Artemisinin biosynthesis and its regulatory enzymes: Progress and perspective

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ABSTRACT

Artemisinin is an endoperoxidized sesquiterpene from the Chinese medicinal plant Artemisia annua, used as an effective anti-malarial drug. Its biosynthesis pathway has been investigated for many years for scientific interest and for potential manufacturing applications to fulfill the market demand. A number of regulatory enzymes of its biosynthesis process, including amorpha-4,11-diene synthase, CYP71AV1 and cytochrome P450 reductase, have been obtained and utilized to increase the content of artemisinin. However, a large knowledge gap still exists, and certain points of controversy have stirred debates within the field. In this review, the progress and perspective of artemisinin biosynthesis and its regulating enzymes are described.

Key words: Artemisinin, biosynthesis pathway, progress and perspective, regulatory enzymes

INTRODUCTION

Artemisinin

Malaria is a leading cause of epidemic and death worldwide with an estimated number of 400 million infections and 1–3 million deaths per year.[1] The disease occurs due to the mosquito-mediated infection of erythrocytes by the malarial parasite *Plasmodium falciparum*.[2] Young children and pregnant women are most vulnerable to contracting this disease. The anti-malarial properties of the traditional Chinese medicine qinghaosu (artemisinin) were discovered by Chinese scientists in 1971 from *Artemisia annua*.[3] Although before the late 1960s, quinine-derived agents controlled malaria efficiently, multi-drug resistant *Plasmodium* strains have made artemisinin the preferred treatment due to its rapid therapeutic efficacy and low toxicity.

Artemisinin is a sesquiterpene lactone with an unusual endoperoxide structure. Numerous derivatives of artemisinin have also been synthesized and tested against malaria parasites,[4] many of which are derivatives at C_{12}. During recent years, a great number of artemisinin analogs have been synthesized, such as trioxolane OZ 277, which was advanced into clinical studies during 2004.[5] This trioxolane compound was orally administered and highly potent, but its development was discontinued prior to phase 3 clinical trials because of its instability in blood.[3] Thus, the need for additional anti-malarial drugs remains immensely urgent.

Artemisinin specifically and selectively inhibits the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) of *P. falciparum* after activation by iron ions.[6] Its specific mechanism is still unclear but the necessity of the endoperoxide bridge for antimalarial activity has been largely established. Besides the anti-malarial activity, artemisinin and its derivatives have exhibited other activities as well, such as anti-schistosomal,[7] anti-viral[8] and anti-cancer activities.[9]

Artemisinin-derived drugs typically have a short half-life and are best used in combination with other anti-malarials such as lumefantrine and sulfadoxime/pyrimethamine in artemisinin-based combination therapies (ACTs).[10] Artemisinin-based combination therapies (ACTs) recommended by WHO (2001) are highly effective in preventing the infection and transmission of MDR malaria.[2]

An estimated number of 400–600 million therapeutic doses of artemisinin will be required for ACT per year, whereas less than 100 million doses per year are presently available.[1] The demand of ACTs has caused artemisinin to fall in short supply. Furthermore, artemisinin on the market is currently produced only from *A. annua*, which contains only small amounts of

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artemisinin at about 0.01% to 0.8%.[11] The yield of artemisinin often depends on additional variable, such as temperature, humidity and soil type, resulting in high market prices for artemisinin range from US $350 to $1700 per kilogram.[3] Consequently, tremendous efforts have been made to increase the production of artemisinin. However, the knowledge about the biosynthetic pathway of artemisinin is not directly lacking.

Biosynthetic pathway of artemisinin

The biosynthesis pathway of artemisinin has been investigated for many years, but the whole process is not completely understood. Overall, two stages can be recognized in the biosynthesis of regular monoterpenes, sesquiterpenes and diterpenes, which involves the production of linear isoprene precursors including geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP), and the formation of cyclic terpenes from linear isoprene precursors.[2] The enzymes responsible for the first stage are conserved across all organisms, while the second stage is catalyzed by enzymes that vary from species to species.

Formation of FPP and enzymes

For decades, the classic cytoplasmic mevanolate (MVA) pathway had been considered as the only pathway for terpenes biosynthesis, which was first discovered by Bloch and Lynen in 1958. It has two isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are biosynthesized via mevalonate originating from acetyl-CoA. However, another biosynthetic pathway was elucidated recently. This plastidic non-MVA pathway, or deoxyxylulose phosphate (DXP)/methylerythritol phosphate (MEP) pathway, stems from glyceric acid-3-phosphate and pyruvate. Initial research indicated that IPP of mevalonate origin served as a precursor of farnesyl diphosphate (FPP), and ultimately, of sesquiterpenes, triterpenes and sterols, whereas the non-mevalonate route provided the precursors of geranyl diphosphate (GPP), geranylgeranyl-diphosphate (GGPP) and ultimately, of monoterpenes, diterpenes and carotenoids.[1] However, more and more evidence now suggests that crosstalk coexists between these two pathways for the biosynthesis of FPP.

Young *A. annua* plants were treated with specific chemical inhibitors of MVA or non-MVA pathway separately, after which the formation of artemisinin was found to decrease in both groups.[12] It demonstrated that precursor IPP was provided not only by the MVA pathway but also by the non-MVA pathway. Recently, a possible scenario is brought forward and supported: DMAPP of mevalonate origin is transferred to the plastid, where an IPP unit of non-mevalonate origin is used for elongation affording geranyl diphosphate (GPP). In the subsequent step, GPP is exported to the cytosolic compartment and converted into FPP using IPP from the mevalonate pathway.[1] The whole formation process of FPP via MVA and non-MVA pathways is shown in Figure 1.

The formation of FPP is catalyzed by a series of enzymes. In the MVA pathway, ATOT (acetoacetyl-CoA thiolase), HMGS

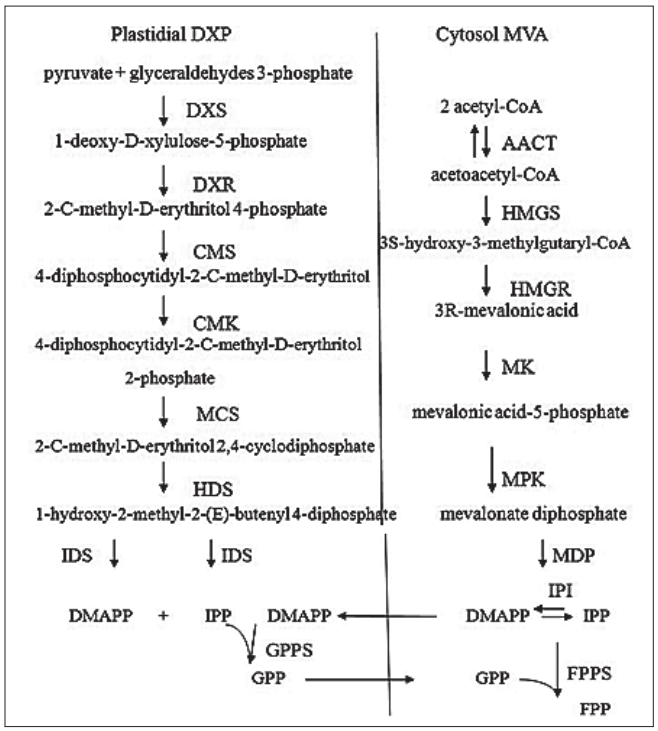

Figure 1: The formation of FPP via both MVA and non-MVA pathways and regulatory enzymes

(3-hydroxyl-3-methyglutaryl CoA synthase), HMGR (3-hydroxyl-3-methyglutaryl CoA reductase), MK (mevalonate kinase), MPK (mevalonate-5-phosphate kinase), MPD (mevalonate pyrophosphate decarboxylase) and IPI (IPP isomerase) all play important roles, while in the non-MVA pathway, enzymes like DXS (1-deoxy-D-xylulose-5-phosphate synthase), DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase), CMS (4-diphosphocytidyl-2C-methyl-D-erythritol 4-phosphate synthase), CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), MCS (2-C-methyl-D- erythritol 2,4-cyclodiphosphate synthase), HDS (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), IDS (IPP/DMAPP synthase) are involved.

HMGR is significant by catalyzing HMG-CoA to MVA, and considered as the first rate-limiting enzyme in MVA pathway, given that MVA formation is an irreversible process. Genome or cDNA cloning products of HMGR in tomato, potato, *Arabidopsis thaliana,* etc. have been reported in the early days, and in *A. annua* till 1995, cloned by Kang. Genes of HMGR and FPPS were co-expressed in transgenic *A. annua* plants, inducing 2.32-fold content increase of artemisinin.[13] Experiments *in vivo* modulated HMGR activity to increase artemisinin biosynthesis through exogenous supply of labeled HMG-CoA, and proved that plant growth regulators, IAA and GA_3, enhanced the artemisinin accumulation by increasing the activity of HMGR.[14] On the contrary, it is also supported by Re *et al* that HMGR does not limit the rate of terpenes biosynthesis in some plant cells.

DXS catalyzed the first step in non-MVA pathway, promoting the formation of DXP from pyruvate and glyceric acid-3-phosphate,

serving as an essential rate-limiting enzyme. DXR is another rate-limiting enzyme in non-MVA pathway, which is an effective target for regulating terpene biosynthesis. The exogenous supply of a competitive inhibitor of DXR, fosmidomycin, inhibited 14.2% of artemisinin accumulation.[14] Furthermore, it has been reported that DXS transcript accumulation was substantially increased when the root cultures of *A. annua* were grown in light as compared to the roots grown in dark.[15]

The sequential head-to-tail condensations of DMAPP and IPP consequently form the linear prenyl diphosphate precursors, GPP and FPP, which are catalyzed by the prenyltransferases: GPP synthase (GPPS) and FPP synthase (FPPS), respectively.[2] These enzymes belong to prenyltransferase (PTS). A cDNA encoding FPPS has been cloned from a cDNA library of *A. annua*, and expressed in *Escherichia coli* cells, whose deduced amino acid sequence was highly similar to that of FPPS from yeast and mammals. Since FPP is the key precursor in the pathway to artemisinin, genetic manipulation of FPPS becomes promising. This suggestion was proven by over-expression of the endogenous FPPS in high-yield *A. annua*; the highest artemisinin content in transgenic *A. annua* was up to 0.9% DW and 34.4% higher than that in non-transgenic *A. annua*.[16] Expression of FPPS was observed in transgenic *A. annua*, exhibiting higher artemisinin content and yield of 2.5- and 3.6-fold, respectively, than that detected in wild plants.[17]

As of now, the biosynthesis of FPP from aceyl-CoA is clear, and a large progress in understanding the process from FPP to artemisinic acid has made. However, how artemisinic acid transforms into artemisinin is still under investigation.

Characteristic pathway of artemisinin biosynthesis and the involved enzymes

Sesquiterpene synthases

A number of cDNAs from *A. annua* encoding different sesquiterpene synthases have been revealed. The cDNA encoding sesquiterpene synthase firstly cloned belongs to the *epi*-cedol synthase (ECS), followed by the genetic cloning of amorpha-4,11-diene synthase (ADS)[18] and β-caryophyllene synthase (CS).[19] After that, scientists reported the cloning of an (E)-β-farnesene synthase (FS) from *A. annua* in Figure 2.[20]

ECS: Epi-cedol synthase; ADS: Amorpha-4,11-diene synthase; CS: β-caryophyllene synthase; FS: (E)-β-farnesene synthase.[20]

All the above-mentioned sesquiterpene synthases have been cloned from *A. annua* leaves. However, to investigate the biosynthesis of sesquiterpenes, Bertea *et al.* firstly isolated total RNA from the glandular trichomes and used it for the construction of a plasmid cDNA library. Thus, a new sesquiterpene synthase, a germacrene A synthase, from a glandular trichome-specific cDNA library of *A. annua* was reported.[21]

The first committed and limiting step of artemisinin biosynthesis should be the cyclization of FPP by ADS. Therefore, the up-regulation of amorphar-4,11-diene synthase is considered a feasible strategy for increasing the production of artemisinin in *A. annua*. ADS gene was cloned and expressed in *A. annua*, resulting in a 2.3-fold increase in artemisinin content compared to non-transgenic *A. annua*.[22] Recently, several groups have cloned some new cDNA of ADS from different strains of *A. annua*. A newly cloned ADS1 cDNA from a special high-yield strain of *A. annua* was reported by Li *et al.*, which is not completely identical to ADS that has been cloned by others after molecular and phylogenetic analysis.[23] Besides, the ADS (3963) gene was obtained from high artemisinin yielding strain of *A. annua*, which was studied by an *in silico* approach.[24] Over-expression of these genes may be possible ways to accumulation of artemisinin.

ADS accumulations in leaves are 16-fold higher than in roots and 10-fold higher than in stems, demonstrating a tissue-specific expression pattern. In general, ADS content remains low in the cells under normal conditions. Interestingly, the flowering field plants and cold-acclimated cultural plants produce higher levels of ADS than non-flowering field plants or untreated cultural plants, indicating the environmental and developmental induction on ADS.[25] It is consistent with the experiments that upon exposure to cold, heat shock or UV light, the transcription levels of ADS were up-regulated compared to untreated plants.[26]

Since squalene synthase (SS) is responsible for sterol biosynthesis, competitive with FPP, it is considered as a regulatory switch. An antisense SS cDNA was introduced into the genome of *A. annua*, resulting in the decline of SS mRNA and simultaneous reduction of total sterols, mRNA coding for amorpha-4,11-diene synthase tremendously elevated.[27]

Cytochrome P_{450}

Cytochrome P_{450} enzymes (CYP/P_{450}) with dehydrogenase and

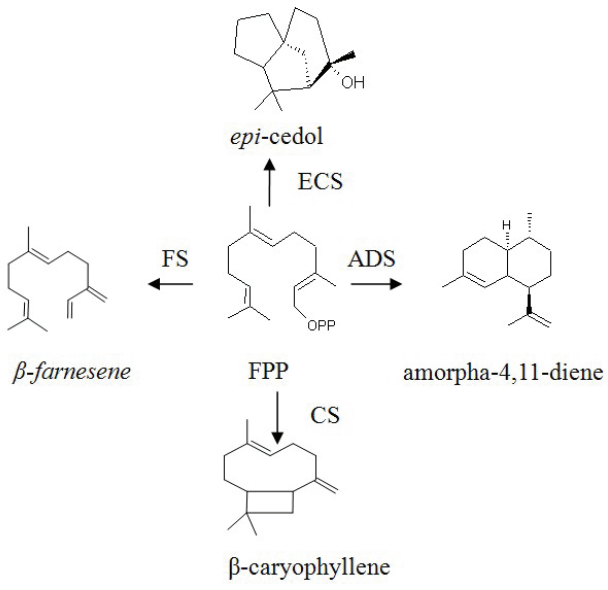

Figure 2: Reactions catalyzed by the four sesquiterpene synthases cloned from *A. annua*

reductase activities were assumed to participate in the oxidation of C_{12} in amorpha-4,11-diene. Moreover, a cDNA clone encoding a cytochrome P_{450} designated CYP71AV1 was characterized by expression in *Saccharomyces cerevisiae* and shown to catalyze the oxidation of the amorpha-4,11-diene, artemisinic alcohol and artemisinic aldehyde,[28] as shown in Figure 3. Indeed, when co-expressed in yeast with genes required for amorphadiene biosynthesis, artemisinic acid was formed in culture.[4]

Note: Steps confirmed are shown in bold arrows.

Expression analysis of CYP71AV1 in *A. annua* tissues indicates that it is most highly expressed in trichomes, moderately expressed in flower buds and lowly expressed in leaves and roots.[28] Zeng *et al* reported that CYP71AV1 accumulates in leaves is eight-fold higher than in roots and four-fold than in stems. Similar with ADS, CYP71AV1 genes is up-regulated to 10- and 7-fold in cultured *A. annua* after being induced by low temperature stress.[25] It is supported by Yin *et al.* that the transcription levels of CYP71AV1 were tremendously enhanced in stress conditions, and artemisinin content increased by 66.7–95.6% in transient pre-chilling plants than untreated plants.[26]

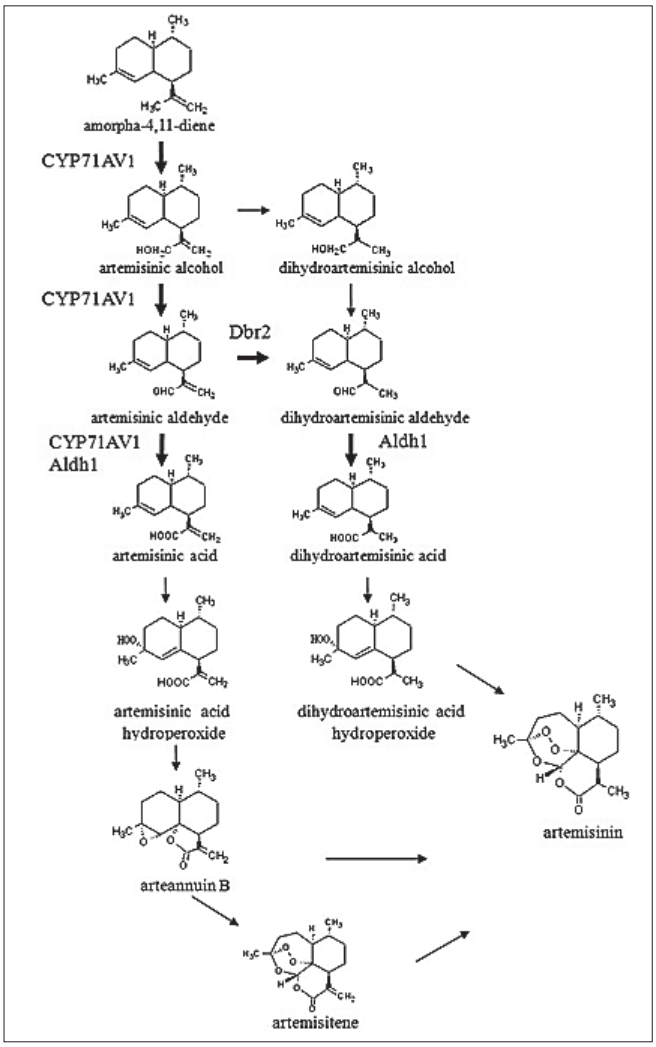

Figure 3: Biosynthesis pathway of artemisinin and enzymes

CPR (cytochrome P_{450} reductase), as the companion body of CYP71AV1, has been cloned and co-expressed with CYP71AV1 and ADS in *S. cerevisiae*, leading to high levels of artemisinic acid.[29] Different from ADS and CYP71AV1, CPR seemed to keep an average transcription level either with or without stress.[26]

Artemisinic aldehyde Δ11(13) reductase

It is obvious that the Δ11(13) double bond in amorpha-4,11-diene is reduced during biosynthesis of artemisinin, which is assumed to occur in artemisinic aldehyde. Recently, a corresponding gene Dbr2 was cloned and characterized, shown to be relatively specific for artemisinic aldehyde and some activity on small α, β-unsaturated carbonyl compounds. Expression in yeast of Dbr2 and other enzymes of artemisinin pathway in yeast resulted in the accumulation of dihydroartemisinic acid.[30] A chemotype of *A. annua* with a high artemisinin level typically has high ratios of dihydroartemisinic acid to artemisinic acid,[31] which indicates that double bond reduction may limit artemisinin accumulation.[32]

Aldehyde dehydrogenase

As mentioned above, cytochrome P_{450} CYP71AV1 was shown to be capable of oxidizing artemisinic alcohol to the corresponding aldehyde and acid. However, the ability of CYP71AV1 oxidizing dihydroartemisinic aldehyde was not detected. A full-length cDNA encoding an aldehyde dehydrogenase homolog was isolated and named Aldh1, which was capable of catalyzing the oxidation artemisinic aldehyde, dihydroartemisinic aldehyde and a limited range of other aldehydes. In addition, the highest expression of Aldh1 in *A. annua* was observed in the glandular trichomes with moderate expression in the flower buds and low expression in the leaves.[33]

Other possible intermediates and enzymes

The entire biosynthetic pathway from amorpha-4,11-diene to artemisinin remains uncertain. The mainly controversial issue on latest steps in artemisinin biosynthesis is whether artemisinic or dihydroartemisinic acid serves as the later precursor.

Artemisinic acid is a common precursor of arteannuin B and artemisinin. Artemisinin was formed after artemisinic acid and arteannuin B incubation with *A. annua* leaves extract, respectively. Scientists purified and characterized an enzyme involved in biochemical transformation of arteannuin B to artemisinin from *A. annua*.[34] However, genes of this enzyme have not been reported. The evidence above suggests the possibility of artemisinin biosynthesis from artemisinic acid. Furthermore, a full-length cDNA (apod1) encoding a peroxidase was isolated from *A. annua*, which favored the bioconversion of artemisinic acid to artemisinin in the cell-free extract of *A. annua* indirectly but no oxidation with artemisinic acid as the only substrate.[35] It has been reported that the level of artemisinin from artemisinic acid was elevated to about one-fold when the horseradish peroxidase was included in cell-free extracts of *A. annua* by phosphate buffer system.[36]

Recently, Brown *et al* have reported that artemisinic acid was

converted *in vivo*, possible non-enzymatically into several compounds including arteannuin B, but not artemisinin. In addition, *in vivo* dihydroartemisinic acid undergoes rapid plant pigment photosensitized oxidation, followed by subsequent spontaneous oxidation to form artemisinin.[37] Dihydroartemisinic acid can act as a quencher of the singlet oxygen to produce dihydroartemisinic acid hydroperoxide, which is later transformed into artemisinin.[38] Notably, perhaps the least spontaneous step in dihydroartemisinic acid oxidation, first singlet oxygen-dependent hydroperoxidation and resulting ring cleavage is very reminiscent of other enzyme reactions, like carotenoid cleavage dioxygenases, which catalyze the cleavage of double bonds to give two carbonyl compounds. Taking into account of above ideas, whether artemisinin biosynthesis from dihydroartemisinic acid involves enzymes is an open question and worthy of being further studies.

Experimental results showed that there was no direct enzymatic conversion of artemisinic acid into dihydroartemisinic acid.[39] Therefore, these two branches during artemisinin biosynthesis remain controversial as shown in Figure 3.

CONCLUSION

The study of artemisinin biosynthesis has made a tremendous progress, especially after a number of regulating enzymes were cloned, isolated and characterized. Since 1995, 12 genes related to artemisinin biosynthesis have been cloned from *A. annua* and their complete or partial mRNA sequences are now accessible in GenBank. Since plant materials are affected by natural environment, and direct extraction is laborious, more and more attentions are now shifting to microbial production. Microbe-based artemisinin production can avoid contamination by other plant-made terpenes, thereby simplifying processing steps for purification.[2] Additionally, with the development of technology, genetic regulation, as a popular method, is adopted all over the world to increase artemisinin production. Consequently, the elucidation of artemisinin biosynthesis pathway is much needed. However, there are still some problems which should be resolved in the future. Any improvements or new discovery of artemisinin biosynthesis are likely to lower the price and bring great benefits to millions around the world.

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