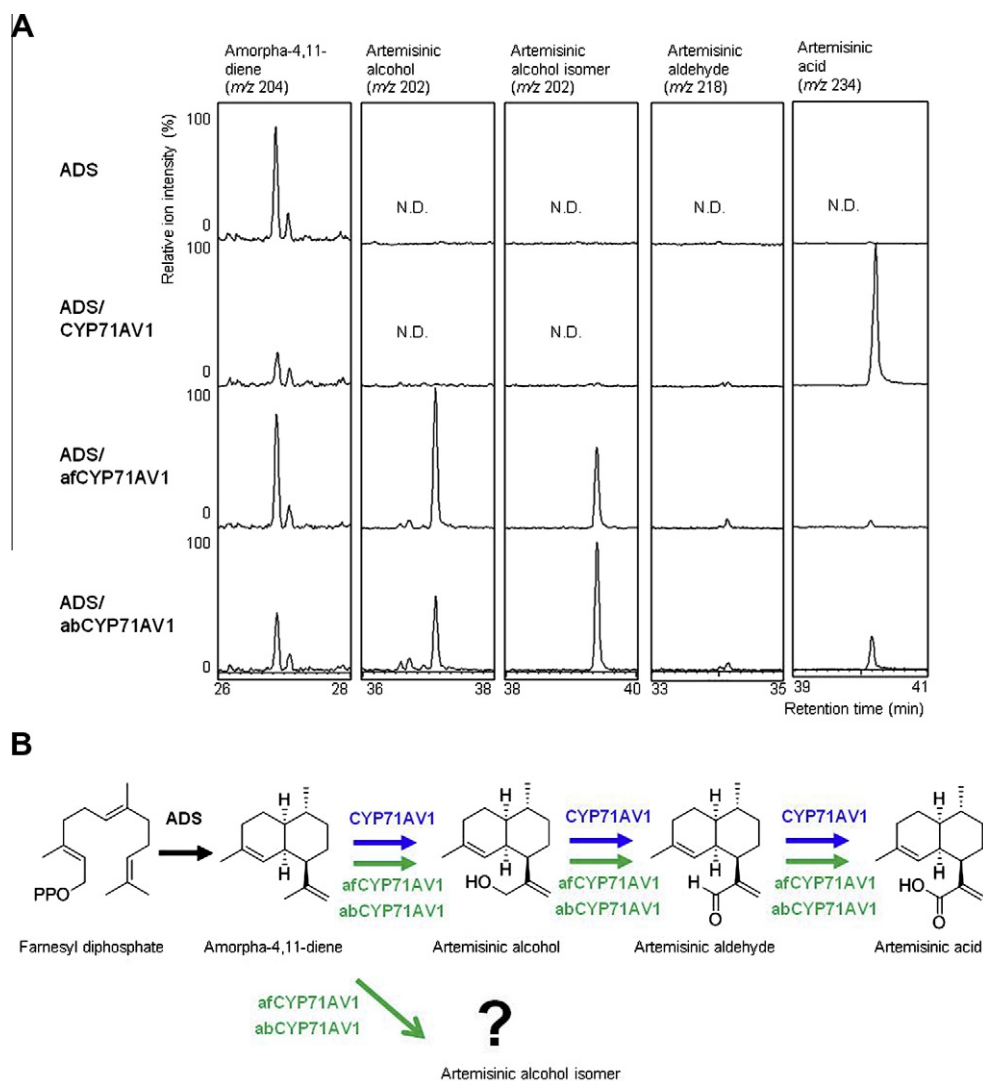

Fig. 2. Armoprha-4,11-diene oxidizing activity of putative CYP71AV1 orthologs. (A) GC profiles of ethyl acetate extracts from yeast strains expressing *CYP71AV1*, *afCYP71AV1*, and *abCYP71AV1*. These P450s were co-expressed with *ADS* and cytochrome P450 reductase (*CPR*). Each box indicates the extracted ion chromatograms at m/z 204, m/z 202, m/z 218, and m/z 234. The detected product name is shown above the boxes. The mass spectra for these products match the authentic standard (Supplementary Fig. 2). N.D., not detectable. (B) Deduced reactions catalyzed by afCYP71AV1 and abCYP71AV1. Blue arrows indicate the CYP71AV1 catalytic pathway. Green arrows denote the afCYP71AV1 and abCYP71AV1 catalytic pathways.
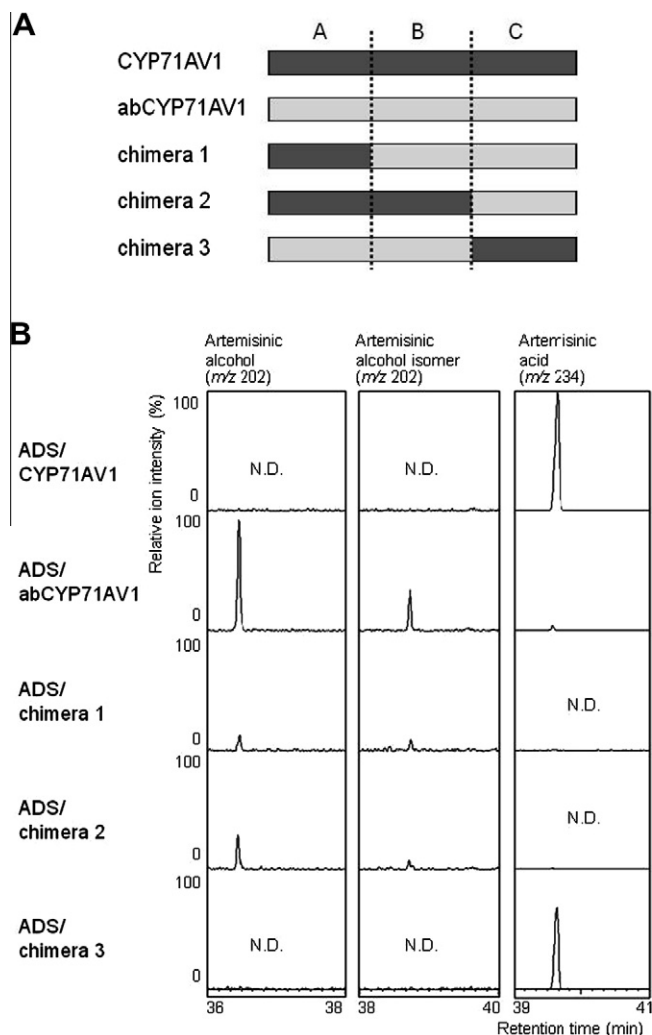
Fig. 3. Amorpha-4,11-diene oxidizing activity of CYP71AV1/abCYP71AV1 chimeric proteins. (A) Chimeric cDNAs are represented by combinations of dark and light-gray boxes representing CYP71AV1 (dark) and abCYP71AV1 (light) coding sequences. The protein regions are indicated by letters. (B) GC profiles of ethyl acetate extracts from yeast strains expressing *ADS*, *CPR*, and each chimeric gene. Each box indicates the extracted ion chromatograms at *m/z* 202 (left and middle) and *m/z* 234 (right). The detected product names are shown above the boxes. The mass spectra for these products match the authentic standard (Supplementary Fig. 2). N.D., not detectable.

alcohol to artemisinic acid via artemisinic aldehyde proceeded weakly (Fig. 2A). Additionally, putative reaction products of both afCYP71AV1 and abCYP71AV1 accumulated at levels comparable to the artemisinic alcohol detected (Fig. 2A). The putative reaction product was not detected from the yeast without expressing *ADS*. Mass spectra of the reaction product revealed a molecular ion of M^+ at *m/z* 220, desorption of $M^+–H_2O$ at *m/z* 202, and α-cleavage of $M^+–CH_2OH$ at *m/z* 189 (Supplementary Fig. 3). Therefore, we presumed that the reaction product was an artemisinic alcohol isomer.

From these results, we suggest that both the oxidation activity and the regioselectivity of CYP71AV1 orthologs are lower than those of CYP71AV1 when comparing product accumulation patterns. This difference in activity could be attributable to the 23 different amino acid residues in CYP71AV1 orthologs.

3.3. Amorpha-4,11-diene oxidizing activity of chimera proteins

To define the residues that define the specificity of the amorpha-4,11-diene oxidizing activity, chimeric cDNAs (within domain
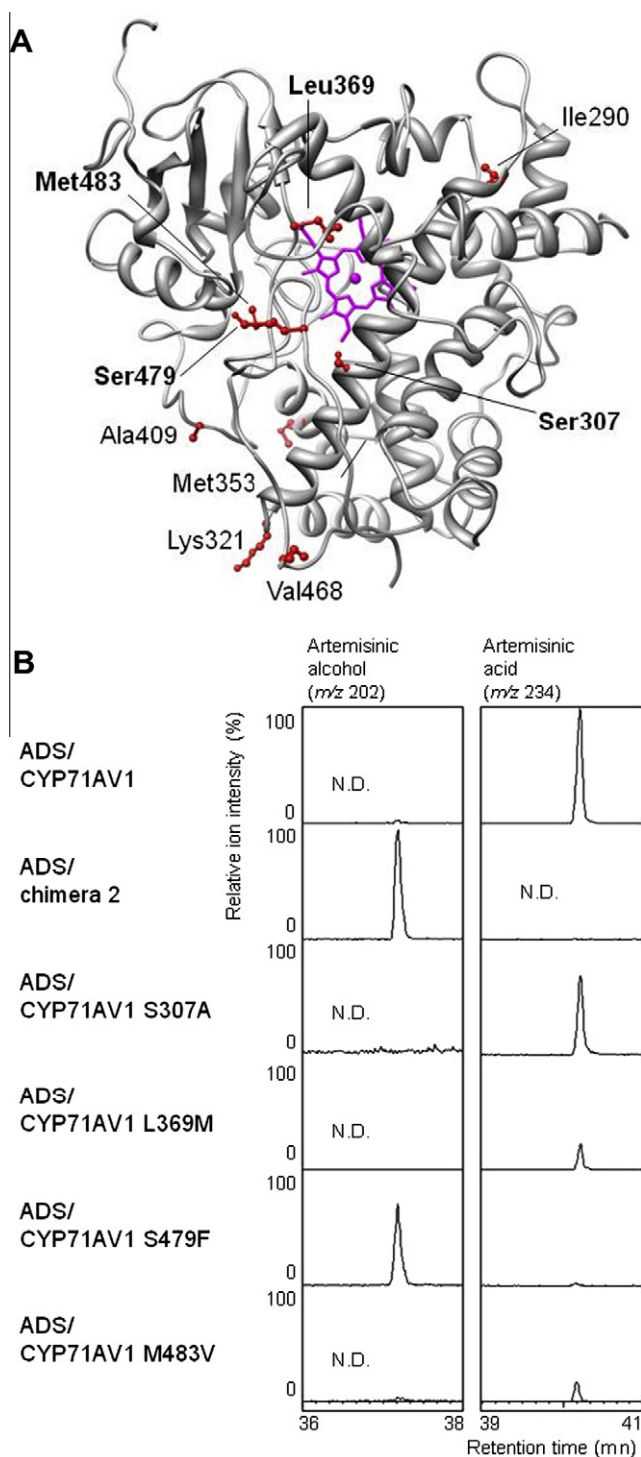

Fig. 4. Effects of site-directed mutation on the successive oxidizing activity of CYP71AV1. (A) Prediction of the 3D model of CYP71AV1 generated using the human microsomal P450 1A2 protein (PDB ID: 2HI4). The heme is shown in magenta. Ile290, Ser307, Lys321, Met353, Leu369, Ala409, Val468, Ser479, and Met483 are shown in red. (B) GC profiles of ethyl acetate extracts from yeast strains expressing *ADS*, *CPR*, and *CYP71AV1* site-directed mutants. Each box indicates the extracted ion chromatograms at *m/z* 202 (left) and *m/z* 234 (right). The detected product names are shown above the boxes. The mass spectra for these products match the authentic standard (Supplementary Fig. 2). N.D., not detectable.

A, B, and C, 10, 4, and 9 of amino acid residues, respectively) were constructed by fusing cDNA fragments of the *CYP71AV1* and *abCYP71AV1*, and were expressed in the *ADS*-expressing yeast (Fig. 3A). In the ethyl acetate extracts of the *chimera1* and

chimera2-expressing yeast, both artemisinic alcohol and artemisinic alcohol isomer were detected, which was a similar product pattern to the abCYP71AV1-expressing yeast (Fig. 3B). However, in chimera3-expressing yeast, only artemisinic acid was detected, which was similar to that of CYP71AV1-expressing yeast (Fig. 3B). Both chimera1 and chimera2 contained the C region from abCYP71AV1, and chimera3 contained the C region from CYP71AV1, suggesting that C regions contain the residues that determine the oxidation activity and the regioselectivity difference between CYP71AV1 and putative CYP71AV1 orthologs.

3.4. Artemisinic alcohol oxidizing activity of CYP71AV1 S479F

The C region of CYP71AV1 contains nine residues of the 23 different amino acid residues. A homology model of CYP71AV1 based on human microsomal P450 (CYP1A2) was constructed for the selection of candidate residues involving the functional activity from the nine different residues (Fig. 4A). The resultant model suggested that four of the nine residues (Ser307, Leu369, Ser479, and Met483) are located in the heme catalytic site (Fig. 4A), indicating that these four residues are most likely the candidate residues for
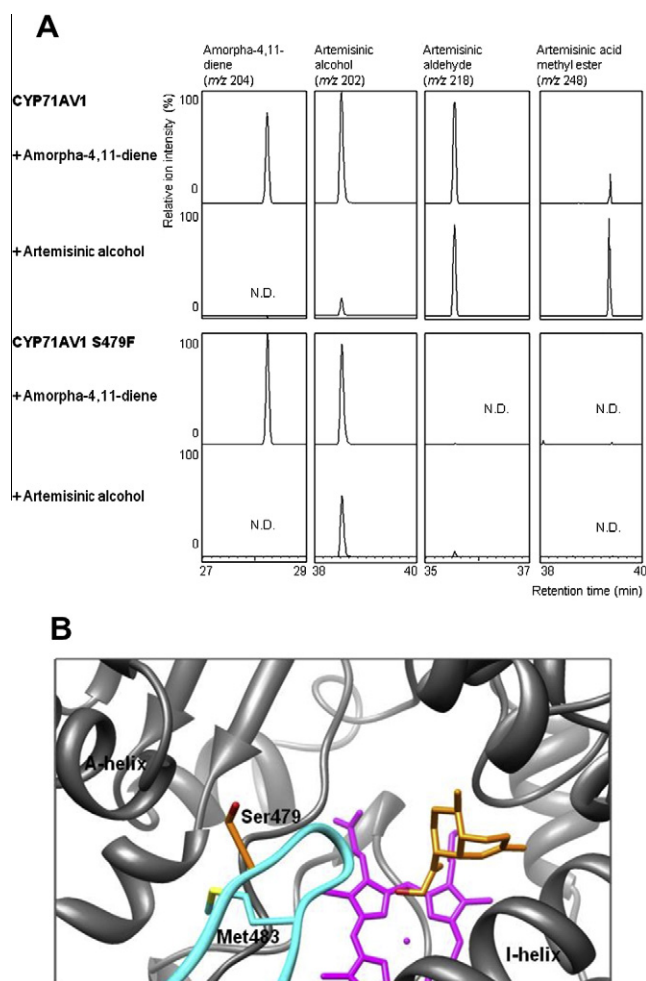

Fig. 5. Artemisinic alcohol oxidizing activity of CYP71AV1 S479F. (A) GC profiles of the extracts from in vitro assays containing amorpha-4,11-diene or artemisinic alcohol as substrate and microsomal fractions isolated from CYP71AV1/CPR or CYP71AV1 S479F/CPR co-expressed yeast. The boxes on the left indicate m/z 204, m/z 202, m/z 218, and m/z 248. The detected product name is shown above the boxes. The mass spectra for these products match the authentic standard (Supplementary Fig. 2). N.D., not detectable. (B) Docking of artemisinic alcohol (orange) into a homology model of CYP71AV1. The C-terminal loop containing Ser479 (orange) and Met483 (cyan) is shown in cyan.

determining the regioselectivity and successive oxidation activity of CYP71AV1.

To further investigate the role of these residues, we performed site-directed mutagenesis experiments. CYP71AV1 was mutated to substitute the four residues for those of abCYP71AV1 and express them in the recombinant yeast. Artemisinic alcohol isomer was not detected in either single point-mutant, indicating that the difference in regioselectivity between CYP71AV1 and putative CYP71AV1 orthologs was probably determined by two or more residues. In CYP71AV1 S479F-expressing yeast, an accumulation of artemisinic alcohol and negligible levels of artemisinic acid were observed (Fig. 4B). However, the other three mutants showed no accumulation of artemisinic alcohol (Fig. 4B).

To verify the results of CYP71AV1 S479F-expressing yeast, we undertook an in vitro assay using yeast microsomal fractions. In the CYP71AV1 S479F-expressing yeast microsomal fractions assayed with amorpha-4,11-diene as a substrate, artemisinic alcohol was detected as a unique product (Fig. 5A). Subsequently, we used artemisinic alcohol as a substrate and calculated the conversion efficiency into artemisinic aldehyde. The microsomal fractions that expressed CYP71AV1 S479F exhibited a 2.9% conversion efficiency, whereas CYP71AV exhibited a 57.8% conversion efficiency (Supplementary Table 2). Therefore, we concluded that Ser479 is important for the second oxidation reaction of CYP71AV1.

4. Discussion

In this study, we isolated putative CYP71AV1 orthologous genes from A. afra and A. absinthium. Comparative functional analysis of CYP71AV1 and its putative orthologs, and homology modeling revealed that Ser479 is important for the successive oxidation reactions of CYP71AV1.

In artemisinin biosynthesis, dihydroartemisinic acid formed from dihydroartemisinic aldhyde is thought to be a late precursor. Therefore, the regulation of CYP71AV1 successive oxidation is interesting because the pathway branches at the formation of artemisinic aldehyde to give dihydroartemisinic aldehyde by the action of artemisinic aldehyde $\Delta 11(13)$ reductase (Dbr2) or artemisinic acid, which appears to be a dead end product, by the action of CYP71AV1 and/or aldehyde dehydrogenase (Aldh1) (Supplementary Fig. 1) [12].

Six putative substrate-recognition sites (SRSs 1-6) for P450s have been proposed in the regions of primary sequences based on the alignment of the CYP2 family with P450cam [28]. Several key amino acid residues that affect the reactivity have been reported within the SRSs. Mutagenesis of residues within SRS6 of multifunctional P450s, 5-epiaristolochene-1,3-dihydroxylase, and flavonoid 3′,5′-hydroxylase was shown to affect the oxidation reaction [29,30]. In CYP71AV1, position 479 corresponds to a region designated SRS6 (Supplementary Fig. 4). The docking of artemisinic alcohol into the CYP71AV1 model demonstrated that a loop located in the C-terminal (C-terminal loop) covering the C12 position of the alcohol. Ser479 is located in the loop and toward the A-helix (Fig. 5B). Conversion of Ser into the large amino acid Phe might result in a conformational change of the C-terminal loop and subsequently affect the substrate binding pocket. These conformational changes may allow the first oxidation reaction but inhibit the second oxidation reaction of CYP71AV1. On the other hand, no clear effect was observed in the replacement of Met by Ver at position 483 in the loop. The similar physical properties of these residue side chains might explain this finding.

The results here show that when Ser479 in CYP71AV1 was substituted to a Phe, a conserved residue in both afCYP71AV1 and abCYP71AV1, the artemisinic alcohol accumulation pattern is altered to a pattern that closely resembles that observed with

afCYP71AV1 or abCYP71AV1. In CYP71AV3-7 of other Asteraceae plants, which show a 80–87% identity to CYP71AV1 and germacrene A oxidation activity [31], the residue corresponding to Ser479 is a Phe resembling afCYP71AV1 and abCYP71AV1 (Supplementary Fig. 4). Nguyen et al. reported that germacrene A oxidase may have subsequently evolved to accommodate amorpha-4,11-diene for the biosynthesis of artemisinic acid [31]. Therefore, the substitution of Phe to Ser may have been a key event in the evolution of ancestral P450s toward CYP71AV1.

A transcript of *ADS* was not detected in *afCYP71AV1*- or *abCYP71AV1*-expressing leaves (Fig. 1A). Additionally, GC–MS analysis could not detect artemisinin and its intermediates in these samples (Suzuki et al., unpublished data). Therefore, the natural substrate of afCYP71AV1 and abCYP71AV1, in planta might be a sesquiterpene other than amorpha-4,11-diene. The fact that CYP71AV3-8 isolated from plants that do not produce artemisinin can oxidize amorpha-4,11-diene and other sesquiterpenoids (germacrene A and/or valencene) [31,32] also supports this hypothesis.

Here, we demonstrated that putative *CYP71AV1* orthologs can be used for investigating the structure–function relationships of CYP71AV1. We also determined that eight species of *Artemisia* have putative *CYP71AV1* orthologous genes, which were shown to have different residues from CYP71AV1, afCYP71AV1, *abCYP71AV1*, and CYP71AV3-8 (Supplementary Fig. 4). Therefore, isolation and functional analysis of the variety of putative CYP71AV1 orthologs distributed in the genus *Artemisia* might enable a more detailed investigation of structure–function relationships and allow the creation of more efficient enzymes that could be useful for the biosynthesis of artemisinin.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.11.031.

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