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Artemisinin-based antimalarial research: application of biotechnology to the production of artemisinin, its mode of action, and the mechanism of resistance of *Plasmodium* parasites

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Abstract Malaria is a worldwide disease caused by *Plasmodium* parasites. A sesquiterpene endoperoxide artemisinin isolated from *Artemisia annua* was discovered and has been accepted for its use in artemisinin-based combinatorial therapies, as the most effective current antimalarial treatment. However, the quantity of this compound produced from the *A. annua* plant is very low, and the availability of artemisinin is insufficient to treat all infected patients. In addition, the emergence of artemisinin-resistant *Plasmodium* has been reported recently. Several techniques have been applied to enhance artemisinin availability, and studies related to its mode of action and the mechanism of resistance of malaria-causing parasites are ongoing. In this review, we summarize the application of modern technologies to improve the production of artemisinin, including our ongoing research on artemisinin biosynthetic genes in other *Artemisia* species. The current understanding of the mode of action of artemisinin as well as the mechanism of resistance against this compound in *Plasmodium* parasites is also presented. Finally, the current situation of malaria infection and the future direction of antimalarial drug development are discussed.

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Introduction

As a worldwide disease, malaria has been one of the main cause of illness and death in humans for over a century, especially in sub-Saharan Africa and Southeast Asia. More than 200 million cases of malaria are reported every year; in 2015, there were 214 million cases and 438,000 related deaths [1]. This disease is caused by five species of *Plasmodium* parasites: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Among these, *P. falciparum* is the major cause of malaria infection in Africa, and *P. vivax* is the most widely distributed malaria-causing parasite globally [1]. Several antimalarial drugs have been developed since the seventeenth century. However, malaria-causing parasites have developed resistance to these conventional drugs, leading to treatment failure.

In response to the urgent need for new antimalarial drugs, Chinese scientists Professor Youyou Tu and her research group discovered artemisinin, the most effective antimalarial drug derived from *Artemisia annua* in 1971 [2]. Artemisinin is a sesquiterpene lactone with an endoperoxide bridge, which is necessary for antimalarial activity during multiple stages of parasite development [3–7]. Owing to its rapid action and high effectiveness against malaria, the combination of artemisinin derivatives and other antimalarial drugs, so-called artemisinin-based combination therapies (ACTs), has been recommended as the first-line treatments against malaria since 2006 [8]. ACTs have become the most powerful strategy to prevent malaria and related deaths. Professor Tu was then awarded

the Nobel Prize in Physiology or Medicine in 2015 for the discovery of this effective antimalarial compound.

The demand for ACTs increases dramatically each year; yet, the production yield of artemisinin from A. annua is very low and varies widely from 0.01 to 2 % dry weight [9]. Alternative approaches, including plant breeding technologies, synthetic biology, and total and semi-syntheses of artemisinin, have been investigated to enhance the production and reduce the cost of this compound. In addition, the recent emergence of artemisinin-resistant Plasmodium parasites has also become a new challenge to scientists in the elucidation of the mechanism of resistance and identification of the new strategies for malaria treatment.

In this review, we summarize recent studies on the enhancement of artemisinin production and on artemisinin biosynthetic genes in other Artemisia species, conducted in our laboratory. In addition, the current understanding of the mode of action of artemisinin against malaria-causing parasites and, in turn, the mechanism of resistance of the parasites to this compound are also presented. Finally, the current situation of malaria infection and future directions, including ongoing studies on antimalarial drug development, are discussed.

Discovery of artemisinin

Before the discovery of artemisinin, powder derived from cinchona tree bark had been used to treat malaria since the seventeenth century. The active compound from this plant, quinine, was first isolated in 1820 and was used as the only effective antimalarial compound until the 1920s. The quinine derivative chloroquine was developed as a new effective antimalarial drug once quinine-resistant Plasmodium strains appeared. During that time, the insecticide DDT was widely used to control the spread of infected mosquitoes as well. However, in the 1960s, increasing of chloroquine-resistant Plasmodium strains and DDT-resistant mosquitoes became a critical sign of the failure of malaria prevention and treatment [10].

In response to the urgent need for new antimalarial drugs, the Chinese government launched a national project against malaria called Project 523 in 1967 [2]. The group, led by Professor Youyou Tu, investigated more than 2000 Chinese herbs used as traditional Chinese medicines to treat fever. Among these herbs, an extract from A. annua showed highly effective inhibition against growth of malaria-causing parasites. The active antimalarial components were then extracted from the leaves of mature plants in 1971 [2, 10–12]. After purification, the active antimalarial compound, named qinghaosu or artemisinin, was obtained as colorless needle-like crystals. Its stereochemistry and chemical and X-ray crystal structures were

determined and reported several years later [2, 10, 11, 13]. Clinical trials involving either a non-toxic A. annua extract or pure artemisinin have been conducted since 1972 by several groups, and all patients in these trials quickly recovered from the disease [11, 12]. These results clearly indicated that artemisinin is an effective antimalarial compound with rapid action and low toxicity.

Despite showing effective antimalarial activity, the low solubility of artemisinin in both oil and water becomes a therapeutic limitation of this compound. To address this problem, many scientists have developed semi-synthetic drugs and synthesized artemisinin derivatives with higher solubility. Some of these artemisinin derivatives, which have been used until the present, include dihydroartemisinin, artemether, and artesunate [14]. In addition, the combination of artemisinin or its derivatives with other conventional antimalarial drugs greatly increased the parasite clearance rate in patients and was first recommended as a new strategy for malaria treatment in 1984 [15]. This strategy, known as ACT, has been recommended by the World Health Organization (WHO) as a first-line treatment for malaria to prevent recurrence and development of resistance in malaria-causing parasites, whereas the monotherapy is considered as an inappropriate treatment [2, 8, 13, 14, 16].

Biosynthesis of artemisinin and expression pattern of artemisinin biosynthetic genes in A. annua

A precursor of artemisinin, farnesyl pyrophosphate (FPP, C_{15}), is synthesized from two C-5 isoprenoid units derived from the cytosolic mevalonate (MVA) pathway and one isoprenoid unit derived from the non-mevalonate (MEP or DXP) pathway [17, 18]. FPP is cyclized to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) [19–21] via the generation of bisabolyl and 4-amorphenyl cation intermediates [22, 23] (Fig. 1). The following step is the oxidation of amorpha-4,11-diene to artemisinic alcohol by amorpha-4,11-diene 12-monooxygenase (CYP71AV1) [24]. This enzyme also catalyzes the oxidation of artemisinic alcohol to artemisinic aldehyde and artemisinic acid. In addition, alcohol dehydrogenase 1 (ADH1) and aldehyde dehydrogenase 1 (ALDH1) also show specific oxidation activity on artemisinic alcohol into artemisinic aldehyde and on artemisinic aldehyde into artemisinic acid, respectively [25, 26]. Artemisinic acid was thought to be the last precursor of artemisinin. However, it has been revealed that this compound is converted non-enzymatically into arteannuin B and related compounds, rather than artemisinin [27]. The next step of artemisinin biosynthesis is the reduction of artemisinic aldehyde into dihydroartemisinic aldehyde by artemisinic aldehyde Δ11(13) reductase (DBR2) [28]. Then,
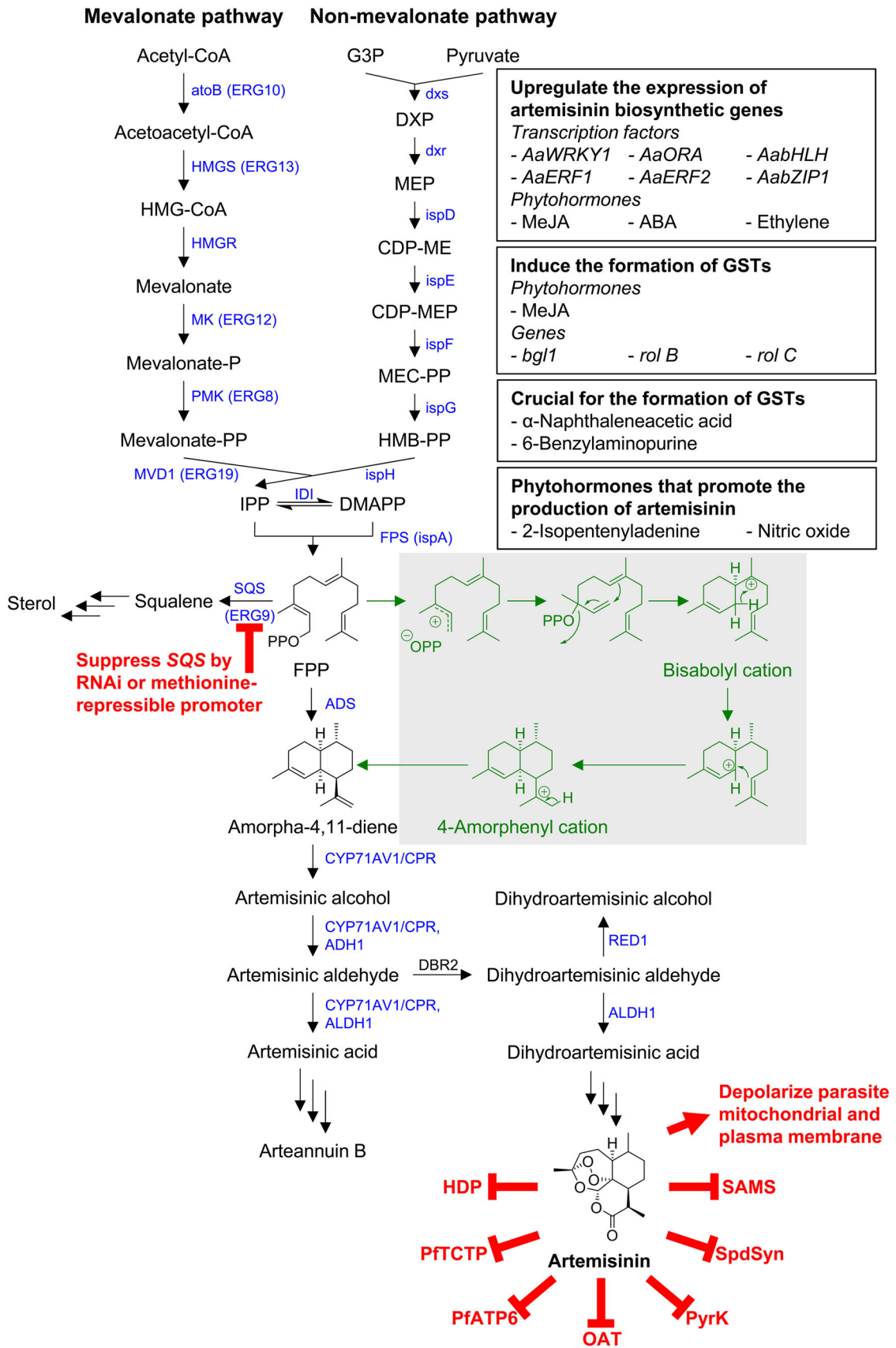
◄ **Fig. 1** Summary of artemisinin biosynthesis, transgenic approaches to enhance artemisinin production, and artemisinin mode of action. The enzymes responsible for each reaction are indicated next to the *arrows*. Suppression of competing pathways and artemisinin activity and its targets are shown in *bold*. Transgenic approaches regulating artemisinin production are shown in black boxes. Cyclization mechanism of FPP to generate amorpha-4,11-diene is highlighted in *gray*. Full names of intermediates and enzymes involved in the pathway are as follows: *HMG-CoA* 3-hydroxy-3-methylglutaryl-coenzyme A, *G3P* glycerol-3-phosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2*C*-methyl-D-erythritol 4-phosphate, *CDP-ME* 4-diphosphocytidyl-2*C*-methyl D-erythritol, *CDP-MEP* CDP-ME 2-phosphate, *MEC-PP* 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, *HMB-PP* (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *atoB* (*ERG10*) acetoacetyl-CoA thiolase, *HMGS* (*ERG13*) HMG-CoA synthase, *HMGR* HMG-CoA reductase, *MK* (*EGR12*) mevalonate kinase, *PMK* (*ERG8*) phosphomevalonate kinase, *MVD1* (*ERG19*) mevalonate pyrophosphate decarboxylase, *dxs* DXP synthase, *dxr* DXP reductase, *ispD* CDP-ME synthase, *ispE* CDP-ME kinase, *ispF* MEC-PP synthase, *ispG* HMB-PP synthase, *ispH* HMB-PP reductase, *IDI* IPP isomerase, *FPS* (*ispA*) farnesyl pyrophosphate (FPP) synthase, *SQS* (*ERG9*) squalene synthase, *ADS* amorpha-4,11-diene synthase, *CYP71AV1* amorpha-4,11-diene 12-monooxygenase, *CPR* cytochrome P450 reductase, *ADH1* alcohol dehydrogenase 1, *ALDH1* aldehyde dehydrogenase 1, *DBR2* artemisinic aldehyde Δ11(13) reductase, *RED1* dihydroartemisinic aldehyde reductase 1 (color figure online)

ALDH1 oxidizes dihydroartemisinic aldehyde into dihydroartemisinic acid, which is converted non-enzymatically into artemisinin [26, 29], as shown in Fig. 1. Rydén et al. [30] discovered dihydroartemisinic aldehyde reductase 1 (RED1), which reduces dihydroartemisinic aldehyde into dihydroartemisinic alcohol. Although the role of RED1 in artemisinin biosynthesis is still unclear, it has been suggested that the silencing of *RED1* might increase the production of artemisinin in *A. annua*.

Artemisinin is produced mainly in glandular secretory trichomes (GSTs) and its accumulation level declines as plants mature. Olofsson et al. [31] showed that GSTs of *A. annua* are found in all aerial tissues of plants, but not in roots or hairy roots. The density of GSTs is highest in flower buds and young leaves and decreases as leaves age.

The expression pattern of genes involved in the artemisinin biosynthetic pathway has been investigated extensively for over a decade. The expression of genes in the upstream pathway shows no correlation with the density of GSTs or the accumulation levels of artemisinin intermediates [32]. In contrast, the expression of genes in the downstream pathway is consistent with the density of GSTs in each tissue. The expression of *ADS* is highest in GSTs, high in flower buds and young leaves, low in stems, negligible in old leaves and hairy roots, and not detected in roots [31, 33–37]. *CYP71AV1*, *DBR2*, and *ALDH1* showed similar expression patterns: highest in GSTs and very low in stems and roots. In hairy roots, the expression levels of

CYP71AV1 and *DBR2* are relatively low, but the expression of *ALDH1* is negligible [24, 26, 28, 31]. The expression levels of *CYP71AV1* and *DBR2* in leaves and flowers show similar patterns, as they are high in leaf primordia and flower buds but decrease as leaves and flowers develop [38–40]. The expression pattern of *ALDH1* in leaves at different stages is similar to those of *CYP71AV1* and *DBR2* [31]. Although there is no report on the expression level of *ALDH1* during different stages of flowering, this gene shows higher expression in flowers than in leaves [26, 31]. The expression of *RED1* is relatively low in flower buds, young leaves, and stems. In contrast, the expression of this gene is much higher in old leaves and roots than in young leaves [30, 31]. Interestingly, the expression of *RED1* is approximately 50-fold higher in hairy roots compared with old leaves. Nevertheless, the function of RED1 in hairy roots has not been established [31].

The expression levels of *ADS* and *ALDH1*, as well as their enzymatic activities in high-artemisinin-producing and low-artemisinin-producing *A. annua* cultivars, show no differences. Even though the expression levels of *CYP71AV1* in these two cultivars are similar, CYP71AV1 in a high-artemisinin-producing cultivar shows lower enzyme activity, which is suitable for the change in metabolic flux to dihydro-analogues and artemisinin production [41]. In contrast, the activity of DBR2 in both cultivars shows no significant difference, but the gene encoding this enzyme shows considerably higher expression levels in high-artemisinin-producing cultivars than in low-artemisinin-producing cultivars [42].

Mode of action of artemisinin

Before artemisinin can exert its action, the endoperoxide bridge has to be activated to generate the free radical species. Two activation pathways of artemisinin have been suggested, namely the mitochondrial and heme-mediated degradation pathways [43]. Mitochondria-activated artemisinin is involved in lipid peroxidation inducing cytotoxicity via the generation of reactive oxygen species (ROS) and depolarization of the parasite mitochondrial and plasma membranes [43–47]. In the heme-mediated pathway, two activation models (i.e., a reductive scission model and an open peroxide model) have been proposed, both of which lead to the generation of an active carbon-centered radical [48]. Even though the non-heme Fe^{2+} ion was suggested to bind and activate artemisinin [7], recent studies showed that heme plays a predominant role in artemisinin activation rather than the Fe^{2+} ion [5]. In *Plasmodium* spp., heme is produced via endogenous heme biosynthesis at the early ring stage and via hemoglobin digestion at the trophozoite stage. However, the level of

heme biosynthesized endogenously in the parasites is much lower than its production via hemoglobin digestion, suggesting that hemoglobin-derived heme plays a major role in artemisinin activation [5, 49]. Recently, Xie et al. [50] reported that falcipains FP2a and FP3 (two main cysteine protease hemoglobinases) are also involved in the potential activation of artemisinin at an early ring stage.

After hemoglobin digestion, the heme detoxification protein (HDP) can trigger the conversion of free heme to hemozoin, which is essential for parasite survival [4, 51]. However, the formation of the artemisinin-free heme complex shows an inhibitory effect on this conversion [51]. A translationally controlled tumor protein (PfTCTP) was also reported as a potential target of artemisinin, as it could form a covalent bond with this protein, resulting in protein malfunction [52, 53]. Eckstein-Ludwig et al. [54] showed that artemisinin specifically mediated the inhibition of PfATP6, an orthologous sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), outside the food vacuole. Recently, five enzymes involved in the key metabolic pathways of the parasite were also reported as potential targets of artemisinin, namely ornithine aminotransferase (OAT), pyruvate kinase (PyrK), L-lactate dehydrogenase (LDH), spermidine synthase (SpdSyn), and *S*-adenosylmethionine synthetase (SAMS). All of them are covalently modified by the interaction with artemisinin, resulting in the irreversible malfunction of enzyme activities [5].

Enhancement of artemisinin production

The demand for artemisinin increases every year. Even though total synthesis of artemisinin from commercially available chemicals or semi-synthesis from its intermediates have been reported, all of those methods are costly and require several synthesis steps [55, 56]. In this review, we summarize recent studies regarding four approaches to enhance the production of artemisinin: (1) plant breeding technologies, (2) overexpression of genes involved in the artemisinin biosynthetic pathway, (3) direct or indirect upregulation of artemisinin biosynthesis, and (4) heterologous production.

Plant breeding technologies

Conventional plant breeding techniques to select high-artemisinin-producing cultivars have been used for decades. These techniques include cultivation of *A. annua* and collection of cultivars with the desired properties. At present, a robust hybrid *A. annua* is generated from the combination of high-artemisinin-producing and vigorous cultivars to increase the production yield of artemisinin to more than 2 % dry weight [57–59]. Recently, an alternative

approach to increase the production of artemisinin from the cultivation of high-artemisinic acid or dihydroartemisinic acid-producing cultivars was proposed, since a method for the semi-synthesis of artemisinin from these two precursors has been developed [56, 60].

Scientists at the University of York used advanced breeding techniques to evaluate the distribution of traits that contribute to artemisinin yield [61]. From the screening of 23,000 strains, they succeeded in identifying genes and molecular markers for fast-track breeding, enabling the construction of a detailed genetic map of *A. annua* with nine linkage groups. The established quantitative trait loci (QTL) map is also applicable for rapid identification of *A. annua* parental lines with useful traits for plant breeding. Two hybrids, called Hybrid 1209r *Shennong* and Hybrid 8001r *Zenith*, were developed with high artemisinin productivity of up to 36.3 and 54.5 kg/ha, respectively. The diallel cross approach to determine the combining ability of the robust parental lines for the production of artemisinin high-yielding *A. annua* hybrids was also developed by the same group and showed consistent results with the QTL-based molecular breeding approach [62].

Hairy root culture is another method to enhance the production of secondary (specialized) metabolites, owing to its rapid growth capabilities [63]. Transformation protocols to obtain hairy roots containing artemisinin from this plant have been reported [64, 65]. In our laboratory, we also attempted to establish the conditions for *A. annua* hairy root cultivation. However, we still could not detect even trace amounts of artemisinin or its intermediates from the extract of hairy root cultures by GC–MS (unpublished data). Artemisinin biosynthetic genes are highly expressed in trichomes but almost negligible in root tissue [31, 33–40], suggesting that the production of this compound by hairy root cultures could be somewhat difficult. Therefore, the most suitable conditions for hairy root cultures to enhance production of artemisinin must be investigated. In addition, the identification of artemisinin production from root extracts requires extreme care, and NMR spectroscopic and mass spectrometric analyses are required.

Overexpression of genes involved in artemisinin biosynthetic pathway

Metabolic engineering of *A. annua* by overexpressing genes involved in artemisinin biosynthesis has been given more attention during the last 20 years. To obtain successful transformants, several parameters for *Agrobacterium tumefaciens*-mediated transformation, such as the concentration of antibiotics, method and duration of cocultivation, and phytohormones supplied for plant regeneration, have been optimized [66–71]. Among various

explants available for transformation, stem internodes and young inflorescence seem to be the most appropriate [70–72]. Phytohormones α-naphthaleneacetic acid and 6-benzylaminopurine are crucial for GST development in young leaves, and root generation also affects GST size [73]. Recently, Kiani et al. [72] developed miniprep methods using *A. tumefaciens*- and *Agrobacterium rhizogenes*-mediated transformation. This method exhibits higher transformation rates with faster development of transformants within 3–4 weeks compared with other methods.

The overexpression of several genes involved in artemisinin biosynthesis in *A. annua* has been evaluated. Overexpression of farnesyl pyrophosphate synthase (*FPS*) increased artemisinin production up to 2- to 3.6-fold higher than that in the control [74, 75]. Overexpressing *CYP71AV1* and its redox partner cytochrome P450 reductase (*CPR*) in artemisinin biosynthesis could increase artemisinin content *in planta* by 38 % [76]. Xiang et al. [77] generated *dxr*- and *CYP71AV1/CPR*-overexpressing *A. annua* and found that both transformants increased the production of artemisinin. The overexpression of *DBR2* increased the production of artemisinin as well as its precursor dihydroartemisinic acid, up to twofold, compared with non-transgenic plants. It also increased production of artemisinic acid up to 5.48- to 9.06-fold and arteannuin B up to twofold [78]. The reason why overexpression of *DBR2* enhanced biosynthesis of artemisinic acid and arteannuin B has not been revealed. However, Yuan et al. [78] hypothesized that excess dihydroartemisinic acid might be converted into artemisinic acid *in planta*.

Overexpression of multiple genes involved in artemisinin biosynthesis could greatly increase the production of artemisinin *in planta*. Chen et al. [79] showed that the co-overexpression of *FPS*, *CYP71AV1*, and *CPR* increased artemisinin levels in *A. annua* up to 3.6-fold. The co-overexpression of *HMGR* and *FPS* increased production of artemisinin up to 1.8-fold higher than that in the control [80]. Alam et al. [81, 82] co-overexpressed *HMGR* and *ADS* in *A. annua* and found greatly increased artemisinin levels, up to 7.65-fold, in this transgenic line.

Suppressing the expression of genes involved in the pathways competing with artemisinin biosynthesis is another approach to enhance artemisinin content *in planta*. Zhang et al. [83] used RNAi techniques to suppress the expression of *SQS*, the first committed gene in sterol biosynthesis. The suppression of this gene enhanced the production of artemisinin up to 3.14-fold.

Direct or indirect upregulation of artemisinin biosynthesis

The effect of several stresses on production of artemisinin in *A. annua* has been analyzed since the 1990s. These stresses usually lead to the generation of ROS (required for the last non-enzymatic step in artemisinin biosynthesis) or upregulate the expression of artemisinin biosynthetic genes [84–87]. Details of the stresses placed on artemisinin production have been summarized previously [88, 89], and the appropriate cultivation conditions of *A. annua* were suggested [9].

Some transcription factors upregulated the expression of artemisinin biosynthetic genes and promoted production of artemisinin in *A. annua*. The WRKY1 transcription factor is thought to bind to the W-box *cis*-acting elements of promoters to promote gene expression. It is also involved in the regulation of plant defense responses and developmental and physiological processes. Ma et al. [33] showed that the transcript levels of *HMGR*, *ADS*, *CYP71AV1*, and *DBR2* were induced in transient *AaWRKY1*-overexpressing leaves. Furthermore, the specific overexpression of this transcription factor in GSTs increased transcript levels of *CYP71AV1* up to 33-fold, compared with the wild type [90]. *AaORA*, one of the APETALA2/ethylene response factor (AP2/ERF) transcription factor involved in plant responses to biotic and abiotic stresses, showed a similar expression pattern to those of *ADS*, *CYP71AV1*, and *DBR2*. The overexpression of this transcription factor led to the upregulation of the expression levels of *ADS*, *CYP71AV1*, and *DBR2 in planta* and promoted artemisinin production [91]. Yu et al. [92] also reported the enhancement of artemisinin production via overexpression of two transcription factors from the same family, *AaERF1* and *AaERF2*, which bind to the promoter regions of *ADS* and *CYP71AV1*. Another transcription factor that positively regulates the biosynthesis of artemisinin is a basic helix-loop-helix (bHLH) transcription factor, involved in metabolic regulation of various hormones, developmental processes, and regulation of light signaling, iron and phosphate homeostasis, and various abiotic stresses [93]. Recently, Zhang et al. [94] reported that a basic leucine zipper transcription factor (AabZIP1) binds to the ABA-responsive elements (ABRE) of *ADS* and *CYP71AV1* promoters and upregulates the expression of *ADS*, *CYP71AV1*, *DBR2*, and *ALDH1*.

Several phytohormones upregulating artemisinin biosynthesis have been reported. Treatment with salicylic acid upregulates the expression of *HMGR* and *ADS*, as well as induces ROS generation, driving the conversion of dihydroartemisinic acid into artemisinin [95]. Methyl jasmonate (MeJA) promotes the formation of GSTs and enhances the expression of several genes involved in the artemisinin biosynthetic pathway and related transcription factors (*ORA* and *ERF1*), leading to the enhancement of artemisinin production [96–98]. This phytohormone also regulates trichome-specific fatty acyl-CoA reductase 1 (*TFAR1*), ABCG transporter unigenes (*AaABCG6* and

AaABCG7), and allene oxide cyclase (*AaAOC*) [96, 99, 100]. TFAR1 is involved in the formation of cuticular wax during GST expansion in *A. annua*. *AaABCG6* and *AaABCG7* are ATP-binding cassette transporter G, involved in the development of trichome cuticle and may share a common regulatory system with *ADS* and *CYP71AV1*. *AaAOC* is involved in JA biosynthesis. The expression of this gene may be upregulated by treatment with not only MeJA but also ABA and ethylene [100]. The overexpression of the ABA receptor, *AaPYL9*, also improves the sensitivity of ABA and promotes artemisinin biosynthesis after ABA treatment [99, 101].

The enhancement of artemisinin production can be achieved by increased GST density. Singh et al. [102] reported that the expression of *bgl1*, encoding β-glucosidase from *Trichoderma reesei*, in *A. annua* improved the density of GSTs in flowers up to 66 % and increased the production of artemisinin up to five-fold compared with the control. The expression of *rolB* and *rolC* of *A. rhizogenes* also increases GST density and upregulates the expression of *ADS*, *CYP71AV1*, *ALDH1*, and *TFAR1*. Artemisinin content is then increased 2- to 9-fold and 4-fold in *rolB*- and *rolC*-expressing plants, respectively [103].

Co-cultivation of an endophytic fungus *Piriformospora indica* and a nitrogen-fixing bacterium *Azotobacter chroococcum* with *A. annua* increases artemisinin content up to 70 % [104]. This dual symbiosis also shows a positive effect on plant height, dry weight, and leaf yield. Another example of using symbiosis to increase the production of artemisinin was reported using *Glomus mosseae* and *Bacillus subtilis* [105]. Although clear evidence for the effect of this symbiosis on the enhancement of artemisinin production is still unknown, Arora et al. [104] suggested that it might be due to improved growth and nutrient status of the plant.

Heterologous production

Metabolic engineering of several platforms, such as *Nicotiana benthamiana* or chloroplasts, has been conducted. Although *ADS* and *CYP71AV1* were introduced into *N. benthamiana*, the production of artemisinic acid 12-β-diglucoside, instead of artemisinic acid, was detected at 39.5 mg/kg fresh weight (FW) [106]. The production yield of artemisinic acid in tobacco chloroplasts was also very low (0.1 mg/g FW) [107].

The production of plant natural compounds in microorganisms is an alternative approach with several advantages. The metabolic pathways in microorganisms could be modified to produce various types of natural compounds, including isoprenoids, alkaloids, and phenylpropanoids. Microorganisms can grow rapidly, allowing shorter production time compared with the biosynthesis of desired natural compounds in plants. Scaling up production to industrial scale is also possible [108].

The production of artemisinin precursors in microorganisms was first reported in 2003. Martin et al. [109] expressed entire genes encoding the MVA pathway from yeast *Saccharomyces cerevisiae* in *Escherichia coli* to increase the intracellular concentration of FPP. To prevent the rapid loss of highly volatile amorpha-4,11-diene during culturing, the culture media was overlaid with dodecane to trap amorpha-4,11-diene, referred to as a two-phase partitioning bioreactor. As a result, they recovered the volatilized amorpha-4,11-diene, improving production titers from 24 mg/L to approximately 500 mg/L in a fed-batch bioreactor [110].

The coexpression of *MevT* operon with extra copies of *HMGR* reduced the accumulation of toxic HMG-CoA and increased production of mevalonate by threefold [111]. The replacement of *lac* by *lacUV5* promoter with a codon-optimized *MevT* and an additional copy of *MK* also led to the increase in artemisinin production [112]. Tsuruta et al. [113] succeeded in enhancing amorpha-4,11-diene production in *E. coli* up to 27.4 g/L by replacing yeast HMGS and HMGR with the equivalent enzymes from gram-positive bacteria *Staphylococcus aureus*.

Engineering of the MEP pathway and membrane efflux transporters to improve the production of amorpha-4,11-diene in *E. coli* has been reported as well [114–117]. However, there are many issues regarding the expression of membrane-bound cytochrome P450s in this bacterium posing a limitation on the production of the subsequent oxidized compounds. To overcome these problems, Chang et al. [118] engineered the N-terminal transmembrane domain of the codon-optimized *CYP71AV1* and coexpressed it with CPR from *A. annua*. As a result, production of artemisinic acid (105 mg/L) in this *E. coli* strain was obtained. Two years later, the same group replaced *CYP71AV1* by engineered P450 from gram-positive bacteria *Bacillus megaterium* ($P450_{BM3}$) and could produce artemisinic-11*S*,12-epoxide at higher than 250 mg/L successfully [119]. From this finding, a novel semi-biosynthetic route for the production of artemisinin stemming from the cleavage of this epoxide followed by several oxidation steps was proposed.

Yeast is another attractive host for the production of artemisinin precursors as it produces FPP for sterol biosynthesis via the MVA pathway. Since the MVA pathway in *S. cerevisiae* has been characterized, *ADS* was introduced into this yeast, and an amorpha-4,11-diene-producing yeast strain was generated successfully [120]. While there are many issues concerning the expression of cytochrome P450s in *E. coli*, the expression of this gene in yeast is much more feasible. Therefore, *CYP71AV1* and *CPR* were coexpressed, and all genes involved in the MVA

pathway were upregulated either directly or indirectly. The competing pathway (sterol biosynthetic pathway) was also downregulated using a methionine-repressible promoter to improve the production of artemisinic acid in the yeast expression system. As a result, this transgenic yeast strain produced artemisinic acid at up to 100 mg/L [121, 122]. Several factors were further optimized for the production of artemisinic acid in an industrial fermenter. For example, the carbon source for growing yeast in a fermenter was switched from glucose to galactose, and the oxygen transfer rate was controlled. With this development, called the galactose fed-batch process controlled by the DO-stat algorithm, the artemisinic acid titer increased to 2.5 g/L [123].

Despite conferring a higher production yield of artemisinic acid, the use of galactose is costly and not applicable, especially in developing countries. Thus, lower-cost chemicals are needed as carbon sources. Yeast with *GAL1*, *GAL7*, *GAL10*, and *GAL80* deletions was generated to exclude the use of galactose, and ethanol was alternatively used as a carbon source. Two additional copies of truncated *HMGR* (*tHMG1*) were integrated into this yeast strain. As a result, the production of amorpha-4,11-diene was increased up to more than 40 g/L [124]. Further development was performed by the introduction of artemisinin biosynthetic genes, *CYP71AV1*, *CPR*, *ADH1*, and *ALDH1*, to oxidize amorpha-4,11-diene into artemisinic acid. Cytochrome b_5 (*CYB5*) was also introduced into this strain as it can accelerate cytochrome P450 reactions [125]. High-level production of artemisinic acid, at 25 g/L, was thereby achieved. The semi-synthesis of artemisinin from artemisinic acid was also optimized, and the overall yield after purification increased to 40–45 % [126, 127]. A potent coupled chromatography–crystallization method to purify artemisinin was then developed, and the recovery yield of this antimalarial compound from the reaction mixture increased to 61.5 %, with 99 % purity [128]. All of the transgenes and modifications to several heterologous hosts mentioned here are summarized in Table 1.

Artemisinin biosynthetic genes in non-artemisinin-producing *Artemisia* species

Some studies reported that artemisinin is produced in other *Artemisia* species [129–134]. However, we attempted to isolate artemisinin from other *Artemisia* species but failed to detect any trace amounts of artemisinin or its intermediates (unpublished data). Thus, we analyzed the expression of genes highly homologous to artemisinin biosynthetic genes in these species. Firstly, we selected *A. afra* and *A. absinthium* as they are widely cultivated in Africa and exhibit anti-plasmodial activity [135–138].

Putative *ADS* orthologs were not expressed in either *A. afra* or *A. absinthium* [139]. However, we detected the expression of putative *CYP71AV1* orthologs in both species. Functional analysis revealed that these orthologous enzymes show similar catalytic activities to their correspondent in *A. annua* on the oxidation of amorpha-4,11-diene into artemisinic acid [139]. We also detected the expression of *DBR2* ortholog in *A. absinthium*, and the encoded enzyme showed comparable activity to that of *A. annua* DBR2 [140]. In addition, we showed that this plant can convert the fed artemisinin intermediates into the following products along the biosynthetic pathway of artemisinin [140]. Our findings suggest that ADS might be a limiting factor for the production of artemisinin *in planta*, and *A. absinthium* could be an alternative host for artemisinin production. The introduction of *ADS* into *A. absinthium* might lead to the generation of artemisinin-producing *A. absinthium*, which could be used as an alternative approach to produce artemisinin in other *Artemisia* species. To prove this hypothesis, this research is now ongoing in our laboratory.

Next challenge: artemisinin-resistant *Plasmodium* parasites

Artemisinin is the most effective antimalarial drug and has been used as an ACT to treat malaria for over a decade. However, the emergence of artemisinin-resistant *Plasmodium* parasites in Southeast Asia, prolonging the parasite clearance rate in patients, has been reported recently and has become a critical issue [141–144]. No correlation between resistance and other previously proposed candidate targets of artemisinin (*PfATP6* and *PfTCTP*) was detected [145]. However, it has been suggested that the resistance occurs predominantly during the early ring stage of parasite development as a result of the multiple forms of mutations in the *PF3D7_1343700* kelch propeller domain (K13-propeller) on chromosome 13 [146–155]. K13-propeller mutations lead to the increase of phosphatidylinositol-3-kinase (PfPI3K), which is required for the mediation of cell signaling and survival [156, 157], and prolong parasite development at the ring stage when the activation level of artemisinin is rather low [5, 7, 158]. The B subfamily of ABC transporters, known as multidrug resistance proteins (MDR), also promotes artemisinin resistance. In artemether–lumefantrine post-treatment infections, alleles of *Pfmdr1* tended to have 86N, 184F, and 1246D, rather than the common YYY haplotype, and increased the number of treatment failures [159]. The deletion of *Pfmdr5* induced greater sensitivity to artemisinin treatment, suggesting that this gene might contribute to artemisinin resistance as well [160].

Table 1 Heterologous production of artemisinin intermediates

Host	No.	Transgenes or modifications	Product	Yield	References
N. benthamiana	1	P_{35S}-*tHMGR-FPS-ADS*, P_{35S}-*CYP71AV1*	Artemisinic acid 12-β-diglucoside	39.5 mg/kg FW	[106]
Tobacco chloroplasts	2	P_{rrn16S}-*atoB-HMGS-HMGR-MK-PMK-MVD1*, P_{psbA}-*E. coli IDI-FPS-ADS-CYP71AV1-AaCPR*	Artemisinic acid	0.1 mg/g FW	[107]
E. coli	3	P_{lac}-*MevT*[a], P_{lac}-*MBIS*[b], P_{trc}-*ADS*	Amorpha-4,11-diene	24 mg/L	[109]
	4	Same as 3 but overlaid with dodecane	Amorpha-4,11-diene	500 mg/L	[110]
	5	P_{BAD}-*MevT*, P_{BAD}-*tHMGR1*	Mevalonate	Threefold from CT[c]	[111]
	6	P_{lacUV5}-*MevT* (codon opt.)-*MBIS*, P_{trc}-*ERG12* (codon opt.)-*ADS*	Amorpha-4,11-diene	293 mg/L	[112]
	7	P_{lacUV5}-*MevT* (codon opt.) with *HMGS* and *HMGR* from *S. aureus*, P_{lac}-*MBIS*, P_{lac}-*ADS*	Amorpha-4,11-diene	27.4 g/L	[113]
	8	P_{BAD}-*dxs-IDI-ispDF*, *ADS* with *Δpts* and optimized medium	Amorpha-4,11-diene	182 mg/L	[114]
	9	P_{TM2}-*galP-glk*, P_{T7}-*dxs-IDI-ispA-ADS*	Amorpha-4,11-diene	201.2 mg/L	[115]
	10	*AcrB*, *TolC* (*x2*), *ADS* (codon opt.)	Amorpha-4,11-diene	404.83 mg/L	[116]
	11	P_{BAD}-*dxs-IDI-ispDF*, P_{araBAD}-*ADS*, P_{TM1}-*macAB-TolC*	Amorpha-4,11-diene	∼30 mg/L/OD	[117]
	12	Same as 3 with *CYP71AV1* (codon opt., engineered N-terminal transmembrane)-*AaCPR*	Artemisinic acid	105 mg/L	[118]
	13	Same as 12 but replaced *CYP71AV1* with $P450_{BM3}$	Artemisinic-11S,12-epoxide	250 mg/L	[119]
S. cerevisiae	14	P_{GAL1}-*ADS*	Amorpha-4,11-diene	600 μg/L	[120]
	15	P_{GAL1}-*tHMGR* P_{GAL1}-*upc2-1* *erg9*::P_{MET3}-*ERG9* P_{GAL1}-*tHMGR* P_{GAL1}-*ERG20*, P_{GAL1}-*ADS* P_{GAL10}-*CYP71AV1* P_{GAL1}-*AaCPR*	Artemisinic acid	100 mg/L	[121, 122]
	16	Same as 15 with optimized culture condition	Artemisinic acid	2.5 g/L	[123]
	17	*gal80Δ*::*nat*[r] *MAT a erg9Δ*::*kan*[r] P_{MET3}-*ERG9*, *leu2-3,112*::*HIS* P_{GAL1}-*MVD1* P_{GAL10}-*ERG8* *his3Δ1*::*HIS* P_{GAL1}-*ERG12* P_{GAL10}-*ERG10ade1Δ*::P_{GAL1}-*tHMG1* P_{GAL10}-*IDI1 ADE1 ura3-52*::P_{GAL1}-*tHMG1* P_{GAL10}-*ERG13 URA3trp1-289*::P_{GAL1}-*tHMG1* P_{GAL10}-*ERG20 TRP1* [pAM322]	Amorpha-4,11-diene	41 g/L	[124]
	18	Same as 17. but replaced *gal80Δ*::*nat*[r] with *gal1Δ gal7Δ gal10Δ*::*hphA*	Amorpha-4,11-diene	37 g/L	[124]
	19	*gal1Δ,gal10Δ,gal7Δ*::P_{GAL3}-*CPR1natA*, *erg9Δ*::*dsdAP*$_{CTR3}$- *ERG9*, *leu2-3,112*::*kanAP*$_{GAL7}$-*AaCYB5P*$_{GAL1}$-*ERG19P*$_{GAL10}$- *ERG8*, *ade1Δ*::P_{GAL1}-*tHMG1P*$_{GAL10}$-*IDI1_ADE1*, *his3Δ1*::*hphAP*$_{GAL7}$-*AaALDH1P*$_{GAL1}$-*ERG12P*$_{GAL10}$-*ERG10*, *ura3-52*:: P_{GAL1}-*tHMG1P*$_{GAL10}$-*ERG13hisG*, *trp1-289*:: P_{GAL1}- *tHMG1P*$_{GAL10}$-*ERG20TRP1*, *ndt80Δ*::P_{TDH1}-*HEM1HIS3PPGK1-CTT1*, *gal80Δ*::*URA3P*$_{GAL7}$-*AaADH1*, [pAM552: 2μ-*LEU2d* P_{GAL1}-*ADS* P_{GAL10}-*CYP71AV1*]	Artemisinic acid	25 g/L	[126, 127]

[a] *MevT* operon consists of *atoB-HMGS-tHMGR*

[b] *MBIS* operon consists of *ERG12-ERG8-MVD1-IDI-ispA*

[c] Production yield as compared to control (CT)

Current situation of malaria infection and ongoing studies on antimalarial drug development

Since ACTs have become the major treatment for malaria and strict preventive measures against parasite-infected mosquitoes have been implemented, the malaria-related mortality rate and case incidence have decreased gradually during the past 10 years [1]. Although artemisinin-resistant *Plasmodium* parasites have emerged and show a significant delay in clearance rate, the response of dihydroartemisinin against either wild-type parasites or mutants exhibits similar K_m values suggesting that dihydroartemisinin does not lose its activity against the mutants [161]. Extending the treatment courses could be an effective strategy to clear resistant parasite infection. However, the parasites can still develop complete resistance against artemisinin-based treatment at any point in the future. In addition, the proportion of malaria-infected patients is concentrated in countries with low national income levels. Among these, more than 68 million infected children do not receive any ACTs [1]. Therefore, large amounts of low-cost artemisinin for ACTs, by either increasing the cultivation of high-artemisinin-producing *A. annua* plants or developing cheaper synthetic biological processes in the long term, are required to prevent any further development of parasites and meet the demand of ACTs worldwide. Moreover, novel effective antimalarial treatments must be developed continually. Recently, low-cost plant-based artemisinin combination therapy (pACT) has driven attention on the production of no semi-synthetic artemisinin *in planta* as this treatment showed higher antimalarial activity, and the synergistic effect of artemisinin and the plant matrix overcame resistance to artemisinin [162–169]. Several scientists have also focused on the investigation of novel potential drug targets [170–180] and on the synthesis of novel antimalarial compounds including artemisinin hybrids [181–186]. Still, further studies on these avenues are required.

Conclusion

Several approaches to enhance the production of artemisinin have been investigated for over a decade. As a result, the availability of artemisinin for ACTs is increasing, and the number of malaria-related deaths is decreasing gradually. Although artemisinin is still effective against malaria-causing parasites, the emergence of artemisinin-resistant strains has posed a new challenge to scientists worldwide. Therefore, elucidating the mode of action of artemisinin and the mechanism of resistance against this compound in *Plasmodium* parasites is important for further development of antimalarial drugs. We hope that the current understanding of artemisinin as summarized in this review will provide clues for further investigation and development of antimalarial treatments to overcome artemisinin resistance in *Plasmodium* parasites in the future.

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