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Functional genomics and the biosynthesis of artemisinin

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Abstract

Artemisinin, a sesquiterpene lactone endoperoxide derived from the glandular secretory trichomes (GSTs) of *Artemisia annua*, provides the basis for the most effective treatments of malaria. The biology and biochemistry of GSTs of the Asteraceae and their biosynthesis of isoprenoids is reviewed. Recent efforts to understand the biosynthesis of artemisinin in *A. annua* GSTs are discussed in detail. This includes the development in the authors' laboratory of an expressed sequence tag (EST) approach to identifying the relevant biosynthetic genes using isolated GST as a source of mRNA. This has lead to the isolation of a cDNA encoding CYP71AV1, a multifunctional cytochrome P450 which catalyzes multiple oxidations of the sesquiterpene intermediate amorpha-4,11-diene to artemisinic acid. Further biochemical and molecular genetic work is required to elucidate the precise route from artemisinic alcohol to artemisinin and to engineer more efficient low cost production of artemisinin-based antimalarial drugs.

Keywords: Artemisia annua; Asteraceae; Artemisinin; Sesquiterpene; Cytochrome P450; Trichome

Contents

1. *Artemisia annua*, artemisinin and malaria

In the early 1970's, the structure of an unusual sesquiterpene lactone from *Artemisia annua* was determined and now bears the name artemisinin **1** (Li et al., 2006; Liu et al., 1979). Today, artemisinin **1** derivatives provide the basis for the most effective treatments for malaria and the compound is one of the most sought after plant-derived drug precursors (Haynes et al., 2006; Li et al., 2006; Mutabingwa, 2005). As a consequence, there is great interest in understanding the biochemistry and molecular biology of artemisinin **1** production in *A. annua* (Abdin et al., 2003; Ro et al., 2006; Teoh et al., 2006). This review considers the recent developments in understanding the biochemistry and molecular genetics of artemisinin **1** biosynthesis with

Abbreviations: AAFB, Full-length flower bud cDNA library; AAGST, Full-length glandular trichome cDNA library; ADS, Amorpha-4,11-diene synthase; CYP, Cytochrome P450; DXP, 1-Deoxy-D-xylulose-5-phosphate; EST, Expressed sequence tag; GST, Glandular secretory trichomes; GSTSUB, Glandular-trichome-minus-flower-bud cDNA library.

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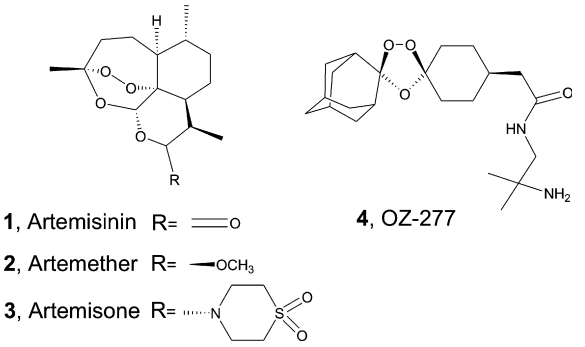

Fig. 1. Artemisinin **1** and related compounds **2**–**4**. Artemisinin **1** is a natural product from *A. annua*; artemether **2** and artemisone **3** are semi-synthetic antimalarial drugs and OZ-277 (compound **4**) is a synthetic antimalarial drug candidate.

emphasis on the functional genomics approaches in the authors' laboratory.

The history leading to the development of artemisinin-based malaria treatment involves both traditional and modern medicine (Ferreira et al., 1997; Hsu, 2006). Both *A. annua* and *Artemisia apiacea* have been used in traditional Chinese medicine for centuries, particularly for the treatment of fevers, probably including those caused by malaria. In the 1960's, Chinese scientists began screening plant extracts for antimalarial activity and found that a diethyl ether extract of *A. annua* had antimalarial activity in mice and monkeys. The chemical agent responsible for this was isolated and characterized; originally, it was named arteannuin, or qinghaosu.[1] Artemisinin **1**, as it is most commonly referred to now, is an amorphane type sesquiterpene lactone with a very unusual endoperoxide structure (see Fig. 1).

The pharmacology of artemisinin **1** and its derivatives has been studied extensively over the last three decades (Davis et al., 2005; Mutabingwa, 2005; O'Neill, 2005). The activity of artemisinin **1**, itself against the malaria parasite *Plasmodium falciparum* has been very well established. Numerous derivatives of artemisinin **1** have also been synthesized and tested against malaria parasites. This has resulted in the registration of a number of related antimalarial drugs, many of which are C10 derivatives of artemisinin **1**. Examples of these include artemether **2**, which is well established for use in combination with lumefantrine, for example, and artemisone **3**, which is currently under development (Haynes et al., 2006; see Fig. 1). The latter compound was developed in part to avoid metabolism to dihydroartemisinin and the possibility of associated neurotoxicity. Recently, analogues of artemisinin **1**, such as OZ-277 (Fig. 1, compound **4**) which include the 1,2,4-trioxane pharmacophore have been synthesized and shown to have antimalarial activity (Vennerstrom et al., 2004). The mode of action of artemisinin-related compounds is still under investigation. While iron-dependent active oxygen-mediated effects and mitochondrial membrane depolarization have been suggested (Li et al., 2005), evidence is emerging for PfATP6, a sarco-endoplasmic reticulum Ca^{2+}-ATPase of the *P. falciparum* parasite, as an important site of action (Krishna et al., 2006; Uhlemann et al., 2005).

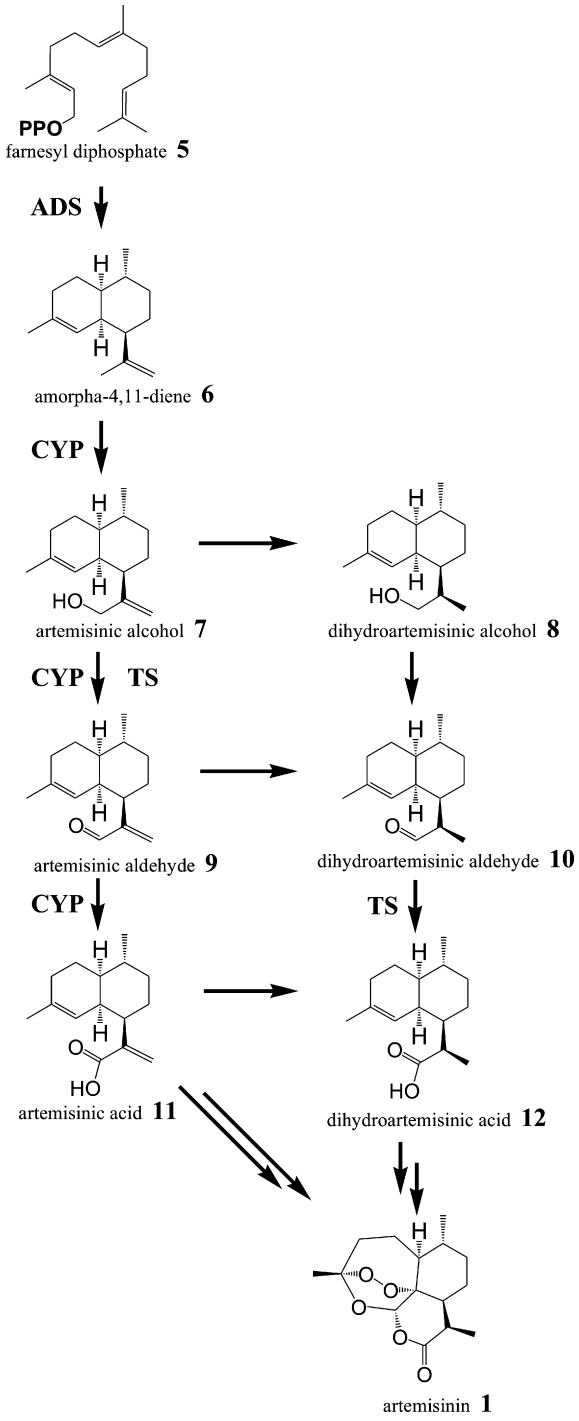

Fig. 2. Theoretical pathways for the biosynthesis of artemsinin 1. "**CYP**", reactions catalyzed by CYP71AV1; "**TS**", ***T***richome ***S***upernatant-catalyzed single step reactions supported by evidence from cell-free GST extracts reported by Bertea et al. (2005).

[1] In older literature, *qinghao* referred to *A. apiacea*; today it is frequently used to refer to *A. annua*.

Artemisinin-derived drugs typically have a short half-life and are best used in combination with other antimalarials such as chloroquine and sulfadoxime/pyrimethamine in artemisinin-based combination therapies (ACTs; Mutabingwa, 2005). The current problem with malaria in the world and the efficacy of ACTs has given rise to an annual demand of over 100 million courses of ACT treatment. The relatively low yield of artemisinin **1** from *A. annua* (0.01–1.5%) has led to difficulties in meeting this demand at an acceptable cost (Mutabingwa, 2005).

Artemisinin **1** and its derivatives also have potential for the treatment of other diseases. For example, artemisinin derivatives are active against other parasites such as *Schistosoma* spp. which cause schistosomiasis (Xiao, 2005), and there are also recent indications that artemisinin **1** will be useful in treating cancer (Efferth, 2006).

2. Glandular secretory trichomes (GSTs) and isoprenoid biosynthesis

The desire to improve the overall supply of artemisinin **1** and reduce its cost has sparked interest in the molecular biology and biochemistry of artemisinin **1** biosynthesis (Bertea et al., 2005; Bouwmeester et al., 1999; Chang et al., 2000) and in the GST structure in which it is thought to be formed (Duke and Paul, 1993). As do other members of the tribe Anthemideae (in the Asteraceae), the aerial surfaces of *A. annua* plants have 10-celled biseriate GSTs which accumulate mono- and sesqui-terpenes. The anatomy, development, isolation and biochemistry of *A. annua* and related species has been the subject of numerous studies (Diettert, 1938; Duke and Paul, 1993; Ferreira and Janick, 1995; Figueiredo and Pais, 1994; Göpfert et al., 2005; Monteiro et al., 2001). The development of GSTs appears to begin very early in leaf development with the anticlinal division of a protruding epidermal cell (Diettert, 1938; Duke and Paul, 1993). Duke and Paul (1993) examined *A. annua* GSTs, showing the ultrastructural distinctions between the basal, stalk and three secretory pairs of cells. By light microscopy, the basal, stalk and apical cell pairs are colourless, whereas the two subapical cell pairs are green (see Fig. 3). This, and the electron microscopy suggests a certain amount of biochemical specialization among the cells within GSTs. The site of artemisinin **1** biosynthesis appears to be primarily GSTs, although transformed root cultures are also reported to produce it as well (Souret et al., 2003).

The majority of essential oil components of *A. annua* and related species is thought to accumulate in the subcuticular space at the apex of the GSTs. This oil consists primarily of mono- and sesqui-terpenes. While the composition varies widely within the species, the major components typically include the monoterpenes α-pinene, artemisia ketone, 1,8-cineole and camphor and to a lesser extent the sesquiterpenes β-caryophyllene and germacrene D (Ferreira et al., 1997; Li et al., 2006; Tellez et al., 1999). GSTs are also thought to be the site of biosynthesis and storage of a wide range of artemisinin-related amorphane type sesquiterpenoid compounds (Li et al., 2006).

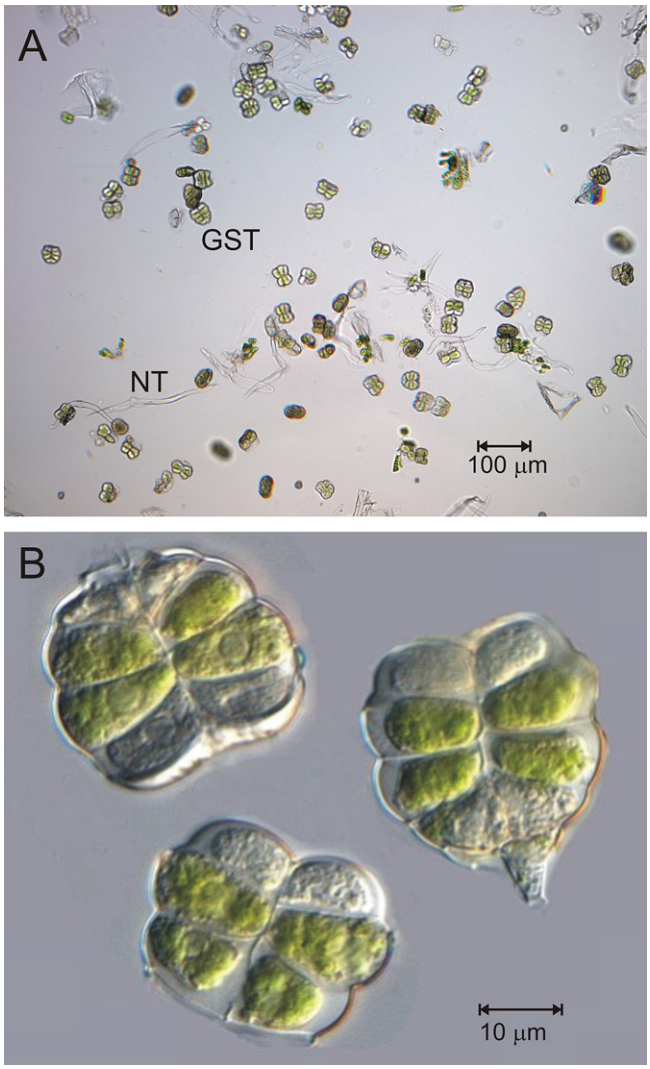

Fig. 3. Example of a floral bud GST preparation. (A) low magnification micrograph; (B) high magnification phase contrast micrograph. GST, glandular secretory trichome; NT, nonglandular trichome. See Teoh et al. (2006) for experimental details.

In a number of species, the ability to isolate GSTs has been an important factor in elucidating the biochemistry in which they specialize. Croteau and coworkers have optimized a method for mint which they report to be suitable for distantly related species including *Tanecetum vulgare* (Asteraceae; Gershenzon et al., 1992). The method is based on the mechanized abrasion of plant tissue followed by filtration. This has recently been adapted for use with *A. annua* (Bertea et al., 2005; Teoh et al., 2006). In the authors' laboratory, as suggested by previous work (Ferreira and Janick, 1995; Slone and Kelsey, 1985), flower buds were found to be a good source of GSTs for which contamination with nonglandular trichomes was minimized (see Fig. 3; Teoh et al., 2006). Using leaf, or in some cases, isolated GSTs as a source of mRNA, a number of cDNAs

encoding enzymes of isoprenoid biosynthesis have been cloned. These include germacrene A synthase (Bertea et al., 2006), (*E*)-β-farnesene synthase (Picaud et al., 2005), epi-cedrol synthase (Mercke et al., 1999) and farnesyl diphosphate synthase (Matsushita et al., 1996).

3. Artemisinin biosynthesis

As with essentially all sesquiterpenes, the biosynthesis of artemisinin **1** is expected to involve the mevalonate pathway and include the cyclization of farnesyl diphosphate. This is supported by the incorporation of mevalonate into artemisinin **1** (Akhila et al., 1987), although it is not clear whether the DXP (1-deoxy-D-xylulose-5-phosphate) pathway[2] can also contribute 5-carbon precursors, as occurs in other sesquiterpene biosynthetic systems (Adam and Zapp, 1998). The formation of the sesquiterpene carbon skeleton, amorpha-4,11-diene **6** is catalyzed by amorpha-4,11-diene synthase (Bouwmeester et al., 1999) for which corresponding cDNAs have been cloned (Chang et al., 2000; Mercke et al., 2000; Wallaart et al., 2001). The non-descript arrangement of the amorphadiene product (see Fig. 2) belies the unique structural features that ultimately allow for the formation of the 1,2,4-trioxane moiety (Sy and Brown, 2002). The numerous amorphane type sesquiterpenes in *A. annua* in which C-12 is oxidized to a carboxyl group and the C11-C13 bond is either single or double suggest early C-12 oxidation. Indeed, Bertea and coworkers (Bertea et al., 2005) showed that *A. annua* leaf microsomes converted amorphadiene **6** to artemisinic alcohol **7** in the presence of NADPH (see Fig. 2). The route from artemisinic alcohol **7** to artemisinin **1** is still not entirely clear. Li et al., for example, have reviewed published data and provide a good indication of the sometimes contradictory and inconclusive data in the literature (Li et al., 2006). In this regard, it is useful to consider the possible route(s) to artemisinin **1** among the pathways shown in Fig. 2. These pathways are based on a few conversions whose order may vary. These conversions include the oxidation of C12 from alcohol to aldehyde and aldehyde to acid, the reduction of the double bond at C11,13 and the formation of the 1,2,4-trioxane moiety.

The latest steps in artemisinin **1** biosynthesis remain controversial and theories differ mainly in their identification of either artemisinic **11** or dihydroartemisinic **12** acids as the later precursor. The evidence for artemisinic acid **11** has been reviewed by Li et al. (2006). This includes the suggestion that C11,13 double bond reduction occurs at the level of an intermediate beyond artemisinic acid **11**, such as arteannuin B or artemisitene. On the other hand, the co-occurrence of dihydroartemisinic acid **12** with high artemisinin **1** levels suggests that even if double bond reduction could occur at a very late intermediate, it also occurs in less oxidized precursors. The double bond reduction at C11,13 is of general interest biochemically, given the relative rarity of enzymes catalyzing double bond reductions (Kasahara et al., 2006).

A case has also been made for dihydroartemisinic acid **12** as a late precursor of artemisinin **1**. Labeled dihydroartemisinic acid **12** is incorporated into artemisinin **1** *in vivo*, a sequence which can occur in the absence of enzymes (Brown and Sy, 2004; Haynes et al., 2006; Sy and Brown, 2002; Wallaart et al., 1999). Upstream of dihydroartemisinic **12** acid, the order of oxidations and reduction of artemisinic alcohol **7** *en route* to dihydroartemisinic acid **12** is still not settled. Bertea et al. (2005) provided biochemical evidence for the fate of artemisinic alcohol **7** in *A. annua* using GST cell-free extracts. Single step conversions labeled "TS" (for trichome supernatant) in Fig. 2 were reported. While the data provided is confounded by the presence of endogenous intermediates, the results were interpreted to indicate that the main pathway to dihydroartemisinic acid **12** is via artemisinic aldehyde **9** and dihydroartemisinic aldehyde **10**, although the direct double bond reduction of artemisinic aldehyde **9**, *per se*, was not observed.

4. Functional genomics and the isolation of a cDNA encoding amorphadiene monooxygenase

In an effort to further elucidate the biosynthesis of artemisinin **1**, as well as the related regulatory and transport processes, the authors have undertaken an approach to gene identification based on ESTs. For this, three cDNA libraries were constructed. Two libraries were constructed from RNA derived from isolated GSTs (AAGST) and from flower buds (AAFB). In addition, a subtracted cDNA library (GSTSUB) enriched in GST-expressed sequences was constructed using GST cDNA as the tester and flower bud cDNA as the driver using suppression subtraction hybridization technology (Diatchenko et al., 1996). For AAGST and AAFB, an effort was made to generate cDNA inserts which were as near full length as possible. For GSTSUB, the cDNA inserts were relatively short (on average) *Rsa I* restriction digestion fragments resulting from the library construction protocol (Teoh et al., 2006). ESTs were generated by sequencing plasmid DNA from randomly picked *Escherichia coli* transformants for a total of 1559, 3674 and 3524 ESTs for AAFB, AAGST and GSTSUB, respectively.

The ESTs generated form the basis for investigation into the genes involved in biosynthesis of artemisinin **1**, as well as other processes relating to GSTs. The two libraries derived from GSTs (GSTSUB and AAGST) are enriched in cDNAs relating to isoprenoid biosynthesis (Teoh et al., 2006). Given the presumptive major role for GSTs in monoterpene biosynthesis, it is not surprising to find that the enzymes of the DXP pathway from DXP synthase

[2] Students of phytochemistry may be interested to note that the DXP pathway, which is apparently active in the relict plastid (apicoplast) of *Plasmodium* spp., is a target for antimalarial drug candidates (Ralph et al., 2004).

to 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase (Rohmer, 2003) were well represented in the two GST-derived libraries relative to AAFB. Enzymes of the mevalonate pathway and amorphadiene synthase were also represented. Thus, the libraries appear to provide useful resources for gene identification relevant to trichome metabolism.

While the first committed step in artemisinin **1** biosynthesis, the cyclization of farnesyl diphosphate is well understood, the later steps in the pathway are less well known. Efforts at gene identification would definitely help expand our knowledge of the relevant enzymes. Bertea et al. (2005) provided evidence for cytochrome P450 involvement in the initial oxidation of amorphadiene **6** and this is a target for gene identification. When cytochrome P450 sequences in the EST collections were examined, it was found that a range of CYP subfamilies were represented at the level of 0.1%, 1.2% and 0.3% in the AAFB, AAGST, and GSTSUB collections, respectively (Teoh et al., 2006). This included a cluster, represented by 1 GSTSUB clone and 11 AAGST clones, with sequences similar to CYP71D subfamily members. Since this subfamily includes a number of terpene hydroxylases, this cluster was considered to be a good candidate to test for amorphadiene hydroxylase activity. By additional DNA sequencing and sequence analysis, the clone AAGST library clone, pKT101 was determined to include a full-length ORF matching the consensus sequence for the 12 highly similar cDNA clones in the cluster. The sequence was designated CYP71AV1 and represents a new CYP71 subfamily. CYP71AV1 shows the highest degree of amino acid sequence similarity to EST contigs from lettuce and sunflower (Ro et al., 2006) and to a number of enzyme of unknown function in the CYP71D subfamily. In terms of enzymes of known function, it is most similar to the monoterpene, diterpene and alkaloid hydroxylases of the CYP71D subfamily which includes CYP71D16, or cembra-2,7,11-triene-4-ol monooxygenase, from *Nicotiana tabacum* (49% identity; Wang et al., 2001).

The activity of CYP71AV1 was investigated by heterologous expression in yeast. The *Saccharomyces cerevisiae* strain WAT11 (Pompon et al., 1996) provides the eukaryotic endoplasmic reticulum membrane environment suitable for plant cytochrome P450's. As well, WAT11 expresses a plant cytochrome P450 reductase to help optimize plant cytochrome P450 activities. Microsomal membranes isolated from WAT11 expressing CYP71AV1 were assayed with a variety of substrates in the presence of NADPH. The most notable substrate tested was amorpha-4,11-diene **6**. Oxidation products were analyzed by GC/MS after trimethylsilylation. Artemisinic alcohol **7** was found to be formed from amorphadiene **6** in a CYP71AV1- and NADPH-dependent manner. Subsequent experiments showed that CYP71AV1 oxidized artemisinic alcohol **7** to the corresponding aldehyde **9** and the aldehyde **9** to artemisinic acid **11**. CYP71AV1 was found to be inactive on a number of other mono- and sesqui-terpenes (Teoh et al., 2006). Thus, CYP71AV1 appears to be a multifunctional cytochrome P450 capable of the oxidation of amorphadiene **6** through to artemisinic acid **11**. This activity is not unlike that of other CYPs which catalyze multiple oxidations of isoprenoids (Aoyama et al., 1989; Ro et al., 2005; Trzaskos et al., 1986). Keasling and coworkers also isolated a cDNA encoding CYP71AV1 and reached the same conclusions about its activity (Ro et al., 2006). Indeed, when co-expressed in yeast with genes required for amorphadiene **6** biosynthesis, artemisinic acid **11** was formed in culture.

Expression analysis of CYP71AV1 in *A. annua* tissues indicates that it is most highly expressed in GSTs (Teoh et al., 2006). The moderate expression observed for flower buds presumably reflects their high density of GSTs. Low but detectable levels of RT-PCR products could be observed for leaves.

The role of CYP71AV1 in the hydroxylation of amorpha-4,11-diene **6** is undoubtedly important in artemisinin **1** biosynthesis. The subsequent route to artemisinin **1** is less clear. Most evidence implicates dihydroartemisinic acid **12** as a late precursor to artemisinin **1** which is derived from artemisinic alcohol **7** by oxidation at C-12 and reduction of the C11–C13 double bond (Fig. 2). This is based on *in vitro* biochemical evidence (Bertea et al., 2005), as well as the conversion of dihydroartemisinic acid **12** to artemisinin **1** both *in vivo* (Brown and Sy, 2004), and *in vitro* in an oxygen-dependent non-enzymatic fashion (Sy and Brown, 2002). The ability of CYP71AV1 to oxidize amorphadiene **6** to artemisinic acid **11** raises some questions about the route to artemisinin **1**. If artemisinic acid **11** represents a branch in the pathway rather than an intermediate to artemisinin **1**, then efficient production of the latter would seem to require a double bond reductase capable of outcompeting CYP71AV1 for C12-oxygenated amorphadienes (artemisinic alcohol **7** and aldehyde **9**).

To help understand the biosynthesis of artemisinin **1**, it is relevant to consider the reaction sequence of CYP71AV1 in the multiple oxidation of amorpha-4,11-diene **6**. The fact that, *in vitro*, CYP71AV1 performs predominantly single oxidations (Teoh et al., 2006), suggest that CYP71AV1 products may be released after a single oxidation, rather than being further oxidized without release from the enzyme. This situation appears to be similar to that of *ent*-kaurene oxidase from *Arabidopsis thaliana* (Helliwell et al., 1999) and is supported by kinetic studies of mammalian sterol C-32 demethylation (Trzaskos et al., 1986). Thus, other enzymes, such as double bond reductases may have access to, and be able to compete with CYP71AV1 for intermediates of amorpha-4,11-diene **6** oxidation.

The available data also point to the possible existence of dehydrogenases involved in artemisinin **1** biosynthesis (Bertea et al., 2005). Further work is required to understand the relative roles of the apparently soluble enzyme activities, such as the artemisinic alcohol dehydrogenase activity of *A. annua* GSTs and that of CYP71AV1.

5. Concluding remarks

The cloning and characterization of CYP71AV1 represents an important advance in understanding artemisinin **1** biosynthesis and towards the engineering of an improved supply of antimalarial drugs. The activity and expression of amorpha-4,11-diene synthase and CYP71AV1 strongly support the notion that artemisinin **1** biosynthesis is localized to GSTs. Our understanding of the compartmentalization of artemisinin **1** biosynthesis would benefit from additional biochemical experiments with isolated GSTs, of the isotope labeling and enzymological sort, as well as experiments to localize relevant gene expression at the cellular level.

The long-term supply of artemisinin **1** for the low-cost treatment of malaria in the Third World is limited by its low levels in the *A. annua*. It is possible that CYP71AV1 could be used in genetic engineering of plants or microorganisms, either through semi-synthesis or in combination with additional genes. Ro et al. (2006) have already demonstrated the feasibility of the production, in yeast, of artemisinic acid **11**, which can be chemically converted to artemisinin **1**. However, genes encoding the putative double bond reductase and possibly other enzymes may also have biotechnological applications. Such genes would presumably allow the bioengineering of dihydroartemisinic acid **12** production (and possibly artemisinin **1**) and avoid the chemical steps required to convert artemisinic acid **11** to dihydroartemisinic acid **12** (Haynes and Vonwiller, 1994; Roth and Acton, 1989). In any case, it will be very interesting to see whether chemical synthesis, microbial ''synthetic biology'', plant biotechnology or conventional agriculture will ultimately provide the optimum system for the production of artemisinin-related antimalarial drugs.

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