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 combinational 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 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.

Keywords Artemisia annua · Artemisinin · Resistant parasites · Malaria · Metabolic engineering · Mode of action

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 technolo-
gies, synthetic biology, and total and semi-syntheses of
artemisinin, have been investigated to enhance the produc-
tion and reduce the cost of this compound. In addition,
the recent emergence of artemisinin-resistant Plasmodium para-
sites 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 develop-
ment, are discussed.

Discovery of artemisinin

Before the discovery of artemisinin, powder derived from
chinchona 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 drug until the 1920s. The quini-
ne derivative chloroquine was developed as a new
effective antimalarial drug once quinine-resistant Plas-
modium 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-resis-
tant 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 compo-
nents were then extracted from the leaves of mature plants
in 1971 [2, 10–12]. After purification, the active anti-
malarial compound, named qinghaosu or artemisinin, was
obtained as colorless needle-like crystals. Its stereochem-
istry 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 dihy-
droartemisinin, 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 recom-
mended 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
strategy for malaria to prevent recurrence and develop-
ment of resistance in malaria-causing parasites, whereas
the monotherapy is considered as an inappropriate treat-
ment [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 inter-
mediates [22, 23] (Fig. 1). The following step is the oxida-
tion 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 arteann-
uin 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,
ALDH1 oxidizes dihydroartemisinic aldehyde into dihydroadamisinic acid, which is converted non-enzymatically into artemisinic [26, 29], as shown in Fig. 1. Rydén et al. [30] discovered dihydroartemisinic aldehyde reductase 1 (RED1), which reduces dihydroartemisinic aldehyde into dihydroadamisinic 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. Olofsen 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 artemisinic 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(II) 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(II) 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
Ca**2+** P/ATP6, an orthologous sarco/endoplasmic reticulum that artemisinin specifically mediated the inhibition of malfunction \[52, 53\]. Eckstein-Ludwig et al. \[54\] showed that artemisinin covalently bonds with this protein, resulting in protein malfunction. However, the formation of the artemisinin-free heme complex shows an inhibitory effect on this conversion \[51\]. A translationally controlled tumor protein (PtTCTP) 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, as it could form a covalent bond with this protein, resulting in protein malfunction \[52, 53\].

**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 biosynthesis 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 diatel 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. tunefaciens- 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 phythohormone also regulates trichome-specific fatty acyl-CoA reductase 1 (TFARI), 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 indica, and a nitrogen-fixing bacterium Azotobacter chroococcum [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 amorph-4,11-diene during culturing, the culture media was overlaid with dodecane to trap amorph-4,11-diene, referred to as a two-phase partitioning bioreactor. As a result, they recovered the volatilized amorph-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 amorph-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 amorph-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 (P450bhm3) and could produce artemisinic-11S,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 amorph-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 \textit{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 \textit{HMGR (hHMG1)} 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, \textit{CYP71AV1, CPR, ADH1, and ALDH1}, to oxidize amorpha-4,11-diene into artemisinic acid. Cytochrome \textit{b5 (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 \textit{Artemisia} species**

Some studies reported that artemisinin is produced in other \textit{Artemisia} species [129–134]. However, we attempted to isolate artemisinin from other \textit{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 \textit{A. afra} and \textit{A. absinthium} as they are widely cultivated in Africa and exhibit anti-plasmodial activity [135–138].

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

**Next challenge: artemisinin-resistant \textit{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 \textit{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 (\textit{PfATP6} and \textit{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 \textit{PF3D7_1343700} kelch propeller domain (K13-propeller) on chromosome 13 [146–155]. K13-propeller mutations lead to the increase of phosphatidylinositol-3-kinase (PI3K), 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 \textit{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 \textit{Pfmdr5} induced greater sensitivity to artemisinin treatment, suggesting that this gene might contribute to artemisinin resistance as well [160].
| Host          | No. | Transgenes or modifications                                                                 | Product                          | Yield                        | References |
|--------------|-----|-----------------------------------------------------------------------------------------------|----------------------------------|------------------------------|------------|
| *N. benthamiana* | 1   | $P_{ASS}^{+}$HMGFR-FPS-ADS, $P_{ASS}^{+}$CYP71AV1                                              | Artemisinic acid 12-β-diglucoside | 39.5 mg/kg FW                | [106]      |
| **Tobacco chloroplasts** | 2   | $P_{mib5}$-atoB-HMGMS-HMG-MK-PMK-MVD1, $P_{pshB}$-E. coli IDI-FPS-ADS-CYP71AV1-AacCPR          | Artemisinic acid                 | 0.1 mg/g FW                   | [107]      |
| *E. coli*    | 3   | $P_{lac}$-MevT*, $P_{lac}$-MBIS*, $P_{lac}$-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, $P_{BAD}$-HMGRI1                                                              | Mevalonate                       | Threefold from CT*            | [111]      |
|              | 6   | $P_{lac}$-PG3-MevT (codon opt.)-MBIS, $P_{lac}$-ERG12 (codon opt.)-ADS                       | Amorpha-4,11-diene               | 293 mg/L                     | [112]      |
|              | 7   | $P_{lac}$-PG3-MevT (codon opt.) with HMGs and HMGs from *S. aureus,* $P_{lac}$-MBIS, $P_{lac}$-ADS | Amorpha-4,11-diene               | 27.4 g/L                     | [113]      |
|              | 8   | $P_{BAD}$-dxx-IDI-ispDF, ADS with Δpts and optimized medium                                  | Amorpha-4,11-diene               | 182 mg/L                     | [114]      |
|              | 9   | $P_{TM2}$-galP-glk, $P_{Tr}$-dxx-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  | $P_{BAD}$-dxx-IDI-ispDF, $P_{mmBAD}$-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)-AacCPR              | Artemisinic acid 11S,12-epoxide  | 105 mg/L                     | [118]      |
|              | 13  | Same as 12 but replaced CYP71AV1 with $P_{450BM3}$                                           | Artemisinic acid                 | 250 mg/L                     | [119]      |
| *S. cerevisiae* | 14  | $P_{GAL1}$-ADS                                                                              | Amorpha-4,11-diene               | 600 μg/L                     | [120]      |
|              | 15  | $P_{GAL1}$-HMG R $P_{GAL1}$-4pc2-1 erg9::$P_{MetP}$-ERG9 $P_{GAL1}$-HMG R $P_{GAL1}$-ERG20, $P_{GAL1}$-ADS $P_{GAL10}$-CYP71AV1 $P_{GAL1}$-AacCPR | 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' MAT a erg9Δ::kan' $P_{METP}$-ERG9, leu2-3,112::His $P_{GAL1}$-MVD1 $P_{GAL10}$-ERG8 hist3Δ1::His $P_{GAL1}$-ERG12 $P_{GAL10}$-ERG10ade1Δ::$P_{GAL1}$-HMG1 $P_{GAL10}$-ID1 ADE1 ura3-52::$P_{GAL1}$-HMG1 $P_{GAL10}$-ERG13 URA3trp1-289::$P_{GAL1}$-HMG1 $P_{GAL10}$-ERG20 TRP1 [pAM322] | Artemisinic acid                 | 41 g/L                   | [124]      |
|              | 18  | Same as 17, but replaced gal80Δ::nat' with gal1Δ gal7A gal10A::hphA                          | Amorpha-4,11-diene               | 37 g/L                       | [124]      |
|              | 19  | gal1Δ,gal10Δ,gal7A::$P_{GALF}$-CPR1natA, erg9Δ::dsdAP365-CPR1-ERG9, leu2-3,112::kanA $P_{GAL7}$-AaCYB5 $P_{GAL7}$-ERG19 $P_{GAL7}$-ERG8, ade1Δ::$P_{GAL1}$-HMG1 $P_{GAL10}$-ID1 ADE1, his3Δ1::hphA $P_{GAL7}$-AaALDH1 $P_{GAL1}$-ERG12 $P_{GAL10}$-ERG10, ura3-52:: $P_{GAL7}$-HMG1 $P_{GAL10}$-ERG13hisG, trp1-289:: $P_{GAL7}$-HMG1 $P_{GAL10}$-ERG20TRP1, nds80Δ::pT7his6-HEM1His3PPGK1-CTT1, gal80Δ::URA3 $P_{GAL7}$-AaADH1, [pAM552: 2µ-LEU2d $P_{GAL7}$-ADS $P_{GAL10}$-CYP71AV1] | Artemisinic acid                 | 25 g/L                   | [126, 127] |

*a MevT operon consists of atoB-HMGs-HMG

*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 Km 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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Conflict of interest The authors declare that they have no conflict of interest.

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