Functional analysis of orthologous artemisinic aldehyde Δ11(13)-reductase reveals potential artemisinin-producing activity in non-artemisinin-producing Artemisia absinthium

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Abstract Artemisin is the most effective antimalarial compound isolated from Artemisia annua. Artemisinic aldehyde Δ11(13)-reductase (DBR2) catalyzes the reduction of artemisinic aldehyde into dihydroartemisinic aldehyde, switching the pathway towards artemisinin production. Although other Artemisia species cannot produce artemisinin, we found a putative DBR2 ortholog expressed in A. absinthium (abDBR2). We examined the catalytic activity of abDBR2 in vitro and found that it shows comparable activity to that of DBR2 based on the reduction of artemisinic aldehyde into dihydroartemisinic aldehyde. Furthermore, we found that dihydroartemisinic aldehyde was detected in the extract of A. absinthium leaves fed with artemisinic aldehyde, suggesting the presence of active abDBR2 in planta. Our results indicate that A. absinthium may be a potential host for the production of artemisinin through metabolic engineering.

Key words: Artemisinin, Artemisia absinthium, DBR2 ortholog, dihydroartemisinic aldehyde, reductase.

Introduction

Artemisin, a sesquiterpene lactone isolated from Artemisia annua, has been recommended by the WHO for use in artemisinin-based combination therapies (ACTs) as a standard treatment for uncomplicated malaria caused by Plasmodium parasites (World Health Organization 2012). However, the production of this effective antimalarial compound in A. annua is very low (ranging from 0.02–2% dry weight) (Cockram et al. 2012; Delabays et al. 2001), and the demand for this compound is approximately two-fold greater than the currently available supply (Noorden 2010). The increasing demand for ACTs and the limited availability of artemisinin from A. annua have led to extensive studies on the artemisinin biosynthetic pathway.

The artemisinin biosynthetic pathway starts with the cyclization of farnesyl diphosphate (FDP) to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) (Chang et al. 2000). Amorpha-4,11-diene is then oxidized to artemisinic aldehyde and artemisinic acid via artemisinic alcohol by amorpha-4,11-diene 12-monoxygenase (CYP71AV1) (Teoh et al. 2006). Artemisinic acid is converted into arteannuin B, a by-product of the pathway, in the presence of light and oxygen (Brown and Sy 2007). Alternatively, artemisinic aldehyde is reduced by artemisinic aldehyde Δ11(13)-reductase (DBR2) to dihydroartemisinic aldehyde (Zhang et al. 2008). Dihydroartemisinic aldehyde is either reduced to dihydroartemisinic alcohol by reductase 1 (RED1) (Rydén et al. 2010a) or further oxidized to dihydroartemisinic acid by aldehyde dehydrogenase 1 (ALDH1) (Teoh et al. 2009). Finally, dihydroartemisinic acid is converted to artemisinin in the presence of light and oxygen (Brown and Sy 2004) (Figure 1).

The Artemisia genus includes over 250 species globally. Among these, artemisinin is produced only in A. annua, and our extensive chemical analyses did not find artemisinin or its intermediates in the extracts of other Artemisia species (Suzuki et al. unpublished data). However, some previous studies reported that extracts of A. absinthium and A. afr?f showed antimalarial activity (Gathirwa et al. 2007; Kraft et al. 2003; Ramazani et al. 2010; Rücker et al. 1991; Valdéz et al. 2008; Zafar et al. 1990). Thus, we investigated the expression of genes involved in artemisinin biosynthesis in these Artemisia species and analyzed the function of orthologous enzymes in a yeast expression system. We found that CYP71AV1 orthologs were expressed in A. absinthium...
(abCYP71AV1) and *A. atra* (afCYP71AV1) and showed similar catalytic activity to *A. annua* CYP71AV1 (Komori et al. 2013).

Among the enzymes involved in artemisinin biosynthesis, DBR2 is a key enzyme that causes a switch in the pathway from production of the by-product arteannuin B to that of artemisinin via dihydroanalogues. In this study, we analyzed the expression of DBR2 in *A. absinthium* and *A. atra* and isolated the putative DBR2 ortholog from *A. absinthium* (abDBR2). We then analyzed the function of abDBR2 using in vitro enzyme assays and examined the conversion to artemisinin production in planta using substrate feeding assays.

**Materials and methods**

*Plant materials*

*A. absinthium* seeds were purchased from a specialty herb store (e-tisanes) in Shizuoka, Japan. *A. atra* seeds were obtained from the University of Pretoria, Pretoria, South Africa. *A. annua* seeds were obtained from the National Institute of Biomedical Innovation, Osaka, Japan. The seeds were germinated and grown in soil in an environment-controlled growth room at 22°C under an 18-h light/6-h dark cycle. After germination and growth in the growth room for 2 months, plants were transferred to the botanical garden in Osaka University, Suita Campus, and allowed to grow until use for in planta substrate feeding experiments. Leaves were collected from *A. annua* plants that had reached approximately 2 m in height (10 months old) and were stored at −80°C until use for transcriptomic analysis.

**Chemicals**

(R)-(−)-Carvone was purchased from Tokyo Chemical Industry, Tokyo, Japan. (−)-α-Pinene was purchased from Wako Pure Chemical Industries, Osaka, Japan. Cyclohexanone and nonanal were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. 2-Cyclohexen-1-one, (+)-dihydrocarvone and trans-2-nononal were purchased from Aldrich, St. Louis, Missouri, USA. Artemisinic acid and dihydroartemisinic acid were isolated and purified from *A. annua* leaves, as described by Vonwiller et al. (1993). Amorpha-4,11-diene, artemisinic alcohol, artemisinic aldehyde, dihydroartemisinic alcohol and dihydroartemisinic aldehyde were synthesized from the isolated artemisinic acid and dihydroartemisinic acid as shown in the Supplementary Information.

**Cloning of DBR2 and gene expression analysis**

Total RNA was extracted from the leaves of *A. absinthium*, *A. atra* and *A. annua* using RNAwiz (Ambion, Carlsbad, CA, USA) and treated with RNase-free DNase (Takara Bio Inc., Shiga, Japan). RNA was then purified further using an RNeasy Plant Mini Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using the SMART RACE cDNA Amplification Kit (Clontech/Takara Bio Inc.) with 1 μg total RNA. To obtain the cDNAs encoding abDBR2 (accession number AB926433) and DBR2 (accession number AB926434), RT-PCR was performed using primers 5′-CAC CAT GTC TGA AAA ACC AAC CTG G-3′ and 5′-GCT CAT AAG ATG CAC CTT ATG AAG-3′ for full-length DBR2 and 5′-ATG CA(A/G)AT(T/C/A)TT(T/C)GTNAA-3′ and 5′-TGNC(G/A)CA(C/T)TT(T/C)TT(T/C)TT-3′ for ubiquitin, the internal control (Cai et al. 2002). The amplified PCR product was cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced.

For gene expression analysis, total RNA was extracted...
from young leaves, old leaves, flowers, stems and roots of *A. absinthium* and *A. annua* using the PureLink™ Plant RNA Reagent (Invitrogen). After treatment with RNase-free DNase (Takara Bio Inc.), first-strand cDNA synthesis was performed using the PrimeScript RT-PCR Kit (Takara Bio Inc.) with 1 µg total RNA as template. Real-time PCR was performed using a LightCycler® Nano (Roche Diagnostics GmbH, Mannheim, Germany) with FastStart Essential DNA Green Master (Roche Diagnostics GmbH). Thermal cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 30 s, and one cycle of 95°C for 10 s, 60°C for 1 min, and 97°C for 1 s. The sequences of the primers used were 5′-TGG GCC AAT ATC ATC AAC AAG C-3′ and 5′-TCT CTA GTG TAA CCA CCG CTG C-3′ for *DBR2* and 5′-GAC GTG ACT TGA CCG ATG CA-3′ and 5′-GTC GAT CCA CCA CTG AGA A-3′ for *actin*, the internal control.

**Heterologous expression of DBR2 proteins**

Full-length cDNA of *abDBR2* and *DBR2* were transferred via pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) into the Gateway-modified version of pCold TF (Takara Bio Inc., Shiga, Japan) using Gateway LR Clonase II Enzyme Mix (Invitrogen, CA, USA) according to the manufacturer’s instructions. The plasmids pCold-abDBR2 and pCold-DBR2 were introduced into *E. coli* Rosetta 2 (DE3) strain (Novagen, Madison, Wisconsin, USA). After culturing in LB medium containing ampicillin and chloramphenicol as selection markers and growth at 15°C with shaking at 170 rpm to an OD₆₀₀ 0.4, they were incubated at 15°C for 30 min followed by induction with IPTG (final concentration 1 mM). Cultures were then incubated at 15°C with shaking at 170 rpm overnight before protein extraction.

IPTG-induced *E. coli* harboring pCold-abDBR2 or pCold-DBR2 were centrifuged at 15,000 rpm and 4°C for 15 min. The resulting cell pellets were re-suspended in binding buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 2 mM imidazole, 0.2 mg mL⁻¹ lysozyme, 2 mM dithiothreitol and 250 µM Cimplete EDTA free (Roche Diagnostics GmbH, Mannheim, Germany) followed by sonication on ice with 10×30-s pulses. The sonicated *E. coli* extracts were then centrifuged under the same conditions to obtain a cell-free supernatant containing DBR2s, as the extract. The mixtures of the protein extracts and Ni-NTA agarose (Qiagen KK, Tokyo, Japan) were incubated at 4°C with rotation at 5 rpm for 1 h using Rotator-RT5 (Taitec, Saitama, Japan). After centrifugation at 8,000 rpm and 4°C for 5 min, the supernatant was removed and the remainder was loaded onto a column. The column was washed three times with 700 µl wash buffer (containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 40 mM imidazole), and the proteins were eluted with 100 µl elution buffer (1 ml wash buffer mixed with 200 µl 2.5 M imidazole). Eluted proteins were desalted using Micro BioSpin™ Chromatography Columns (Bio-Rad, Osaka, Japan) according to the manufacturer’s instructions. SDS-PAGE was performed and CBB Stain One (Nacalai Tesque, Osaka, Japan) was used for visualization.

**In vitro enzyme assays**

In *in vitro* enzyme assays were performed as described in Zhang et al. (2008) with minor modifications. To screen the substrate specificity of abDBR2 compared with DBR2, 0.5 mM substrate was added to a solution containing 50 mM Tris-HCl pH 7.5, 1 mM NADPH, 2 mM dithiothreitol and 0.6 µg purified enzyme. The mixtures were incubated at 30°C with shaking at 500 rpm for 30 min before stopping the reactions using 10 µl glacial acetic acid, followed by extraction using 150 µl EtOAc for GC-MS analysis. Reactions with boiled abDBR2 or without NADPH were used as negative controls. To analyze enzyme kinetics, the reaction was incubated for 15 min with different concentration ranges of the following compounds: artemisinic aldehyde, 50–500 µM (in the presence of 1 mM NADPH); NADPH, 20–500 µM (in the presence of 0.5 mM artemisinic aldehyde); and NADH, 250–1,500 µM (in the presence of 0.5 mM artemisinic aldehyde). Tetradecane was used as an internal standard for quantitative analysis. Each reaction was performed in triplicate, and all kinetic parameters were calculated using Hanes-Woolf linearization. Statistical differences among the kinetic parameters were determined by t-test using the *proc ttest* of the Statistical Analysis System, SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

**In planta substrate feeding assays**

Artemisinin intermediates were dissolved in acetone to a final concentration of 10,000 ppm and used as substrates for *in planta* substrate feeding experiments. For feeding with amorpha-4,11-diene, the final concentration was adjusted to 400 ppm. Feeding with acetone was used as a control experiment. One leaf of *A. absinthium* or *A. afra* (100–200 mg) was placed in the hermetic plastic box containing tissue paper immersed in water. After feeding each substrate (100 µl) to the leaf, the box was covered tightly and kept in the growth room at 22°C under an 18-h light/6-h dark cycle for 7 days. The leaf was then extracted using hexane and analyzed using GC-MS. To analyze the presence of artemisinic acid and dihydroartemisinic acid in the leaf extract, methylation was performed as described in Bertia et al. (2005) prior to GC-MS analysis.

**Gas Chromatography-Mass Spectrometry (GC-MS) analysis**

GC-MS analysis was performed using a JMS-AM SUN200 mass spectrometer (JEOL, Tokyo, Japan) connected to a gas chromatograph (6890 A, Agilent Technologies, Santa Clara, CA, USA) and HP-InnoWax (30 m×0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Santa Clara, CA, USA) or HP-5MS (30 m×0.25 mm i.d., film thickness 0.25 µm, J&W Scientific) capillary columns with a fused silica capillary tube (1 m×0.25 mm i.d., GL-Sciences, Tokyo, Japan). For *in vitro*
enzyme assays of artemisinin intermediates, the HP-InnoWax capillary column was programmed to increase from 40 to 240°C at a rate of 5°C min⁻¹ and a held at 240°C for 15 min. For in vitro enzyme assays of other substrates, the HP-5MS capillary column was programmed at an initial temperature of 50°C for 2 min, increased to 200°C at a rate of 10°C min⁻¹ and a held at 200°C for 5 min. For in planta substrate feeding assays, the HP-InnoWax capillary column was programmed to increase from 46°C to 240°C at a rate of 3°C min⁻¹ and a held at 240°C for 15 min 20 s. The HP-5MS capillary column was programmed to increase from 60 to 280°C at a rate of 3°C min⁻¹, followed by a held at 280°C for 6 min 40 s. In all analyses, the injection port temperature was set at 220°C for HP-InnoWax and at 250°C for HP-5MS. The ion source temperature was maintained at 250°C. The interface temperature was set at 220°C for HP-InnoWax and at 280°C for HP-5MS. The carrier gas was He, and the flow rate was set to 0.7 ml min⁻¹. Split injection (1 µl) was made at a ratio of 1:10. The scanning speed of the mass spectrometer was 2 scans min⁻¹ from m/z 40–350 at an electron voltage of 70 eV. Peaks were identified by comparison of retention times and mass spectra with the corresponding authentic standards.

Results

A putative DBR2 ortholog in A. absinthium showed high homology to A. annua DBR2

To investigate the presence of putative DBR2 orthologs in A. absinthium and A. atra, RT-PCR was performed using RNA isolated from A. absinthium and A. atra leaves with primers designed based on the full sequence of A. annua DBR2. RT-PCR products of DBR2 were detected in A. absinthium leaves, but not in A. atra leaves (Figure 2A). Expression of the putative DBR2 ortholog of A. absinthium (designated here as abDBR2) was analyzed in young leaves, old leaves, stems, flowers and roots from A. absinthium and compared with the expression of DBR2 in the same tissues of A. annua. The expression of abDBR2 was relatively high in young leaves and stems but low in flowers and negligible in roots (Figure 2B). Full length cDNA encoded a polypeptide of 392 amino acid residues with a predicted molecular mass of 43.02 kDa that differed by seven amino acids from DBR2 amino acid residues with a predicted molecular mass of 24.35 kDa. Full length cDNA encoded a polypeptide of 392 amino acid residues with a predicted molecular mass of 24.35 kDa. Full length cDNA encoded a polypeptide of 392 amino acid residues with a predicted molecular mass of 24.35 kDa.

Amino acid sequence of abDBR2 shows high homology to plant 12-oxophytodienoate reductases

BLASTP analysis showed that the amino acid sequence of abDBR2 is highly homologous to plant 12-oxophytodienoate reductases (OPRs). All catalytic amino acid residues of OPRs (Breithaupt et al. 2006) were found in the corresponding positions of abDBR2 (Figure S1). Phylogenetic analysis of abDBR2 and plant OPRs based on amino acid sequences showed that abDBR2 and DBR2 were located within the same branch as Solanum lycopersicum and Arabidopsis thaliana OPR3s (Figure S2).

In vitro enzyme assays

In vitro enzyme assays of abDBR2 or DBR2 were performed using artemisinic aldehyde as the substrate, and the reaction products were analyzed using GC-MS. To identify the product peak, dihydroartemisinic aldehyde was synthesized from dihydroartemisinic acid and used as an authentic standard. The structure of the synthesized dihydroartemisinic aldehyde was confirmed using NMR analysis, and its configuration was assigned as 11R (Figure S3).

As shown in Figure 3, a peak corresponding to (11R)-dihydroartemisinic aldehyde was observed in the chromatogram of the artemisinic aldehyde reduction products formed via abDBR2, but not in that of the reaction products obtained either in the presence of boiled abDBR2 or in the absence of NADPH. Both the retention time and mass spectrum of the detected products matched well with those of the detected product from reaction with DBR2 and the authentic (11R)-dihydroartemisinic aldehyde standard (retention time= 40:58 min). This indicates that abDBR2 exhibited a catalytic activity similar to that of DBR2. Although the $K_m$ value of abDBR2 was slightly higher and its catalytic efficiency ($k_{cat}/K_m$) apparently lower than those of DBR2 (Table 1), no statistical differences were detected in the kinetic parameters between these two enzymes for artemisinic aldehyde ($p≥0.05$, Table S1).

The average $V_{max}$, $K_m$ and $k_{cat}$ values of abDBR2 for

Figure 2. Expression of DBR2 and putative DBR2 orthologs. (A) RT-PCR analysis of DBR2s in A. absinthium, A. atra, and A. annua leaves. Ubiquitin (UBQ) was included as an internal control. (B) Relative expression of DBR2 in A. absinthium and A. annua tissues measured using real-time PCR. The expression of DBR2 in old leaves of A. absinthium and A. annua was normalized to 1. The expression of DBR2 in all other tissues was calculated relative to that in old leaves. Relative expression levels shown in the graph represent the means, and the vertical bars indicate the standard deviation calculated from triplicate experiments.
The values are mean ± SE calculated from triplicated reactions. No statistical difference was detected (p≥0.05) (Table S1).

NADPH differed from those of DBR2 by approximately two-fold (Table 1), and the $k_{cat}/K_m$ of abDBR2 was apparently lower than that of DBR2. However, statistical analyses clearly indicated no statistical differences in the kinetic parameters (p≥0.05, Table S1). In case of NADH, the kinetic parameters of both enzymes were closed to each other (Table 1), and no statistical differences were detected (p≥0.05, Table S1).

In addition, the activities of both abDBR2 and DBR2 were also tested against other artemisinin intermediates: amorpha-4,11-diene, artemisinic alcohol, and artemisinic acid and other substrates, including (−)-α-pinene, 2-cyclohexen-1-one, (R)-(−)-carvone, and trans-2-nonenal. Neither enzyme showed activity against other artemisinin intermediates and (−)-α-pinene (data not shown), but did towards the reduction of 2-cyclohexen-1-one, (R)-(−)-carvone, and trans-2-nonenal into cyclohexanone, (−)-dihydrocarvone, and nonanal, respectively (Figure S5).

In planta substrate feeding assays
To determine whether artemisinin production can be detected in planta, amorpha-4,11-diene, artemisinic alcohol, artemisinic aldehyde, and artemisinic acid were administered to either A. absinthium or A. afra leaves, and the extracts were analyzed using GC-MS. No converted product was detected from the extracts of leaves that were fed with artemisinic acid (data not shown). A peak corresponding to artemisinic alcohol was detected from the extract of A. absinthium leaves fed with amorpha-4,11-diene (Figure S6). Peaks corresponding to artemisinic aldehyde and artemisinic acid were detected in the extracts of either A. absinthium or A. afra leaves fed with artemisinic alcohol (Figure 4A). Peaks corresponding to artemisinic acid, dihydroartemisinic alcohol, dihydroartemisinic aldehyde and dihydroartemisinic acid were detected from the extract of A. absinthium leaves fed with artemisinic aldehyde (Figure 4B). Although a peak corresponding to artemisinic acid was detected from the extract of A. afra leaves fed with artemisinic alcohol, peaks corresponding to dihydroartemisinic alcohol, dihydroartemisinic aldehyde and dihydroartemisinic acid were not detected (Figure 4B). Mass spectra of all detected peaks are shown in Figure S7.
Discussion

Among over 250 species in the *Artemisia* genus distributed globally, artemisinin is produced only in *A. annua*. Nevertheless, some of the artemisinin biosynthetic enzyme orthologs are expressed in other *Artemisia* species, including *A. absinthium* and *A. afra*, and show similar catalytic activity to those of the corresponding enzymes in *A. annua* (Komori et al. 2013). In this study, we examined the expression of *DBR2*, the enzyme involved in the branching point step of the artemisinin biosynthetic pathway, in *A. absinthium* and *A. afra*. We found that a putative *DBR2* ortholog was expressed in *A. absinthium* (*abDBR2*), but not in *A. afra*. This *abDBR2* also showed comparable activity to that of *A. annua* *DBR2* and may function in planta.

Gene expression analysis of *abDBR2* showed that the relative expression of this orthologous gene was high in young leaves and stems, compared with those in flowers and roots, and was similar to that of *DBR2* (as shown in Figure 2B; Oloffson et al. 2011; Zhang et al. 2008). All catalytic residues of plant OPRs were detected in the corresponding residues of *abDBR2* (Breithaupt et al. 2006) (Figure S1). This putative *DBR2* ortholog was also located within the same branch as *S. lycopersicum* and *A. thaliana* OPR3s, which showed activity on the reduction of α,β-unsaturated carbonyl compounds (Müssig et al. 2000; Schaller et al. 2000; Strassner et al. 2002) (Figure S2). Thus, we hypothesized that this enzyme has a catalytic activity similar to those of *DBR2* and OPR3s.

*In vitro* analyses showed that *abDBR2* catalyzes the reduction of artemisinic aldehyde into dihydroartemisinic aldehyde, as does *DBR2* (Figure 3). *abDBR2* also generated product with the same stereochemical configuration as that produced by catalysis via *DBR2*. The cofactor preference of *abDBR2* was also evaluated. Although *abDBR2* was active in the presence of NADH, its *K_m* was approximately 7-fold higher, and its *k_cat/K_m* approximately 30-fold lower than those observed in the presence of NADPH. This result suggests that *abDBR2* prefers NADPH as a cofactor over NADH. A similar preference was observed with *DBR2* (Table 1; Zhang et al. 2008). The kinetic parameters of *abDBR2* for artemisinic aldehyde and dihydroartemisinic aldehyde, and dihydroartemisinic acid.

Figure 4. *In planta* substrate feeding assays of (A) artemisinic alcohol and (B) artemisinic aldehyde administered to *A. absinthium* and *A. afra* leaves. Each box indicates the extracted ion chromatograms with different *m/z* values as shown above the boxes. The names of the detected compounds are shown above the boxes. Bold arrows indicate the detected converted product peaks. Artemisinic acid and dihydroartemisinic acid were detected as methyl esters after methylation. N.D., not detected. The HP-5MS capillary column was used for the analysis of artemisinic alcohol, artemisinic aldehyde, and artemisinic acid. The HP-InnoWax capillary column was used for the analysis of dihydroartemisinic alcohol, dihydroartemisinic aldehyde, and dihydroartemisinic acid.
tested cofactors showed no statistical differences when compared with those of DBR2 (p≥0.05, Table 1 and S1). Both enzymes also exhibited catalytic reduction of other α,β-unsaturated carbonyl compounds, including the conversions of 2-cyclohexen-1-one, (R)-(-)-carvone and trans-2-nonenal into cyclohexanone, (+)-dihydrocarvone and nonanal, respectively (Figure S5). Thus, the catalytic activity of abDBR2 is comparable to that of DBR2.

Two enzymes required for artemisinin production are expressed in A. absinthium (abCYP71AV1 and abDBR2) and show similar catalytic activities to the corresponding enzymes in A. annua. We hypothesized that if these orthologous enzymes are active in planta, then in the presence of artemisinin intermediates, partial conversion of these compounds to artemisinin should be detected in the extracts. Thus, we fed each of the artemisinin intermediates into A. absinthium leaves and analyzed the extracts using GC-MS. Since only one enzyme required for artemisinin production (afCYP71AV1) was detected in A. atra, to compare with A. absinthium, we also included this plant in our substrate feeding assays. Although artemisinic acid can be converted into arteannuin B in the presence of light and oxygen (Brown and Sy 2007), no converted product was detected from the extracts of either A. absinthium or A. atra administered with this compound (data not shown). This indicates that our experimental conditions may not be suitable for replicating the non-enzymatic conversion of artemisinic acid.

As reported by Komori et al. (2013), CYP71AV1 orthologs from A. atra and A. absinthium catalyze the conversion of amorpha-4,11-diene into artemisinic acid. When either artemisinic alcohol or artemisinic aldehyde was administered to A. atra or A. absinthium leaves, a peak corresponding to artemisinic acid was detected in both extracts (Figure 4). Therefore, the conversion of artemisinic alcohol and artemisinic aldehyde into artemisinic acid detected in this experiment may result from the activity of CYP71AV1 homologs.

Interestingly, peaks corresponding to dihydroartemisinic alcohol, dihydroartemisinic aldehyde and dihydroartemisinic acid were also detected from the extract of A. absinthium leaves fed with artemisinic aldehyde. In contrast, no dihydro-analogue was detected from the extract of A. atra leaves fed with artemisinic aldehyde (Figure 4B). These results are supported by the expression analysis of the DBR2 orthologs in A. absinthium and A. atra, as shown in Figure 2A. Based on this result, we suggest that the detected conversion of artemisinic aldehyde into dihydroartemisinic aldehyde could result from the activity of abDBR2.

Rydén et al. (2010b) reported that CYP71AV1 can catalyze the conversion of dihydroartemisinic alcohol into dihydroartemisinic aldehyde and dihydroartemisinic acid. Although it remains unknown whether the ALDH1 ortholog is expressed in A. absinthium, the conversion of dihydroartemisinic aldehyde into dihydroartemisinic acid detected in our feeding experiments (Figure 4B) could be the result of abCYP71AV1 activity.

Based on our preliminary results using in planta substrate feeding assays with amorpha-4,11-diene, we could not detect any converted product from the extracts of either A. atra or A. absinthium leaves fed with this compound (data not shown). Amorpha-4,11-diene has lower solubility than other artemisinin intermediates. Therefore, we hypothesized that it may be more difficult for amorpha-4,11-diene to enter the cell and react with CYP71AV1 orthologs. Thus, we decreased the concentration of fed amorpha-4,11-diene to 400 ppm and performed the substrate feeding assays again. As a result, a small peak of artemisinic alcohol was detected from the extract of A. absinthium (Figure S6).

The chemical composition of A. absinthium essential oils was reported previously (Blagojević et al. 2006; Judžentienë and Mockutë 2004). Among these, monoterpenoids and sesquiterpenoids are the most predominant. Other compounds such as coumarins, lignans, phenols, or flavonoids were also reported in A. absinthium (Aberham et al. 2010; Asghar et al. 2011; Canadanovic-Brunet et al. 2005; Craciunescu et al. 2012; Ramazani et al. 2010; Singh et al. 2012). Since these reported compounds, except the flavone artemisietin, do not contain the α,β-unsaturated carbonyl group in their structure, none of them appear to be an endogenous substrate for abDBR2. Although the endogenous substrate of this homologous enzyme has not been identified, we found that abDBR2 shows comparable activity to DBR2 and may be active in planta.

Studies on plant-based artemisinin combination therapy have received increasing attention recently (Weathers et al. 2014) due to its high antimalarial activity compared with pure artemisinin (Elfawal et al. 2012; Weathers et al. 2011). Although the ADS ortholog was not expressed in A. absinthium (Komori et al. 2013), as shown in this study, endogenous enzymes in A. absinthium can partially catalyze pathways of artemisinin biosynthesis in planta (Figure 4). Based on these findings, the introduction of ADS into A. absinthium could lead to the generation of artemisinin-producing plants, which may be used as a plant-based artemisinin combinatorial therapy.

**Conclusion**

DBR2 is a key enzyme that causes a switch in the pathway from production of arteannuin B to that of artemisinin. In this study, the function of abDBR2 was analyzed, and its reducing activity of artemisinic aldehyde into dihydroartemisinic aldehyde was
comparable to that of DBR2. This orthologous enzyme also showed reducing activity towards other substrates containing the α,β-unsaturated carbonyl group, such as 2-cyclohexen-1-one, (R)-(-)-carvone and trans-2-nonenal. In addition, we showed that if artemisinin intermediates are present, A. absinthium can convert them towards the production of artemisinin, suggestive of active abDBR2 and abCYP71AV1 in planta. These results strongly suggest that ADS may be the limiting factor in the artemisinin-producing ability of A. absinthium. Therefore, it is possible to produce artemisinin-producing A. absinthium through the introduction of ADS.

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Supplementary Materials and Methods

General methods

$^1$H- and $^{13}$C-NMR spectra were recorded using a JEOL JNM-ECS400 NMR spectrometer ($^1$H: 400 MHz; $^{13}$C: 100 MHz). Chemical shifts were reported as $\delta$ (ppm) values relative to internal tetramethyilsilane ($\delta = 0.00$ ppm). Artemisinic acid and dihydroartemisinic acid were isolated from *Artemisia annua* (Vonwiller et al. 1993). Reagents and solvents were purchased from Sigma Aldrich, Wako Pure Chemical Industries, Kanto Chemical Industry, GL Sciences, Merck-Schuchardt, and Kishida Chemical. Column chromatography was performed using a Silica Gel 60N (spherical neutral, particle size 40-50 μm, Kanto Chemical Industry, Tokyo, Japan). All artemisinin intermediates were synthesized from the isolated artemisinic acid and dihydroartemisinic acid as described previously (Bertea et al. 2005; Chang et al. 2000; Zhang et al. 2008) with minor modifications. The structures of the synthesized compounds were confirmed by NMR analyses and compared with those of previous reports (Bertea et al. 2005; Kim et al. 1996; Misra et al. 1993; Ngo and Brown 1999; Sy et al. 1998; Sy et al. 1999).

Artemisinic alcohol.

Excess diazomethane in diethyl ether was added dropwise into a flask containing artemisinic acid (117.65 mg, 0.502 mmol). The reaction mixture was stirred at room temperature for 15 minutes and concentrated under reduced pressure to yield artemisinic acid methyl ester as a colorless oil, which was used directly for the next reaction.

Diisobutylaluminium hydride (DIBAL-H) solution (1.9 mL, 1.90 mmol, 1 M $n$-hexane solution) was added dropwise to a stirred solution of the resulting ester in dry toluene (4.0 mL) at -78°C under a nitrogen atmosphere. After stirring for 3 hours at -78°C, the reaction mixture was allowed to warm to room temperature and quenched with sat. aq. ammonium chloride (0.5 mL). After filtration through celite and anhydrous Na$_2$SO$_4$ and evaporation...
under reduced pressure, the residue was purified using silica gel column chromatography (hexane:EtOAc = 4:1) to yield artemisinic alcohol (68.52 mg, 63% 2 steps) as a colorless oil:

$^1$H-NMR (CDCl$_3$) $\delta$: 0.89 (3H, d, $J = 5.95$ Hz, H-14), 1.01 (1H, ddd, $J = 24.27, 12.82, 3.66$ Hz, H-9), 1.34 (1H, ddd, $J = 25.42, 12.82, 3.21$ Hz, H-8), 1.36 (1H, m, overlapped, H-1), 1.41 (1H, m, overlapped, H-10), 1.49 (1H, ddd, $J = 12.82, 3.21, 1.83$ Hz, H-8), 1.54 (1H, dd, $J = 6.41, 2.75$ Hz, H-2), 1.60 (3H, s, H-15), 1.70 (1H, ddd, $J = 12.82, 6.87, 3.21$ Hz, H-9), 1.79 (1H, br, H-3), 1.88 (1H, dd, $J = 11.91, 5.04$ Hz, H-3), 1.96 (1H, br, m, H-2), 2.22 (1H, d, $J = 12.36$ Hz, H-7), 2.50 (1H, t, $J = 1.83$ Hz, H-6), 4.12 (2H, s, H-12), 4.85 (1H, s, H-13), 5.06 (1H, d, $J = 1.37$ Hz, H-5), 5.19 (1H, t, $J = 1.37$ Hz, H-13); $^{13}$C-NMR (CDCl$_3$) $\delta$: 19.8 (C-14), 23.6 (C-15), 25.7 (C-2), 25.8 (C-8), 26.4 (C-3), 27.8 (C-10), 35.3 (C-9), 37.7 (C-6), 41.6 (C-1), 43.4 (C-7), 65.6 (C-12), 109.9 (C-13), 120.4 (C-5), 135.0 (C-4), 151.1 (C-11).

**Artemisinic aldehyde.**

Excess manganese (IV) oxide was added to a stirred solution of artemisinic alcohol (20.64 mg, 0.094 mmol) in CH$_2$Cl$_2$:benzene (= 1:1 2 mL). The reaction was stirred at room temperature for 10 hours. The resulting mixture was filtered and concentrated under reduced pressure. The residue was purified using silica gel column chromatography (hexane:EtOAc = 9:1) to yield artemisinic aldehyde (17.20 mg, 84%) as a colorless oil: $^1$H-NMR (CDCl$_3$) $\delta$: 0.90 (3H, d, $J = 5.95$ Hz, H-14), 1.07 (1H, ddd, $J = 23.81, 12.36, 3.66$ Hz, H-9), 1.39 (2H, m, overlapped, H-8), 1.44 (1H, br, overlapped, H-10), 1.45 (1H, br, overlapped, H-1), 1.53 (1H, br, m, H-2), 1.59 (3H, s, H-15), 1.71 (1H, dq, $J = 12.36, 3.21$ Hz, H-9), 1.78 (1H, br, H-3), 1.86 (1H, br, m, H-3), 1.91 (1H, br, m, H-2), 2.53 (1H, s, H-6), 2.71 (1H, dt, $J = 11.89, 3.66$ Hz, H-7), 4.88 (1H, s, H-5), 6.13 (1H, s, H-13), 6.18 (1H, s, H-13), 9.53 (1H, s, H-12); $^{13}$C-NMR (CDCl$_3$) $\delta$: 19.8 (C-14), 23.6 (C-15), 25.3 (C-8), 25.4 (C-2), 26.3 (C-3), 27.6 (C-10), 35.1 (C-9), 37.4 (C-6), 39.5 (C-7), 41.2 (C-1), 120.1 (C-5), 134.7 (C-13), 135.1 (C-4), 152.5 (C-11), 194.7 (C-12).
Amorpha-4,11-diene.

Triethylamine (20.0 μL, 0.143 mmol) and methanesulfonyl chloride (6.0 μL, 0.077 mmol) were added to a stirred solution of artemisinic alcohol (7.11 mg, 0.032 mmol) in a mixture of EtOAc:toluene (= 1:1, 3 mL) at 0°C under a nitrogen atmosphere. After stirring for 2 hours at 0°C, the reaction mixture was filtered and concentrated under reduced pressure to yield methanesulfonyl artemisinate as a colorless oil, which was used directly for the next reaction.

Lithium aluminium hydride (3.67 mg, 0.097 mmol) was added to a stirred solution of the resulting mesylate in dry tetrahydrofuran (2 mL) at 0°C. After stirring for 4 hours at 0°C, the reaction was quenched with 1 M HCl (10 mL). The reaction mixture was filtered and extracted with diethyl ether (3 × 10 mL). After washing with brine (10 mL), the combined extracts were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The residue was purified using 10% silver (I) nitrate impregnated silica gel column chromatography (hexane:EtOAc = 50:1) to yield amorpha-4,11-diene (6.35 mg, 97% 2 steps) as a colorless oil: ¹H-NMR (CDCl₃) δ : 0.89 (3H, d, J = 6.41 Hz, H-14), 0.97 (1H, ddd, J = 24.73, 12.82, 3.21 Hz, H-9), 1.25 (2H, dd, J = 26.65, 3.21 Hz, H-8), 1.32 (1H, m, J = 3.21 Hz, H-1), 1.40 (1H, br, m, H-10), 1.50 (1H, ddd, J = 12.82, 3.21, 1.83 Hz, H-8), 1.56 (1H, br, overlapped, H-2), 1.60 (3H, s, H-15), 1.67 (1H, ddd, J = 12.82, 6.87, 3.66 Hz, H-9), 1.74 (3H, s, H-12), 1.79 (1H, br, H-3), 1.93 (1H, br, overlapped, H-3), 1.97 (1H, br, overlapped, H-2), 2.00 (1H, br, overlapped, H-7), 2.55 (1H, br, m, H-6), 4.64 (1H, s, H-13), 4.87 (1H, m, J = 1.37, 0.92 Hz, H-13), 5.06 (1H, s, H-5); ¹³C-NMR (CDCl₃) δ : 19.8 (C-14), 22.6 (C-12), 23.6 (C-15), 25.8 (C-2), 26.0 (C-8), 26.4 (C-3), 27.8 (C-10), 35.4 (C-9), 37.6 (C-6), 41.8 (C-1), 47.6 (C-7), 109.8 (C-13), 120.8 (C-5), 134.6 (C-4), 148.0 (C-11).
Dihydroartemisinic alcohol.

Excess diazomethane in diethyl ether was added dropwise into a flask containing dihydroartemisinic acid (10.81 mg, 0.046 mmol). The reaction mixture was stirred for 2 hours at room temperature and evaporated to dryness to yield dihydroartemisinic acid methyl ester as a colorless oil, which was directly used for the next reaction.

DIBAL-H (137.2 μL, 0.137 mmol, 1 M n-hexane solution) was added dropwise to a stirred solution of the resulting ester in dry toluene (3 mL) at -78ºC under nitrogen atmosphere. After stirring for 4 hours at -78ºC, the reaction mixture was allowed to warm to room temperature. The reaction was quenched with sat. aq. ammonium chloride (0.5 mL). After filtration through celite and anhydrous Na$_2$SO$_4$ and evaporation under reduced pressure, the residue was purified using silica gel column chromatography (hexane:EtOAc = 4:1) to yield dihydroartemisinic alcohol (9.63 mg, 95% 2 steps) as a white powder: $^1$H-NMR (CDCl$_3$) δ : 0.87 (3H, d, J = 6.41 Hz, H-14), 0.93 (1H, m, overlapped, H-9), 0.97 (1H, m, overlapped, H-8), 1.00 (3H, d, J = 6.87 Hz, H-13), 1.21 (2H, br, overlapped, H-1, H-7), 1.41 (1H, br, m, H-10), 1.54 (1H, dd, J = 6.41, 2.75 Hz, H-2), 1.60 (1H, br, overlapped, H-8), 1.63 (5H, br, overlapped, H-9, H-11, H-13), 1.80 (1H, br, m, H-3), 1.91 (1H, br, m, H-3), 1.95 (1H, ddd, J = 5.95, 3.21, 1.37 Hz, H-2), 2.47 (1H, br, m, H-6), 3.53 (1H, dd, J = 10.53, 5.95 Hz, H-12), 3.74 (1H, dd, J = 10.53, 3.21 Hz, H-12), 5.21 (1H, d, J = 1.00 Hz, H-5); $^{13}$C-NMR (CDCl$_3$) δ : 15.0 (C-13), 19.8 (C-14), 23.9 (C-15), 25.9 (C-2), 26.3 (C-8), 26.7 (C-3), 27.7 (C-10), 35.6 (C-9), 36.6 (C-11), 37.5 (C-6), 42.1 (C-1), 42.7 (C-7), 66.8 (C-12), 120.7 (C-5), 135.2 (C-4).
Dihydroartemisinic aldehyde.

Pyridinium dichromate (58.24 mg, 0.155 mmol) was added to a stirred solution of dihydroartemisinic alcohol (7.73 mg, 0.035 mmol) in dry CH₂Cl₂ (3 mL) at room temperature under nitrogen atmosphere. After stirring for 7 hours, the reaction mixture was treated with water (10 mL) and extracted with EtOAc (3 × 10 mL). The combined extracts were washed with brine (10 mL) and dried over anhydrous Na₂SO₄. After filtering and concentrating under reduced pressure, the residue was purified using silica gel column chromatography (hexane:EtOAc = 9:1) to yield dihydroartemisinic aldehyde (4.93 mg, 65%) as a colorless oil: 

\[ \text{H-NMR (CDCl}_3) \delta : 0.88 (3H, d, J = 6.41 \text{ Hz}, H-14), 0.96 (1H, ddd, J = 24.73, 12.82, 3.21 \text{ Hz}, H-9), 1.07 (3H, d, J = 6.87 \text{ Hz}, H-13), 1.11 (1H, dd, J = 12.82, 3.21 \text{ Hz}, H-8), 1.29 (1H, br, m, H-1), 1.41 (2H, br, overlapped, H-8, H-10), 1.55 (1H, br, m, H-2), 1.60 (2H, br, overlapped, H-7, H-9), 1.64 (3H, s, H-15), 1.83 (1H, br, m, H-3), 1.92 (1H, br, m, H-3), 1.97 (1H, br, m, H-2), 2.37 (1H, m, J = 4.12, 3.66, 3.21, 2.75 Hz, H-11), 2.49 (1H, br, m, H-6), 5.13 (1H, d, J = 1.37 Hz, H-5), 9.58 (1H, d, J = 3.66 Hz, H-12); \text{C-NMR (CDCl}_3) \delta : 11.7(C-13), 19.7 (C-14), 23.8 (C-15), 25.7 (C-2), 26.6 (C-3), 27.3 (C-8), 27.7 (C-10), 35.2 (C-9), 36.6 (C-6), 41.4 (C-7), 41.8 (C-1), 48.4 (C-11), 119.5 (C-5), 136.1 (C-4), 206.1 (C-12).
Table S1 Statistical differences of all kinetic parameters between DBR2 and abDBR2

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<th>Substrate</th>
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<th>T value</th>
<th>P value</th>
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<td>Artemisinic aldehyde</td>
<td>$V_{\text{max}}$ ($\mu\text{M min}^{-1}$)</td>
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Statistical differences of the kinetic parameters between abDBR2 and DBR2 were analyzed by t-test using the `proc ttest` of the Statistical Analysis System, SAS 9.2 software.
Figure S1 (cont.)
Alignment of deduced amino acid sequences of abDBR2, DBR2, and plant 12-oxophytodienoate reductases (OPRs). DBR2 cloned in this study was named AaDBR2 HS. DBR2, which was reported previously (Zhang et al. 2008), was named AaDBR2 YZ. The amino acid sequences of plant OPRs used in this alignment showed higher than 50% amino acid identity to DBR2. Yeast old yellow enzymes (OYEs) were also included in this alignment. Asterisks and underlined residues indicate structurally important catalytic amino acid residues reported by Breithaupt et al. (2006). Black shading indicates completely conserved amino acid residues. Gray shading indicates amino acid residues with greater than 51% conservation.

Figure S1
Figure S2 Phylogenetic analysis of DBR2s and plant OPRs. Phylogenetic analysis based on abDBR2 and DBR2 (cloned in this study) amino acid sequences compared with those of reported plant OPRs was performed as described previously (Dereeper, et al., 2008). The species and GenBank™ accession numbers of the enzymes are as follows: Aegilops tauschii OPR3 (EMT31315.1); Arabidopsis lyrata OPR3 (EFH62072.1); Arabidopsis thaliana OPR1 (NM106318), OPR2 (NM106319), OPR3 (NM001084415); A. absinthium DBR2 (AB926433); A. annua DBR2 HS (AB926434); A. annua DBR2 YZ (ACH61780.1); Cicer arietinum OPR3 (XP_004507991.1); Elaeis oleifera OPR (ACO24430.1); Fragaria vesca OPR3 (XP_004287627.1); Glycine max OPR3 (XP_003542357.1); Hevea brasiliensis OPR (AAY27752); Isodon eriocalyx OPR (AEZ53110.1); Ipomoea nil OPR3 (ADN92997.2); Prunus persica OPR (EMJ05582.1); Populus trichocarpa OPR (ERP49797.1) Ricinus communis OPR (EEF46289.1) Saccharomyces cerevisiae OYE2 (EDN62417), OYE3 (CAA97878); Solanum lycopersicum OPR1 (Q9XG54), OPR3 (Q9FEW9); Theobroma cacao OPR2 (EOY26077.1), OPR3 (EOY30181.1); Triticum urartu OPR3 (EMS55956.1); Vitis vinifera OPR3 (AFG73688.1); and Zea mays OPR7 (AAY26527), OPR8 (AAY26528).

The numbers indicate the bootstrap values (%) from 1,000 replications. The scale represents a substitution frequency of 0.5 amino acids per site. The asterisk indicates the DBR2 ortholog...
from *A. absinthium* evaluated in this study. The clear circle indicates the DBR2 cloned in this study. The black circle indicates the DBR2 cloned and reported by Zhang et al. (2008). *S. cerevisiae* OYE2 and OYE3 were used as an out group. The right square bracket indicates the branch discussed in the main text.
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**Figure S3** NMR analysis of (11R)-dihydroartemisinic aldehyde. (A) $^1$H, $^{13}$C, and NOESY spectral data of (11R)-dihydroartemisinic aldehyde and (B) representative NOESY correlations of (11R)-dihydroartemisinic aldehyde.
Figure S4 SDS-PAGE of the recombinant abDBR2 and DBR2 after purification. The molecular weight of the target protein was seemingly larger than its actual weight due to fused expression with a trigger factor of pCold TF vector (~60 kDa). The samples in lanes 1 to 3 are Precision Plus Protein™ Dual Color Standards (Bio-Rad, Osaka, Japan), abDBR2, and DBR2, respectively. The molecular weight (kDa) of the protein is indicated.
Figure S5 *In vitro* enzyme substrate specificity assays of abDBR2 and *A. annua* DBR2. Each column shows the reactions against 2-cyclohexen-1-one (left), (R)-(−)-carvone (middle) or trans-2-nonenal (right). The TICs of *in vitro* assay reaction products are shown. Chromatograms, from the top, show the reaction with abDBR2, DBR2, boiled abDBR2 and abDBR2 without NADPH (as negative controls), substrate standards and product authentic standards, respectively. Bold arrows indicate the product peaks.
Figure S6 In planta substrate feeding assays with amorpha-4,11-diene administered to A. absinthium and A. afra leaves. (A) EIC chromatograms of the extracted products of in planta substrate feeding assays with amorpha-4,11-diene. Acetone administration was used as a
control experiment. Each box indicates the extracted ion chromatograms with different $m/z$ values as shown above the boxes. The names of the detected compounds are shown above the boxes. The bold arrow indicates the detected converted product peak. Artemisinic acid was detected as a methyl ester after methylation. N.D., not detected. (B) The mass spectrum of detected artemisinic alcohol compared with the authentic standard.
Figure S7 Comparison of the mass spectra of the detected converted products (right) with the respective authentic standards (left) in Figure 4.
Supplementary References


