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REVIEW

Transgenic approach to increase artemisinin content in *Artemisia annua* L.

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Abstract Artemisinin, the endoperoxide sesquiterpene lactone, is an effective antimalarial drug isolated from the Chinese medicinal plant *Artemisia annua* L. Due to its effectiveness against multi-drug-resistant cerebral malaria, it becomes the essential components of the artemisinin-based combination therapies which are recommended by the World Health Organization as the preferred choice for malaria tropica treatments. To date, plant *A. annua* is still the main commercial source of artemisinin. Although semi-synthesis of artemisinin via artemisinic acid in yeast is feasible at present, another promising approach to reduce the price of artemisinin is using plant metabolic engineering to obtain a higher content of artemisinin in transgenic plants. In the past years, an *Agrobacterium*-mediated transformation system of *A. annua* has been established by which a number of genes related to artemisinin biosynthesis have been successfully transferred into *A. annua* plants. In this review, the progress on increasing artemisinin content in *A. annua* by transgenic approach and its future prospect are summarized and discussed.

Keywords Artemisinin · Artemisinin biosynthetic pathway · *Artemisia annua* L. · Metabolic regulation · Secondary metabolism

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Introduction

Malaria, one of the most serious health problems in many tropical countries, is responsible for more than 660,000 deaths every year (World Malaria Report 2012, World Health Organization, WHO). Moreover, an estimated 220 million people are at risk of malaria in 2011. An endoperoxide sesquiterpene lactone called artemisinin, isolated from the herb *Artemisia annua* L, is effective against drug-resistant malaria. Artemisinin-based combination therapies (ACTs) are recommended by WHO to be the best choice for acute malaria (Graham et al. 2010; Mutabingwa 2005; Weathers et al. 2011). Like many other secondary metabolites, trace amounts of artemisinin at a range of 0.1–1 % dry leaf weight of *A. annua* plant result in short supply and high costs of this effective drug (Duke et al. 1994; Kumar et al. 2004).

Through recent work from several research groups, the biosynthesis of artemisinin is almost completely elucidated (Fig. 1). Figure 1 shows the biosynthetic pathway leading to artemisinin as it is best understood today along with other pathways of terpene metabolism in *A. annua* (Newman and Chappell 1999; Srivastava and Akhila 2011). The biosynthetic pathway of artemisinin belongs to the isoprenoid pathway. Therefore, farnesyl diphosphate (FPP) occupies the central position, which is generated from its precursor isopentenyl diphosphate (IPP). There are two independent pathways leading to IPP in the plant: the mevalonate pathway (MVA) occurring in the cytosol and the non-MVA pathway or methylerythritol phosphate (MEP) pathway located in the plastid (Arsenault et al. 2008; Nguyen et al. 2011; Olofsson et al. 2011; Srivastava and Akhila 2011; Weathers et al. 2006). Artemisinin is synthesized in the glandular trichome. The cyclization of FPP to generate amorpha-4,11-diene is catalyzed by amorpha-4,11-diene

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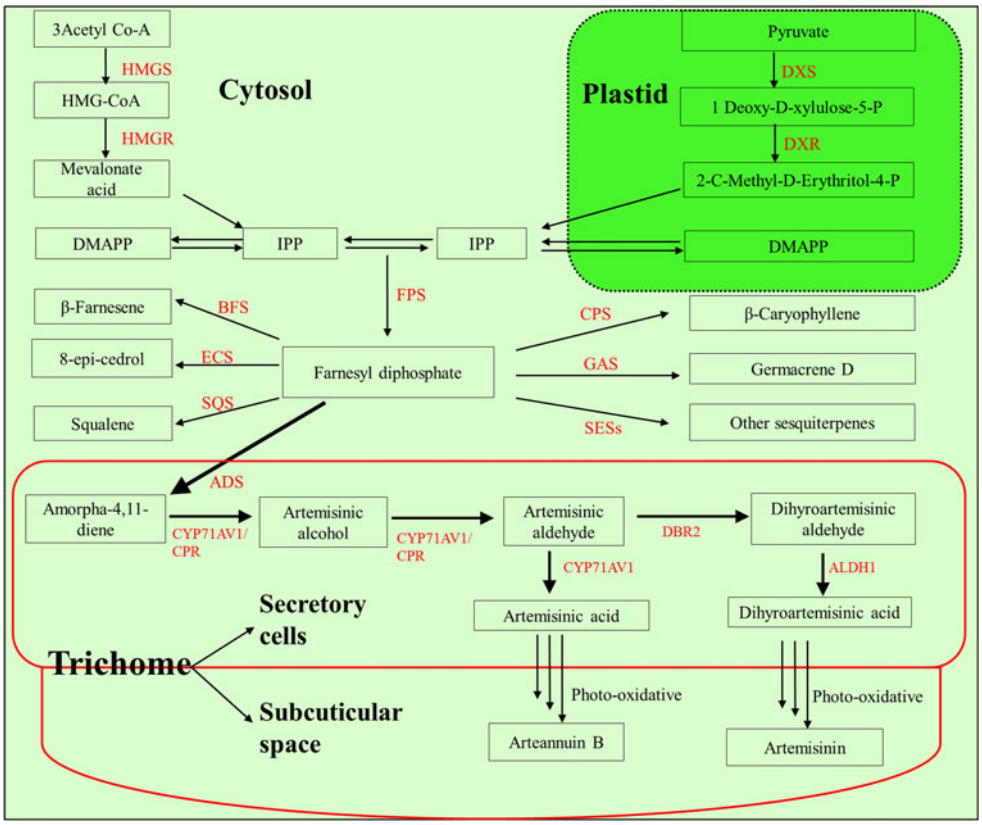

Fig. 1 Biosynthetic pathways of artemisinin and some terpenoids in *A. annua*. *HMGS* 3-hydroxy-3-methyl-glutaryl coenzyme A synthase, *HMGR* 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, *DXS* 1-deoxy-D-xylulose-5-phosphate synthase, *DXR* 1-deoxy-D-xylulose-5-phosphate reductoisomerase, *FPS* farnesyl diphosphate synthase, *BFS* β-farnesene synthase; *CPS* β-caryophyllene synthase, *ECS* *epi*-cedrol synthase, *GAS* germacrene A synthase, *SQS* squalene synthase, *ADS* amorpha-4,11-diene synthase, *CYP71AV1* cytochrome P450 monooxygenase, *CPR* cytochrome P450 reductase, *DBR2* artemisinic aldehyde Δ11(13) reductase, *ALDH1* aldehyde dehydrogenase 1

synthase (ADS), which is specifically expressed in the glandular trichome, and is thought to be the first committed step in artemisinin biosynthesis (Kim et al. 2008; Mercke et al. 2000; Wang et al. 2011a). Subsequently, amorpha-4,11-diene is hydroxylated to artemisinic alcohol, artemisinic aldehyde and artemisinic acid by three steps that are catalyzed by the multi-function cytochrome P450 monooxygenase (CYP71AV1), which is also trichome specific (Teoh et al. 2006; Wang et al. 2011b, 2013). The artemisinin and artemisinic acid biosynthesis are divided by the key enzyme artemisinic aldehyde Δ11 (13) reductase (DBR2) (Zhang et al. 2008), which converts artemisinic aldehyde into dihydroartemisinic aldehyde, then dihydroartemisinic aldehyde is converted into dihydroartemisinic acid by aldehyde dehydrogenase (ALDH1) (Teoh et al. 2009). Dihydroartemisinic acid is regarded as the direct precursor of artemisinin. Thus far, no enzyme has been found to catalyze the conversion of dihydroartemisinic acid to artemisinin. Rather, the reaction appears to be non-enzymatic photo oxidation in the cell-free cuticular space which is outside of the glandular trichome secretory cells (Brown and Sy 2004; Sy and Brown 2002). Similarly, artemisinic acid is converted into arteannuin B via a photo-oxidative enzyme-independent reaction (Brown and Sy 2004; Sy and Brown 2002).

Due to the short supply and high costs of artemisinin, different strategies were applied in increasing artemisinin supply such as organic total synthesis, semi-synthesis of artemisinin from microbially sourced artemisinic acid, and transgenic approach to increase artemisinin accumulation in *A. annua*. Organic total synthesis is difficult and costly, although Zhu and Cook reported a concise synthesis of artemisinin from inexpensive starting material cyclohexenone and achieved on a gram scale, a lot of work still needs to do before large-scale production (Zhu and Cook 2012). Recently, semi-synthesis of artemisinin achieved a tremendous success. Keasling's group reports that they can produce 25 grams per litre of artemisinic acid in *Saccharomyces cerevisiae*, leading to the production of artemisinin in industrial scale (Paddon et al. 2013; Ro et al. 2006, 2008). In the meantime, great progress has also been made on increasing artemisinin content in *A. annua* plant, providing an effective way to lower down the artemisinin price. In this review, the progress on transgenic approaches to enhance artemisinin content in *A. annua* is reviewed and summarized.

Overexpressing key enzyme genes of artemisinin biosynthetic pathway in *A. annua*

The *HMGR* genes

Artemisinin is derived from the condensation of three 5-carbon isoprenoid molecules that originate from both the plastid and cytosol (Ram et al. 2010; Schramek et al. 2010;

Towler and Weathers 2007). The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which shunts HMG-CoA into the isoprenoid pathway, is deemed to be the key enzyme in artemisinin biosynthesis. It has been demonstrated that HMGR activity limits artemisinin biosynthesis and its accumulation in *A. annua* plants (Ram et al. 2010), and several research groups tried to increase artemisinin contents by overexpressing the *HMGR* gene driven by CaMV 35S promoter in *A. annua*. In their reports, they transferred *HMGR* gene from *Catharanthus roseus* (L.) G. into *A. annua* and found that the transgenic lines possessed significantly higher HMGR activity compared with wild-type controls. As a result, the transgenic lines depicted an increase of 22.5–38.9 % artemisinin content compared with wild-type control plants (Aquil et al. 2009; Nafis et al. 2011).

Furthermore, the *A. annua HMGR* gene has also proved important in synthetic biology that produces artemisinic acid, a precursor of artemisinin, in *S. cerevisiae* (baker's yeast). Engineered yeast imported with three copies of *HMGR* genes was demonstrated to have significantly higher artemisinic acid content than that with only one copy of the *HMGR* gene (Paddon et al. 2013; Ro et al. 2006).

The *DXR* gene

In plastids, deoxy-D-xylulose-5-phosphate synthase (DXS) catalyzes the condensation of pyruvate and D-glyceraldehyde-3-phosphate, and represents the first enzymatic step of the mevalonate-independent pathway leading to the formation of 1-deoxy-D-xylulose-5-phosphate (Croteau et al. 2000). The first committed step leading to IPP is the step, which produces 2-*C*-methyl-D-erythritol-4-phosphate via the action of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) using 1-deoxy-D-xylulose-5-phosphate as its substrate (Carretero-Paulet et al. 2002). According to the genetic map of *A. annua* constructed by Graham et al. (2010), the *DXR* gene is tightly linked with high levels of artemisinin. Schramek et al. (2010) used isotopologue profiling of *A. annua* plants with $^{13}CO_2$, and confirmed that the farnesyl diphosphate, which is the precursor of amorpha-4,11-diene, composed of two isoprene units from the MVA pathway and one isoprene unit from the MEP pathway (Towler and Weathers 2007). Therefore, DXR plays an important role in the control of plastid isoprenoid biosynthesis. To comprehensively evaluate the effect of the *DXR* gene in artemisinin biosynthesis, *DXR* gene driven by CaMV 35S promoter was introduced into *A. annua* and HPLC analysis between overexpressing *DXR* transgenic and non-transgenic plants was undertaken, and results indicated that the transgenic plants produced 1.21–2.35 more artemisinin than the controls (Xiang et al. 2012). Fosmidomycin inhibits production of 2-*C*-methyl-D-erythritol-4-phosphate (MEP) from 1-deoxy-D-xylulose-5-P (DXP) and specifically blocks the MEP pathway (Rodríguez-Concepción et al. 2004). Artemisinin content in *A. annua* plants exposed to 100 μM fosmidomycin for 14 days declined 25 % compared with the control ones (Towler and Weathers 2007). These results indicate that the plastid MEP pathway also contributes to artemisinin biosynthesis, and that along with HMGR, DXR plays a regulatory role in artemisinin biosynthesis.

The heterologous and homologous *FPS* genes

Farnesyl diphosphate synthase (FPS) is a prenyltransferase that catalyzes the two sequential 1–4 condensations of IPP with DMAPP to produce farnesyl diphosphate (FPP). FPP is the branching point of a large variety of essential isoprenoid end products, including artemisinin, sesquiterpenoids, and triterpenoids (Newman and Chappell 1999). When the heterologous *Gossypium arboreum FPS* gene driven by CaMV 35S promoter was introduced into *A. annua,* transgenic plants accumulated artemisinin up to 1.0 % dry weight (DW), which was 2–3 times higher than that in non-transgenic control plants (Chen et al. 2000). Other groups introduced the *A. annua FPS* gene driven by CaMV 35S promoter into *A. annua* plants and the *FPS* overexpressing lines exhibited higher artemisinin content, with 2–2.5 fold than that detected in wild-type plants. A relatively high correlation ($R^2 = 0.78$) was observed between level of expression of *FPS* gene and artemisinin content (Banyai et al. 2010; Han et al. 2006). Moreover, one group found that the copy numbers of *FPS* in *A. annua* genome also affected the artemisinin content. Most of the transgenic lines contained single copies and resulted in higher gene expression and higher artemisinin, whereas the transgenic lines that contained double copies of *FPS* showed lower gene expression even than the wild type, coinstantaneous with lower artemisinin content (Banyai et al. 2010). Although FPP is a common precursor for a wide range of isoprene products, it does influence the metabolic flow to artemisinin in *A. annua*.

The *ADS* gene

The branching point that switches farnesyl diphosphate (FPP) to artemisinin biosynthesis is catalyzed by amorpha-4,11-diene synthase (ADS), which produces the amorpha-4,11-diene metabolite (Bouwmeester et al. 1999; Mercke et al. 2000). ADS is considered the first rate-limiting enzyme in the artemisinin biosynthetic pathway. By investigating the ADS promoter, two groups revealed that the *ADS* gene was specifically expressed in glandular trichome cells. Artemisinin is produced and stored in the

glandular trichomes of *A. annua* (Kim et al. 2008; Wang et al. 2011a). Therefore, the *ADS* gene is a reasonable target for artemisinin metabolic engineering. Although, Arsenault et al. (2010) found that by spraying artemisinic acid to *A. annua* plants the transcripts of *ADS* decreased tenfold, suggesting that there are some feedback inhibitions on artemisinin accumulation. The results of GC × GC–MS showed that the contents of artemisinin, artemisinic acid and dihydroartemisinic acid were increased by about 82, 65 and 59 %, respectively, in the *ADS* overexpressing transgenic plant lines (Ma et al. 2009a); however, it was also found that there was no significant change in the content of amorpha-4,11-diene between transgenic lines and the control ones. A possible reason is that amorpha-4,11-diene was quickly consumed by the downstream enzymes (Bouwmeester et al. 1999).

The *CYP71AV1* and *CPR* genes

Cytochrome P450 monooxygenase (CYP71AV1) is a multifunctional sesquiterpene oxidase with a key role in the biosynthesis of artemisinin. By three steps, it converts amorpha-4,11-diene to artemisinic acid via artemisinic alcohol and artemisinic aldehyde middle metabolites (Maes et al. 2011; Teoh et al. 2006). Cytochrome P450 oxidoreductase (CPR) has also been isolated from *A. annua*, and was identified as a redox partner of CYP71AV1 that helps CYP71AV1 to catalyze the conversion of amorpha-4,11-diene to more oxygenated products in vivo (Ro et al. 2006). Like *ADS*, *CYP71AV1* is also specifically expressed in the glandular trichomes of *A. annua* (Wang et al. 2011b, 2013); meanwhile, the transcripts of *CYP71AV1* gene declined significantly when the plants were sprayed with artemisinin or artemisinic acid (Arsenault et al. 2010). Although, the metabolites catalyzed by CYP71AV1 mainly flow toward artemisinic acid, *CYP71AV1* is still thought to be very important in artemisinin biosynthesis. To enhance artemisinin content, *CYP71AV1* and *CPR* genes cloned from *A. annua* and driven by CaMV35S promoters were introduced into *A. annua*, and artemisinin content in transgenic lines reached 980 ± 108 μg/g FW (fresh weight), which was 38 % higher than that in the controls (708 ± 18 μg/g) (Shen et al. 2012; Xiang et al. 2012). These studies demonstrate that co-overexpressing genes *CYP71AV1* and *CPR* could indeed increase artemisinin content in *A. annua*.

The combination of key enzyme genes

Previous work by producing amorpha-4,11-diene or artemisinic acid in *Escherichia coli* and *S. cerevisiae* showed that there was more than one bottleneck in the artemisinin biosynthetic pathway (Anthony et al. 2009; Ro et al. 2006; Tsuruta et al. 2009); therefore, overexpressing a single pathway gene may not be sufficient to maximize artemisinin accumulation in plant. On the basis of above considerations, several research groups tried to increase artemisinin content by co-overexpressing two or even more genes in *A. annua*. HMG-CoA reductase (HMGR) gene from *C. roseus* and amorpha-4,11-diene synthase (ADS) gene from *A. annua* were overexpressed in *A. annua* and the HPLC analyses showed that the transgenic lines had greater artemisinin content than the non-transgenic lines. The maximum artemisinin content observed in transgenic line was 1.73 mg/g (DW) that was 7.65-fold higher than the control ones (Alam and Abdin 2011).

Co-overexpressing *HMGR* and *FPS* genes in transgenic *A. annua* plant were also found to lead to higher artemisinin content (9 mg/g DW, about 1.8-fold higher) than that in controls (Wang et al. 2011c). However, compared with previous investigations in which a single-enzyme gene (HMGR or FPS) was overexpressed, simultaneous overexpression of *HMGR* and *FPS* genes did not result in a significant improvement in artemisinin content compared with the single-gene (HMGR or FPS) overexpressed transgenic *A. annua* plants.

In another example, co-overexpression of *FPS*, *CYP71AV1* and *CPR* genes leads to the increased artemisinin content in transgenic lines. Those three genes were in independent expression cassette joined one by one in pCAMBIA2300 vector as their backbone and they were all driven by CaMV35 promoters (Chen et al. 2012). Transgenic plants overexpressing the combination of the three genes exhibited greater amounts of artemisinin content, 3.6-fold higher than that of the control plants, demonstrating this combination to be a feasible method for increasing artemisinin content compared to single gene engineering (Chen et al. 2012). Furthermore, artemisinin was also increased by co-overexpressing *ADS*, *CYP71AV1* and *CPR* genes. HPLC analyses showed one of the transgenic plants contained 2.4-fold higher (15.1 mg/g DW) artemisinin than the control plants (Lu et al. 2013a).

Enhancing artemisinin biosynthesis by blocking artemisinin biosynthesis competitive pathway key enzyme genes

RNAi *SQS* gene

In *A. annua*, farnesyl diphosphate (FPP) serves as a common precursor of amorpha-4,11-diene and of other sesquiterpenes diverted by different sesquiterpene synthases through competitive pathways.

SQS (squalene synthase) is the key enzyme catalyzing the first step of the sterol biosynthetic pathway, which is in competition with artemisinin biosynthesis pathway (Liu

et al. 2003). In 2009, a study showed for the first time that utilization of hpRNA-mediated (hairpin RNA) RNAi technology by suppressing the expression of *SQS* significantly increased artemisinin content in transgenic *A. annua* and the highest artemisinin content reached 31.4 mg/g (DW), about 3.14-fold of non-transgenic *A. annua* (Zhang et al. 2009). A GC–MS analysis between *hpSQS* transgenic lines and non-transgenic lines indicated that the contents of four major sterols, campesterol, stigmasterol, β-sitosterol and ergosterol were decreased in *hpSQS* transgenic lines in which the artemisinin contents were significantly increased. Sterols are very important in the development of plants; however, in most of the analyzed *hpSQS* transgenic lines the expression of SQS was not suppressed entirely, at most by 60 %, and did not alter the plant growth significantly so the total plant biomass did not show big difference (Zhang et al. 2009). This study demonstrates that RNAi technology is an effective means for increasing artemisinin content in plants.

Antisense *CPS* gene

The β-caryophyllene synthase (CPS) is an enzyme that convents farnesyl diphosphate (FPP) into β-caryophyllene, so it is in a competitive pathway of artemisinin biosynthesis (Cai et al. 2002). Down-regulation of the expression of the *CPS* gene was achieved by introducing the antisense fragment of β-caryophyllene synthase cDNA (asCPS) into *A. annua* plants. In an analysis of the content of β-caryophyllene and artemisinin in transgenic asCPS and non-transgenic control plants, all transgenic plants exhibited significantly lower β-caryophyllene content, around a 40–60 % reduction compared with the controls, while in some transgenic plants the content of artemisinin was increased by 54.9 % (Chen et al. 2011). This study demonstrates that antisense technology is also an effective way to increase the artemisinin content in plants.

Taken together, compared with the overexpression of key enzymes, blocking competitive biosynthetic pathways is also an effective approach for enhancing the artemisinin content in plants.

Regulation by transcription factor genes

Plant transcription factors often regulate a series of genes in a specific pathway and overexpression of these factors has been proposed as a promising approach for more efficiently regulating plant secondary metabolic pathways (Verpoorte and Memelink 2002). ORCA3, a jasmonate-responsive transcription factor, has been shown to regulate more than five genes related to the terpenoid indole alkaloid (TIA) biosynthetic pathway in *Catharanthus roseus*. Overexpression of *ORCA3* resulted in increased accumulation of some terpenoid indole alkaloids in transgenic cell lines and plants of *C. roseus* (van der Fits and Memelink 2000, 2001; Pan et al. 2012).

AaWRKY1, the first *A. annua* transcription factor isolated and characterized, has been shown to bind to the W boxes in both *ADS* and *CYP71AV1* promoters, and activates the expression of artemisinin biosynthetic pathway key enzyme genes (Ma et al. 2009b). Overexpression of *AaWRKY1* activated the expression of several genes in the artemisinin biosynthetic pathway, and HPLC analysis showed that the artemisinin content in transgenic lines reached 24.5 mg/g (DW), which is 4.4-fold that of the control lines (Tang et al. 2012c).

Two JA-responsive AP2 family transcription factors, *AaERF1* and *AaERF2*, were cloned from *A. annua* and found to bind to the CRTDREHVCBF2 (CBF2) and RAV1AAT (RAA) motifs present in both *ADS* and *CYP71AV1* promoters (Yu et al. 2012). HPLC analysis showed that overexpressing either of the transcription factors increased accumulation of artemisinin and artemisinic acid in transgenic plants. By contrast, the content of these two metabolites was reduced in the RNAi transgenic lines in which expression of *AaERF1* or *AaERF2* was suppressed (Yu et al. 2012).

AaORA, a trichome-specific AP2/ERF transcription factor in *A. annua*, is also demonstrated to regulate the expression of several genes in artemisinin biosynthetic pathway. By overexpression of *AaORA* driven by CaMV 35S promoter in *A. annua*, the expression levels of *ADS*, *CYP71AV1*, *DBR2* and *AaERF1* were significantly up-regulated, resulting in increased artemisinin and dihydroartemisinic acid accumulation. On the contrary, down-regulation of *AaORA* expression by hairpin-mediated RNAi in *A. annua* resulted in the significantly down-regulated expression levels of *ADS*, *CYP71AV1*, *DBR2* and *AaERF1*, accompanied by the decrease of artemisinin and dihydroartemisinic acid accumulation. The highest artemisinin content in overexpressing transgenic lines was 11.9 mg/g (DW), 53 % higher than that in the control ones (Lu et al. 2013b).

So far, four transcription factors have been cloned from *A. annua* and shown to up-regulate expression of key enzyme genes in artemisinin biosynthetic pathway, resulting in increased artemisinin content in transgenic plants (Lu et al. 2013b; Ma et al. 2009b; Yu et al. 2012). Artemisinin stored in the glandular trichomes located mainly on the surface of leaves, buds and sepals (Olofsson et al. 2011, 2012), so it is generally thought that trichome-specific expression genes may play important roles in artemisinin biosynthesis. All cloned transcription factors bind to the *ADS* and *CYP71AV1* promoters (Ma et al. 2009b; Yu et al. 2012), which were glandular trichome-specific promoters.

Recently, the DBR2 promoter also showed to be trichome specific, and several *cis*-elements present in ADS and CYP71AV1 promoters were also found in the DBR2 promoter region (Jiang et al. 2013). Therefore, transcription factors AaWRKY1 and AaORA may also bind to the promoter of DBR2; this requires further investigation.

Indirect regulation of artemisinin biosynthesis

The phytohormone jasmonic acid (JA) and its derivatives are essential signaling molecules that in addition to plant secondary metabolic regulation processes coordinate plant response to biotic and abiotic challenges (Chini et al. 2009; Galis et al. 2009; Wasternack and Kombrink 2010). Exogenous JA treatments increased the content of artemisinin by 49 % (Wang et al. 2010), so it was speculated that artemisinin content could also be increased by enhancing the endogenous JA content. Jasmonates are generated via one specific branch of oxylipin biosynthesis and the allene oxide cyclase (AOC) is a key enzyme in the biosynthetic pathway that catalyzes allene oxide 12, 13(S)-EOT into (9S, 13S)-OPDA (Wasternack and Kombrink 2010). The *AaAOC* gene was recently cloned and characterized from *A. annua* (Lu et al. 2011). Transgenic *A. annua* overexpressing the *AaAOC* gene was generated by *Agrobacterium*-mediated transformation. GC–MS analysis showed that the jasmonate content in transgenic lines was 2–4.7-fold that of the control ones. The expression levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* were also increased, and interestingly the glandular trichome density was also increased in the transgenic lines. As a result, the artemisinin content was enhanced with an increase of 87.9 % in some *AaAOC* overexpressing transgenic lines (Lu et al. unpublished). These results were consistent with earlier studies that showed that exogenous JA treatments of *A. annua* for several weeks increased the glandular trichome density (Maes and Goossens 2010; Maes et al. 2011).

The phytohormone abscisic acid (ABA) plays an important role in plant development and environmental stress response, including drought, salt, osmotic and cold stresses (Zhu 2002). Jing et al. (2009) showed that artemisinin content was increased by spraying plant leaves with exogenous ABA before flowering. Recently, an ortholog ABA receptor gene, *AaPYL9*, was cloned and characterized from *A. annua*. Transgenic *A. annua* lines overexpressing *AaPYL9* showed a hypersensitive phenotype under ABA induction and had an enhanced tolerance to drought, induced stomatal closure and reduced rates of transpiration (Zhang et al. 2013). HPLC analysis revealed that without ABA treatment the transgenic lines showed no difference in artemisinin content. However, when treated with ABA, wild type showed 33 % increase in artemisinin content, while artemisinin was increased by 74–95 % in transgenic lines (Zhang et al. 2013). This study demonstrates that overexpression of *AaPYL9* in *A. annua* enhanced ABA sensitivity after ABA treatment, and improved artemisinin content in plants.

In *Arabidopsis*, cryptochrome 1 (CRY1) is one of the key receptors that perceive light signals, and its overexpression promotes accumulation of secondary metabolites in many plants (Chatterjee et al. 2006; Giliberto et al. 2005). Overexpression of *Arabidopsis CRY1* in *A. annua* resulted in increased accumulation of both artemisinin and anthocyanins. Artemisinin content in most transgenic lines was increased by 30–40 %, with the maximum transgenic line increasing about 74 % (Hong et al. 2009). The finding that the higher artemisinin accumulation was accompanied with more anthocyanin contents suggests that overexpression of *Arabidopsis CRY1* in transgenic *A. annua* could stimulate secondary metabolism of divert pathways.

The summary of transgenic *A. annua* studies is shown in Table 1.

Conclusion

Much of Africa is still at risk of malaria, and in 2011 more than 660,000 people died from malaria (World Malaria Report 2012, WHO, http://www.who.int/malaria/publications/world_malaria_report_2012/en/index.html). The relatively low yield of artemisinin in *A. annua* is one of the major obstacles for greater commercial production and clinical use. Although semi-synthesis of artemisinin by yeast engineering shows the great potential to increase the production, extracting artemisinin from plant *A. annua* is still the major source right now (Barbacka and Baer-Dubowska 2011; Paddon et al. 2013; Ro et al. 2006; Tsuruta et al. 2009). Organic total synthesis was difficult and costly (Avery et al. 1992; Constantino et al. 1996), and was considered difficult for commercial production. An inspiration achievement was reported last year, that is, concise total synthesis of artemisinin using readily available and affordable cyclohexenone as the key starting material was achieved on a gram scale (Zhu and Cook 2012), and hopefully on large-scale production in the future. Therefore, great efforts are still needed currently to focus on enhancement of the production of artemisinin both in vivo and in vitro. More and more transgenic *A. annua* plants have been reported and *agrobacterium*-mediated transformation of *A. annua* is not a difficult task nowadays. Almost all the reports indicate that transgenic *A. annua* plants show no significant change in biomass compared with the wild-type controls, including those transgenic *A. annua* plants blocking down the expression of *SQS* (Zhang et al. 2009). There are many ways to manipulate *A. annua* by transgenic

Table 1 Summary of genes used in genetic engineering in *A. annua*

Enzyme types	Enzyme names	Gene sources	GenBank No.	Wildtype artemisinin content	Transgenic artemisinin content	References
Artemisinin biosynthesis pathway key enzymes	HMGR	*Catharanthus roseus*	AY623812	0.37 mg/g (DW)	0.6 mg/g (DW)	Nafis et al. (2011)
				1.1 mg/g (DW)	1.7 mg/g (DW)	Aquil et al. (2009)
	DXR	*A. annua*	AF182287.2	0.52 mg/g (DW)	1.21 mg/g (DW)	Xiang et al. (2012)
	FPS	*A. annua*	AF112881	5.0 mg/g (DW)	13.0 mg/g (DW)	Banyai et al. (2010)
						Han et al. (2006)
		Gossypium arboreum	None	3.1 mg/g (DW)	10.0 mg/g (DW)	Chen et al. (2000)
	ADS	*A. annua*	EF197888	0.65 mg/g (DW)	1.18 mg/g (DW)	Ma et al. (2009a)
	CYP71AV1/CPR	*A. annua*	DQ268763/JN594507	0.70 mg/g (FW)	0.98 mg/g (FW)	Shen et al. (2012)
						Xiang et al. (2012)
	DBR2	*A. annua*	EU704257	7.90 mg/g (DW)	22.35 mg/g (DW)	Tang et al. (2012b)
	ALDH1	*A. annua*	FJ809784	7.92 mg/g (DW)	25.34 mg/g (DW)	Tang et al. (2012c)
	HMGR, ADS	*hmgr C. roseus/ads A. annua*	AY623812/EF197888	0.2 mg/g (DW)	1.73 mg/g (DW)	Alam and Abdin (2011)
	HMGR, FPS	*A. annua*	AF142473.1/AF112881	5.0 mg/g (DW)	9.0 mg/g (DW)	Wang et al. (2011c)
	ADS, CYP71AV1, CPR	*A. annua*	EF197888/DQ268763/ JN594507	6.4 mg/g (DW)	15.1 mg/g (DW)	Lu et al. (2013a)
	FPS, CYP71AV1, CPR	*A. annua*	AF112881/DQ268763/ JN594507	0.83 mg/g (FW)	2.90 mg/g (FW)	Chen et al. (2012)
Artemisinin biosynthesis competitive pathway enzymes	SQS	*A. annua*	AF302464.2	10 mg/g (DW)	31.4 mg/g (DW)	Zhang et al. (2009)
	CPS	*A. annua*	AF472361	2.3 mg/g (DW)	3.56 mg/g (DW)	Chen et al. (2011)
Transcription factors	AaWRKY	*A. annua*	FJ390842.1	5.5 mg/g(DW)	24.5 mg/g (DW)	Tang et al. (2012a)
	AaERF1	*A. annua*	JN162091	5.4 mg/g (DW)	9.1 mg/g (DW)	Yu et al. (2012)
	AaERF2	*A. annua*	JN162092	5.4 mg/g (DW)	8.1 mg/g (DW)	Yu et al. (2012)
	AaORA	*A. annua*	JQ797708	7.9 mg/g (DW)	11.9 mg/g (DW)	Lu et al. (2013b)
Others	AaAOC	*A. annua*	HM189219	9.1 mg/g (DW)	17.1 mg/g (DW)	Our lab data (unpublished)
	AaPYL9	*A. annua*	None	0.61 mg/g (FW)	1.18 mg/g (FW)	Zhang et al. (2013)
	AtCRY1	*Arabidopsis thaliana*	NM_116961	0.9 mg/g (DW)	1.65 mg/g (DW)	Hong et al. (2009)

technology, such as overexpression of key enzyme genes, RNAi or antisense of the competitive pathways genes, overexpression of transcription factors or genes that can regulate artemisinin biosynthesis by indirect ways. All these approaches are demonstrated to be effective, but there is still a big space to further increase artemisinin content. Nearly, all the genes overexpressed or blocked in *A. annua* reported earlier were driven by CaMV 35S promoters. As we know that artemisinin is synthesized and stored in the glandular trichome of *A. annua*, and the artemisinin biosynthesis key enzyme genes such as *ADS*, *CYP71AV1* and *DBR2* are all trichome specific. Therefore, overexpression of those genes driven by trichome-specific promoter may be more effective, especially for co-overexpression of two or more genes. To further increase the artemisinin content in transgenic *A. annua*, the strategy used in yeast engineering for producing artemisinin precursor can be adopted in transgenic *A. annua* by utilizing multiple strategies simultaneously, such as overexpressing multiple downstream genes in artemisinin biosynthetic pathway such as *ADS, DBR2, CYP71AV1,CPR* and *ALDH1* together with the upstream genes such as *HMGR* driven by different promoters, combined with blocking the competitive pathways such as using antisense or RNAi of SQS and β-farnesene synthase, manipulating the indirect pathways and controlling the copy number of inserted genes, etc.

Artemisinin biosynthesis in *A. annua* plants is complicated, and can be affected or regulated by many factors, such as the culture conditions, phytohormone, biomass, glandular trichome density, roots and growth stage (Banyai et al. 2011; Caretto et al. 2011; Graham et al. 2010; Maes et al. 2011; Mannan et al. 2010; Nguyen et al.2011; Pu et al. 2009). Moreover, Arsenault et al. (2010) reported that spaying with exogenous artemisinin or artemisinic acid inhibited the transcriptions of genes *ADS* and *CYP71AV1*, and possibly feedback inhibited the biosynthesis of artemisinin in *A. annua*. As artemisinin is synthesized and stored in glandular trichomes, trichome density was reported to be highly consistent with artemisinin content (Lommen et al. 2006). A higher (1.4-fold more) trichome density was observed in the leaves of high-artemisinin producer *A. annua* cultivar than low-artemisinin producer *A. annua* cultivar (Maes et al. 2011). It may be possible to increase the artemisinin content tremendously by increasing the numbers of trichomes through manipulation of trichome development relevant genes. Another possible strategy to further increase artemisinin production is to increase the biomass of *A. annua* through manipulation of some genes regulating plant biomass. Taken together, more and combined transgenic strategies are needed for further investigation to finally solve the world shortage of artemisinin.

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