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Effects of overexpression of *AaWRKY1* on artemisinin biosynthesis in transgenic *Artemisia annua* plants

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ABSTRACT

The effective anti-malarial medicine artemisinin is costly because of the low content in *Artemisia annua*. Genetic engineering of *A. annua* is one of the most promising approaches to improve the yield of artemisinin. In this work, the transcription factor *AaWRKY1*, which is thought to be involved in the regulation of artemisinin biosynthesis, was cloned from *A. annua* var. Chongqing and overexpressed using the CaMV35S promoter or the trichome-specific CYP71AV1 promoter in stably transformed *A. annua* plants. The transcript level of *AaWRKY1* was increased more than one hundred times under the CaMV35S promoter and about 40 times under the CYP71AV1 promoter. The overexpressed *AaWRKY1* activated the transcription of *CYP71AV1* and moreover the trichome-specific overexpression of *AaWRKY1* improved the transcription of *CYP71AV1* much more effectively than the constitutive overexpression of *AaWRKY1*, i.e. up to 33 times as compared to the wild-type plant. However the transcription levels of FDS, ADS, and DBR2 did not change significantly in transgenic plants. The significantly up-regulated *CYP71AV1* promoted artemisinin biosynthesis, i.e. up to about 1.8 times as compared to the wild-type plant. It is demonstrated that trichome-specific overexpression of *AaWRKY1* can significantly activate the transcription of *CYP71AV1* and the up-regulated CYP71AV1 promotes artemisinin biosynthesis.

Introduction

Malaria, caused by the genus *Plasmodium*, has been a life-threatening disease for thousands of years. There are more than 380 million cases of malaria each year and more than 1 million deaths, according to the report from the World Health Organization (WHO, 2006). The traditional antimalarial medicines have the severe problem of drug resistance. In the 1970s, Chinese scientists found a new effective antimalarial medicine, i.e. artemisinin, which is extracted from the Chinese traditional medicinal herb *Artemisia annua* L. (Collaboration research group for Qinghaosu, 1979). Artemisinin is active against all *Plasmodium* species. Artemisinin and its derivatives, delivered in the form of artemisinin-based combination therapies (ACTs), are currently the best treatment option for malaria (WHO, 2006). Besides the antimalarial activity, artemisinin has antiviral, anticancer, and antischistosomal activity (Arsenault et al., 2008). Unfortunately the artemisinin content in *A. annua* is low, ranging from 0.01 to 1.0% dry wt., which results in a relatively high cost of artemisinin-based treatments. It is a serious problem for economically disadvantaged people in developing countries where malaria frequently occurs. Therefore a number of efforts have been made to enhance the production of artemisinin, including chemosynthesis, cell and tissue culture, plant genetic engineering, synthetic biology, and breeding of high artemisinin yielding plants (Covello, 2008; Graham et al., 2010; Liu et al., 2011). A yeast-based production of artemisinic acid, which can be converted to artemisinin in relatively good yields (40–45%), has been developed recently (Paddon et al., 2013). This process has been scaled up and the commercial production of artemisinin has been announced by Sanofi Aventis (http://www.path.org/news/press-room/422/). The yearly production will be around 50–60 tons of artemisinin, which, however, is not sufficient to cover the demand of this drug. Consequently, the plant will remain an important source for artemisinin.

In the last decade, there has been significant progress in elucidating the pathway of artemisinin biosynthesis (Fig. 1). Artemisinin is synthesized through the isoprenoid metabolic pathway.

Abbreviations: ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; CaMV, cauliflower mosaic virus; CPR, cytochrome P450 reductase; CYP71AV1, amorpha-4,11-diene 12-hydroxylase; DBR2, artemisinic aldehyde Δ11(13) reductase; FDS, farnesyl diphosphate synthase; GST, glandular secretory trichome; JA, jasmonate; SA, salicylic acid; WRKY, WRKY transcription factor.

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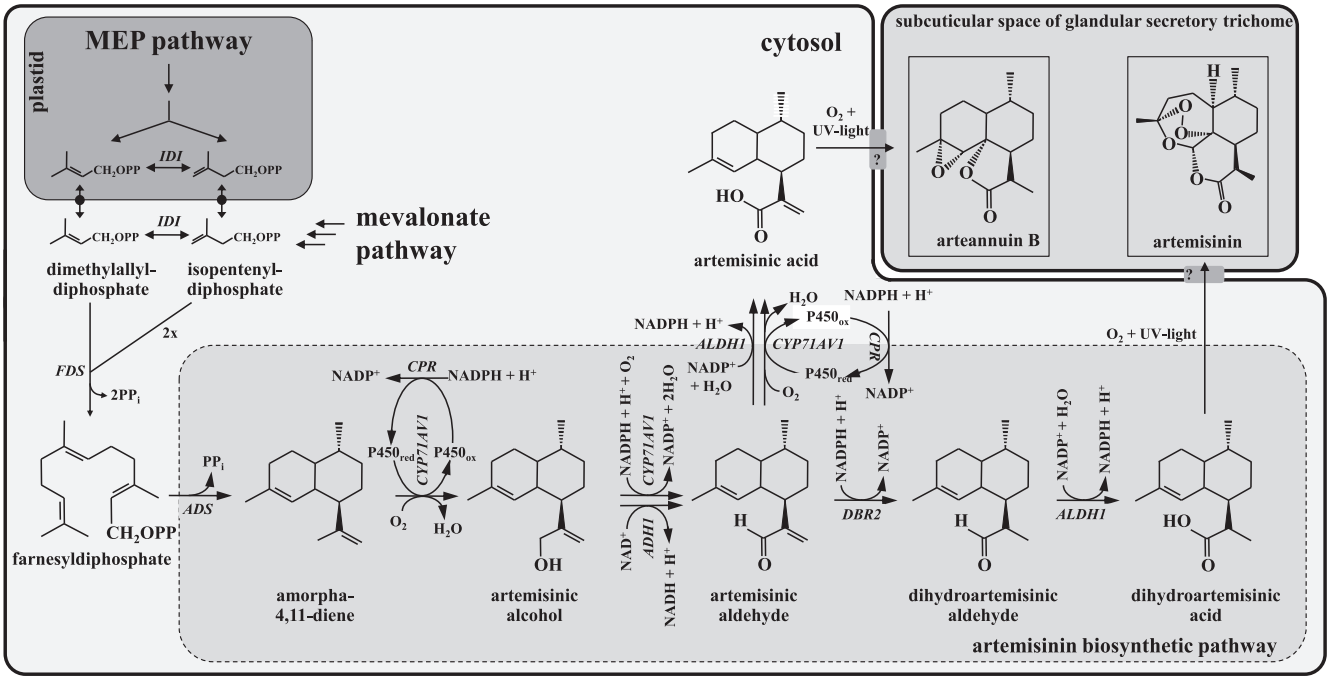

Fig. 1. Artemisinin biosynthetic pathway. FDS: farnesyl diphosphate synthase; ADS: amorpha-4,11-diene synthase; CYP71AV1: amorpha-4,11-diene 12-hydroxylase; CPR: cytochrome P450 reductase; ADH1: alcohol dehydrogenase 1; DBR2: artemisinic aldehyde Δ11(13) reductase; ALDH1: aldehyde dehydrogenase 1.

Cyclization of farnesyl diphosphate to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) is the initial step of the artemisinin biosynthesis (Bouwmeester et al., 1999; Mercke et al., 2000; Wallaart et al., 2001). In the following step, a cytochrome P450 dependent amorpha-4,11-diene 12-hydroxylase (CYP71AV1) catalyzes the oxidation of amorpha-4,11-diene to produce artemisinic alcohol, which is oxidized to artemisinic aldehyde by CYP71AV1 and/or alcohol dehydrogenase 1 (ADH1) (Paddon et al., 2013; Teoh et al., 2006). Next, the artemisinic aldehyde is reduced to dihydroartemisinic aldehyde by artemisinic aldehyde Δ11(13) reductase (DBR2) and subsequently oxidized to dihydroartemisinic acid by aldehyde dehydrogenase 1 (ALDH1) (Teoh et al., 2009; Zhang et al., 2008). The artemisinin biosynthetic pathway is branched and the intermediate artemisinic aldehyde may be oxidized to artemisinic acid by CYP71AV1 and/or ALDH1 (Teoh et al., 2006, 2009). The intermediates dihydroartemisinic acid and artemisinic acid are converted to artemisinin and arteannuin B, respectively, in a non-enzymatic reaction (Brown, 2010).

All the genes encoding the enzymes mentioned above have been cloned and genetic engineering of *A. annua* is becoming one of the most promising approaches to improve artemisinin yield. Overexpression of *Gossypium arboretum* or *A. annua* farnesyl diphosphate synthase (FDS) in *A. annua* plants increased the artemisinin content (from 0.3 to 0.9–1.0% dry wt.) (Chen et al., 2000; Han et al., 2006). Overexpression of ADS in *A. annua* plants increased the amounts of artemisinin, dihydroartemisinic acid, and artemisinic acid around 100%, 59%, 65%, respectively, and the absolute content of artemisinin was 0.9–1.0% dry wt. (Ma et al., 2009a). In transgenic *A. annua* plants in which HMGR was overexpressed alone or together with ADS, the artemisinin content was increased 1.22- and 6.65-fold, respectively, compared to the non-transgenic line (artemisinin content 0.02% dry wt.) (Alam and Abdin, 2011; Aquil et al., 2009). In transgenic *A. annua* plants in which the *CYP71AV1* and cytochrome P450 reductase (*CPR*) genes were overexpressed, the artemisinin content was increased 38%, as compared to the non-transgenic line of *A. annua* (700 μg/g fresh weight) (Shen et al., 2012). Since multiple enzymes are involved in artemisinin biosynthesis, it is more appropriate to up-regulate the activities of artemisinin biosynthetic enzymes simultaneously to effectively improve artemisinin synthesis.

Artemisinin synthesis was promoted by jasmonate (JA) and salicylic acid (SA) (Maes et al., 2011; Pu et al., 2009). The transcriptional level of FDS, ADS, CYP71AV1, DBR2, and CPR were increased when *A. annua* plants were treated with JA (Maes et al., 2011). It indicates that a transcriptional control network is involved in artemisinin biosynthesis. Transcription factors play an important role in controlling the transcription of biosynthetic genes and it is a major mechanism regulating secondary metabolite production in plants (Endt et al., 2002). It has been reported that WRKY transcription factor and AP2/ERF transcription factors are involved in artemisinin synthesis (Lu et al., 2013; Ma et al., 2009b; Yu et al., 2011). The WRKY family is among the ten largest families of transcription factors in higher plants. These transcription factors carry the WRKY domain which is a 60-amino acid stretch containing a conserved amino acid sequence of WRKYGQK together with a zinc-finger like motif. WRKY proteins specifically bind to a *cis-acting* DNA element, i.e. the W-box (TTGACC/T) to realize their biological function. The WRKY proteins are involved in regulating defense responses and developmental and physiological processes of plants, such as trichome initiation, senescence, and metabolism (Rushton et al., 2010). In *Arabidopsis*, MPK3/MPK6 signaling leads directly to phosphorylation of AtWRKY33 and then the phosphorylated WRKY protein binds to the promoter of *PAD3*, which encodes a P450 enzyme (CYP71B15) that carries out the last step of camalexin biosynthesis, a major phytoalexin in *A. thaliana* (Mao et al., 2011). CjWRKY1, which was cloned from *Coptis japonica*, plays a specific and comprehensive role in the pathway of benzylisoquinoline alkaloid biosynthesis (Kato et al., 2007). CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Suttipanta et al., 2011). In cotton plants, the transcription factor GaWRKY1 activates the transcription of the sesquiterpene synthase gene (+)-δ-cadinene synthase-A (CAD1-A) by means of interacting with the W-box element of the CAD1-A promoter (Xu et al., 2004). *AaWRKY1* was isolated from a cDNA library of the glandular secretory trichomes of *A. annua*; AaWRKY1 protein was capable of binding to the