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REVIEW

Production of artemisinin by genetically-modified microbes

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Abstract Artemisinin, an endoperoxidized sesquiterpene originally extracted from the medicinal plant *Artemisia annua* L., is a potent malaria-killing agent. Due to the urgent demand and short supply of this new antimalarial drug, engineering enhanced production of artemisinin by genetically-modified or transgenic microbes is currently being explored. Cloning and expression of the artemisinin biosynthetic genes in *Saccharomyces cerevisiae* and *Escherichia coli* have led to large-scale microbial production of the artemisinin precursors such as amorpha-4,11-diene and artemisinic acid. Although reconstruction of the complete biosynthetic pathway toward artemisinin in transgenic yeast and bacteria has not been achieved, artemisinic acid available from these transgenic microbes facilitates the subsequent partial synthesis of artemisinin by either chemical or biotransformational process, thereby providing an attractive strategy alternative to the direct extraction of artemisinin from *A.annua* L. In this review, we update the current trends and summarize the future prospects on genetic engineering of the microorganisms capable of accumulating artemisinin precursors through heterologous and functional expression of the artemisinin biosynthetic genes.

Keywords Amorpha-4,11-diene · Artemisinic acid · Artemisinin · Genetic engineering · Malaria · Metabolic engineering

Abbreviations

ACTs	Artemisinin-based combination therapies
ADS	Amorpha-4,11-diene synthase
AMO	Amorpha-4,11-diene oxidase
CPR	Cytochrome P450 reductase
CYP/P450	Cytochrome P450 enzymes
CYP71AV1	Cytochrome P450 monooxygenase
DMAPP	Dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
ECS	Epi-cedrol synthase
EST	Expressed sequence tag
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate

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HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IPP	Isopentenyl diphosphate
MDR	Multi-drug resistant
MEP	Methylerythritol phosphate
MVA	Mevanolate
RT-PCR	Reverse transcription-polymerase chain reaction
SS	Squalene synthase

Introduction

Malaria is a severe human disease in the tropical areas of Africa, Southeast Asia, Central and South America, which threatens more than one third of the global population and kills approximately two million people annually (Korenromp et al. 2005). The disease occurs due to the mosquito-mediated infection of erythrocytes mainly by the malarial parasite *Plasmodium falciparum*. Although quinine-derived antimalarial agents have efficiently controlled malaria for decades, multi-drug resistant (MDR) malarial strains have appeared in recent years which have led to a substantial increase of mortality in malaria patients (Newton and White 1999). Fortunately, artemisinin-based combination therapies (ACTs) recommended by the World Health Organization (WHO 2001) are highly effective in preventing the infection and transmission of MDR malaria. However, ACTs are not always available for every malaria victim in the endemic regions because artemisinin is produced in *Artemisia annua* L. (sweet wormwood) in trace amounts (Duke and Paul 1993, 1994), which makes it a substantial bottleneck for meeting the demand of over 100 million courses of ACTs each year (Mutabingwa 2005). The shift of artemisinin manufacture from plants to microbes will allow the industrial process of artemisinin synthesis, ultimately enabling ACTs accessible at an affordable price to all patients in poor nations. For this purpose, practical approaches have been developed to transplant artemisinin biosynthetic genes from *A. annua* L. into genetically tractable microbial hosts, for example, *Escherichia coli* and *Saccharomyces cerevisiae* (Khosla and Keasling 2003). Such metabolic engineering platform is readily established for terpene production as most catalytic steps for terpene biosynthesis are conserved among the higher plants and even in prokaryotic and eukaryotic microbes.

Elucidation of the artemisinin biosynthetic pathway

Artemisinin, also known as qinghaosu or arteannuin and first identified by Chinese scientists (Liu et al. 1979), is a sesquiterpene lactone endoperoxide that specifically accumulates in the glandular trichomes on leaves and floral buds of *A. annua* L. and has been categorized as a terpenoid or isoprenoid (Connolly and Hill 1991). The structurally diverse terpenes comprise monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), and polyterpenes (C45-C500), which share the common precursors of five-carbon (C5) isoprene units, i.e. isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Croteau et al. 2000).

Two stages can be recognized in the biosynthesis of regular monoterpenes, sesquiterpenes and diterpenes regardless of their biological origin. The first phase involves the production of linear isoprene precursors, including geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). These reactions are catalyzed by the corresponding synthases that appear to be conserved in all organisms. The second phase involves the production of cyclic terpenes from linear isoprene precursors. The reaction is catalyzed by a terpene synthase/terpene cyclase that may be specific to the species (Covello et al. 2007). Many of these enzymes have been isolated and identified from various plant species and their functional expression in microorganisms is well documented (Chang and Keasling 2006). The representative examples among the medicinal plants are monoterpene limonene synthase from *Mentha piperita*, sesquiterpene amorpha-4,11-diene synthase from *A. annua* L., and triterpene taxadiene synthase from *Catharanthus roseus*.

Common steps toward FPP biosynthesis in all organisms

Terpenes are generated by the condensation of two isoprene building block isomers, IPP and DMAPP (Croteau et al. 2000). In plant cells, two chemically

distinct and spatially compartmented pathways exist for the biosynthesis of IPP and DMAPP, i.e. the classic cytoplasmic mevanolate (MVA) pathway originating from acetyl-CoA (Cane 1981), and the more recently discovered plastidic non-MVA pathway, or deoxyxylulose phosphate (DXP)/methylerythritol phosphate (MEP) pathway stemming from glyceric acid-3-phosphate and pyruvate (Newman and Chappell 1999).

While the MVA pathway typically exists in eukaryotes, the DXP/MEP pathway usually distributes in prokaryotes with some exceptions (Lange et al. 2000). The sequential head-to-tail condensations of DMAPP and IPP consequently form the linear prenyl diphosphate precursors, GPP, FPP and GGPP, which are catalyzed by the prenyltransferases GPP synthase (GPPS), FPP synthase (FPPS) and GGPP synthase (GGPPS), respectively. In the plant cytoplasm, FPP is a common precursor for the biosynthesis of sesquiterpenes, triterpenes (including sterols) and polyterpenes. In plastids, GPP is as a precursor of monoterpene, while GGPP as a precursor of diterpenes (including phytol), tetraterpenes (such as carotenoids) and plastoquinone. In addition, ubiquinones with nona- and deca-prenyl chains found in mitochondria are derived from cytosolic IPP and DMAPP (Mahmoud and Croteau 2002).

Evidence suggests crosstalk between the cytosolic MVA pathway and plastidic DXP/MEP pathway (Croteau et al. 2000; Laule et al. 2003), and whether the plastidic IPP contributes to artemisinin biosynthesis in the cytoplasm of *A.annua* L. as it does in other sesquiterpene-rich plants (Adam and Zapp 1998; Dudareva et al. 2005) has been addressed. The isotopic labeling study by Wu et al. (2006) has suggested little, if any, IPP exchange between such two subcellular compartments. The combined application of pathway-specific inhibitors, lovastatin and fosmidomycin, has been used to confirm the derivation of artemisinin from IPP originated from both the MVA pathway and DXP/MEP pathways (Towler and Weathers 2007).

Unique steps involved in artemisinic acid biosynthesis in A. annua *L.*

The involvement of MVA pathway in artemisinin biosynthesis has been previously deduced from the incorporation of isotope-labeled mevalonate into artemisinin (Akhila et al. 1987). Afterwards, the cyclization of FPP to generate amorpha-4,11-diene catalyzed by amorpha-4,11-diene synthase (ADS) has been suggested as the first committed step by Bouwmeester et al. (1999) and a putative reaction mechanism for ADS has been proposed by Mercke et al. (2000). However, the entire biosynthetic pathway from amorpha-4,11-diene to artemisinin is still unclear. It has been proposed that the sequential oxidations of C12 in amorpha-4,11-diene are multistep enzymatic reactions catalyzed by cytochrome P450 enzymes (CYP/P450) with dehydrogenase and reductase activities (Wallaart et al. 2000).

A cytochrome P450 monooxygenase (CYP71AV1)/amorpha-4,11-diene oxidase (AMO) for the oxidation of amorpha-4,11-diene has recently been independently identified by the Covello group (Teoh et al. 2006) and the Keasling group (Ro et al. 2006). The endoplasmic reticulum-bound CYP71AV1/AMO is a multifunctional enzyme capable of oxidizing amorpha-4,11-diene to generate artemisinic acid via artemisinic alcohol and artemisinic aldehyde intermediates. Such three-step oxidations by CYPs have also been demonstrated in the biosynthesis of the plant hormone gibberellin (Helliwell et al. 1999, 2001).

Cloning of artemisinin biosynthetic genes

Since 1995, 12 genes related to artemisinin biosynthesis have been cloned from *A. annua* L. and their complete or partial mRNA sequences are accessible in GenBank (Table 1). At present, the functional genomics regarding artemisinin biosynthesis in *A. annua* L. have been emphasized in several laboratories (Bertea et al. 2006; Covello et al. 2007; Zeng et al. 2008), which should lead to the discovery of a large batch of useful genes encoding structural proteins and regulatory factors involved in the metabolism of artemisinin and other secondary metabolites.

While two plastidic enzymes, DXS and DXR, in the DXP/MEP pathway may be indirectly responsible for artemisinin biosynthesis, other cytoplasmic enzymes including ADS and CYP71AV1 are particularly relevant to artemisinin biosynthesis and are therefore the focus of discussion in this review.

Table 1 Artemisinin biosynthetic genes cloned and accessed in GenBank

Gene	Enzyme/location/sequence type	GenBank accession	Submitter
HMGR/HMG	3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, cytosol		
	mRNA, complete cds	AAU14624	Kang et al. (1995)
	mRNA, complete cds	AAU14625	Kang et al. (1995)
	mRNA, partial cds	AF142473	Chen et al. (1999)
	mRNA, complete cds	AF156854	Chen et al. (1999)
FDPS/FPS	Farnesyl diphosphate (FDP) synthase, cytosol		
	mRNA, complete cds	AF136602	Chen et al. (1999)
	mRNA, complete cds	AF149257	Liu et al. (1999)
	mRNA, complete cds	AF112881	Chen et al. (2000)
DXS	1-deoxy-D-xylulose-5-phosphate synthase (DXS), plastid		
	mRNA, complete cds	AF182286	Wobbe et al. (2000)
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), plastid		
	mRNA, partial cds	AF182286	Wobbe et al. (2000)
	mRNA, complete cds	AF182287	Wobbe et al. (2000)
ADS	Amorpha-4,11-diene synthase (ADS), cytosol		
	mRNA, complete cds	AF138959	Mercke et al. (2000)
	mRNA, complete cds	AF327527	Liu et al. (2001)
	mRNA, complete cds	AY006482	Wallaart et al. (2001)
	mRNA, complete cds	AJ251751	Chang et al. (2005)
	mRNA, complete cds	DQ241826	Huang et al. (2005)
	mRNA, complete cds	EF197888	Kong et al. (2007)
CYP71AV1	Cytochrome P450 monooxygenase (CYP71AV1), endoplasmic reticulum		
	mRNA, complete cds	DQ268763	Ro et al. (2006)
	mRNA, complete cds	DQ315671	Teoh et al. (2006)
	mRNA, complete cds	DQ453967	Olsson et al. (2006)
	mRNA, complete cds	DQ872632	Yin et al. (2006)
	mRNA, complete cds	DQ667171	Kong et al. (2006)
	mRNA, complete cds	EF197889	Kong et al. (2007)
CPR	Cytochrome P450 reductase (CPR), cytosol		
	mRNA, complete cds	DQ104642	Ro et al. (2006)
	mRNA, complete cds	DQ318192	Yin et al. (2006)
	mRNA, complete cds	EF197890	Kong et al. (2007)
SS/SQS	Squalene synthase (SS), endoplasmic reticulum		
	mRNA, complete cds	AF181557	Wobbe et al. (1999)
	mRNA, complete cds	AF405310	Liu et al. (2001)
	mRNA, complete cds	AY445505	Zeng et al. (2003)

Bouwmeester et al. (1999) first reported the partial purification of natural ADS protein from *A. annua* L. This enzyme has a pH optimum around 6.5–7.0, a K_m of 0.6 μM for FPP, and a molecular weight of 56 kDa. The *ADS* gene has been identified and cloned by several groups independently (Mercke et al. 2000; Chang et al. 2000; Wallaart et al. 2001). The recombinant ADS prepared from *E. coli* by Mercke et al. (2000) has a broad pH optimum between 7.5–9.0 and a K_m of 0.9 μM for FPP at pH 7.5 and a molecular mass of 63.9 kDa that is higher than the native plant ADS identified by Bouwmeester et al. (1999). Expression of the *ADS* gene in *E. coli* leads to the generation of a number of terpene

precursors, predominantly amorpha-4,11-diene (91.2%, w/w). Picaud et al. (2005) have reported that the recombinant ADS expressed in *E. coli* gives rise to amorpha-4,11-diene as a major product, but 15 sesquiterpenes in total are simultaneously produced (see below for explanation).

As one of the largest plant gene families, the *CYP* family has tremendous gene sequence diversity (Schuler and Werck-Reichhart 2003). Teoh et al. (2006) have currently isolated a cDNA clone from an *A. annua* L. trichome library. This gene, designated as *CYP71AV1*, belongs to the *CYP71D* subfamily that includes a number of terpene hydroxylases. The yeast-expressed and microsomal membrane-bound recombinant CYP71AV1 has been assayed with a variety of substrates in the presence of NADPH. When amorpha-4,11-diene has been supplemented as a substrate, artemisinic alcohol is synthesized in a NADPH-dependent manner. Their experiments have also suggested that CYP71AV1 is a multifunctional enzyme that oxidizes artemisinic alcohol to generate artemisinic acid via artemisinic aldehyde intermediates.

By analyzing the expressed sequence tag (EST) database generated by The Compositae Genomic Projects (CGP), which focuses on *Lactuca*, *Helianthus* and other Compositae plants (http://www.cgpdb.ucdavis.edu), Ro et al. (2006) have identified two major *CYP* subfamilies, *CYP71* and *CYP82*. Using degenerate primers designed based on the conserved sequence within the *CYP71* subfamily, a full-length cDNA, *CYP71AV1*, encoding an open reading frame of 495 amino acids, has been isolated from *A. annua* L. When *CYP71AV1* is co-expressed with *ADS* in *S. cerevisiae*, the cells oxidized amorpha-4,11-diene to produce artemisinic acid.

Production of artemisinin precursors by genetically modified yeast

As a eukaryotic microbe, *S. cerevisiae* offers promise for heterologous expression of plant genes including those encoding the membrane-bound proteins that have difficulty functionalizing in bacterial systems (DeJong et al. 2006). One of the early successful examples is expression of the gene coding for taxane 10β-hydroxylase from *Taxus* in *S. cerevisiae* (Schoendorf et al. 2001). Once a yeast has been chosen for high-level production of artemisinin, however, its known high rate of isoprene metabolism must be taken into account. An ideally engineered yeast strain should have increased FPP production and decreased sterol accumulation.

The Brodelius group has compared the effects of two different formats of *A. annua* L. *ADS* gene expression in yeast on the production of amorpha-4,11-diene. First, the *ADS* gene has been introduced into yeast cells on an episomal plasmid using a galactose-inducible promoter. Second, the *ADS* gene has been inserted into the yeast genome by homologous recombination. The plasmid-harboring and genome-integrated yeast strains with functionally expressed *ADS* gene produce approximately 600 μg amorpha-4,11-diene/l and 100 μg amorpha-4,11-diene/l, respectively, in 16-day batch cultivations (Lindahl et al. 2006). It is apparent that the production of sesquiterpene in yeast is positively correlated with the gene dosage, but the insufficient substrate (FPP) pool is most likely a limiting factor.

Engineered yeast strains capable of producing artemisinic acid have been achieved through multi-gene transfer as reported by Ro et al. (2007). In their studies as illustrated by Fig. 1, the *ADS* and *HMGR* genes have been transferred into yeast cells for generating amorpha-4,11-diene and increasing the FPP pool, respectively. Further augmented levels of FPP and amorpha-4,11-diene have been achieved through the reduction of sterol biosynthesis by repressing *SS* gene expression with a methionine-repressible promoter. Then, *CYP71AV1* and *CPR* genes have been introduced into yeast cells to enable a three-step oxidation from amorpha-4,11-diene producing artemisinic acid. When evaluated step-by-step, the expression of *ADS* gene alone only results in a relatively lower quantity of amorpha-4,11-diene (4.4 mg amorpha-4,11-diene/l), while co-expression of a truncated *HMGR12* gene improves the yield of amorpha-4,11-diene by approximately fivefold. Introduction of a methionine repressible promoter down-regulates the sterol biosynthetic gene, *ERG9*, and leads to an additional twofold increase of amorpha-4,11-diene. Down-regulation of the *ERG9* gene and overexpression of *upc2-1*, a dominant mutated gene encoding the Upc2p transcription factor capable of regulating sterol biosynthesis, increases amorpha-4,11-diene production to 105 mg amorpha-4,11-diene/l. Integration of an additional copy of the *HMGR* gene into the chromosome further increases amorpha-4,11-diene production to 149 mg amorpha-4,11-diene/l. Overexpression of the

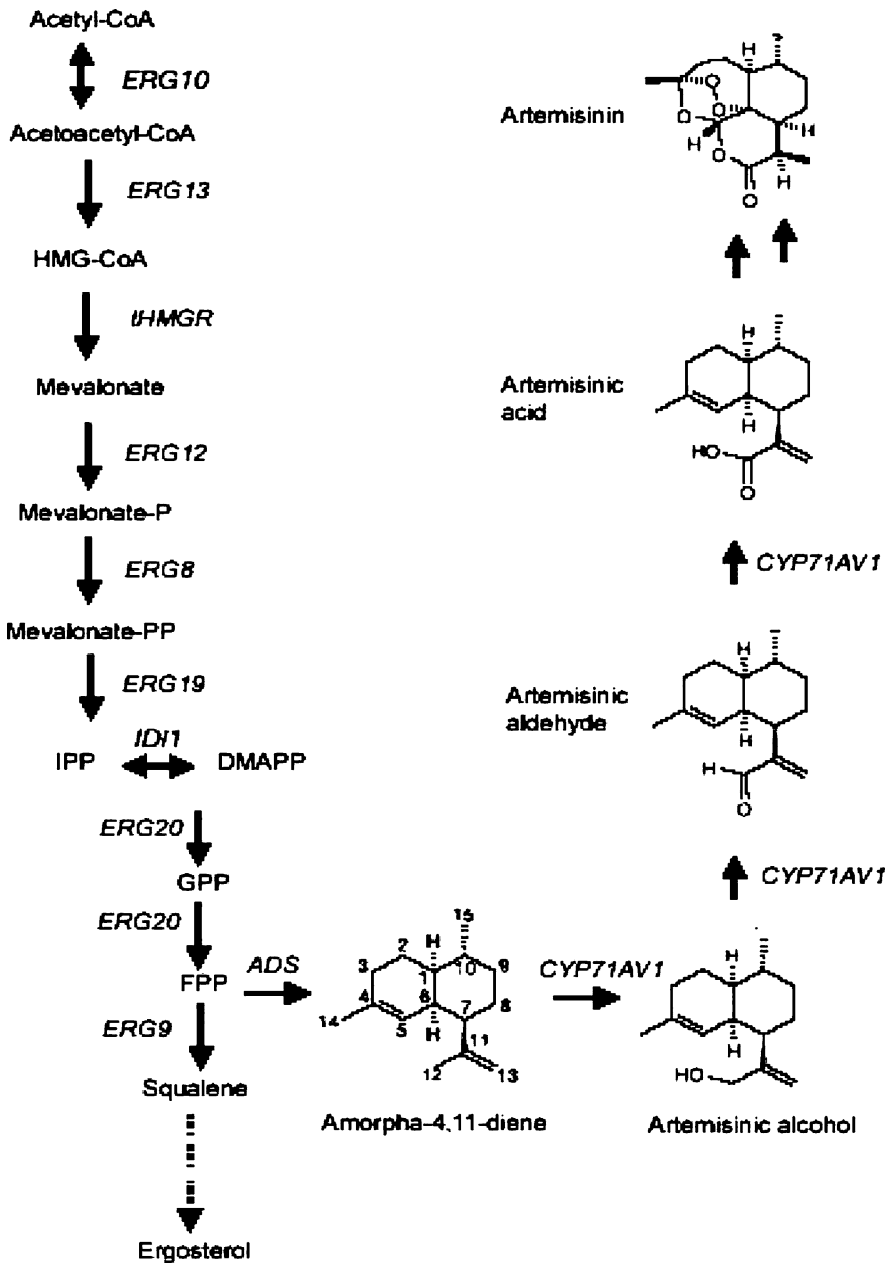

Fig. 1 Engineered artemisinic acid biosynthetic pathway in *S. cerevisiae* adopted from Ro et al. (2006) with modifications

FPPS (*ERG20*) gene decreases cell density and, in turn, increases amorpha-4,11-diene yield by about additional 10% (w/w). All these multi-gene manipulations lead to a total enhancement in amorpha-4,11-diene biosynthesis up to 153 mg amorpha-4,11-diene/l, an elevation of nearly 500-fold of what has been reported previously (Jackson et al. 2003).

In the next step, two additional genes, *CYP71AV1* and *CPR*, have been introduced into yeast cells to make it possible for the conversion from amorpha-4,11-diene to artemisinic acid as a final product. In a shake flask, approximately 32 mg artemisinic acid/l is produced, but only negligible artemisinic alcohol and artemisinic aldehyde intermediates are accumulated in the culture medium as well as in cell pellets.

S. cerevisiae has previously been employed to express a single epi-cedrol synthase gene (*ECS*) of *A. annua* L. to produce a corresponding sesquiterpene, epi-cedrol (Jackson et al. 2003). The yeast cells expressing the *ECS* gene convert their endogenous FPP to produce

epi-cedrol up to 90 μg epi-cedrol/l. Further optimization by introducing the *upc2-1* gene increases the availability of FPP and yields 370 μg epi-cedrol/l, a sixfold increase in production compared to what has been reported in *E. coli* (Cane et al. 1993; Martin et al. 2001). This is a convincing example of how transcription factor(s), alone or in concert with additional pathway enzymes, can be used to manipulate and/or optimize secondary metabolite biosynthesis.

Transgenic yeast can produce comparable levels of artemisinic acid as *A. annua* L. on a biomass basis but during a much shorter time (4–5 days for yeast versus several months for *A. annua* L.). As such, the productivity of the engineered yeast is nearly two orders of magnitude greater than *A. annua* L., demonstrating an advantage for the large-scale production of artemisinic acid and other plant terpenes in yeast. In addition, the secretion of artemisinic acid to the medium by the yeast cells simplifies fractionation and purification of the product, representing another potential cost-saving advantage in industrial processing.

Production of artemisinin precursors by genetically modified bacteria

E. coli remains an amenable host for production of secondary metabolites due to its rapid growth rate and ease in genetic modification. However, the difficulty in reassembly of cytochrome P450 renders the use of *E. coli* for isoprene production extremely challenging, in spite of successful heterologous production of certain secondary metabolites such as carotenoids (Lee and Schmidt-Dannert 2002).

Expression of genes coding for common-step enzymes

Most efforts in engineering *E. coli* for isoprene production have been focused on manipulation of the DXP/MEP pathway. However, the metabolic flux of this pathway is subject to intracellular regulatory control. Thus, for rebuilding an artemisinin biosynthetic pathway in *E. coli*, Martin et al (2003) have attempted to bypass the endogenous DXP/MEP pathway by introduction of an entire suite of exogenous genes of MVA pathway from yeast. The MVA genes have been organized into two synthetic operons, in which the *MevT* operon is responsible for the three-step conversion of acetyl-CoA to mevanolate, and the *MBIS* operon converts mevanolate to FPP. Construction of the MVA pathway in *E. coli*, however, results in severe growth inhibition due to excessive accumulation of FPP, IPP and/or DMAPP intermediates. Co-expression of a codon optimized *ADS* gene successfully alleviates this toxicity and yields amorpha-4,11-diene with a titer of 280–480 mg amorpha-4,11-diene/l in a fed-batch bioreactor (Newman et al. 2006).

While expression of the *MevT* operon leads to HMG-CoA toxicity in *E. coli*, co-expression of the *HMGR* gene prevents HMG-CoA buildup and increases mevalonate production twofold (Pitera et al. 2007). Manipulation of the intergenic regions of *MevT* operon appears to have effects on balancing the expression of individual genes and results in a sevenfold increase in mevalonate titer (Pfleger et al. 2006). Enhancement of gene stability by chromosomal integration of the MVA pathway genes also greatly elevates mevalonate production (Yuan et al. 2006). These efforts have demonstrated that carbon sources can be effectively channeled to an exogenous isoprenoid pathway in engineered *E. coli* while the flux of native isoprenoid biosynthesis remains low.

Expression of genes coding for unique-step enzymes

The recombinant sequiterpene synthases appear to lack the specificity of their native enzymes. For example, bacteria expressed *Abies grandis* δ-selinene synthase and γ-humulene synthase give rise to 34 and 52 sesquiterpenes, respectively (Steele et al. 1998). Similarly, the recombinant *A. annua* L. ADS catalyzes the synthesis of up to 16 sesquiterpenes. However, the fidelity of ADS reaction is improved considerably in the presence of Mn^{2+} or Co^{2+} (Picaud et al. 2005). The byproduct (δ-cadinene) is eliminated in the presence of Mn^{2+} for (*E*)-β-farnesene synthase from peppermint (Crock et al. 1997). Two recombinant maize sesquiterpene synthases catalyze the production of a complex mixture of at least 23 sesquiterpenes, but the product spectrum shifts toward (*E*)-β-farnesene in the presence of Mn^{2+} (Kollner et al. 2004).

The functional expression of plant *CYP* genes in *E. coli* is crucial to the production of plant terpenes, and represents a challenge due to difficulties in proper peptide folding, membrane insertion, cofactor

incorporation, and post-translational modification as well as other factor interactions (Carter et al. 2003). By using plant-derived *CYPs*, Chang et al. (2007) have recently demonstrated that *E. coli* can be genetically engineered to produce high levels of complex sesquiterpenes. Although the expression of native *CYP71AV1* gene only leads to undetectable in vivo or in vitro CYP activity, codon optimization coupled with *N*-terminal transmembrane domain modification results in the generation of artemisinic alcohol at low concentrations (0.18–0.45 mg artemisinic alcohol/l). Replacement of *Candida tropicalis CPR* gene (*ctCPR*) with *A. annua* L. *CPR* gene (*aaCPRct*) from the same native host as CYP increases artemisinic alcohol production 12-fold to 5.6 mg artemisinic alcohol/l. Furthermore, the choice of plasmid vectors and *E. coli* strains strongly affects the titer of artemisinic acid. The use of pCWori plasmid and *E. coli* strain DH1 further increases oxidation of amorpha-4,11-diene by more than 1,000-fold to 553 mg amorpha-4,11-diene/l.

From artemisinic acid to artemisinin: a puzzle

Based on the detection of intermediates in the essential oil of *A. annua* L. and considering the previous suggestions by others (Woerdenbag et al. 1990, 1993; Boumeester et al. 1999; Wallaart et al. 1999; Bertea et al. 2005). Lommen et al. (2006) have suggested an integrative artemisinin biosynthetic pathway which illustrates two distinct branches either from artemisinic acid or from hydroartemisinic acid toward artemisinin (Fig 2).

Artemisinic acid is a common precursor of arteannuin B and artemisinin shown by radioactive isotope labeling (Wang et al. 1988) and supported by another report (Sangwan et al. 1993). At the same time, Nair and Basile (1993) have realized the in vitro bioconversion of arteannuin B to artemisinin by incubation with a leaf extract of *A. annua* L., suggesting that arteannuin B may be an intermediate in the bioconversion of artemisinin from artemisinic acid. Later, Bharel et al. (1998) have found that the leaf extract of *A. annua* L. converts artemisinic acid and arteannuin B into artemisinin. Dhingra et al. (2000), Dhingra and Narasu (2001) have purified and characterized an enzyme from *A. annua* L. that is involved in the bioconversion of arteannuin B to artemisinin. It is highly desirable to further determine the biochemical and enzymatic bases for the conversion of artemisinic acid to artemisinin and finally clone the corresponding genes responsible for such conversion.

Wallaart et al. (1999) have hypothesized that dihydroartemisinic acid may act as a scavenger of singlet oxygen ($^1O_2$), which is often released from the plant cells as exposed to oxidative stress, to generate dihydroartemisinic acid hydroperoxide and then yield artemisinin as a final product by auto-oxidation. Indeed, the enhancement of artemisinin biosynthesis in *A. annua* L. by night frost (Wallaart et al. 2000) or by post-harvest drying (Laughlin 2002) has been observed. Wallaart et al. (2001) have also found that the *ADS* cDNA can only be amplified by the reverse transcription-polymerase chain reaction (RT-PCR) from RNA isolated from drought and photo stressed *A. annua* L. shoots but not from the non-stressed plant tissues. Sy and Brown (2002) have shown that in vitro dihydroartemisinic acid undergoes slow spontaneous auto-oxidation to form artemisinin and that the first step of auto-oxidation requires light whereas the second step takes place in the dark, implicating a relevance of artemisinin biosynthesis with environmental factors. Nevertheless, direct evidence supporting the above hypothesis is still absent, and the relationship between oxidative stress and artemisinin production is not understood. Perhaps the induction of $^1O_2$ *in planta* or the reaction of artemisinic acid with $^1O_2$ in vitro may shed light into the developmental and environmental control of artemisinin biosynthesis. We have recently observed that low-temperature stress induces the up-regulation of *ADS* and *CYP71AV1* genes up to ten- and sevenfold in cultured *A. annua* L. (Yin et al. 2008).

Despite quantitative measurement data are still unavailable, there are some hints that *ADS* mRNA may be present in all tissues of *A. annua* L. Weathers et al. (2006) have suggested that the regulation of *ADS* may occur at the level of translation or post-translation rather than transcription, i.e. the *ADS* mRNA may not be translated in all tissues or the enzyme may not be active in all tissues. This tentative conclusion, however, needs more detailed analysis on the exact patterns of temporal and spatial gene expression in *A. annua* L.

The question as to the two distinct branches during artemisinin biosynthesis via intermediates arteannuin

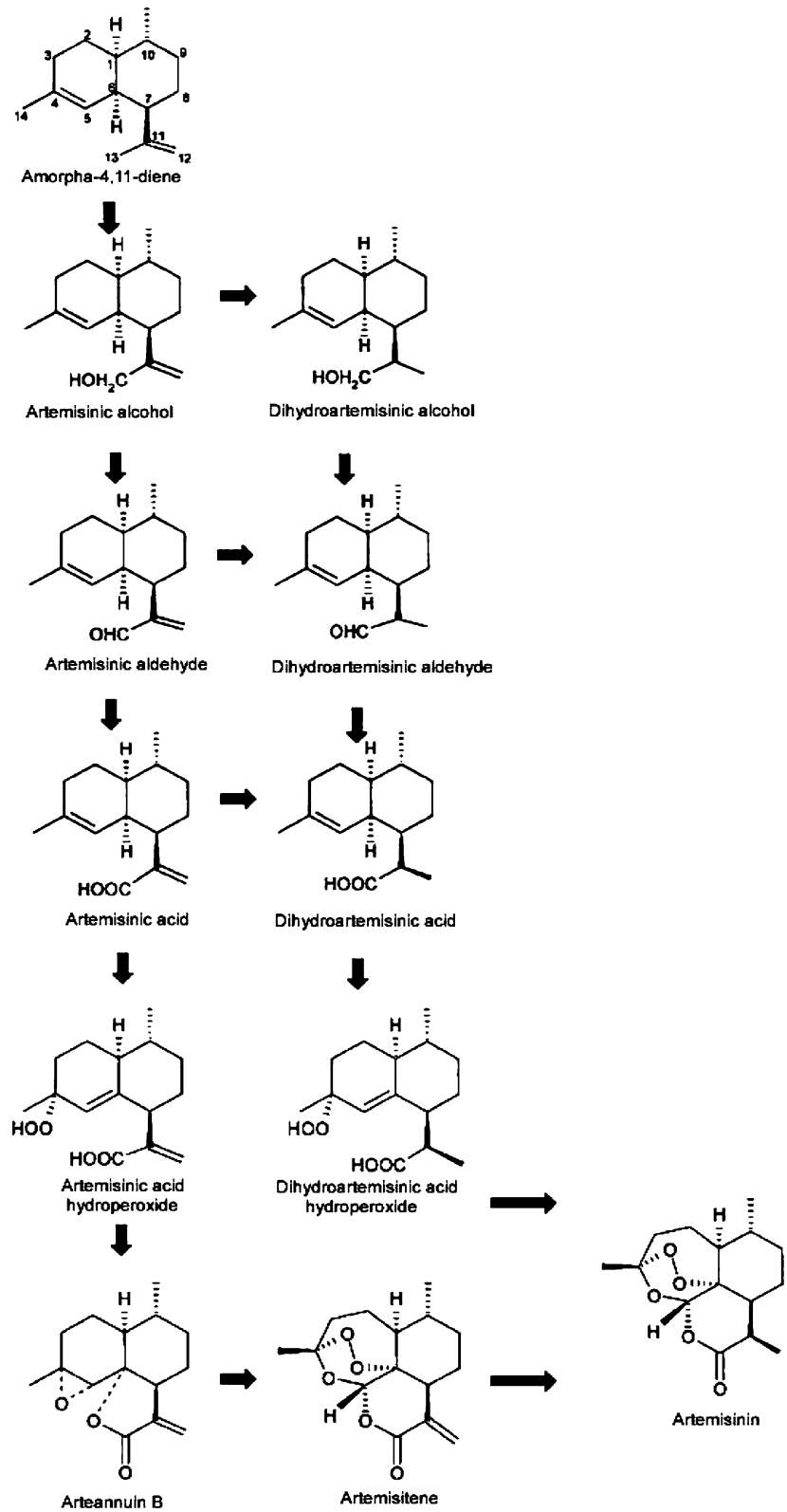

Fig. 2 An integrative artemisinin biosynthetic pathway in *A. annua* L. adopted from Lommen et al. (2005) with modifications

B and artemisitene or dihydroartemisinic acid and dihydroartemisinic acid hydroperoxide has been raised. Until now, which pathway is predominant or if in fact both are cooperative remains unanswered. Therefore, it is unclear whether the special endoperoxide bridge structure in artemisinin is derived by photochemical conversion from dihydroartemisinic acid hydroperoxide or enzymatic catalysis from artemisitene. Brown and Sy (2004) have detecte much a lower proportion of isotope-labeled artemisinin in *A. annua* L. after isotope-labeled dihydroartemisinic acid feeding, implying that the artemisinin accumulated in *A. annua* L. is totally converted from artemisinic acid rather than dihydroartemisinic acid. If this conclusion is true, we may infer that both the enzymatic and non-enzymatic branches described above exist concomitantly; perhaps the former is predominant in *A. annua* L. Nonetheless, the biochemical characterization of cellular fractions subjected to oxidative stress or identification of genetic mutants may be employed in the future to elucidate the mechanism underlying the bioconversion of artemisinin from artemisinic acid and other precursors.

Conclusions

Engineering the microbial terpene pathway to yield artemisinic acid provides a practical route for the semi-synthesis of artemisinin by chemical or biotransformational methods and represents a novel approach to the scaleable and cost-effective production of this valuable drug. Upon recovering artemisinic acid from culture medium, chemical conversion of artemisinic acid to artemisinin can be achieved in two steps with an overall yield of 30–40% (w/w) (Roth and Acton 1989). Compared to the direct extraction from plant materials, the microbial process is conducted in a controlled industrial environment, and is thus less affected by natural environmental variability than field-grown plants encounter. Microbe-based artemisinin production can also avoid contamination by other plant-made terpenes, thereby simplifying processing steps for purification.

Today, we have only limited ability to predict the outcomes of metabolic-pathway reconstruction in microbes. The challenges we face include optimization for expression of many plant genes in microbes and understanding of the biochemical mechanism underlying artemisinic acid-artemisinin conversion. Still, a great deal of biochemical and biotechnological knowledge has been and continues to be gained from the microbial engineering for sesquiterpene synthesis, which will guide our future maneuvers to develop more efficient and low-cost processes for artemisinin production.

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