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Review

Metabolic regulation of the artemisinin biosynthetic pathway in *Artemisia annua* L.

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Artemisinin was the most effective antimalarial drug in the world and it was a sesquiterpene lactone extracted from a traditional Chinese medicinal herb, *Artemisia annua* L., Due to its great antimalarial activity but extremely low content of wild-type *A. annua* plants, many research groups had focused on enhancing the content of artemisinin in *A. annua* plants. In this review, it mainly discussed the recent research advances on several different metabolic regulation approaches for enhanced artemisinin production.

Key words: *Artemisia annua*, artemisinin, metabolic regulation, biosynthetic pathway.

INTRODUCTION

As we all know, being the world's most severe disease caused by *Plasmodium falciparum* infection, malaria had affected at least 300 million people especially in developing nations, such as African, and it had caused more than a million deaths annually, which brought great loss to people not only economically but also spiritually (Greenwood and Mutabingwa, 2002). Although many efforts had been tried to control malaria, no apparent result had been obtained except the artemisinin combination therapies recommended by the world health organization (WHO) (Graham et al., 2010). Due to its high efficacy, fast action, and no serious side effect, artemisinin was regarded as a most promising and potential antimalarial drug, especially in the treatment of cerebral malaria. Besides its antimalarial activity, artemisinin also had great activity against hepatitis B, schistosomiasis, small-cell lung carcinomas and drug-resistant cancers and so on (Romero et al., 2005; Sadava et al., 2002). The ancient Chinese scripts had once described the use of *Artemisia annua* for treating diseases. In all, it is not hard to see the significance of researching on artemisinin. Artemisinin was first extracted from a traditional Chinese medicinal herb, *A. annua* L., primarily from the aerial parts, such as leaves and flowers, then the stems, but nearly no artemisinin was detected in roots in our experiments. A latest research found that artemisinin was biosynthesized in glandular trichomes of *A. annua* (Wang et al., 2009). At the same time, some researchers had detected the content of artemisinin at different stages during life cycle, and it came out at two stages with the highest content, one stage was just before flowering, while another was just during the full flowering period (Abdin et al., 2003; Wang et al., 2004).

Furthermore, someone once reported that artemisinin content was highly heritable with various *A. annua* strains (Delabays et al., 2001). So, it can easily come to a conclusion that the artemisinin content varied considerably from different tissues, different life stages and even different strains.

However, the content of artemisinin and its active derivatives mainly extracted from the wild plants of *A. annua* is so limited, ranging from 0.01 to 0.5% of dry weight, which makes them so expensive (Wallaart et al., 2000).

Additionally, artemisinin as a complex molecule can be

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chemically synthesized, but it is not as that economical, and also toxiferous with very limited production. Due to the foregoing reasons, it is hard for artemisinin to become commercialization and meet the increasing demand of the world market. Anyway, artemisinin is in seriously short supply. In recent years, with the development of genetics, cell biology and molecular biology, the biosynthetic pathways of artemisinin have been made clear gradually. Some key enzyme genes in the pathways have been cloned, characterized and even transgenic *A. annua* plants with high content artemisinin have been obtained. Furthermore, many methods have been tried to enhance the production of artemisinin, such as overexpression of the endogenous genes or exogenous genes, even transcriptional factor in *A. annua*, and treatment of *A. annua* with exogenous hormone. In all, the foregoing metabolic regulation methods have been paid increasing attention and made great progress, which will make the price of artemisinin-based antimalarial drugs affordable by more and more patients. Therefore, any breakthrough in this field may bring great benefits to the world.

THE BIOSYNTHETIC PATHWAYS OF ARTEMISININ

Different kinds of terpenoids in nature shared a common precursor, isopentenyl diphosphate (IPP), of course, artemisinin recognized as a sesquiterpene lactone was not an exception (Croteau et al., 2000). In artemisinin biosynthesis, there were two independent pathways producing IPP. One was the classical cytosolic mevalonate pathway (MVA) originating from acetyl CoA, the other was the methylerythritol phosphate pathway (MEP), starting from pyruvate. Although it was generally accepted that IPP was provided by the cytosolic MVA pathway, it found that the plastidial MEP pathway also could produce IPP for biosynthesis of sesquiterpene in *A. annua* (Olofsson et al., 2011). In a previous experiment, it demonstrated that the MVA pathway provided the major part of the carbon skeleton for artemisinin biosynthesis (Ram et al., 2010). Anyway, both the MVA and MEP pathways are involved in artemisinin biosynthesis (Figure 1). Besides, there was an artemisinin-specific pathway starting from the formation of FPP with IPP and DMAPP as substrates (Weathers et al., 2006) (Figure 2). Much biological information about the genes of enzymes in the three pathways was available to us by now (Table 1).

METABOLIC REGULATION OF THE BIOSYNTHETIC PATHWAYS FOR ENHANCED ARTEMISININ

In recent years, to break the bottle-neck reactions involved in the targeted pathway through overexpression of the rate-limiting enzymes is one of the most successful strategies in metabolic engineering. Some functional genes involved in the artemisinin biosynthetic pathways have been over expressed in *A. annua* with notably elevated artemisinin, such as *hmgr*, *fps*, *ads*, *cyp71av1*, *cpr* and *antsqs*.

Overexpression of *hmgr* in *A. annua*

It is available to produce transgenic plants of *A. annua* with exogenous gene to improved artemisinin. In a report, it transformed *hmgr* gene from *Catharanthus roseus* (L.) G. into *A. annua,* and the transgenic plants were obtained successfully. The most important was that artemisinin content of one transgenic *A. annua* improved for 22.5% rather than the wild-type, (Aquil et al., 2009; Nafis et al., 2010).

Furthermore, another example was that overexpression of the blue light receptor gene cryptochrome1 *(cry1)* derived from *arabidopsis* in *A. annua,* which resulted in increased accumulation of both artemisinin and anthocyanins, at the same time, the expression of three important genes *ads*, *cyp71av1*, and *fps* were promoted (Hong et al., 2009). Through the previous two examples, it is easy to think that overexpression other species' genes in *A. annua* can also bring us with surprising enhanced artimisinin.

Overexpression of *fps* in *A. annua*

Overexpression of the endogenous *fps* gene in *A. annua* led to highly enhanced artemisinin, which the content was 34.4% higher than that in non-transgenic *A. annua,* approximately 0.9% of dry weight (Han et al., 2006; Chen et al., 2000). Later, a paper published in 2010 furthered the result; it found that there was also reduced artemisnin content of transgenic *A*. *annua* plants due to gene silence of *fps* (Banyai et al., 2010a). So, it was necessary to screen the transgenic clones in which the transgenic *fps* gene was not silenced.

Overexpression of *cyp71av1* in *A. annua*

CYP71AV1 is a multifunctional enzyme which can catalyze the formation of artemisinic alcohol, artemisinic aldehyde, and artemisinic acid. To enhance artemisinin content, *cyp71avl* and *cpr* were cloned from *A. annua*, the binary vector was constructed, and with agrobacterium-mediated transformation, transgenic plants were obtained which showed a highest artemisinin content reached 2.4-fold than that of control (Jing et al., 2008). It just demonstrated that co-expression of *cyp71avl* and *cpr* in *A. annua* was an effective method in metabolic regulation for artemisinin production.

Over expression of *antsqs* in *A. annua*

Inhibition of the enzyme SQS catalyzing the competitive

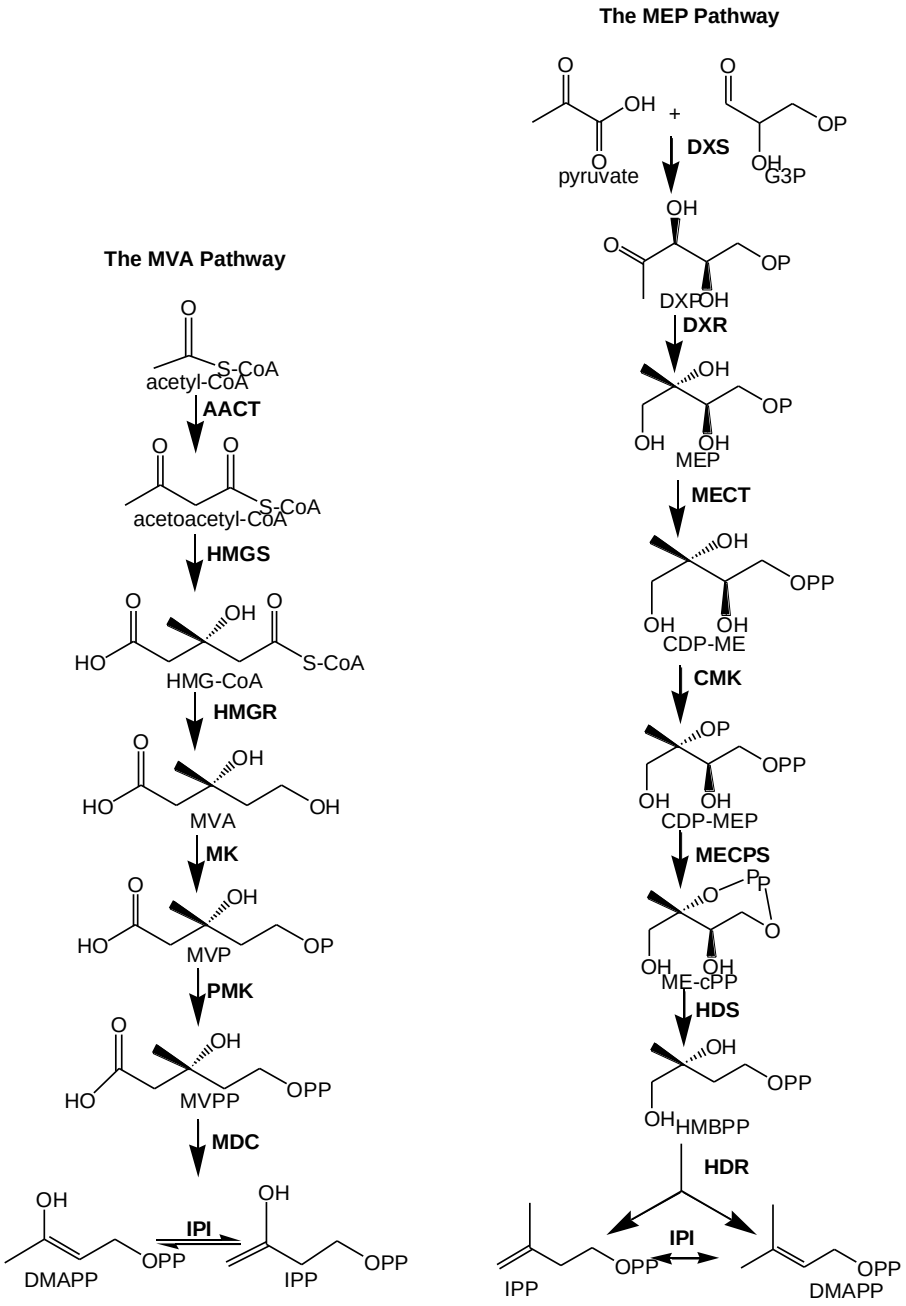

Figure 1. The MVA and MEP pathways. There are seven enzymatic reactions in the up-stream MVA and MEP pathways of artemisinin biosynthetic pathway, respectively.

sterol biosynthesis is an available method in metabolic regulation, which the branch pathway may be blocked, on the contrary, the artemisinin content can be improved by using of anti-sense RNA and/or RNA interference (RNAi) technology.

The antisense squalene synthase (antSQS) gene was cloned and introduced into the genome of *A. annua*, with the synchronous decline of *sqs* mRNA and total sterols, mRNA coding for *ads, cyp71av1* and *cpr* elevated and presented a bias in redirection of partially blocked metabolic flow to amorpha-4,11-diene with overproduced artemisinin (Li et al., 2009; Yang et al., 2008). One more example was hairpin-RNA-mediated RNAi (RNA interference) technique, which could also increase the artemisnin content by suppress the expression of *sqs*, and a highest artemisinin content reached 31.4 mg/g of dry weight, about 3.14-fold of non-transgenic *A. annua* (Zhang et al., 2009). In all, the competitive pathway had

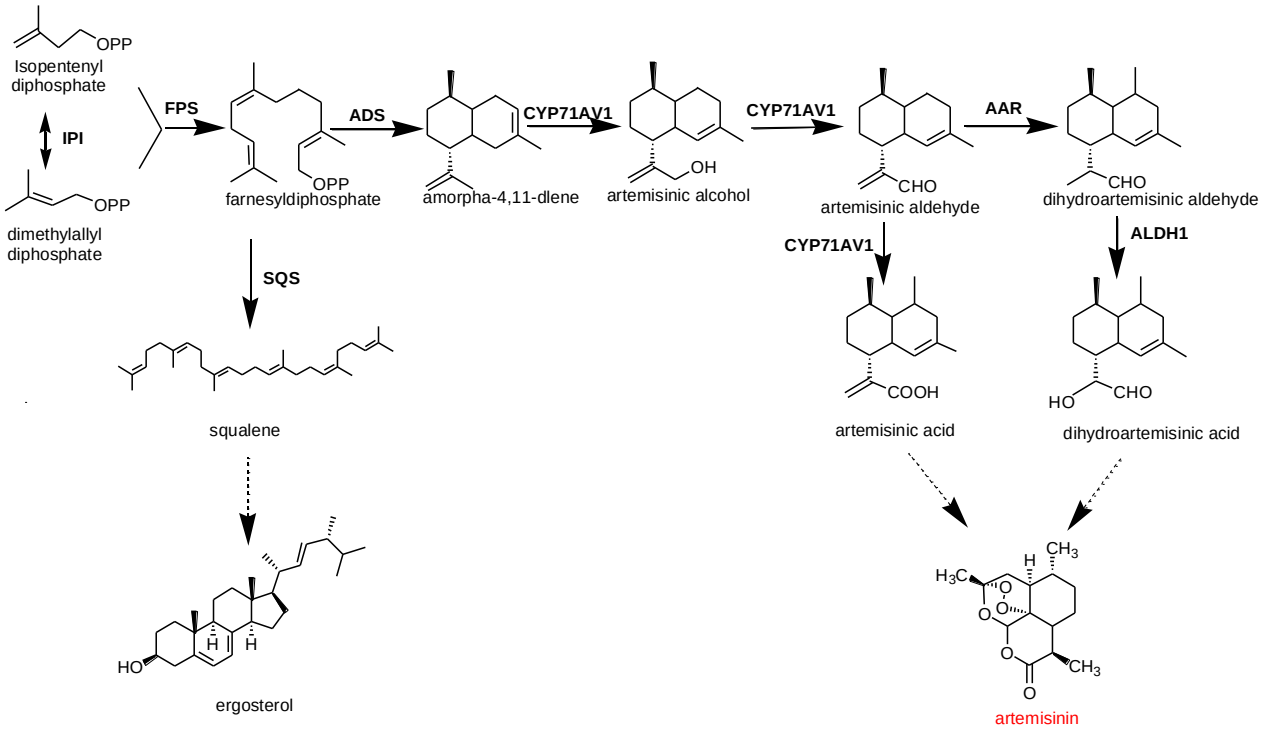

Figure 2. The artemisinin-specific pathway. There are five enzymes involved in the pathway, FPS, ADS, CYP71AV1, AAR and ALDH1. While, SQS catalyzes the competitive pathway.

been down-regulated, while the artemisinin biosynthetic pathway had been up-regulated, which demonstrated that the blocking method was just an efficient method in metabolic engineering for artemisinin production.

Overexpression transcription factors in *A. annua*

The WRKY transcriptional factor of *A. annua* was named AaWRKY. AaWRKY was overexpressed in *A. annua,* which resulted in activation of *hmgr*, *cyp71av1*, and especially the *ads* gene (Ma et al., 2009). Therefore, AaWRKY had participated in the metabolic regulation of artemisinin biosynthesis, and the thoughts to produce transgenic *A. annua* plants with transcriptional factor is a promising method for high content of artemisinin.

EXOGENOUS HORMONE TREATMENT FOR HIGH CONTENT ARTEMISININ

One more supplemental method of metabolic regulation for enhanced artemisinin is that which use the exogenous hormone to treat the wild-type *A. annua*, and the transcription level of relative endogenous genes can be elevated. Four commonly researched exogenous hormones are abscisic acid (ABA), methyl jasmonate (MeJA), gibberellic acid (GA_3) and salicylic acid (SA).

Firstly, the artemisinin content was increased significantly in ABA-treated *A. annua* plants, and *hmgr*, *fps*, *cyp71av1*, *cpr* were induced remarkably (Jing et al., 2009). Secondly, the artemisinin content in MeJA treated *A. annua* plants increased 49%, together with an increase in artemisinin acid and dihydroartemisinic acid, at the same time, the *ads* gene was up-regulated (Wang et al., 2010; Guo et al., 2010). Thirdly, a paper published in 2009 reported the result of SA treatment on *A. annua.* It demonstrated that the expression of *hmgr* and *ads* was increased gradually (Pu et al., 2009). Fourthly, in a GA3 treatment experiment, both the wild and transgenic *A. annua* were treated with GA3, and the expression of *fps*, *ads* and *cyp71av1* was dramatically increased in both type of plants, but the artemisinin content showed a delayed increase (Banyai et al., 2010b). In all, exogenous hormone treatment can promote artemisinin biosynthesis both in transgenic and non-transgenic *A. annua* plants.

PERSPECTIVES

Firstly, several metabolic regulation approaches, including breaking the bottle-neck of artemisinin biosynthetic pathway through overexpression genes or transcriptional factor, blocking the competitive branch pathway, and treating A. annua with exogenous hormone, have been reviewed in this article, and all are available for producing enhanced artemisinin. Secondly, the transcription factor mediated transformation is a very

Table 1. Biological information of enzymes involved in artemisinin biosynthesis. All enzymes involved in the MVA, MEP pathways and artemisinin-specific-pathway are presented as follows.

Enzymes	Substrates	Products	Plant source	GenBank No.	References
ACCT	Acety-CoA	Acetoacety-CoA	*A. thaliana*	AF364059	Gual et al. (1992)
HMGS	Acetoacety-CoA	HMG-CoA	*A. thaliana*	NM_117251	Luskey et al. (1985)
HMGR	HMG-CoA	MVA	*Catharanthus roseus*	AY623812	Abdin et al. (2003)
			Artemisia annua	AF142473	Chen et al. (2000)
MK	MVA	MVP	*A. thaliana*	NP_198097.1	Lluch et al. (2000)
PMK	MVP	MVPP	*A. thaliana*	AC079041.4	Tsay et al. (1991)
MDC	MVPP	IPP	*A .thaliana*	NM_115285.1	Dhe-Paganon et al. (1994)
IPI	IPP and DMAPP	IPP or DMAPP	*A. thaliana*	U49259	Wouters et al. (2003)
DXS	Pyruvate and G3P	DXP	*Artemisia annua*	AF182286.2	Sprenger et al. (1997)
DXR	DXP	MEP	*Artemisia annua*	AF182287.2	Graham et al. (2010)
MECT	MEP	CDP-ME	*Taxus x media*	EF534010	Rohdich et al. (2000)
CMK	CDP-ME	CDP-MEP	*A. thaliana*	AAG01340	Steinbacher et al. (2003)
MECPS	CDP-MEP	ME-cPP	*Ginkgo biloba*	AY971576	Herz et al. (2000)
HDS	ME-cPP	HMBPP	*Artemisia annua*	FJ479720	Baker et al. (1992)
HDR	HMBPP	IPP or DMAPP	*Artemisia annua*	GQ119345	Peng et al. (2011)
FPS	IPP and DMAPP	FPP	*Artemisia annua*	AF112881	Matsushita et al. (1996)
ADS	FPP	Amorpha-4, 11-diene	*Artemisia annua*	EF197888	Mercke et al. (2000)
CYP71AV1	Amorpha-4, 11-diene	Artemisinic alcohol, artemisinic aldehyde or artemisinic acid	*Artemisia annua*	DQ268763	Ro et al. (2005) and Teoh et al. (2006)
AAR	Artemisinic aldehyde	Dihydroartemisinic aldehyde	*Artemisia annua*	EU704257	Zhang et al. (2008)
ALDH1	Dihydroartemisinic aldehyde	Dihydroartemisinic acid	*Arabidopsis thaliana*	NP_566749	Teoh et al. (2009)
SQS	Farnesyl diphosphate	Squalene	*Artemisia annua*	AY445506	Yang et al. (2008)
CPR			*Artemisia annua*	DQ984181	Jing et al. (2008)
AaWRKY			*Artemisia annua*	FJ390842	Ma et al. (2009)

very promising method in the metabolic regulation of artemisnin biosynthesis which can activate several genes in the pathway and it has wide world market, but more information is needed in this field, only one transcription factor named AaWRKY has been isolated from *A. annua* by now.

Thirdly, *hmgr*, *dxr*, *hds* and *hdr* on the up-stream pathways, MVA or MEP pathway, have been cloned from *A. annua,* and only exogenous *hmgr* has been transformed into *A. annua.* Although, transgenic plants of *fps*, *cyp71av1*, and anti sense sqs on the artimisinin-specific-pathway have been obtained, the transgenic *A. annua* plants with *ads*, *aar* and *aldh1* have not been gotten.

In all, future work can turn to those un-reached fields which would complement the metabolic regulation of artemisinin biosynthesis.

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Abbreviations: AACT, **Acety-CoA,** Acetyl-CoA C-acetyltransferase; **AAR,** artemisinic aldehyde Δ11(13) reductase; **ADS,** amorpha-4,11-diene synthase; **ALDH1,** aldehyde dehydrogenase; **CDP-ME,** 4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol; **CDP-MEP,** 4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol 2-phosphate; **CMK,** 4-(Cytidine5'- diphospho)-2-C-methyl- D-erythritol kinase; **CPR,** cytochrome P-450 reductase; **CYP71AV1,** amorpha-4,11-diene hydroxylase; **DMAPP,** dimethylallyl diphosphate; **DXP,** 1-Deoxy-D-xylulose 5-phosphate; **DXR,** 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; **DXS,** 1-Deoxy-D-xylulose 5-phosphate synthase; **FPP,** farnesyl diphosphate; **FPS,** farnesyl diphosphate synthase; **G3P,** glyceraldehyde 3-phosphate; **HDS,** hydroxymethylbutenyl 4-diphosphate synthase; **HMBPP,** 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphoaphate; **HMGR,** 3-hydroxy-3-methylglutaryl-CoA reductase; **HMGS,** 3-hydroxy-3-methylglutaryl-CoA synthase; **HMG-CoA,** 3S-Hydroxy-3-methylglutaryl-CoA; **HDR,** IPP/DMAPP synthase; **IPI,** isopentenyl diphosphate isomerase; **IPP,** isopentenyl diphosphate; **MCT,** 2-C-Methyl-D-erythritol 4-phosphate cytidyltransferas; **MDC,** mevalonate 5-diphosphate decarboxylase; **ME-cPP,** 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; **MECPS,** 2-C-Methylerythritol 2, 4-cyclodiphosphate synthase; **MEP,** 2-C-Methy-D-erythritol 4-phosphate; MK: mevalonate kinase; MVA: 3R-Mevalonic acid; MVP: mevalonic acid-5-phosphate; **MVPP,** mevalonate diphosphate; **PMK,** mevalonate 5-diphosphate kinase; **SQS,** squalene synthase.

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