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REVIEW

Metabolic engineering of artemisinin biosynthesis in *Artemisia annua* L.

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Abstract Artemisinin, a sesquiterpene lactone isolated from the Chinese medicinal plant *Artemisia annua* L., is an effective antimalarial agent, especially for multi-drug resistant and cerebral malaria. To date, *A. annua* is still the only commercial source of artemisinin. The low concentration of artemisinin in *A. annua*, ranging from 0.01 to 0.8% of the plant dry weight, makes artemisinin relatively expensive and difficult to meet the demand of over 100 million courses of artemisinin-based combinational therapies per year. Since the chemical synthesis of artemisinin is not commercially feasible at present, another promising approach to reduce the price of artemisinin-based antimalarial drugs is metabolic engineering of the plant to obtain a higher content of artemisinin in transgenic plants. In the past decade, we have established an *Agrobacterium*-mediated transformation system of *A. annua*, and have successfully transferred a number of genes related to artemisinin biosynthesis into the plant. The various aspects of these efforts are discussed in this review.

Keywords *Artemisia annua* · Artemisinin · Metabolic engineering · Metabolic profiling

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Introduction

Artemisinin (also called Qinghaosu) is a sesquiterpene lactone with an unusual endoperoxide structure (see Fig. 1). It was isolated from the herb *Artemisia annua* L. by Chinese scientists when searching for novel antimalarial drugs in the 1970s (Li et al. 2006a; Liu et al. 1979). Today, artemisinin derivatives provide the basis for the most effective treatments for malaria, particularly in the form of artemisinin-based combination therapies (ACTs) which are advocated by the World Health Organization (WHO) in order to reduce the odds of developing resistance (Duffy and Mutabingwa 2006; White 2008). Since the identification of the antimalarial activity of artemisinin, significant efforts have been made to increase the production of this compound, including chemical synthesis, plant cell cultures, hairy root cultures and fermentation of the engineered microorganism. However, none of these methods is commercially feasible and the *A. annua* plant is currently still the only commercial source of artemisinins. The low artemisinin content in *A. annua*, ranging from 0.01 to 0.8% of the plant dry weight, makes artemisinin a relatively expensive drug, especially for economically disadvantaged people in developing countries where malaria frequently occurs. Therefore, in the last decade, we initiated metabolic engineering of the plant in an attempt to obtain a higher content of artemisinin in transgenic plants to reduce the price of artemisinin. The different aspects of this work are summarized in this review.

The project leading to the development of artemisinin-based malaria treatment started on May 23, 1967, and was called the "523 Project". It was a large joint project, and 10 provinces, 60 institutions, and 500 scientists were involved in the project. They examined 10,000 secret recipes and

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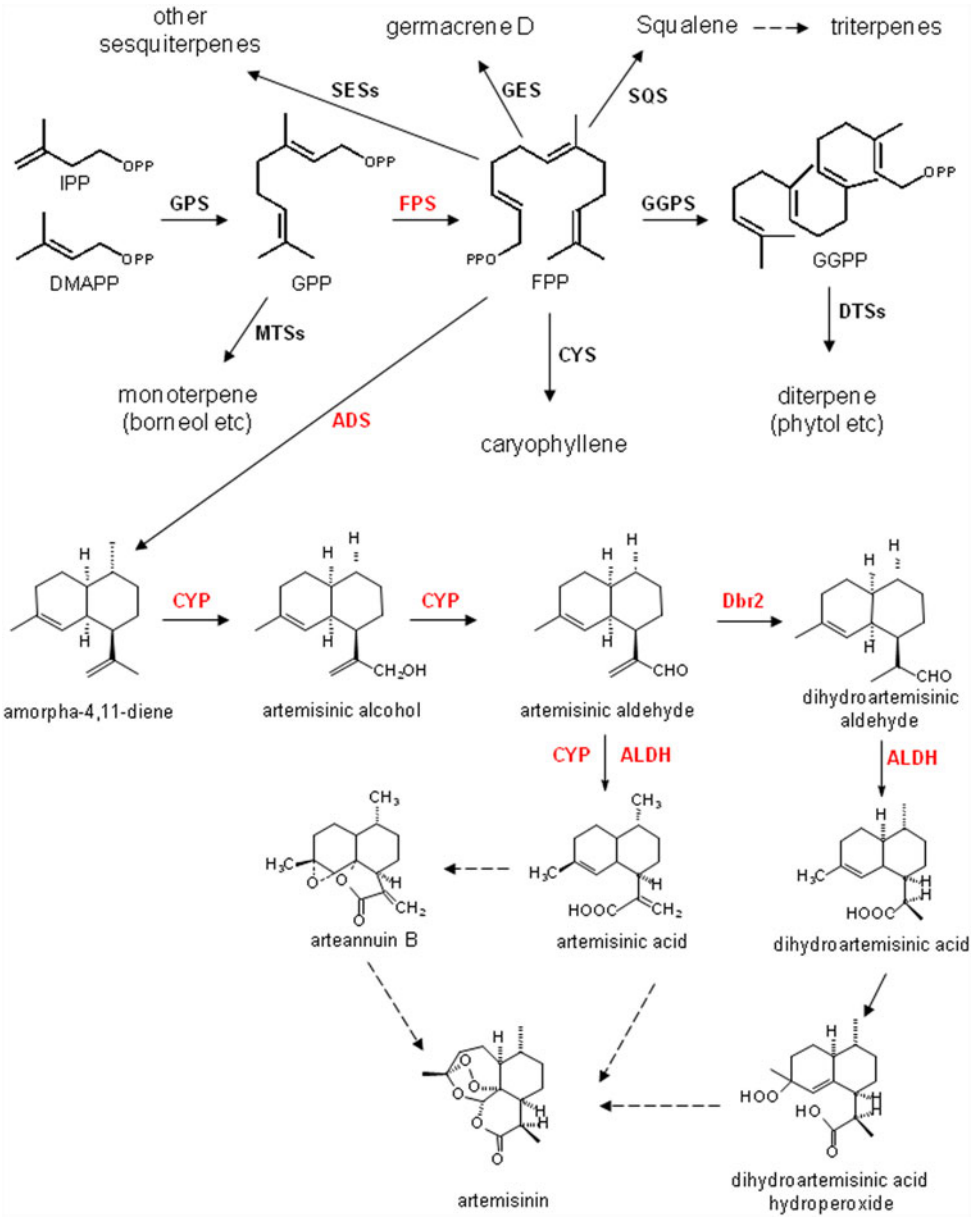

Fig. 1 Biosynthetic pathways of some terpenoids and their relationship to artemisinin biosynthesis. *ADS* amorpha-4,11-diene synthase, *ALDH* aldehyde dehydrogenase, *CYP* CYP71AV1 (p450 enzymes) *CYS* caryophyllene synthase, *Dbr*2 artemisinic aldehyde Δ11(13) double bond reductase, *DMAPP* dimethylallyl diphosphate, *DTS* diterpene synthase, *FAS* farnesene synthase, *FPP* farnesyl diphosphate, *FPS* farnesyl diphosphate synthase, *GES* germacrene D synthase, *GGPP* geranylgeranyl diphosphate, *GGPS* geranylgeranyl diphosphate synthase, *GPP* geranyl diphosphate, *GPS* geranyl diphosphate synthase, *IPP* isopentenyl diphosphate, *MTS* monoterpene synthase, *SES* sesquiterpene synthase, *SQS* squalene synthase

screened 5,000 herbs and 40,000 compounds. Inspired by the description of "a handful of Qinghao immersed with 2 liters of water, get juice and drink it." in the "Handbook of Prescriptions For Emergency Treatment" (肘后备急方 in Chinese) written in 340 A.D. by Hong Ge, Professor Youyou Tu found that the neutral extracts of *A. annua* inhibited 100% of the parasites of mouse and monkey malaria in October 1971 at Beijing Institute of Chinese Medicine. The principal compound responsible for this activity was isolated and characterized as a sesquiterpene lactone with a very unusual endoperoxide bridge, and was originally named "qinghaosu" or "arteannuin", but is now most commonly referred to as artemisinin.

Establishment of the *Agrobacterium*-mediated transformation system

The earliest reports of successful *Agrobacterium tumefaciens*- and *Agrobacterium rhizogenes*-mediated transformation of *A. annua* appeared more than 10 years ago (Banerjee et al. 1997; Vergauwe et al. 1996). Although we have transferred a number of genes into *A. annua* using these methods (Chen et al. 1999, 2000; Geng et al. 2001; Wang et al. 2004, 2007), the transformation of *A. annua* has proved to be very difficult, as it was laborious, time-consuming and more importantly the transformation efficiency was very low, and was significantly affected by

parameters such as explant type, *Agrobacterium* strains, age of explants, and other factors (Teixeira da Silva 2003; Vergauwe et al. 1998). Therefore, we optimized the *Agrobacterium*-mediated transformation system of *A. annua* including the *Agrobacterium* strain, plant genotype, preculture time, composition of the infecting bacterial suspension, methods of co-cultivation, and co-cultivation time. Under optimized conditions, the frequency of transformation reached approximately 4–10%. Because one cluster of fascicled shoots consists of 10–40 shoots, the transformation efficiency of this system is quite high (Han et al. 2005). Compared with Vergauwe's transformation system (Vergauwe et al. 1996, 1998), the new transformation system takes less time and overcomes the difficulty of choosing appropriate decontaminating antibiotics. The establishment of the new highly efficient transformation system should facilitate the genetic engineering of *A. annua*.

Overexpression of heterologous genes in *A. annua*

With the optimized transformation system, we have successfully transferred a number of heterologous genes into *A. annua*. These genes include the isopentenyl transferase gene (*ipt*) from *Agrobacterium tumefaciens* and the farnesyl diphosphate synthase gene (*fps*) from *Gossypium arboreum*.

The *A. tumefaciens* cytokinin biosynthetic gene codes for the enzyme, isopentenyl transferase (IPT), and this enzyme catalyzes the condensation of isopentenyl diphosphate (IPP) with adenosine monophosphate to give isopentenyl AMP (iPMP) (Akiyoshi et al. 1984). A number of reports have shown that overexpression of IPT increases the content of chlorophyll (Smigocki et al. 1993; Smigocki 1991), and artemisinin content is higher in *A. annua* plants with higher chlorophyll. Therefore, we transferred the IPT gene isolated from T-DNA of *A. tumefaciens* into *A. annua.* Transgenic plants exhibited an extreme shoot phenotype with little root development similar to cucumber (Mihalka et al. 2003) and tobacco (Smigocki 1991). The cytokinin (iPA and iP) content was elevated two- to threefold, the chlorophyll content increased by 20–60%, and artemisinin content increased by 30–70% in the transgenic plants. A direct positive correlation was found between the contents of cytokinin, chlorophyll and artemisinin (Geng et al. 2001). Elevated endogenous cytokinin levels improve the biosynthesis of chlorophyll in transgenic plants, which leads to an increase in artemisinin content.

Farnesyl diphosphate synthase (FPS) is a prenyltransferase that catalyzes the two sequential 1–4 condensations of IPP with DMADP to produce farnesyl diphosphate (FPP). FPP is the branching point of different branches of the pathway, leading to the synthesis of artemisinin, other sesquiterpenoids, and triterpenoids (Fig. 1). After elicitor treatment, the accumulation of sesquiterpenoid phytoalexins was correlated with increased *FPS* mRNA and protein levels, and enzymatic activity (Hugueney et al. 1996; Liu et al. 1999). Thus, overexpression of FPS in *A. annua* may increase the accumulation of artemisinin and related sesquiterpenoids. This hypothesis was proven to be correct by our studies, as overexpression of *Gossypium arboreum* FPS in hairy roots and transgenic plants resulted in a three- to fourfold and a two- to threefold increase in artemisinin content, respectively (Chen et al. 1999, 2000). On the other hand, overexpression of the cadinene synthase (CAD) gene which was believed to be the cyclase specific for artemisinin biosynthesis (Brown 1994) in hairy roots had no influence on artemisinin content (unpublished data). These results indicated that overexpression of genes related to artemisinin biosynthesis could increase artemisinin content, and that FPS plays a regulatory role in artemisinin biosynthesis.

Overexpression of the genes of *Artemisia annua* related to artemisinin biosynthesis

At the end of the 1990s, there was a significant breakthrough in artemisinin biosynthesis. Biochemical and molecular biological studies showed that amorpha-4,11-diene synthase (ADS) catalyzes the first commit step of artemisinin biosynthesis (Bouwmeester et al. 1999; Chang et al. 2000; Li et al. 2006b; Mercke et al. 1999; Wallaart et al. 1999). In order to further increase the artemisinin content in transgenic plants, we amplified the cDNA of FPS and ADS from *A. annua* and overexpressed them in *A. annua* again under the control of the CaMV 35S promoter. The terpenoid profiling of the transgenic plants was detected by GC–MS and GC × GC–MS.

When endogenous FPS from *A. annua* was overexpressed in high-yielding *A. annua* (artemisinin content around 0.65% DW), the FPS activity in transgenic plants was 2–3 times that of the control, however, the highest artemisinin content in transgenic *A. annua* (0.9% DW) was only 34.4% higher than that in the control (Han et al. 2006). It is quite interesting to compare this result with that of overexpressing heterogeneous (*G. arboreum*) FPS in *A. annua* (artemisinin content around 0.3% DW) which caused a twofold increase in artemisinin content (Chen et al. 2000). Although the increment in artemisinin content resulting from overexpression of *G. arboreum* FPS was higher than that caused by overexpression of endogenous FPS, the highest artemisinin content in transgenic *A. annua* was similar in both cases (1.0 vs. 0.9%). One possible reason for this finding is that there was a limitation in the

artemisinin content in *A. annua* because artemisinin has phytotoxic properties and inhibits root growth and cell division (Dayan et al. 1999).

In order to comprehensively evaluate the effects of overexpressing FPS in transgenic plants, the terpenoid profiling of *A. annua* plants was detected by GC–MS. The results indicated that there was a clear difference in metabolic profiling between transgenic plants and the control in the late stage of development (Ma et al. 2008). Among the main compounds which contributed to the separation of the two types of plants, most of them were terpenoids, which meant that overexpressing FPS indeed altered terpenoid metabolism. Detailed kinetic analysis of artemisinin and its biosynthetic precursors, artemisinic acid, dihydroartemisinic acid and arteannuin B, revealed that overexpression of FPS could dramatically increase the content of arteannuin B, but did not increase artemisinin content obviously (Ma et al. 2008). There are two possible explanations for this result. One is that there is another rate-limiting step from arteannuin B to artemisinin if arteannuin B is a precursor of artemisinin biosynthesis (Li et al. 2006a). The other is that arteannuin B is another end product of the pathway, which competes with artemisinin for precursors (Fig. 1). Recent biochemical and molecular biological results support the second explanation (Bertea et al. 2005; Brown and Sy 2004, 2007; Teoh et al. 2009; Zhang et al. 2008). Thus, we should try to regulate the carbon flux from artemisinic aldehyde to dihydroartemisinic aldehyde—dihydroartemisinic acid—artemisinin (Fig. 1) in order to increase artemisinin content.

Amorpha-4,11-diene synthase is one of many different terpene cyclases in terpenoid metabolism (Fig. 1). It catalyzes the conversion of FPP into amorpha-4,11-diene, which is the commit step of artemisinin biosynthesis (Bouwmeester et al. 1999), so it is a reasonable target for artemisinin metabolic engineering. The results of GC × GC–MS indicated that the content of artemisinin, artemisinic acid and dihydroartemisinic acid increased by around 100, 65 and 59%, respectively, in the transgenic plant line overexpressing ADS (Ma et al. 2009a). The absolute concentration of artemisinin was similar to the transgenic plant line overexpressing FPS. These results indicated that artemisinin biosynthesis is strictly regulated in the plant, and that there is another rate limiting step (either enzymatic or nonenzymatic) between dihydroartemisinic acid and artemisinin. There was no significant change in the content of amorpha-4,11-diene between transgenic lines overexpressing ADS and the control overexpressing GUS (Ma et al. 2009a). A possible reason for this is that amorpha-4,11-diene was quickly consumed by the downstream enzymes (Bouwmeester et al. 1999). However, significant changes in other sesquiterpenes were observed. The concentrations of germacrene D and (E)-β-farnesene were lower in transgenic plants overexpressing ADS and higher in transgenic plants suppressing ADS than those in the control plants. Another interesting result was that the contents of borneol (monoterpene) and phytol (diterpene) increased in transgenic plants overexpressing ADS. These results indicated that regulation of one sesquiterpene cyclase (ADS) had an effect on the original carbon flux distribution in the terpenoid metabolic networks. Similar to the case of amorpha-4,11-diene, the contents of artemisinic alcohol, artemisinic aldehyde and dihydroartemisinic aldehyde in transgenic lines were not altered significantly, which means that these steps are not rate limiting steps in artemisinin biosynthesis. The contents of deoxyartemisinin, dihydrodeoxyarteannuin B and arteannuin B were also increased in transgenic plants overexpressing ADS, and there was a positive correlation between the four sesquiterpenoids (including artemisinin) and the two acids. These results indirectly indicated the possible transformation from the two acids to the four terpenoids. In other words, artemisinic acid and dihydroartemisinic acid are precursors for the four sesquiterpenoids. These data are consistent with the related literature (Brown and Sy 2004, 2007).

Conclusions and perspectives

Our artemisinin metabolic engineering studies showed that it is possible to regulate artemisinin biosynthesis at the gene level. However, overexpressing a single gene is not sufficient to increase the final artemisinin content. This phenomenon has been reported in other metabolic studies of plant secondary metabolites (Zhang et al. 2004). The metabolic profiling results also indicate that there is more than one bottle-neck in the artemisinin biosynthetic pathway, and it might be necessary to overexpress these key genes to improve artemisinin production. Among the known genes of artemisinin biosynthesis, artemisinic aldehyde reductase (Dbr2) might be a good candidate, with higher Dbr2 activity more dihydroartemisinic acid will be formed, which will lead to an increase in artemisinin production. Furthermore, there are still unknown enzymatic (or non-enzymatic) steps between dihydroartemisinic acid and artemisinin in the artemisinin biosynthesis. Elucidation of these steps will be helpful in precise metabolic engineering strategies to improve artemisinin production. A metabolic engineering study by Aquil et al. (2009) indicated that overexpression of the HMGR gene of the MAV pathway promoted artemisinin production. Since our labeling (Schramek et al. 2010) and inhibition studies (Towler and Weathers 2007) showed that the MEP pathway also contributed isoprenyl units to artemisinin biosynthesis, overexpressing genes of the MEP pathway might also promote artemisinin biosynthesis. Transcriptional

factors often regulate a number of genes in a specific pathway (Borevitz et al. 2000) and overexpression of transcriptional factors has been proposed as a promising approach to manipulate secondary metabolic pathways more efficiently (Petersen 2007; Verpoorte and Memelink 2002). We have recently isolated and characterized the first *A. annua* transcriptional factor—AaWRKY1, which could activate several genes of the artemisinin biosynthetic pathway in the transient expression system (Ma et al. 2009b). Overexpression of AaWRKY1 may activate the whole artemisinin biosynthesis pathway.

Artemisinin is synthesized and stored in glandular secretory trichomes on the surface of leaves and flowers (Duke and Paul 1993; Olsson et al. 2009). It is reasonable to assume that the artemisinin content will be increased if the number of trichomes is increased. There are reports that some genes can promote initiation and development of trichomes in tobacco (Payne et al. 1999; Perez-Rodriguez et al. 2005). If they can also promote trichome development in *A. annua*, it will very likely increase the accumulation of artemisinin in transgenic plants.

In tobacco, overexpressing FPS and ADS in plastids leads to a 1,000-fold increase in amorpha-4,11-diene production compared to overexpression of the two enzymes in cytosol (Wu et al. 2006). If this is also true in *A. annua*, more artemisinin will be produced because all the other factors for artemisinin production are present.

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