Original Paper

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   Artemisinin 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

Artemisinin, 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-monooxygenase (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. afra* 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*
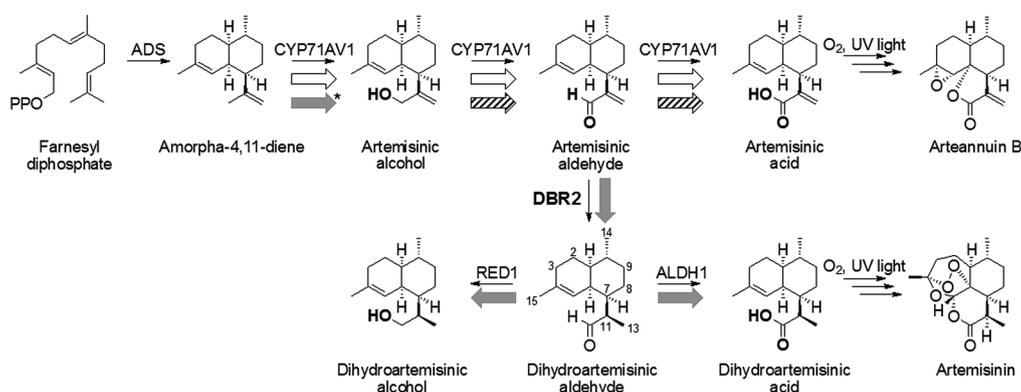
Figure 1.   Biosynthetic pathway of artemisinin. The enzymes responsible for each reaction are indicated by black arrows. White arrows indicate the conversion of artemisinin intermediates by abCYP71AV1 or afCYP71AV1 as reported by Komori et al. (2013). Striped arrows and gray arrows indicate the detected conversion of artemisinin intermediates in substrate feeding assays in this study. Conversion detected in both *A. absinthium* and *A. afra* is shown by striped arrows. Conversion detected only in *A. absinthium* leaves is shown by gray arrows. The asterisk indicates barely detectable conversion in this experiment. Atom numbering for all compounds was assigned as exemplified with dihydroartemisinic aldehyde. ADS, amorpha-4,11-diene synthase; CYP71AV1, amorpha-4,11-diene 12-monooxygenase; DBR2, artemisinic aldehyde Δ11(13)-reductase; RED1, reductase 1; ALDH1, aldehyde dehydrogenase 1.

(*abCYP71AV1*) and *A. afra* (*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 dihydro-analogues. In this study, we analyzed the expression of *DBR2* in *A. absinthium* and *A. afra* 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. afra* 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 artemisinic acid and dihydroartemisinic acid extraction. Flowers of *A. absinthium* and *A. annua* were collected from 10-month-old plants. Other plant tissues were collected from 6-month-old plants. All collected tissues were frozen and stored at −80°C until analyses by RT-PCR and real-time PCR.

Chemicals

(*R*)-(−)-Carvone was purchased from Tokyo Chemical Industry, Tokyo, Japan. (−)-α-Pinene was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Cyclohexanone and nonanal were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. 2-Cyclohexen-1-one, (+)-dihydrocarvone and *trans*-2-nonenal 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. afra* 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 CTT G-3′ and 5′-GCT CAT AAG ATG CAC CTT AAT AAG-3′ for full-length *DBR2* and 5′-ATG CA(A/G)AT(T/C/A)TT(T/C)GTNAA-3′ and 5-TGNCC(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 $1\,\mu g$ 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′-TGC 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 37°C with shaking at 170 rpm to an OD_{600} 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 was 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 $250\,\mu M$ cOmplete 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 condition 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 $700\,\mu l$ wash buffer (containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 40 mM imidazole), and the proteins were eluted with $100\,\mu l$ elution buffer (1 ml wash buffer mixed with $200\,\mu l$ 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. The purity of the obtained proteins was analyzed using the Molecular Imager Gel Doc XR+ Imaging System (Bio-Rad, Osaka, Japan).

In vitro enzyme assays

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 $0.6\,\mu g$ purified enzyme. The mixtures were incubated at 30°C with shaking at 500 rpm for 30 min before stopping the reactions using $10\,\mu l$ glacial acetic acid, followed by extraction using $150\,\mu l$ 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–$500\,\mu M$ (in the presence of 1 mM NADPH); NADPH, 20–$500\,\mu M$ (in the presence of 0.5 mM artemisinic aldehyde); and NADH, 250–$1,500\,\mu M$ (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 ($100\,\mu l$) 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 Bertea 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 $0.25\,\mu m$; J&W Scientific, Santa Clara, CA, USA) or HP-5MS (30 m×0.25 mm i.d., film thickness $0.25\,\mu m$, 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. afra*, RT-PCR was performed using RNA isolated from *A. absinthium* and *A. afra* 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. afra* 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 (Figure S1).

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 *OPR*s based on amino acid sequences showed that *abDBR2* and *DBR2* were located within the same branch
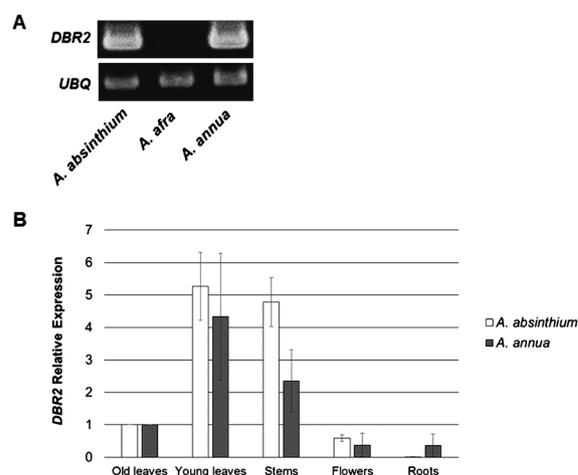

Figure 2.   Expression of *DBR2* and putative *DBR2* orthologs. (A) RT-PCR analysis of *DBR2*s in *A. absinthium*, *A. afra*, and *A. annua* leaves. *Ubiquitin* (*UBQ*) was included as an internal control. (B) Relative expression of *DBR2* in *A. absinthium* and *A. annua* tissues measured using real-time 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.

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 11*R* (Figure S3).

As shown in Figure 3, a peak corresponding to (11*R*)-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 (11*R*)-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 ($p \geq 0.05$, Table S1).

The average V_{max}, K_m and k_{cat} values of abDBR2 for
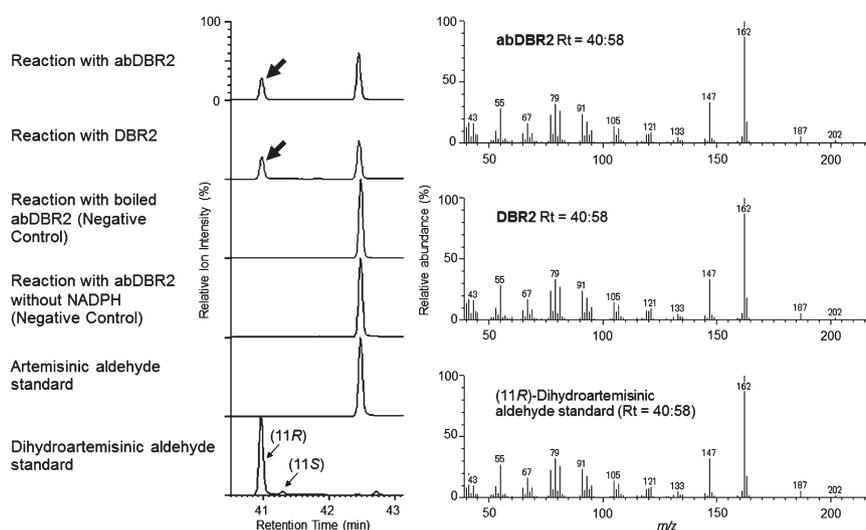
Figure 3. *In vitro* enzyme assays of abDBR2 and DBR2 against artemisinic aldehyde. The TICs of *in vitro* assay reaction products are shown. Chromatograms, from the top, show the reactions with abDBR2, DBR2, boiled abDBR2 and abDBR2 without NADPH (as negative controls), artemisinic aldehyde and dihydroartemisinic aldehyde authentic standards, respectively. Dihydroartemisinic aldehyde was detected in the reaction using either abDBR2 or DBR2. Mass spectra of the product peaks are compared with the standard. Bold arrows indicate the product peaks.

Table 1. Apparent kinetic parameters for abDBR2 and DBR2.

Substrate	Enzymes	V_{max} (μM min^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Artemisinic aldehyde	abDBR2	18.1$\pm$1.4	58.5$\pm$11.6	6.48$\pm$0.50	116$\pm$12
	DBR2	15.3$\pm$1.1	39.6$\pm$14.3	5.49$\pm$0.39	189$\pm$74
NADPH	abDBR2	23.3$\pm$4.5	47.9$\pm$7.5	8.36$\pm$1.62	184$\pm$49
	DBR2	12.6$\pm$1.9	28.0$\pm$14.7	4.53$\pm$0.68	234$\pm$75
NADH	abDBR2	9.53$\pm$0.20	356$\pm$47	2.05$\pm$0.04	5.96$\pm$0.75
	DBR2	8.76$\pm$0.39	306$\pm$26	1.89$\pm$0.08	6.21$\pm$0.24

The values are mean$\pm$SE calculated from triplicated reactions. No statistical difference was detected ($p \geq 0.05$) (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 ($p \geq 0.05$, 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 ($p \geq 0.05$, 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 aldehyde, 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.
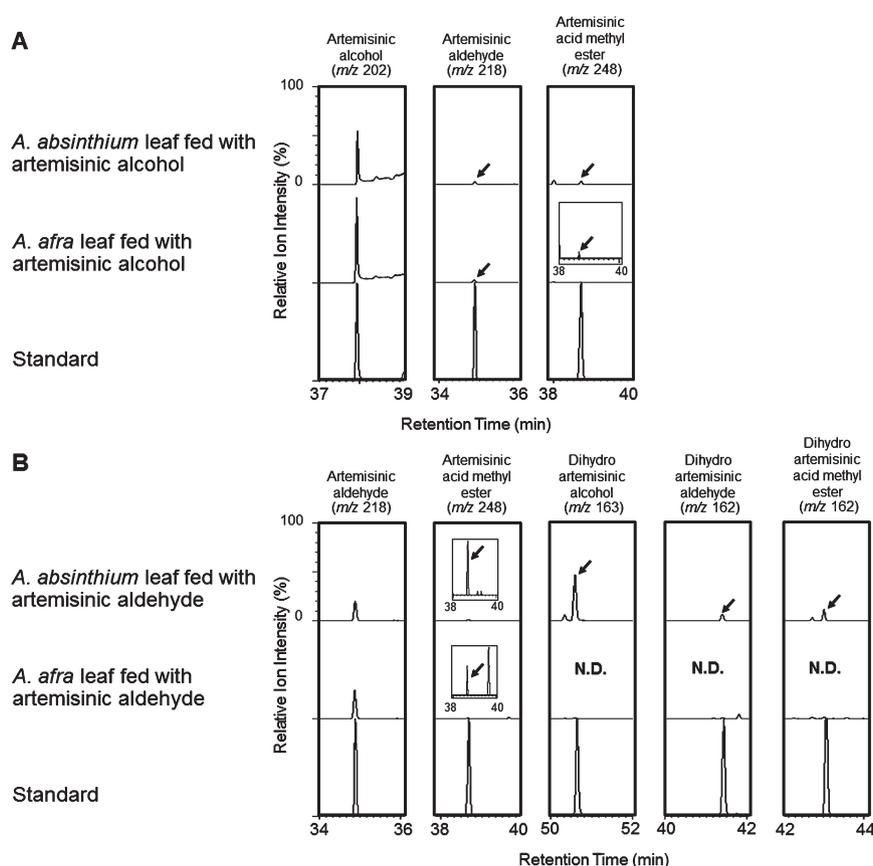
Figure 4. *In planta* substrate feeding assays of (A) artemisinic alcohol and (B) artemisinic aldehyde administered to *A. absinthium* and *A. afra* leaves. Each box indicates the extracted ion chromatograms with different *m/z* values as shown above the boxes. The names of the detected compounds are 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.

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

tested cofactors showed no statistical differences when compared with those of DBR2 ($p \geq 0.05$, 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. afra*, 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. afra* 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. afra* 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. afra* 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. afra* leaves fed with artemisinic aldehyde (Figure 4B). These results are supported by the expression analysis of the *DBR2* orthologs in *A. absinthium* and *A. afra*, 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. afra* 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; Judþentienë 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 artemisetin, 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 (Elfwal 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*.

Acknowledgements

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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 δ (ppm) values relative to internal tetramethylsilane (δ = 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_2SO_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$_3$) δ : 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$_3$) δ : 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$_3$) δ : 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$_3$) δ : 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 Na_2SO_4 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: ^{1}H-NMR (CDCl$_3$) 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); ^{13}C-NMR (CDCl$_3$) 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_2SO_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 CH_2Cl_2 (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 Na_2SO_4. 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: ^{1}H-NMR (CDCl$_3$) δ : 0.88 (3H, d, J = 6.41 Hz, H-14), 0.96 (1H, ddd, J = 24.73, 12.82, 3.21 Hz, H-9), 1.07 (3H, d, J = 6.87 Hz, H-13), 1.11 (1H, dd, J = 12.82, 3.21 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); ^{13}C-NMR (CDCl$_3$) δ : 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

Substrate	Kinetic parameters	Degree of Freedom	*T* value	*P* value
Artemisinic aldehyde	V_{max} (μM min^{-1})	4	-1.56	0.1938
	K_m (μM)	4	-1.02	0.3643
	k_{cat} (s^{-1})	4	-1.55	0.1951
	k_{cat}/K_m (mM^{-1} s^{-1})	4	0.97	0.3860
NADPH	V_{max} (μM min^{-1})	4	-1.88	0.1325
	K_m (μM)	4	0.07	0.9452
	k_{cat} (s^{-1})	4	-1.87	0.1351
	k_{cat}/K_m (mM^{-1} s^{-1})	4	-0.68	0.5355
NADH	V_{max} (μM min^{-1})	4	-1.75	0.1549
	K_m (μM)	4	-0.94	0.4023
	k_{cat} (s^{-1})	4	-1.74	0.1574
	k_{cat}/K_m (mM^{-1} s^{-1})	4	0.33	0.7607

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.

```
abDBR2.seq      1  --------------MSEKPTLFCPYKMGNFNLSHRVVLAPMTRCRAIN--AIPNEA-LVEYYRQRST-AGGFLITEGTMISPSSAGFPHV   72
AaDBR2 HS.seq   1  --------------MSEKPTLFSPYKMGNFNLSHRVVLAPMTRCRAIN--AIPNEA-LVEYYQQRST-AGGFLITEGTMISPSSAGFPHV   72
AaDBR2 YZ.seq   1  --------------MSEKPTLFSAYKMGKFNLSHRVVLAPMTRCRAIN--AIPNEA-LVEYYRQRST-AGGFLITEGTMISPSSAGFPHV   72
AlOPR3.seq      1  --------MTAAQKNSNETLFSSYKMGRFDLSHRVVLAPMTRCRALN--GVPNAA-LAEYYAQRTT-PGGFLISEGTMVSPGSAGFPHV   77
AtaOPR3.seq     1  ----------MDFLATFGAAVDGRSARSPSRHHALNVDGEWSVYDNAG--CPTLVS-MSTDWSLVRG-QMRKRIYGRLTNKTSLDRFPHV   76
AtOPR1.seq      1  -------MENGEAKQSVPLLTPYKMGRFNLSHRVVLAPLTRQRSYG--NVPQPH-AAIYYSQRTT-PGGFLITEATGVSDTAQGYQDT   77
AtOPR2.seq      1  -------MEMVNAEAKQSVPLLTPYKMGRFNLSHRVVLAPLTRQKSYG--SVPQPH-AILYYSQRTS-PGGFLIAEATGVSDTAQGYPDT   79
AtOPR3.seq      1  --------MTAAQGNSNETLFSSYKMGRFDLSHRVVLAPMTRCRALN--GVPNAA-LAEYYAQRTT-PGGFLISEGTMVSPGSAGFPHV   77
CaOPR3.seq      1  -----MADNSSSRQGELGSTLFSPYNVGNFNLSHRIVLAPMTRCRALK--GIPNAA-VGEYYGQRST-PGGLLISEGTSISPTAAGFPHV   81
EoOPR.seq       1  ------------------------------------------------------------VISPTGAGFPHC   12
FvOPR3.seq      1  ---------MADSSSTGGLTLFSPYKMGKFNLSHRVVLAPLTRCRALN--QLPNSA-LVEYYSQRST-PGGLLITEGTWPSDQSAGFPHV   77
GmOPR3.seq      1  -------MADNATTQGSNTLFSPYKMAKFNLSHRVVLAPMTRCRALN--WIPQAA-LAEYYAQRST-PGGFLISEGTLISPTAPGFPHV   78
HbOPR.seq       1  ------MAETGTEGTGITTLFSPYKMGKFSLSHRVVLAPMTRCRALN--GIPNAA-LVDYYTQRST-PGGFLITEGTLVSPTAPGFPHV   79
IeOPR.seq       1  -------------MAETTPALFSPYKMGRFRLSHRVVLAPMTRCRALN--TMPNSA-LAEYYAQRAT-DGGFLITEGTMISPYSAGFPHV   73
InOPR3.seq      1  ------MARTSSLDQGSTPSLFSPYKMGRFNLSHRVVLAPCTRCRAIN--GIPQAA-LAEYYSQRAT-QGGFLITEGTMIDPTSAGFPHV   80
PpOPR.seq       1  -------MAEASSQGPTLFSPFKMGKFNLSHRVVLAPMTRCRALN--GLPQPA-LAEYYTQRST-NGGFLITEGTLVSDTGAGFPHV   76
PtOPR.seq       1  ----------MAENRTGTSLFSPCKMGKFSLSHRVVLAPMTRCRALN--GIPGDA-LAEYYAQRST-PGGFLITEGALISPTAPGFPHV   75
RcOPR.seq       1  ---------------MAETTLFSPYKMGRFNLSHRVVLAPMTRCRALN--GIPNAA-LAEYYTQRST-PGGFLITEGTLVSPTAPGFPHV   71
ScOYE2.seq      1  ----MPFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRAQHPGNIPNRDWAVEYYYAQRAQRPGTLIITEGTFPSPQSGGYDNA   86
ScOYE3.seq      1  ----MPFVKGFEPISLRDTNLFEPIKIGNTQLAHRAVMPPLTRMRATHPGNIPNKEWAAVYYGQRAQRPGTMIITEGTFISPQAGGYDNA   86
SlOPR1.seq      1  -----MENKVVEEKQVDKIPLMSPCKMGKFELCHRVVLAPLTRQRSYG--YIPQPH-AILHYSQRST-NGGLLIGEATVISETGIGYKDV   81
SlOPR3.seq      1  -------MASSAQDGNNP-LFSPYKMGKFNLSHRVVLAPMTRCRALN--NIPQAA-LGEYYEQRAT-AGGFLITEGTMISPTSAGFPHV   77
TcOPR2.seq      1  -MGGTKQTQQQQQENVTTQPLLTPYKFGKFNLSHRVVLAPLTRQRSYN--NVPQPH-AILYYSQRTS-RGGFMISEATGVSDTAQGYAHT   85
TcOPR3.seq      1  ---------MAETQSQG--APTLFSPYKMGKFNLSHRVVLAPMTRCRALN--GIPRPA-LAEYYTQRST-PGGFLITEGTLVSNTGAGFPHV   77
TuOPR3.seq      1  -------------------------------------------MG--GLPGPA-LAEYYSQRAT-QGGLLISEGTVVSPAGPGFPHV   40
VvOPR3.seq      1  -------MAESSAEG--TTTLFSPYKMGRFQLSHRVALAPMTRCRAMN--GLPQPA-LAEYYSQRST-NGGFLITEGTLISPTAAGFPHV   77
ZmOPR7.seq      1  MASTDRSTPAEDEQQQQKRPSLFSPYQMPRFRLAHRVVLAPMTRCRAPD--AVPGPA-LAEYYAQRST-DGGLLISEGTIISPSGPGFPRV   86
ZmOPR8.seq      1  MASTDRSAPAEDQQQPQRPSLFSPYQMPHFRLAHRVVLAPMTRCRAPD--ALPGPA-LAEYYAQRST-EGGLLISEGTIISPAGPGFPRV   86
                                                                                 *                        *
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abDBR2.seq      73  PGIFTKEQVAGVKKVVDAAIKEGAVIFCQLWHVGRASHQVYQPGGA-APISSTSKPIS-KKWKILMPDATYGAYPEPRPLAANEILEVVE  160
AaDBR2 HS.seq   73  PGIFTKEQVEGVKKVVDAAIKEGAVIFCQLWHVGRASHQVYQPGGA-APISSTSKPIS-KKWKILMPDATYGTYPEPRPLAANEILEVVE  160
AaDBR2 YZ.seq   73  PGIFTKEQVEGVKKVVDAAIKEGAVIFCQLWHVGRASHQVYQPGGA-APISSTSKPIS-KKWEILLPDATYGTYPEPRPLAANEILEVVE  160
AlOPR3.seq      78  PGIYSDEQVEAVKQVVDAVIAKGGFIFCQLWHVGRASHQVYQPNGG-SPISSTNIPISEKRWRVLLPDGSYAKYPKPRALEASEIPRVVE  166
AtaOPR3.seq     77  PGIYNEQIDGVKKVVDAVIAKGGIFFCQLWHVGRASHQVYQPDGA-APISSTDKPIS-ARWKILLPDGSYGTYPTRLATSEIPDIVE  164
AtOPR1.seq      78  PGINTKEHVEAVKPIVDAVIAKGGIFFCQIWHVGRVSNSGFQPNGK-APISCSDKPLM-PQIRSNGIDE--ALFTPRRLGIEEIPGIVN  163
AtOPR2.seq      80  PGINTKEHVEAVKPIVDAVIAKGGIFFCQIWHVGRVSNRGFQPRRQ-APISCTGKPIM-PQMRANGIDE--ARFTPRRLSIEEIPGIVN  165
AtOPR3.seq      78  PGIYSDEQVEAVKQVVEAVIAKGGFIFCQLWHVGRASHAVYQPNGG-SPISSNPISENRWRVLLPDGSHVKYPKPRALEASEIPRVVE  166
CaOPR3.seq      82  PGIYSEEQVEAVKNVVNAVIAKGSIIFCQLWHVGRASHSLLQPGGV-APISSTSKPLS-KRWRVLLPDGSYAVYPEPRPLTTSEIQQIVQ  169
EoOPR.seq       13  PGIYTQEQIDAVKKVVDAVIAKGSIIFCQLWHVGRASNQVYQPGGA-APISSTDKPIS-GRWRILMPDGTYGRYPAPRKLATSEIAEIVQ  100
FvOPR3.seq      78  PGIYTDEQVEAVKNVVDAVIAKGAIIFCQLWHVGRASHQVFQPGGG-SPISSTSNPIS-KKWKVALPDGSLDVHSEPRRLETQEIPQIVE  165
GmOPR3.seq      79  PGIYSDEQVEAVRNIVDAVIAKGSIIFCQLWHVGRASHPVYQPGGA-PPISSTSKPIS-ARWRILLPDGSYGVYPEPRALSTSEIPEIVQ  166
HbOPR.seq       80  PGIYTEEQAEAVKRVVDAVIAKGSIIFCQLWHVGRASHQVYQPNGA-SPISSTGKAIS-NRWRILMPDGSYGKYPTPRPLETPEILEVVK  167
IeOPR.seq       74  PGIFSKEQVEAVKETVERVIAKGAVIFCQLWHVGRASHPELQLGGV-APISSTDKPIS-KRWRVLLPDGKYQLYPKPRKLEAYELPELVE  161
InOPR3.seq      81  PGIFTKEQVEAVKKVVDAVIAKGAIIFCQLWHVGRASHEVYQPGGG-SPISSTDVPIS-RRWRILLPDGSHGIYPKPRAVGVDDIANVVE  168
PpOPR.seq       77  PGIYNDEQVEAVKKVVDAVIAKGAIIFCQLWHVGRASHEVYQPGGG-SPISSTDVPIS-RRWRILLPDASHATYPKPRRLETPEILQVVE  164
PtOPR.seq       76  PGIYSDAQVEAVKKVVDEVIAKGSVIFCQLWHVGRASHQVYQPGGV-PPISSTNKPIS-NRWRILMPDGTYGIYPAPRALKTSEILELVE  163
RcOPR.seq       72  PGIYTEEQVEAVKKVVDEVIAKGSIIFCQLWHVGRASHQVYQPGGA-APISSTSKAIS-NRWRILMPDGSYGYSPTPRALETSDIQEVVE  159
ScOYE2.seq      87  PGIWSEEQIKEVTKIFKAIIEKKSFAWVQLWVLGWAAFPDTLARDG-LRYDSASDNVY---MNAEQEEKAKKANNPQHSITKDEIKQYVK  172
ScOYE3.seq      87  PGIWSDEQVAEWKNIFLAIIDCQSFAWVQLWSLGWASFPDVLARDG-LRYDCASDRVY---MNATLQEKAKDANNLEHSLTKDDIKQYIK  172
SlOPR1.seq      82  PGINTKEQVEAVKPIVDAVIAKGGIFFCQIWHVGRVSNKDFQPNGE-DPISCTDRGLT-PQIRSNGIDI--AHFTPRRLTTDEIPQIVN  167
SlOPR3.seq      78  PGIFTKEQVREVKKIVDVVIAKGGIFFCQLWHVGRASHPVQPGGA-APISSTEKPIS-NRWRILMPDGTHGIYPKPRAIGTYEISQVVE  165
TcOPR2.seq      86  PGINTKEQVEAVKPIVDAVIAKGGIIFCQIWHVGRVSNYGFQPNGQ-APISSTDKSLT-PQIRANGVDV--AQFSPPRRLRTDEIPQIVN  171
TcOPR3.seq      78  PGIYNEEQVEAVKNIVDAVIAKGSFIFCQLWHVGRASHSVYQPDGA-APISSTGKPIS-KRWRILMPDGSYGIYPKPRALEIPEIQEAVE  165
TuOPR3.seq      41  PGIYNQEQIDGVKKVVDAVIAKGGIFFCQLWHVGRASHQVYQPDGA-APISSTDKPIS-ARWKILLPDGSYGTYPTRRLATLEIPDIVE  128
VvOPR3.seq      78  PGIYTGEQVEAVKKVVEAVIAKGAIIFCQLWHVGRASHQVYQPGGT-GAPISSTSKPIA-GRWRILMPDGSHGKYPAPRALKTSEIPEVVE  166
ZmOPR7.seq      87  PGIYNQEQTDAVRKVVDAVIAKGAIFFCQLWHVGRASHQVYQPGAA-APISSTDKPIS-SRWRILMPDGSYGKYPTPRRLATSEIPEIVE  174
ZmOPR8.seq      87  PGIYNQEQTDAVKKVVDAVIAKGAIFFCQLWHVGRASHQVYQPGGS-APISSTDKPIS-SRWRILMPDGSYGKYPTPRRLATSEIPEIVE  174
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abDBR2.seq     161  DYRVAAIINAIEAGFDGIEIHGAHGYLLDQFMKDGINDRIDEYGGSLENRCKFILQVVQAVAAIGADRVGLRIISPAIDHLDAMDSDPRSL  250
AaDBR2 HS.seq  161  DYRIAAIINAIEAGFDGIEIHGAHGYLLDQFMKDGINDRIDEYGGSLENRCKFILQVVQAVSAAIGADRVGLRIISPAIDHLDAMDSDPRSL  250
AaDBR2 YZ.seq  161  DYRVAAIINAIEAGFDGIEIHGAHGYLLDQFMKDGINDRIDEYGGSLENRCKFILQVVQAVSAAIGTDRVGIRIISPAIDHTDAMDSDPRSL  250
AlOPR3.seq     167  DYCRSALNAIRAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSIANRCRFLKQVVEGVTRAVLSAIGASKVGVRVSPAIDHLDATDSDPLSL  256
AtaOPR3.seq    165  QYRQAAIINAIKAGFDGIEIHGAHGYIIDQFLKDGINDRIDEYGGSLTNRCKVLLEGTRAVLSAIGAERTAVRVSPAIDHLDAYDSNPMQL  254
AtOPR1.seq     164  DFRLAARNAMEAGFDGVEIHGANGYLIDQFMKDTVINDRIDEYGGSLQNRCKFPLEIVDAVAKEIGPDRVGIRLSPFADYMESGDTNPGAL  253
AtOPR2.seq     166  DFRLAARNAMEAGFDGVEIHGAHGYLIDQFMKDKDVINDRIDEYGGSLQNRCKFALEVVDAVAKEIGPDRVGIRLSPFADYMESGDTNPEAL  255
AtOPR3.seq     167  DYCLSALNAIRAGFDGIEIHGAHGYLIDQFLKDGINDRIDOYGGSIANRCRFLKQVVEGVVSAIGASKVGVRVSPAIDHLDATDSDPLSL  256
CaOPR3.seq     170  HYRHSAIINAIRAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSVENRCRFLIQVVRTVVSAIGAERVGVRIISPAIDHLDATDSNPLGL  259
EoOPR.seq      101  QYRQAALNAIRAGFDGIEIHGAHGYLIDQFLKDGINRSDAYGGSLNRCRFLSEITRAVVSAIGPDRVGVRIISPAIDHLDAYDSDPLGL  190
FvOPR3.seq     166  QYRQAAIINAIRAGFDGIEIHSAHGYLIDQFLKDGVINDRIDEYGGSLANRCFLIQTVEAVVESLGADRVAVRIISPAIDFYDATDADPIGL  255
GmOPR3.seq     167  HYRQAAIINAIRAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSLANRCRFLMQVVQAVVSAIGAERVAVRIISPAIDHLDAIDSDPLKL  256
HbOPR.seq      168  NYRQSALNAIRAGFDGIEVIGAHGYLIDQFLKDGINDRIDEYGGSINNRCRFLMQVIQAVVAAIGDRVGFRVSPAIDHLDAIDSDPLNL  257
IeOPR.seq      162  QYRQAAIINAIEAGFDGIEIHGAHGYLLDQFMKDGINERIDEYGGSLENRCKFIIQVVQAVASAIGDRVGVRIISPAIDHLDAMDSDPLNL  251
InOPR3.seq     169  YYRIAAIINAIEAGDGVEIHGAHGYLIDQFLKDGINNRKDEYGGSLANRCKFIMQVVQAVATAIGADRLGVRISPAIDHLDAMDSSPLSL  258
PpOPR.seq      165  HYRQAALNAIRAGFDGIEIHGAHGYLIDQFLLQVVGAVGAVDRVGVRIISPAIDHLDAVDSAPLTL  254
PtOPR.seq      164  HYSQAALNAIRAGFDGIEIHGAHGYLIDQFLKNGINDRVDEYGGSINRCRFMMQVIQAVVSAVGAERVAFRVSPAIDHLDATDSDPLNL  253
RcOPR.seq      160  TYRQAALNAIRAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSINRCRFMQVIEAVVAAIGADRVGFRVSPAIDHLDAMDSDPLNL  249
ScOYE2.seq     173  EYVQAAKNSIAACADGVEIHSANGYLNQFLDPHSNRIDEYGGSIENRARFTLEVVDAVVDAIGPEKVGLRLSPYGVFNSMSGGAETGI  262
ScOYE3.seq     173  DYIHAAKNSIAACADGVEIHSANGYLLNQFLDPHSNRIDEYGGTIENRARFTLEVVDALIETIGPERVGLRLSPYGTFNSMSGGAEPGI  262
SlOPR1.seq     168  EFRVAARNAMEAGFDGVEIHGAHGYLIDQFMKDQVINDRSDKYGGSLENRCRFALEIVEAVANEIGSDRVGIRISPFAHYNEAGDTNPTAL  257
SlOPR3.seq     166  DYRRSALNAIRAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSLANRCKFITQVVGAVVSAIGADRVGVRIISPAIDHLDAMDSNPLSL  255
TcOPR2.seq     172  DFRTAANAMEAGFDGVEIHGAHGYLIDQFLKDQVINDRIDEYGGSLENRCRFALEIVEAVANEIGADRVGIRLSPFTDFIEAVDSDPKAL  261
TcOPR3.seq     166  HYCRAALNAIRAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSLANRCKFLMQIVQAVVAAIGADRVAVRIISPAIDHLDATDSNPLNL  255
TuOPR3.seq     129  QYRQAAINAIKAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSLENRCKFLLEVTRAVVSAIRAERTAVRVSPAIDHLDAYDSNPMQL  218
VvOPR3.seq     167  HYRQSALNAIAIAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSISNRCKFLVQVVQAVAAIGADRVGVRVSPAIDHLEATDSNPLNL  256
ZmOPR7.seq     175  QYRQAAVNAIKAGFDGIEIHGAHGYLIDQFLKDGINDRIDEYGGSLNRCRFLLEVTRAVVSAIGADRVAVRVSPAIDHLDAYDSNPLQL  264
ZmOPR8.seq     175  QYRQAAINAIKAGFDGIEIHGAHGYLIDQFLKDGINRADEYGGSLNRCRFLLEVTRAVVSAIGADRVAVRVSPAIDHLDAYDSNPLQL  264
                                * **                                                *      *
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Figure S1 (cont.)

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abDBR2.seq       251 GLAVIERLNKLQFKL--GSRLAYLHVTQPRYTA--YGQTEAGAHGSEEEVAQLMKTWRGAYVGTFICSGGYT-RELGLQAVAQGDADLVA 335
AaDBR2 HS.seq    251 GLAVIERLNKLQFKL--GSRLAYLHVTQPRYTA--YGQTEAGAHGSEEEVAQLMKAWRGAYVGTFICSGGYT-RELGLQAVAQGDADLVA 335
AaDBR2 YZ.seq    251 GLAVIERLNKLQFKL--GSRLAYLHVTQPRYTADGHGQTEAGANGSEEEVAQLMKTWRGAYVGTFICCGGYT-RELGLQAVAQGDADLVA 337
AlOPR3.seq       257 GLAVVDLLNKLQDVN--GSKLSYLHVTQPRYHA--YGQTESGRQGSDEEEAKLMRSLRMAYNGTFMSSGGFN-KDLGMQAIQQGDADLVS 341
AtaOPR3.seq      255 GMAVVERLNALQRDA--VQP-AYLRVTQPRYAA--YGQTESGPHGSAEEESRLMRTLRGAYRGTFMCSGGYT-RELGLEAVESGDADLVS 338
AtOPR1.seq       254 GLYMAESLNKYGILY--CHVIEARMKTMGEVHACPHTLMPMRKAFKGTFISAGGFTREDGNEAVSKGRTDLV-AYGRWFLANPDLPKRFQ 340
AtOPR2.seq       256 GLYMVESLNKYGILY--CHMIEPRMKTVGEIAACSHTLMPMREAFKGTFISAGGFTREDGNEAVAKGRTDLV-AYGRWFLANPDLPKRFQ 342
AtOPR3.seq       257 GLAVVGMLNKLQGVN--GSKLSYLHVTQPRYHA--YGQTESGRQGSDEEEAKLMKSLRMAYNGTFMSSGGFN-KELGMQAVQQGDADLVS 341
CaOPR3.seq       260 GLAVIERLNNIQKEF--GKKLSYLHVTQPRYIA--YRKTKSGR---EEEEAHLIRNLRKAYEGTFMCSGGFT-RKLGIEAVAQGDADLVS 341
EoOPR.seq        191 GLAVIGRLNAIQRDA--GERLAYLHVTQPRYTA--YGQTESGRQGSVDEESRLMRRGRTDTRGPFYVGAGLR-GSWGWNGDTGGRRSGVP 275
FvOPR3.seq       256 GLAVIERLNKLQHNI--GSKLAYLHVTRPRLTL-TAIMTSVPAEVDEDREAQFMRTLREAYEGAFMASGGFT-RELGMKTVSSGDADLVS 341
GmOPR3.seq       257 GLAVVERLNNFQKEL--GRKLTYLHVTQPRYTA--YGQTESGRPGSEEEEAHLMQNLRKAYEGTFMCSGGFT-RKLGMEAVAEGHADLVS 341
HbOPR.seq        258 GLAVIERLNKLQLNL--GSKLTYLHVTQPRYTA--YGQTESGRHGTEEEEARLMRTWRRAYKGTFICSGGFT-RELGMEAIAQDDADLVS 342
IeOPR.seq        252 GLAVIERLNKLQLDC--GSKLAYLHITQPRYTA--YGQTESGRHGTADEEAQLLRTWRRAHQGTFICSGGFT-RELGIEAVAVGDADLVS 336
InOPR3.seq       259 GLAVVERLNKLQVDI--RTKLAYLHVTQPRYVA--YGQTESGKQGSEEEEARLMLTLRNTYQGTFICSGGFT-RELGIEAIAQGDADLVS 343
PpOPR.seq        255 GLGVIERLNKLQQDW--GSKLTYLHVTQPRYAA--YGQTESGKPGSDEEEAVFMRTLRNAYRGTFVASGGYT-RELGIHAVASRDADLVS 339
PtOPR.seq        254 GLSVIERINKLQLQV--GSKLTYLHVTQPRYTA--SGQTESRRTGSEDEEAQMIRTWRRAYQGTFMCSGGFT-RELGIQAVTEGDADLVS 338
RcOPR.seq        250 GLAVIERLNKLQLNL--GSKLTYLHVTQPRYTA--YGQTESGRHGTEDEEARLMRTWRNGYQGTFICSGGFT-RELGIEAIAEGNADLVS 334
ScOYE2.seq       263 VAQYAYVLGELERRAKAGKRLAFVHLVEPRVTN---PFLTEGEGEYNGGSNEFAYSIW---KGPIIRAGNFALHPEVVREEVKDPRTLIG 346
ScOYE3.seq       263 IAQYSYVLGELERKAKAGKRLAFVHLVEPRVTD---PSLVEGEGEYSEGTNDFAYSIW---KGPIIRAGNYALHPEVVREQVKDPRTLIG 346
SlOPR1.seq       258 GLYMVESLNKYDLAY--CHVVEPRMKTAWEKIECTESLVPMRKAYKGTFIVAGGYDREDGNRALIEDRADLV-AYGRLFISNPDLPKRFE 344
SlOPR3.seq       256 GLAVVERLNKIQLHS--GSKLAYLHVTQPRYVA--YGQTEAGRLGSEEEEARLMRTLRNAYQGTFICSGGFT-RELGIEAVAQGDADLVS 340
TcOPR2.seq       262 ALYMVEALNKYGILY--CHMIEPRLKMMGDVSECSHTLLPMRKAFNGTFIAAGGYTKEDGNKAVAENRADLV-TYGRLFLANPDLPRRFE 348
TcOPR3.seq       256 GLAVIERLNQLQLQL--GSKLAYLHVTQPRYHA--YGQTESGQHGSEDEEAHLMRTLKRGYQGTFMCSGGFT-RELGMQAVAEGDADLVS 340
TuOPR3.seq       219 GMAVVERLNALQ-----------------------TESGPHGSAEEESRLMRTLRGAYRGTFMCSGGYT-RELGLEAVESGDADLVS 281
VvOPR3.seq       257 GLAVIERLNKLQLDW--GSKLTYLHVTQPRYTA--YGQTESGRHGSEEEEAQLMRTWRRAYQGTFICSGGFT-RELGIEAVALGDADLVS 341
ZmOPR7.seq       265 GLAVVERLNALQQEA--G-RLAYLHVTQPRYTA--YGQTESGQHGSAEEESRLMRAVRGAYRGTFMCSGGYT-RELGVEAIESGDADLVS 348
ZmOPR8.seq       265 GLAVVDRLNALQEET--G-RLAYLHVTQPRYTA--YGQTESGQHGSAEEESRLMRALRGAYRGTFMCSGGYT-RELGVEAVESWDADLVS 348
                                                            *  ** **                                    *

abDBR2.seq       336 FGRHFISNPDLVLRLKLNAPLNRYVRATFYTHDPVVGYTDYPSLDKGNVGVERLSRL 392
AaDBR2 HS.seq    336 FGRHFISNPDLVLRLKLNAPLNRYVRATFYTHDPVVGYTDYPSLDKGNVGVERLSRL 392
AaDBR2 YZ.seq    338 FGRYFVSNPDLVLRLKLNAPLNRYDRATFYTHDPVVGYTDYPSLDKGSLL------- 387
AlOPR3.seq       342 YGRLFIANPDLVSRFKIDAKLNKYNRKTFYSQDPVVGYTDYPFLAAFSRL------- 391
AtaOPR3.seq      339 FGRLFISNPDLVERLRLNAGLNKYVRKTFYTPDPVVGYTDYPFLGKPKARM------ 389
AtOPR1.seq       341 VDAPLNKYDRPTFYTSDPVVGYTDYPFLESTA------------------------ 372
AtOPR2.seq       343 LDAPLNKYNRSTFYTSDPVVGYTDYPSLESTA------------------------ 374
AtOPR3.seq       342 YGRLFIANPDLVSRFKIDGELNKYNRKTFYTQDPVVGYTDYPFLAPFSRL------- 391
CaOPR3.seq       342 FGRLFISNPDLVLRFKLNAPLNKYNRKTFYTYDPVIGYTDYPFLNKGNGTKQIVARL 398
EoOPR.seq        276 TGGFFFSNPDLALRFKVSGDLNRYKRATFYTHDPVVGYTDYPFLGEHRSRL------ 326
FvOPR3.seq       342 YGRDFISNPDLVLRFKLNAPLNKYNRETFYTHDPVVGYTDYPFLNIES--------- 389
GmOPR3.seq       342 YGRLFISNPDLVLRLKLNAPLTKYNRKTFYTQDPVIGYTDYPFLSKESETKEPRARL 398
HbOPR.seq        343 YGRLFISNPDLVLRFKLNAPLNKYVRKTFYTQDPVVGYTDYPFFRKVDGSQEPRSRL 399
IeOPR.seq        337 YGRLFISNPDLVLRLKLDAPLTKYVRATFYTHDPVVGYTDYPFLKPEAASRF----- 388
InOPR3.seq       344 YGRLFIANPDLVLRFKLDAPLNKYNRKTFYTQDPVVGYTDYPFLNKERGSNRPFSRL 400
PpOPR.seq        340 YGRLFISNPDLVLRLKLNAPLTRYNRKTFYTQDPVVGYTDYPFLSNANGKEEPLSRL 396
PtOPR.seq        339 YGRLFISNPDLVLRLKLNAPLNKYIRKTFYTQDPVVGYTDYPFLSKANGVQVPLSRL 395
RcOPR.seq        335 YGRVFISNPDLVLRLKLNAPLNKYIRKTFYTPDPVVGYTDYPFLSKENGSQQLLSRL 391
ScOYE2.seq       347 YGRFFISNPDLVDRLEKGLPLNKYDRDTFYKMSA-EGYIDYPTYEEALKLGWDKN-- 400
ScOYE3.seq       347 YGRFFISNPDLVYRLEEGLPLNKYDRSTFYTMSA-EGYIDYPTYEEAVDLGWNKN-- 400
SlOPR1.seq       345 LNAPLNKYNRDTFYTSDPIVGYTDYPFLETMT------------------------ 376
SlOPR3.seq       341 YGRLFISNPDLVMRIKLNAPLNKYNRKTFYTQDPVVGYTDYPFLQ-GNGSNGPLSRL 396
TcOPR2.seq       349 LNAPLNKYDRNTFYTSDPVIGYTDYPFLEANGDA---------------------- 382
TcOPR3.seq       341 YGRLFISNPDLVLRLKVNAPLSRYIRKTFYTQDPVVGYTDYPFLSEEKGTQFQSRL- 396
TuOPR3.seq       282 YGRLFISNPDLVERLRLNAGLNKYVRKTFYTPDPVVGYTDYPFLGKPKARM------ 332
VvOPR3.seq       342 YGRLFISNPDLVLRLKVNAPLNRYIRAYFYTQDPVKGYTDYPFLNKGSESHEPLSRL 398
ZmOPR7.seq       349 YGRLFIANPDLVERFRRDAPLNKYVRKTFYTPDPVVGYTDYTFLGQPKARM------ 399
ZmOPR8.seq       349 YGRLFIANPDLVERFRRDAPLNRYVRKTFYTPDPVVGYTDYPFLGQPKARM------ 399
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Figure S1 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.
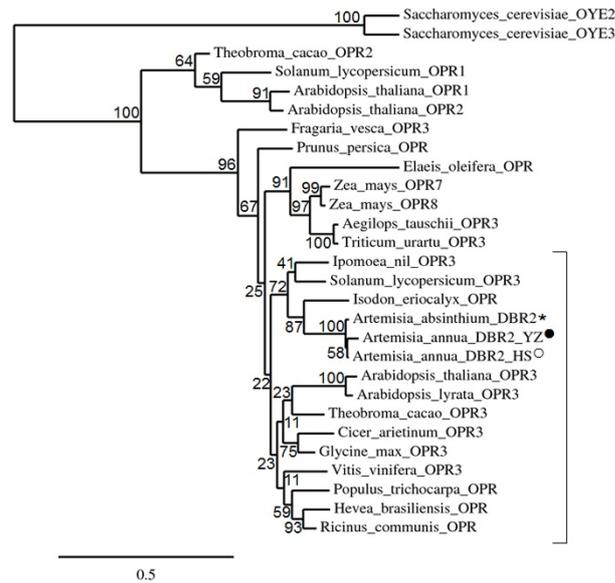
Figure S2 Phylogenetic analysis of DBR2s and plant OPRs. Phylogenetic analysis based on abDBR2 and DBR2 (cloned in this study) amino acid sequences compared with those of reported plant OPRs was performed as described previously (Dereeper, *et al.*, 2008). The species and GenBank[TM] accession numbers 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.

A

| Position	δ_H (mult, J in Hz)	δ_C	NOESY
1	1.29 (br, m)	41.8	H-2$_{ax}$, H-6, H-7, H-9$_{ax}$, H-14
2$_{ax}$	1.55 (br, m)	25.7	H-2$_{eq}$, H-3$_{eq}$, H-6
2$_{eq}$	1.97 (br, m)		H-1, H-2$_{ax}$, H-3$_{ax}$, H-14
3$_{ax}$	1.92 (br, m)	26.6	H-10, H-14
3$_{eq}$	1.83 (br, m)		H-2$_{ax}$
4	-	136.1	-
5	5.13 (d, 1.37)	119.5	H-6, H-11, H-13, H-15
6	2.49 (br, m)	36.6	H-1, H-2$_{ax}$, H-5, H-7, H-13
7	1.60 (br, overlapped)	41.43	H-1, H-6, H-8$_{eq}$, H-9$_{ax}$, H-12, H-13
8$_{ax}$	1.11 (dd, 12.82, 3.21)	27.3	H-8$_{eq}$, H-9$_{eq}$, H-10, H-11
8$_{eq}$	1.41 (br, overlapped)		H-8$_{ax}$, H-9$_{ax}$, H-12
9$_{ax}$	0.96 (ddd, 24.73, 12.82, 3.21)	35.2	H-1, H-7, H-8$_{eq}$, H-9$_{eq}$
9$_{eq}$	1.60 (br, overlapped)		H-8$_{ax}$, H-9$_{ax}$, H-10
10	1.41 (br, overlapped)	27.7	H-3$_{ax}$, H-9$_{eq}$, H-14
11	2.37 (m, 4.12, 3.66, 3.21, 2.75)	48.4	H-5, H-8$_{ax}$, H-12, H-13
12	9.58 (d, 3.66)	206.1	H-7, H-8$_{eq}$, H-11, H-13
13	1.07 (d, 6.87)	11.7	H-5, H-6, H-7, H-11, H-12
14	0.88 (d, 6.41)	19.7	H-1, H-2$_{eq}$, H-3$_{ax}$, H-10
15	1.64 (s)	23.8	H-5

B
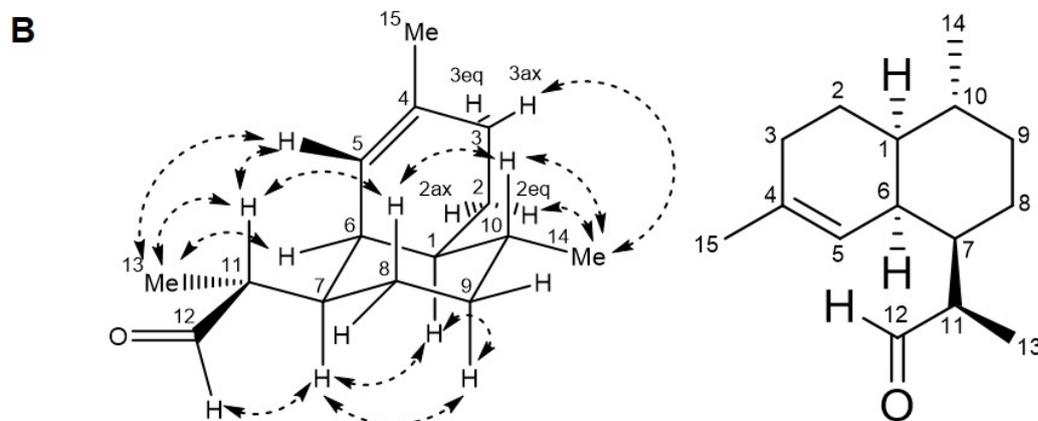
Figure S3 NMR analysis of (11R)-dihydroartemisinic aldehyde. (A) ^{1}H, ^{13}C, and NOESY spectral data of (11R)-dihydroartemisinic aldehyde and (B) representative NOESY correlations of (11R)-dihydroartemisinic aldehyde.
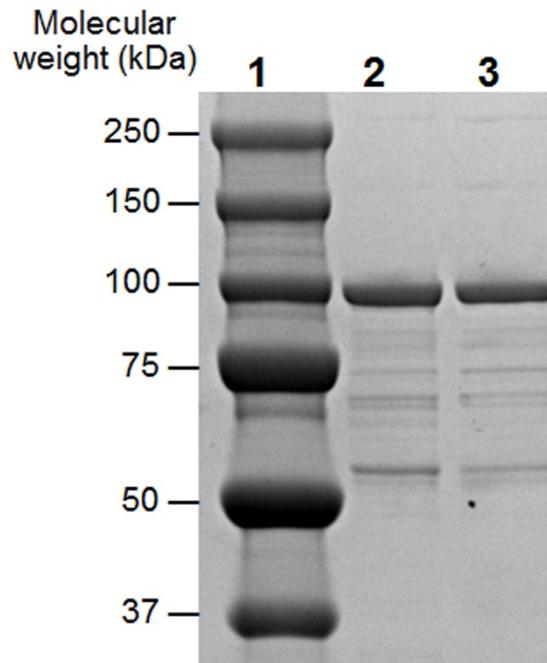
Figure S4 SDS-PAGE of the recombinant abDBR2 and DBR2 after purification. The molecular weight of the target protein was seemingly larger than its actual weight due to fused expression with a trigger factor of pCold TF vector (~60 kDa). The samples in lanes 1 to 3 are Precision Plus Protein[TM] Dual Color Standards (Bio-Rad, Osaka, Japan), abDBR2, and DBR2, respectively. The molecular weight (kDa) of the protein is indicated.
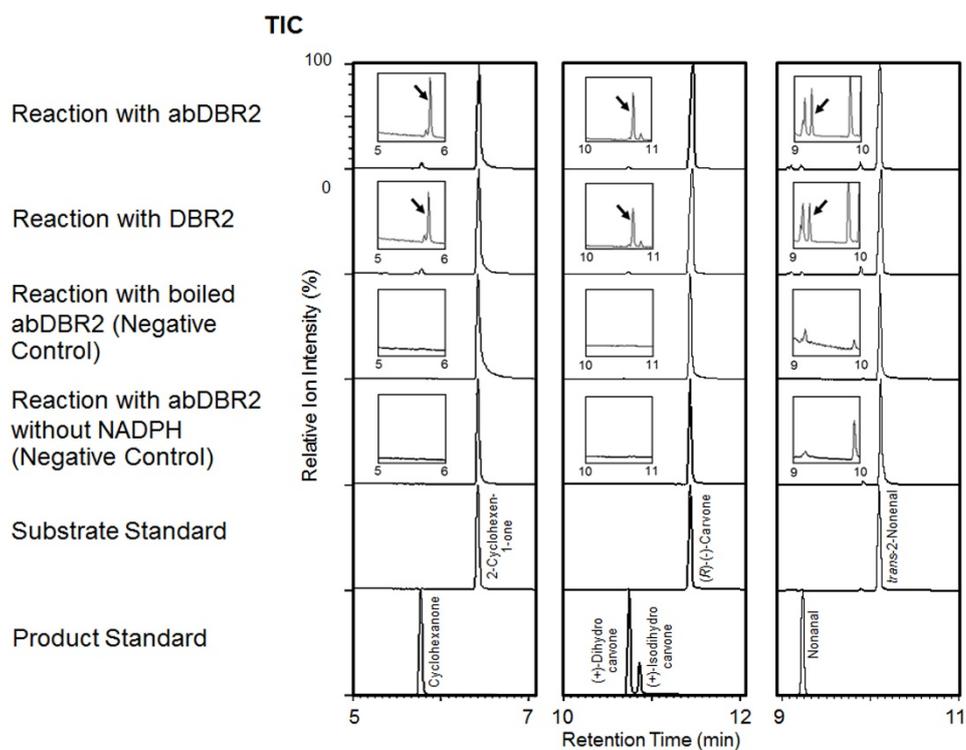
Figure S5 *In vitro* enzyme substrate specificity assays of abDBR2 and *A. annua* DBR2. Each column shows the reactions against 2-cyclohexen-1-one (left), (*R*)-(-)-carvone (middle) or *trans*-2-nonenal (right). The TICs of *in vitro* assay reaction products are shown. Chromatograms, from the top, show 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.
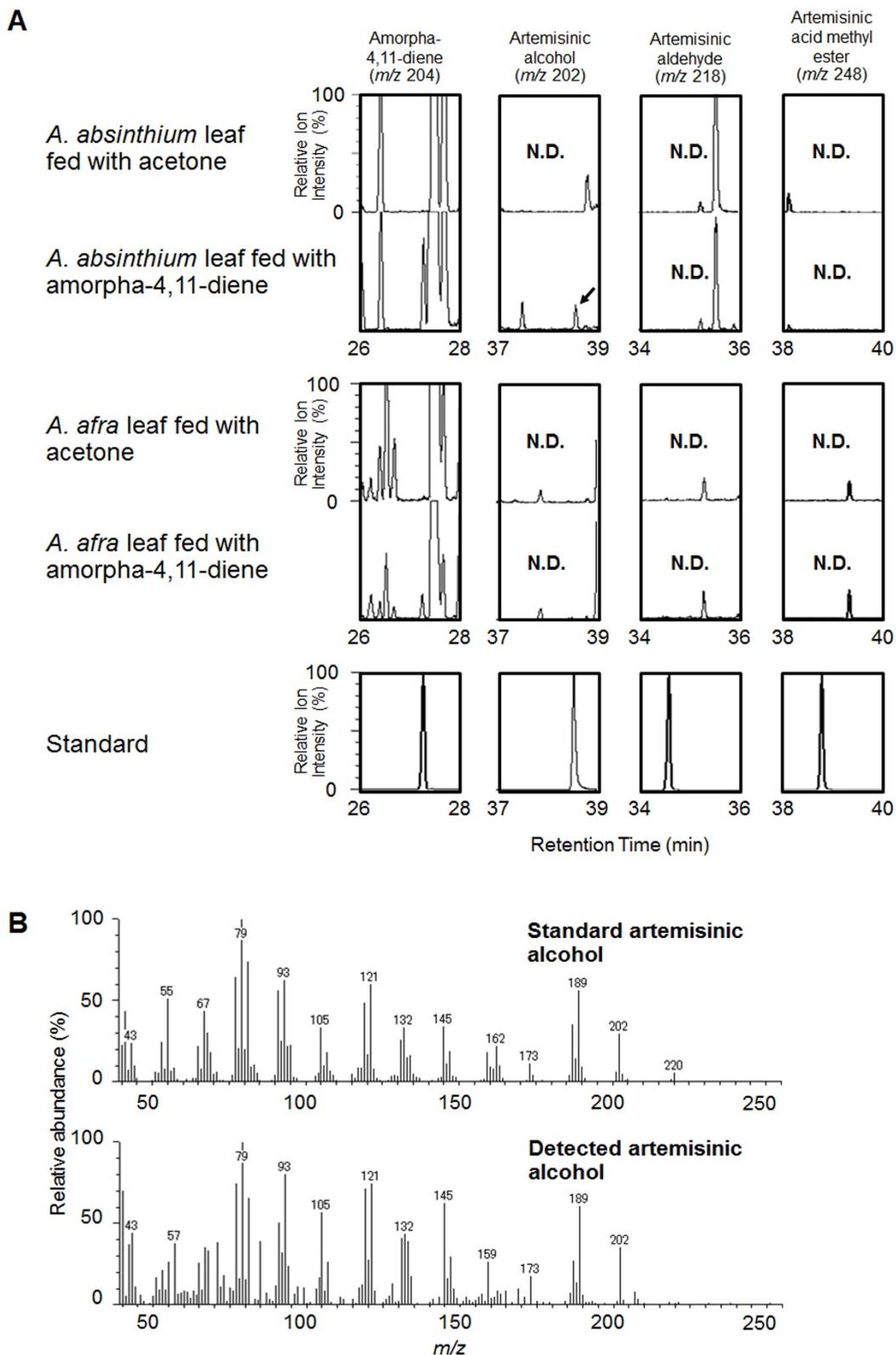
Figure S6 *In planta* substrate feeding assays with amorpha-4,11-diene administered to *A. absinthium* and *A. afra* leaves. (A) EIC chromatograms of the extracted products of *in planta* substrate feeding assays with amorpha-4,11-diene. Acetone administration was used as a

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.
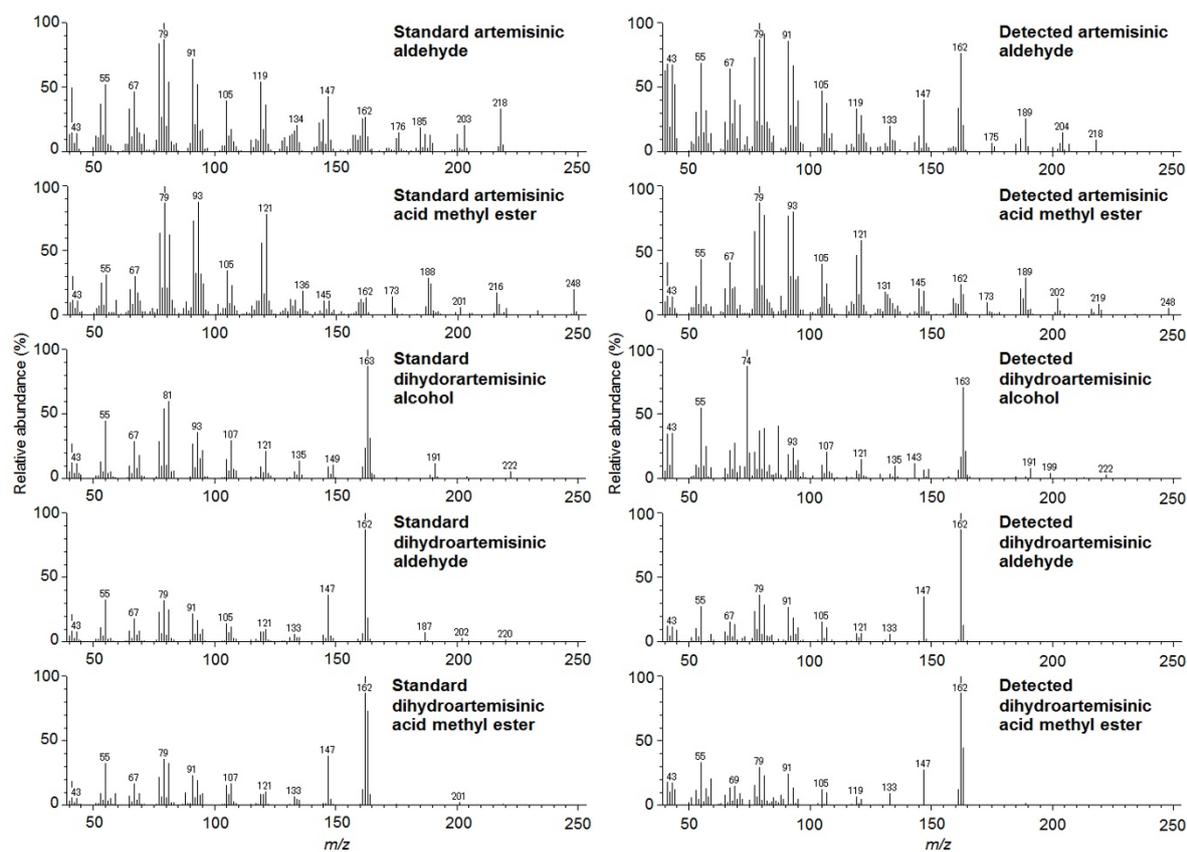
Figure S7 Comparison of the mass spectra of the detected converted products (right) with the respective authentic standards (left) in Figure 4.

Supplementary References

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