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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.**
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## 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

*Abbreviations:* *abCYP71AV1*, *CYP71AV1* isolated from *A. absinthium*; Aldh1, aldehyde dehydrogenase; ADS, amorpha-4,11-diene synthase; *afCYP71AV1*, *CYP71AV1* isolated from *A. afra*; CPR, cytochrome P450 reductase; Dbr2, artemisinic aldehyde Δ11(13) reductase; FDP, farnesyl diphosphate; SRSs, substrate-recognition sites

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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 ($^{1}H$ NMR), carbon nuclear magnetic resonance spectroscopy ($^{13}C$ 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×g for 10 min at 4 °C to remove cellular debris followed by centrifugation at 100,000×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).

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