THE MOLECULAR CLONING OF ARTEMISINIC ALDEHYDE Δ11(13) REDUCTASE AND ITS ROLE IN GLANDULAR TRICHOME-DEPENDENT BIOSYNTHESIS OF ARTEMISININ IN ARTEMISIA ANNUA

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At some point during biosynthesis of the antimalarial artemisinin in glandular trichomes of Artemisia annua, the Δ11(13) double bond originating in amorpha-4,11-diene is reduced. This is thought to occur in artemisinic aldehyde, but other intermediates have been suggested. In an effort to understand double bond reduction in artemisinin biosynthesis, extracts of A. annua flower buds were investigated and found to contain artemisinic aldehyde Δ11(13) double bond reductase activity. Through a combination of partial protein purification, mass spectrometry and expressed sequence tag analysis, a cDNA clone corresponding to the enzyme was isolated. The corresponding gene Dbr2, encoding a member of the enoate reductase family with similarity to plant 12-oxophytodienoate reductases, was found to be highly expressed in glandular trichomes. Recombinant Dbr2 was subsequently characterized and shown to be relatively specific for artemisinic aldehyde and to have some activity on small α,β-unsaturated carbonyl compounds. Expression in yeast of Dbr2 and genes encoding four other enzymes in the artemisinin pathway resulted in the accumulation of dihydroartemisinic acid. The relevance of Dbr2 to trichome-specific artemisinin biosynthesis is discussed.

Since its discovery in the 1970’s (1), the sesquiterpene lactone artemisinin (see Figure 1) from Artemisia annua has become a key factor in the drive to control malaria (2-4). Semi-synthetic derivatives of plant-derived artemisinin form the basis for artemisinin-based combination therapies (ACTs) which are the treatment of choice for most forms of the disease. Given the pharmaceutical importance of artemisinin and its singular plant source, there is considerable interest in maintaining a reliable and low cost supply (5). A more complete knowledge of artemisinin biosynthesis, and the genes involved, is likely to provide ways of increasing production and lowering cost through crop improvement or microbial engineering (6;7).

As in other members of the Asteraceae, A. annua has 10-celled biseriate glandular trichomes which appear on the surfaces of aerial parts of the plant (8-10). Along with other isoprenoids, the sesquiterpene lactone artemisinin accumulates to levels of 0.01 to 2% dry weight (11). While progress is being made in understanding the biosynthesis of artemisinin, considerable gaps in our knowledge remain (6;12). The formation of amorpha-4,11-diene by amorpha-4,11-diene synthase is the first committed step in the pathway. This is followed by oxidation at C12 of amorpha-4,11-diene by the cytochrome P450, Cyp71av1 to give artemisinic alcohol. These steps are well-supported from biochemical studies and by the molecular cloning of genes encoding the relevant enzymes (12-14). The pathway beyond artemisinic alcohol is somewhat less well-established (6;7;15). However, there is biochemical evidence supporting a route to dihydroartemisinic acid via artemisinic aldehyde (12). This route includes the proposed reduction of the Δ11(13) double bond of artemisinic aldehyde (see Figure 1). Artemisinin per se, appears to be derived from dihydroartemisinic acid in a series of reactions which may not be enzyme-dependent (16;17). Alternatively, based on labeling studies, artemisinic acid and derivatives, such as...
arteannuin B and artemisitene, have been suggested as precursors to artemisinin (6;7;15). Clearly, a more detailed knowledge of the biochemistry of Δ11(13) double bond reduction during artemisinin formation is important in differentiating the proposed routes which can be considered “early” and “late” reduction pathways.

As part of an effort to understand the isoprenoid metabolism in glandular trichomes of the *Artemisia annua* L., we have undertaken an expressed sequence tag (EST)-based approach to identify relevant genes (6;14). This led to the identification of Cyp71av1, a multifunctional cytochrome P450 capable of multiple oxidations of amorpha-4,11-diene. In a continuation of our investigation of the enzymes involved in artemisinin biosynthesis, we report here on the combined use of enzyme purification, mass spectrometry and EST analysis leading to the molecular cloning and characterization of a sesquiterpenoid double bond reductase from *A. annua*. The characterization of this enzyme provides important support for the “early” Δ11(13) reduction in artemisinin biosynthesis.

**Experimental Procedures**

**Plant materials**- *A. annua* seeds were obtained from Pedro Melillo de Magalhães (State University of Campinas, Brazil; line 2/39). The *A. annua* 2/39 line is characterized as a high artemisinin chemotype with characteristically high dihydroartemisinic acid to artemisinic acid ratio (approximately 20:1 in flower buds, data not shown) (18). Seeds were germinated and grown in soil in a controlled environment chamber with 16 hour/24 °C days and 8 hour/21 °C nights. Plants that had reached the height of approximately 1.2 m (after about 3 months) were transferred to a flowering chamber with 8 h/24 °C days and 16 h/21 °C nights. Roots, leaves (mixtures of upper, middle and lower leaves), flower buds (27-29 days after flower induction under short days) were harvested and stored at -80 °C for subsequent RNA isolations and enzyme assays. Glandular trichomes were prepared from flower buds as described previously (14).

**Chemicals**- Artemisinin, pyridinium chlorochromate, coniferyl aldehyde, 2-cyclohexene-1-one, 2E-hexenal, hexanal, 2E-nonenal, nonanal, (+)-α-pinene, and (+)-pulegone were obtained from Aldrich and (+)-carvone, cyclohexanone, dihydrocarvone were obtained from Sigma. 12-oxophytodienoic acid was obtained from Cedarlane Laboratories (Burlington, Canada). Arteannuin B and artemisitene were kindly provided by Dieter Deforce (University of Ghent). Artemisinic alcohol preparation was described previously (14). Sabinone was synthesized from sabinyl acetate obtained from the PBI terpene collection (19) by saponification of the sabinyl acetate to d-sabinol followed by oxidation of the alcohol using pyridinium chlorochromate (20). The product was verified by mass spectral analysis. Isolation and/or semi-synthesis of artemisinic acid and artemisinic aldehyde, dihydroartemisinic acid and three preparations containing dihydroartemisinic aldehyde epimers were prepared as described in the Supplemental Data.

**GC/MS analysis**- GC/MS analyses were performed on an Agilent 6890N gas chromatograph coupled to an Agilent 5973N mass selective detector in electron impact mode (70 eV) with either an InnoWax column (30 m x 0.25 mm i.d., Agilent) for sesquiterpenoid analysis or a DB-5 column (30 m x 0.25 mm i.d., J&W Scientific) for other compounds analyzed. The InnoWax column was temperature programmed from 125°C to 300°C at 5°C min⁻¹; the DB-5 column was programmed at an initial temperature of 40°C for 5 min, then increased to 240°C at 5°C min⁻¹. GC/MS analysis of reductase assays using arteannuin B and artemisitene was accomplished using a short DB-5 column (10 m x 0.25 mm i.d., Agilent) with all heated zones set to <150 °C to prevent thermal decomposition of any heat labile compounds (including artemisinin ) and an oven temperature program of 120°C to 150°C at 2°C min⁻¹. Under these conditions no degradation of a standard of artemisinin was detected.

**Reductase assays**- Unless otherwise stated, double bond reductase assays were carried out as follows. Reactions were initiated by adding 0.5 mM substrate to a 300 µL reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM NADPH, 2 mM DTT and various amounts of enzyme preparation. Protein in enzyme preparations was quantified by Bio-Rad protein assay (Bio-Rad laboratories, Inc.). Negative controls were carried out with boiled proteins and without NADPH. Reactions were
allowed to proceed for 30 minutes at 30°C with shaking (500 rpm), stopped by adding 15 µL glacial acetic acid and extracted with 150 µL ethyl acetate. The ethyl acetate extracts were used directly for GC/MS analysis. The reaction products were confirmed by comparing GC retention time and MS data with those of standards. For quantitation, 3 µg of octadecane was used as an internal standard.

For details of the reductase measurements in plant tissues, the partial purification of the reductase and related protein MS, see Supplemental Data.

Phylogenetic analysis- Phylogenetic analysis of selected amino acid sequences showing similarity to Dbr2 was performed as described previously (21).

Characterization of recombinant artemisinic aldehyde Δ11(13) reductase- Recombinant Dbr2 was prepared as described in Supplemental Data. For characterization of the enzyme, reaction mixtures were pre-warmed to 30°C and reactions were initiated by addition of Dbr2. The pH optimum of the purified Dbr2 was determined to be 7.5 in assay that included one of three 50 mM buffers (MES, HEPES, and Tris-HCl) adjusted to between pH 5.5 and 9.0 in 0.5 unit intervals and 0.5 mM artemisinic aldehyde and 0.48 µg purified recombinant Dbr2. Apparent kinetic parameters were determined under conditions which limited conversion to less than 10% as follows. Concentrations of artemisinic aldehyde (6-250 µM; 0.24 µg Dbr2, 2 min), 2-cyclohexen-1-one (80-10,000 µM; 0.48 µg Dbr2; 10 min) and (+)-carvone (20-5000 µM; 0.48 µg Dbr2; 10 min) were varied in the presence of 1 mM NADPH. To assess cofactor specificity, concentrations of NADPH (10-640 µM; 0.48 µg Dbr2, 2 min) and NADH (0.2-13 mM; 1.9 µg Dbr2; 2 min) were varied in the presence of 0.5 mM artemisinic aldehyde. The ethyl acetate extracts from triplicate reaction mixtures were directly analyzed by GC-MS. Octadecane was used as an internal standard to quantify the products formed from the reactions using response factors determined using standards for each enzyme product. \( K_m \) and \( k_{cat} \) were determined by non-linear regression analysis using GraphPad software (GraphPad Software Inc. San Diego, CA).

RNA isolation and gene expression analysis- Total RNA was extracted from plant tissues using RNeasy Plant RNA isolation kit (Qiagen). For qRT-PCR analysis of Dbr2 expression, first strand cDNA was prepared using Superscript II reverse transcriptase (Invitrogen) using 2 µg of total RNA as the template. Quantitative RT-PCR was performed using a MX3005P Cycler (Stratagene) with SYBR® GreenER qPCR SuperMix Universal kit (Invitrogen). The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 4 min, followed by 40 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 40 s, and one cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. The primers 5'-CTTGGTTACAAGCTTGCGTCAAG-3' and 5'-ATATAATCAAAAACACTAGAGGAGTGAC C-3' were used to amplify a 209 bp fragment of the Dbr2 transcript. The primers 5'-CCGTCGCTTTCATTCCGAT-3' and 5'-CGCCATCCTTCTGTGAGCT-3' were used to amplify a 300 bp fragment of a gene named A. annua Actin1 by the authors. These were designed based on the sequence of a cDNA fragment corresponding to A. annua Actin1 cloned from an RT-PCR product using the degenerate primers 5'-AGCAACTGGGATGACATGGAG-3' and 5'-CACCTTCGATCTTCTCATGCT-3' and A. annua leaf cDNA.

Subcellular localization of Dbr2- In order to investigate the subcellular localization of artemisinic aldehyde Δ11(13) reductase, Dbr2 and A. thaliana Opr3 were introduced into the vector pER420. The vector pER420, obtained from Raju Datla (Plant Biotechnology Institute), was derived from pK7WG2 (22) by the introduction of a sequence encoding smGFP immediately downstream of the 35S promoter, and a sequence encoding an E-tag immediately upstream of the 35S terminator.

The open reading frame of A. thaliana Opr3 was first obtained by RT-PCR using the primers 5'-CACCATGAGCGCCGCAAAGGGAAGTC-3' and 5'-TCAGAGGCGGGAAAAAG GAGC-3'. The resulting PCR product was cloned using pENTR/D to give pENTR/D-AtOPR3. The open reading frames of Dbr2 and Opr3 were introduced separately into pER420 using LR recombination reactions (Invitrogen) with pENTR/D-AaDBR2 and pENTR/D-AtOPR3 to give the plasmids pER420-AaDBR2 and pER420-AtOPR3, respectively.
Both expression vectors were transferred to the *Agrobacterium tumefaciens* strain C58C1[pMP90] for transformation of *A. thaliana* cotyledons using a protocol developed by J.-D. Faure and colleagues \(^5\). Transformation was achieved by vacuum infiltration, after which infiltrated cotyledons were left for 4 days in liquid growth medium (Murashige and Skoog medium without sucrose) to allow transient expression of the fusion proteins. GFP localisation was examined with a 100M confocal microscope with software package LSM 510 version 3.2 (Zeiss) equipped with a 63X water-corrected objective. Dual GFP fluorescence and chlorophyll autofluorescence was imaged in a multichannel setting with 488 nm and 543 nm light for GFP and chlorophyll excitation, respectively. Fluorescence emission was captured in the frame-scanning mode alternating GFP fluorescence via a 500- to 550-nm bandpass filter and chlorophyll via a 560-nm cutoff filter.

**Yeast engineering** - The preparation of the plasmids pESC-HIS-FPS-ADS, pESC-LEU-CYP-CPR, and pYESDEST52-AaDBR2 and their use in transformed yeast is described in Supplemental Data.

**RESULTS**

Artemisinic aldehyde \(\Delta11(13)\) reductase activity in *A. annua* extracts. The nature of the enzyme involved in the sesquiterpenoid \(\Delta11(13)\) double bond reduction was initially investigated in extracts of *A. annua* 2/39 tissues. Preliminary work with flower buds showed reductase activity in the presence of artemisinic aldehyde and NADPH (see Figure 2, D and E). This assay resulted in the detection of a product peak by GC/MS which was identified as (11R)-dihydroartemisinic aldehyde (Figure 2E) by comparison with natural (Figure 2A) and semi-synthetic (Figure 2B and C) preparations of dihydroartemisinic aldehyde epimers. This peak had a retention time (Figure 2E) and mass spectrum (see Supplemental Data Figure S4) identical to (11R)-dihydroartemisinic aldehyde synthesized from natural dihydroartemisinic acid (Figure 2B and Supplemental Data Figure S4). The retention time was distinct from the (11S)-aldehyde in the mixture formed in the chemical reduction of methyl artemisinate (Figure 2C). Thus, given the \(R\) configuration of artemisinin at C11, the stereochemistry of the enzyme activity found is consistent with a role in artemisinin biosynthesis.

Assay conditions for the reductase reaction were optimized (see Experimental Procedures and Supplemental Data) and extracts from roots, leaves, flower buds and glandular trichomes were tested. The results for the four tissues are shown in Figure 3A. Compared to trichome extracts, reductase levels in extracts of flower buds and leaves were at least 10-fold lower. In root extracts, reductase activity was negligible. Given the presence of glandular trichomes on leaf surfaces and at higher densities on floret surfaces, the data from the plant extracts are consistent with a trichome-localized enzyme.

Partial purification and mass spectral analysis of artemisinic aldehyde \(\Delta11(13)\) reductase from *A. annua*. With a view towards complete characterization of the reductase, its purification from plant material was attempted. Flower buds were chosen for convenience, in lieu of the more active glandular trichomes. Artemisinic aldehyde \(\Delta11(13)\) reductase was partially purified from the flower buds of *A. annua* line 2/39 using fractional ammonium sulfate precipitation, ion-exchange and size exclusion chromatography, and dye affinity batch adsorption (see Supplemental Data for experimental details). This resulted in an approximately 100-fold purification on a protein basis relative to the ammonium sulfate precipitate (See Supplemental Data, Table 1S). The size exclusion chromatography indicated that the native molecular weight of artemisinic aldehyde \(\Delta11(13)\) reductase was approximately 44 kDa (data not shown). The partially purified enzyme was subjected to SDS-PAGE followed by silver staining (See Supplemental Data, Figure S1). The most densely staining bands were excised from a polyacrylamide gel, digested with trypsin, and analyzed by LC-MS/MS. When the resulting data from a band corresponding to approximately 44 kDa was used to search a database of *A. annua* expressed sequence tags (EST), the best match was to a cDNA clone “GSTSUB_026_G01” derived from the *A. annua* trichome-specific cDNA library called GSTSUB (14). The mass spectral data corresponded to the four predicted...
peptide sequences YFVSNPDLVLR, ELGLQAVAQGDADLVAFGR, ATFYTHDPVVGTYTDYPSLDK and GAYVGTFICCGGYTR. The gene corresponding to this EST was named Dbr2. Analysis of A. annua ESTs (14) indicated the presence of 0, 8 and 9 ESTs corresponding to Dbr2 derived from flower bud (AAFB), glandular trichome (AAGST) and “trichome-minus-bud” (GSTSUB) libraries, respectively. This suggested a relatively high expression of Dbr2 in trichomes relative to flower buds.

Isolation of a full-length Dbr2 cDNA from A. annua 2/39. To allow the characterization of the product of Dbr2, a full-length cDNA clone was obtained by SMART-RACE-PCR. The Dbr2 ORF is 1331 bp long and encodes a 415-amino acid protein with a predicted molecular mass of 45.6 kDa. A BLASTP search of Genbank with the predicted amino acid protein sequence of Dbr2 revealed greatest sequence identity with plant 12-oxophytodienoate reductases (OPRs) and related enzymes. In particular, Dbr2 showed high amino acid sequence identity to tomato (77%; GenBank Accession Number Q9FEW9) and Arabidopsis (68%; GenBank Accession Number AAG15379) OPR3’s, both of which have been shown to reduce the 12-oxophytodienoate isomer involved in jasmonate biosynthesis (23). Figure 4 illustrates the results of phylogenetic analysis of Dbr2 and a selection of related sequences based on amino acid sequence alignment (see Supplemental Data, Figure S2). Dbr2 lies within a branch which includes the tomato and Arabidopsis OPR3s, as well as an OPR3 homologue from Hevea brasiliensis and the maize OPR7 and OPR8 proteins. Based on these sequence comparisons, there is strong support for inclusion of Dbr2 in the \( \alpha/\beta \) barrel fold family of FMN-containing oxidoreductases (24) which include fungal old yellow enzymes (OYEs) (25;26). This protein family is also known as the enolate reductases.

Sequence analysis revealed that Dbr2 contains His, His and Tyr at positions 180, 183 and 185, respectively (see Supplemental Data, Figure S2). The residues are highly conserved in OYEs and OPR-like enzymes and are thought to be involved in catalysis. Conserved amino acids in the substrate binding cavity of OPR3s (27) were also found in corresponding positions of Dbr2 (His239 and Phe69). However, the amino acids that form a loop near the active site of Arabidopsis OPR3, “AYG” (27), are occupied by “ADGHG” in Dbr2 (A281-G285; see Supplemental Data, Figure S2). The pronounced structural differences in the loops adjacent to the active site are thought to be responsible for the differences in substrate specificity of OPRs and OYE (27). In addition, the carboxy-terminus (Ser-Arg-Leu) that most likely accounts for the peroxisomal location of tomato and Arabidopsis OPR3s (23;28) is replaced with Ser-Leu-Leu in the C-terminus of Dbr2. This suggests a different subcellular localization of Dbr2 in A. annua (see below).

Recombinant Dbr2 shows artemisinic aldehyde \( \Delta 11(13) \) reductase activity. For functional characterization of Dbr2, the recombinant enzyme was purified from E. coli cells harbouring the plasmid pDEST17-Dbr2. The sizes of Dbr2 determined from sequence-based prediction, electrophoresis (see Supplemental Data, Figure S1) and gel filtration indicate that the enzyme functions as a monomer. The purified Dbr2 protein was assayed with various substrates followed by GC/MS analysis. Similar to plant extracts, purified Dbr2 showed NADPH-dependent formation of \( (11R) \)-dihydroartemisinic aldehyde as the major product using artemisinic aldehyde as a substrate (see Supplemental Data, Figure S3). Control assays with boiled enzyme preparation (not shown) and in the absence of NADPH did not support the production of dihydroartemisinic aldehyde.

Given the similarity of Dbr2 to tomato and Arabidopsis 12-oxophytodienoate reductase 3, Dbr2 was tested for activity with 12-oxophytodienoic acid. No products were found in this assay (data not shown). Dbr2 was also tested with other potential substrates, including artemannin B, artemisinic acid, artemisinic alcohol, artemisitene, (+)-carvone, coniferyl aldehyde, 2-cyclohexen-1-one, 2E-hexenal, 2E-nonenal, (+)-\( \alpha \)-pinene, (+)-pulegone, and sabinine. The results indicate that, in addition to artemisinic aldehyde, Dbr2 has activity on 2-cyclohexen-1-one, (+)-carvone and low activity on 2E-nonenal (approximately 12% of the rate for artemisinic aldehyde; see Supplemental Data, Figure S3). The GC/MS-derived retention times and mass spectra of the products of artemisinic aldehyde, (+)-carvone, 2-cyclohexen-1-one and 2E-nonenal matched \( (11R) \)-dihydroartemisinic aldehyde,
dihydrocarvone, cyclohexanone and nonanal standards, respectively (see Supplemental Data Figures S3 and S4). Based on comparison to a standard 5:1 mixture of (+)-dihydrocarvone/(+)-isodihydrocarvone and assuming that the configuration at C4 was retained, the major and minor products of (+)-carvone were identified as (−)-(1R,4S)-isodihydrocarvone and (−)-(1S,4S)-dihydrocarvone, which were produced in a 5:1 ratio. No activity was detected with arteannuin B, artemisinic acid, artemisinic alcohol, artemisitene, coniferyl aldehyde, 2E-nonenal, (+)−α-pinene, (+)−pulegone, and sabinone (data not shown).

The pH optimum of Dbr2 was determined to be pH 7.5. At pH 5.5, the enzyme activity was completely inhibited; at pH 9.0, it retained ~ 25% of its activity at pH 7.5. Preliminary experiments indicated that the enzyme was also active in the presence of NADH. Kinetic parameters were determined for artemisinic aldehyde, 2-cyclohexen-1-one, (+)-carvone, NADPH and NADH. Relative to 2-cyclohexen-1-one and (+)-carvone, the reductase was very specific for artemisinic aldehyde for which the Km was more than 30-fold lower (Table 1). The enzyme is also highly specific for NADPH with a Km of 95 µM, as compared to more than 770 µM for NADH.

Dbr2 expression and subcellular localization. The expression of Dbr2 in various A. annua tissues was studied using qRT-PCR. As indicated in Figure 3B, Dbr2 expression is relatively high in glandular trichomes, less so in leaves and flower buds, and negligible in roots. This pattern is striking similar to the observed reductase activities in these tissues (Figure 3A) and supports the in vivo role of Dbr2 in artemisinin biosynthesis in glandular trichomes.

The subcellular localization of Dbr2 was investigated by transient expression of GFP fusion proteins in A. thaliana cotyledons. The Dbr2 fusion protein localized primarily to the cytosol (Figure 5), whereas the Arabidopsis Opr3 fusion protein localized exclusively to the peroxisomes, confirming the importance of the Ser-Arg-Leu tripeptide sequence as a peroxisomal targeting signal (24).

Dbr2-dependent production of dihydroartemisinic acid in yeast. The possible applications of Dbr2 include the engineering of microbes (and plants) for the production of artemisinin. This concept was tested by expression of multiple genes in S. cerevisiae. In preliminary experiments, dihydroartemisinic aldehyde supplied to wild type yeast cells was converted to dihydroartemisinic acid, presumably as a result of endogenous aldehyde dehydrogenase activity (data not shown). Based on this, it was reasoned that the de novo production of dihydroartemisinic acid could be realized by co-expression of farnesyl diphosphate synthase (Fps2), amorpha-4,11-diene synthase (Ads), amorpha-4,11-diene monooxygenase (Cyp71av1), cytochrome P450 reductase, and artemisinic aldehyde Δ11(13) reductase (Dbr2) in yeast. In order to test this, three yeast strains were developed: a control strain containing three empty vectors; a strain which reconstitutes the artemisinin pathway up to artemisinic acid by expressing Fps2, Ads, Cyp71av1 and Cpr, and a strain which additionally expresses Dbr2. As indicated in Figure 6 unlike the control strain, the strain expressing Fps2 through Cyp71av1 (but not Dbr2) accumulated artemisinic acid to a level of 29.4 (+/-4.7) µg/mL culture. This is comparable to levels found in similar previously reported experiments (29). In the strain additionally expressing Dbr2, artemisinic acid accumulated to 11.8 (+/-2.8) µg/mL and, in addition, dihydroartemisinic acid was found at a level of 15.7 (+/-1.4) µg/mL culture.

DISCUSSION

The molecular cloning of Dbr2 is an example of the power of the combined use of protein purification, mass spectrometry and expressed sequence tag analysis. In particular, the availability of ESTs and limited biochemical knowledge allowed the identification of an enzyme using relatively impure extracts. Modern protein mass spectrometry methods allowed the connection to be made between components of a protein mixture and corresponding cDNA sequences encoding, in this case, oxidoreductases.

The current understanding of the biosynthesis of artemisinin is still somewhat limited (6;7;15). Some data remain in conflict, particularly concerning the nature of the late precursor of artemisinin. Some work has suggested that compounds such as artemisinic acid, arteannuin B, artemisitene and related
compounds are precursors. However, other evidence strongly supports the notion that artemisinic acid and dihydroartemisinic acid represent two branches in the pathway, with only the latter giving rise to artemisinin. This is supported by in vitro work (12) and in vivo biochemical studies (16). A key difference in the proposed pathways to artemisinin is the point at which the Δ11(13) carbon-carbon double bond is reduced. In the “early reduction” pathway, it has been suggested to occur in the conversion of artemisinic aldehyde to dihydroartemisinic aldehyde. On the other hand “late reduction” pathways would require Δ11(13) reduction at artemisinic acid, arteannuin B or artemisitene or derivatives thereof (7).

The characterization of Dbr2 has shed light on the nature of the pathway to artemisinin. In particular, given that artemisinic alcohol is almost certainly a precursor to artemisinin, the specificity of Dbr2 for artemisinic aldehyde over artemisinic alcohol and acid, as well as arteannuin B and artemisitene is very strong support for the “early reduction” pathway. To have such an enzyme highly expressed in trichomes indicates that at least some artemisinin is made via its product, dihydroartemisinic aldehyde and subsequently dihydroartemisinic acid. It remains possible that a “late reduction” pathway is also in operation; however, at present it appears that such a route may be negligible.

It is notable that two broad chemotypes have been identified within A. annua which differ in artemisinin content and the related acids (18). Low artemisinin chemotypes tend to have high levels of artemisinic acid; high artemisinin chemotypes tend to have high dihydroartemisinic acid. It is of course tempting to speculate that variation in Dbr2 expression is responsible for this phytochemical differences and further work is in progress to investigate this. In any case, it suggests that Dbr2 activity may be a limiting factor in artemisinin biosynthesis, an important consideration for engineering enhanced artemisinin production. With this in mind, one can imagine that Dbr2 would be useful both for marker-assisted selection of high-expressers of Dbr2 and for the genetic modification of plants for high Dbr2 expression. Both approaches would be expected to aid in the enhancement of artemisinin yields.

The biochemistry of carbon-carbon double bond reduction often includes enzymes which act on α,β-unsaturated carbonyls. Indeed, where it has been investigated the carbonyl group is thought to be intimately involved in the reaction mechanism. This is true of the family to which Dbr2 belongs, which includes the old yellow enzyme (26). Thus, by analogy, His180 and His183 would be predicted to hydrogen bond to the carbonyl of artemisinic aldehyde (27). Likewise, hydride and proton transfer to the β (C13) and α (C11) carbons is likely to involve flavin N-5 and Tyr185, respectively.

Some of the first terpene double bond reductases characterized were the glandular trichome-specific (-)-isopiperitone and (+)-pulegone reductases from peppermint (30). These enzymes are members of the short- and medium-chain dehydrogenase/reductases superfamilies, respectively. Dbr2 represents a third distinct superfamily associated with terpene double bond reduction – the FMN-linked oxidoreductases. Within this superfamily, the enzymes most closely related to Dbr2 are the plant OPRs. Some of these, including tomato and Arabidopsis OPR3s, act on the α,β-unsaturated ketone 12-oxophytodienoic acid as part of the pathway to jasmonic acid. However, the in vivo role of most other plant OPR-like genes is unknown. In fact, the sesquiterpenoid reductase activity of Dbr2 appears to be one of the first examples of an activity other than 12-oxophytodienoic acid reduction which can be attributed to an in vivo pathway. This suggests the general possibility that OPRs have been recruited during evolution for functions in secondary metabolism. Thus, one should probably think quite broadly about the possible roles of other OPRs, even those in the OPR3 branch (see Figure 4).

The cloning and characterization of Dbr2 present some biotechnological possibilities. In this paper, we have included a proof-of-concept for the microbial production of dihydroartemisinic acid. By the simultaneous expression of five A. annua genes in yeast, we were able to demonstrate dihydroartemisinic acid production at levels comparable to those previously reported for artemisinic acid (29). In both cases, the sesquiterpene acid accumulated can be converted chemically to artemisinin. However, the conversion of dihydroartemisinic acid to
artemisinin is simpler, requiring only oxygen under the appropriate conditions.
REFERENCES


**FOOTNOTES**

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Sequence data relevant to this article can be found in the GenBank database under the following accession numbers: *Arabidopsis thaliana OPR1* (GenBank Accession Number NM_106318), *OPR2* (GenBank Accession Number NM_106319) and *OPR3* (GenBank Accession Number NM_001084415); *Artemisia annua Actin1* (GenBank Accession Number EU531837, submitted by authors), *Ads* (GenBank Accession Number AF138959), *Cpr* (GenBank Accession Number EF104642), *Cyp71av1* (GenBank Accession Number DQ315671), *Dbr2* (GenBank Accession Number EU704257; submitted by authors) and *Fps* (GenBank Accession Number AF136602); *Hevea brasiliensis Opr* (GenBank Accession Number AAY27752); *Saccharomyces cerevisiae OYE2* (GenBank Accession Number EDN62417); *Solanum lycopersicum Opr1* (GenBank Accession Number Q9XG54) and *Opr3* (GenBank Accession Number Q9FEW9); *Zea mays Opr7* (GenBank Accession Number AAY26527) and *Opr8* (GenBank Accession Number AAY26528).

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The abbreviations used here are: AA; artemisinic acid, AAA; artemisinic aldehyde, *Ads*; amorpha-4,11-diene synthase, *AtOPR*; *Arabidopsis thaliana* 12-oxophytodienoate reductase, *Cpr*; *A. annua*cytochrome P450 reductase, *Cyp71av1*; amorpha-4,11-diene monoxygenase, *Dbr2*; artemisinic aldehyde Δ11(13) reductase, DHAA; dihydroartemisinic acid, DHAAA; dihydroadamisinic aldehyde, EST; expressed sequence tag, *Fps2*; *A. annua* farnesyl diphosphate synthase, ScOYE2; *Saccharomyces cerevisiae* old yellow enzyme 2, SIOPRs; *Solanum lycopersicum* 12-oxophytodienoate reductase, ZmOPRs; *Zea mays* 12-oxophytodienoate reductase.
Table 1. Apparent kinetic parameters for *Dbr2*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>artemisinic aldehyde</td>
<td>19</td>
<td>2.6</td>
<td>140</td>
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<tr>
<td>2-cyclohexen-1-one</td>
<td>790</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>(+)-carvone</td>
<td>650</td>
<td>0.86</td>
<td>1.3</td>
</tr>
<tr>
<td>NADPH</td>
<td>95</td>
<td>2.5</td>
<td>26</td>
</tr>
<tr>
<td>NADH</td>
<td>770</td>
<td>1.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Fig. 1. Proposed pathways for the biosynthesis of artemisinin from artemisinic alcohol in *A. annua*.

Fig. 2. Stereoselective reduction of artemisinic aldehyde by *A. annua* extracts. GC/MS analyses of three standards and two enzymes assays are shown as follows: a diethyl ether extract from *A. annua* flower buds indicating the predominance of the 11R-epimer of dihydroartemisinic aldehyde (A); [predominantly (11R)-] dihydroartemisinic aldehyde synthesized from dihydroartemisinic acid extracted from *A. annua* (B); a mixture of dihydroartemisinic aldehyde (mixed 11-epimers) and artemisinic aldehyde synthesized from artemisinic acid extracted from *A. annua* (C); an ethyl acetate extract of an *A. annua* flower bud extract assayed with artemisinic aldehyde in the absence (D) and presence (E) of NADPH. AAA, artemisinic aldehyde. R, (11R)-dihydroartemisinic aldehyde. S, (11S)-dihydroartemisinic aldehyde. See Supplemental Data for associated mass spectra.

Fig. 3. Artemisinic aldehyde Δ11(13) reductase activity and gene expression in *A. annua* tissues. The specific activities of artemisinic aldehyde Δ11(13) reductase (A) and *Dbr2* expression as measured by qRT-PCR (B) for extracts of various tissues are indicated as the means and standard deviations of single experiments performed in triplicate.

Fig. 4. An unrooted phylogram based on Dbr2-like enzymes. The species and Genbank accession numbers of the enzymes are as follows: AaDBR2, *A. annua* Dbr2 (this study); AtOPR1, AtOPR2 and AtOPR3, *Arabidopsis thaliana* OPR1, OPR2 and OPR3, respectively; HbOPR, *Hevea brasiliensis* Opr; ScOYE2, *Saccharomyces cerevisiae* OYE2; SIOPR1 and SIOPR3, *Solanum lycopersicum* Opr1 and Opr3, respectively; ZmOPR7 and ZmOPR8, *Zea mays* Opr7 and Opr8, respectively. Bootstrap values greater than 70 out of 100 are indicated.

Fig. 5. Subcellular localisation of the DBR2 protein. Transient expression of AtOPR3-GFP (left) and AaDBR2-GFP (right) in Arabidopsis cotyledon cells detected by confocal laser scanning microscopy. From top to bottom, images represent bright-field, red channel, green channel and the overlay of all channels, respectively. Chlorophyll autofluorescence is detected in the red channel and GFP fluorescence in the green channel. Chloroplasts (chl), peroxisomes (per), and cytosol (cyt) are indicated by arrows. White bars at the bottom right of each image represent 20 µm.

Fig. 6. *Dbr2*-dependent production of dihydroartemisinic acid in engineered yeast. GC/MS analysis is shown for methylated extracts of yeast strains containing the three control plasmid vectors pESC-HIS, pESC-LEU, and pYESDEST52-GUS (A), the plasmids pESC-HIS-FPS-ADS, pESC-LEU-CYP-CPR, and pYESDEST52-GUS (B), and the plasmids pESC-HIS-FPS-ADS, pESC-LEU-CYP-CPR, and pYESDEST52-AaDBR2 (C). Chromatograms correspond to equivalent volumes of yeast culture. The largest peak with a retention time of ~16 min corresponds to methyl cis-9-hexadecenoate.
Figure 3

(A) Specific activity (pmol/min/μg protein)

(B) Dbr2 relative expression level
Figure 4 Phylogenetic Analysis
Figure 6

A

B

C

Time (min)

(11R)-DHAA

AA

AA
The molecular cloning of artemisinic aldehyde δ11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in artemisia annua
Yansheng Zhang, Keat H. Teoh, Darwin W. Reed, Lies Maes, Alain Goossens, Douglas J. H. Olson, Andrew R. S. Ross and Patrick S. Covello

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