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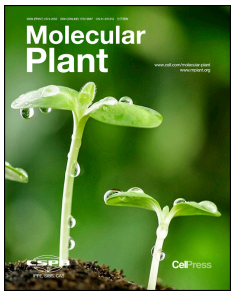


Promoting artemisinin biosynthesis in Artemisia annua plants by substrate channeling

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**Dear Editor**

Professor Youyou Tu, China Academy of Chinese Medical Sciences, Beijing, was awarded the 2015 Nobel Prize in Physiology or Medicine for "*her discoveries concerning a novel therapy against malaria*" (https://www.nobelprize.org/nobel_prizes/medicine/laureates/2015/press.html). This therapy is based on the sesquiterpenoid artemisinin, isolated from *Artemisia annua* L. Artemisinin and its derivatives, administrated in the form of artemisinin-based combination therapies (ACTs), are currently the best treatment of malaria.

In the past decade, the study of artemisinin biosynthesis has advanced considerably and the biosynthetic pathway has been elucidated (Figure 1A). A key enzyme of this pathway is amorpha-4,11-diene synthase (ADS), which is the first enzyme of the pathway converting farnesyldiphosphate (FDP) to the aliphatic sesquiterpene amorpha-4,11-diene. This intermediate has the right carbon skeleton for the biosynthesis of artemisinin. ADS is a rate-limiting enzyme with a very low turnover number, which is a general feature of terpene synthases.

Numerous efforts have been made to enhance the production of artemisinin, including synthetic biology, plant genetic engineering and breeding of high artemisinin yielding plants. Some genes of artemisinin biosynthesis (Figure 1A) have been overexpressed in *A. annua* and increased levels of artemisinin have been reported. However, low artemisinin producers (LAPs) have been used in these studies as summarized in Supplementary Table 1.

We have investigated a new strategy, *i.e.* substrate channeling, by overexpressing fused genes in two relatively high artemisinin producing varieties (HAPs) of *A. annua* (var. Anamed and var. Chongqing) (*cf.* supplementary Table 1). Substrate channeling is a process of directly transferring the product of one enzyme to the next enzyme of the pathway without equilibration with the bulk cytosol. Reaction rates can be significantly accelerated and therefore substrate channeling has great potential in metabolic engineering (Zhang, 2011). Enzyme complexes and multifunctional enzymes can be constructed through scaffolds or fusion of genes. Our group has previously reported on the fusion enzyme of farnesyldiphosphate synthase (FDS) and *epi*-aristolochene synthase (EAS), a sesquiterpene synthase involved in the biosynthesis of phytoalexins in tobacco (Brodelius et al., 2002). The amount of *epi*-aristolochene produced *in vitro* by the fusion enzyme was considerably higher than when the two single enzymes were used. In this study, ADS, the first and rate-limiting enzyme of artemisinin biosynthesis, and FDS were fused. For constitutive or trichome-specific expression of the fusion gene we used the

CaMV35S or the recombinant promoter of amorphadiene 12-hydroxylase (CYP71AV1), respectively (Wang et al., 2013).

The substrate for ADS, *i.e.* FDP, is the precursor of many primary and secondary metabolites is the plant. The fused enzyme ADS-FDS will convert IDP + DMADP to amorpha-4,11-diene (shaded box in Figure 1A). The fused enzyme was constructed in the same way as reported for the *EAS::FDS* construct (Brodelius et al., 2002). The *ADS::FDS* construct was introduced into a modified *pCAMBIA 1381Z* (Wang et al., 2010) in order to obtain two transformation vectors with different promoters, *i.e. pCAMBIA-1381Z-M::pCYP71AV1::[ADS-FDS]* (Figure 1B) and *pCAMBIA-1381Z-M::pCaMV35S::[ADS-FDS]* (Figure 1C). These vectors were used to transform two different varities of *A. annua* (var. Chongqing and var. Anamed) using the procedure previously reported (Han et al., 2005).

Twelve transgenic lines were analyzed by Southern blotting (supplementary Figure 1), *i.e.* transgenic lines of *A. annua* var. Chongqing (CYP-701, CYP-702, CYP-703) and var. Anamed (CYP-301, CYP-302, CYP-303), transformed with the vector *pCAMBIA-1381Z-M::pCYP71AV1::[ADS-FDS]* and transgenic lines of var. Chongqing (35S-801, 35S-802, 35S-803) and var. Anamed (35S-401, 35S-402, 35S-403), transformed with the vector *pCAMBIA-1381Z-M::pCaMV35S::[ADS-FDS]*.

A pair of primers giving a fragment (142 bp) spanning the fusion site of the fusion gene *[ADS-FDS]* was designed to analyze the transcripts level of the fusion gene (supplementary Table 2). The fusion gene, under the control of the CaMV35S promoter, was expressed at a much higher level than that controlled by the CYP71AV1 promoter (Figure 1D and 1E). Compared to the wild-type *A. annua* var. Chongqing, the transcript level of ADS was increased up to 24 and 4 times, respectively, when the CaMV35S promoter and CYP71AV1 promoter was used and in the Anamed variety the corresponding figures were 6.5 and 2 times, respectively. However, the transcript level of FDS was not increased to any great extent in any of the analyzed transgenic *A. annua* plants. The first enzyme of artemisinin biosynthesis, ADS, is specifically expressed in glandular secretory trichomes (GSTs) of *A. annua* (Wang et al., 2011), while FDS, whose product FDP is the substrate for the biosynthesis of various terpenoids such as sterols and sesquiterpenes, is constitutively expressed in plants. The transcript level of FDS is high in wild-type *A. annua* ($C_t \approx 20$ for both varieties) and therefore there is only a subtle increase of the FDS transcript level since the fusion gene *[ADS-FDS]* was expressed at much lower levels. For both

varieties, the recorded $C_t$ values for the fusion gene were around 28 and 24 for the CYP71AV1 and CaMV35S promoter, respectively. The transcript level of ADS in wild-type *A. annua* var. Anamed ($C_t \approx 22$) was higher than that in wild-type *A. annua* var. Chongqing ($C_t \approx 27$), which may explain why the observed increase of the transcript level of ADS in transgenic var. Chongqing was significantly higher than in transgenic var. Anamed.

The biosynthesis of artemisinin is located to GSTs of *A. annua* since enzymes of artemisinin biosynthesis, *i.e.* ADS, CYP71AV1 and DBR2, are specifically expressed in GSTs (Wang et al., 2011, 2013; Jiang et al., 2014). Our results showed that the dihydroartemisinic acid and artemisinin content of transgenic plants was increased significantly when the fusion gene *[ADS-FPS]* was specifically expressed in GSTs (Figures 1F and 1G). Artemisinin content was increased up to 2.5 and 2.0 times for var. Chongqing and var. Anamed, respectively, and the absolute content was around 2.5% DW in both varieties, which is a relatively high yield (cf. supplementary Table 1). Compared with the CYP71AV1 promoter, the CaMV35S promoter was not so effective to improve artemisinin content (Figures 1F and 1G) even though the expression levels of the fusion gene were much higher (Figures 1D and 1E). These results are explained by the fact that most of the recombinant fusion enzyme in this case is found in tissues where there is no artemisinin biosynthesis. In the case of the CYP71AV1 promoter, the recombinant fusion enzyme is located to trichomes where all the enzymes of artemisinin biosynthesis are expressed.

It may be pointed out that the levels of artemisinic acid and arteannuin B (*c.f.* Figure 1A) were only marginally increased in plants overexpressing the *ADS-FDS* gene (data not shown). Obviously the increased carbon flow in these transgenic high artemisinin-producing plants is directed towards dihydroartemisinic acid and artemisinin biosynthesis. DBR2, located at the branching point (*c.f.* Figure 1A), is the key enzyme of this channeling and it was recently shown that the promoters of the DBR2 gene of high artemisinin-producing varieties of *A. annua* including var. Chongqing and var. Anamed carry a unique nucleotide sequence believed to be involved in the up-regulation of the DBR2 gene (Yang et al., 2015).

It may be of interest to look at the kinetic properties of the two fused enzymes. In general, the $k_{cat}$-values for sesquiterpene synthases are very low *in vitro* ($< 1.0$ min$^{-1}$) and most likely also *in vivo* compared to those of prenyltransferases such as FDS as exemplified by the enzyme from *Artemisia tridentata* ($> 150$ min$^{-1}$) (Hemmerlin et al., 2003). FDS is a homodimer and we assume that a dimer of the fusion protein is formed *in vivo*. In the study on the fusion of FDS and EAS, it

was reported that the $K_m$ value for FDP with the recombinant tobacco EAS and the two fusion enzymes EAS/FDS and FDS/EAS was 1.7, 1.6 and 2.6 μM, respectively, indicating that the dimerization does not affect the steady state kinetics of the fusion enzyme to any great extent (Brodelius et al., 2002). The $K_m$-value for recombinant ADS has been reported to be 2.0 μM (Picaud et al., 2005) and that of FDS 5.5, 7.1 and 1.6 μM for DMADP, IDP and GDP, respectively (Hemmerlin et al., 2003). We suggest that the fact that FDS exhibits a much higher turnover rate than ADS is beneficial for the overall conversion of IDP/DMADP to amorpha-4,11-diene. It is likely that due to the relatively high potential turnover capacity of the FDS and proximity of the two enzymes, the ADS of the fusion protein will experience a higher local FDP concentration than the native ADS present in the cytosol. It is likely that the ADS of the fusion enzyme operates at substrate saturation, which results in a maximum production of amorpha-4,11-diene by the fusion enzyme resulting in significantly increased artemisinin production.

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**AUTHOR CONTRIBUTIONS**

J.H. and P.E.B. conceived and designed the experiments. J.H., H.W., S.K., M.H. and A.L. performed the experiments. J.H. and P.E.B. analyzed the data. J.H. and P.E.B. wrote the manuscript.

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Figure 1

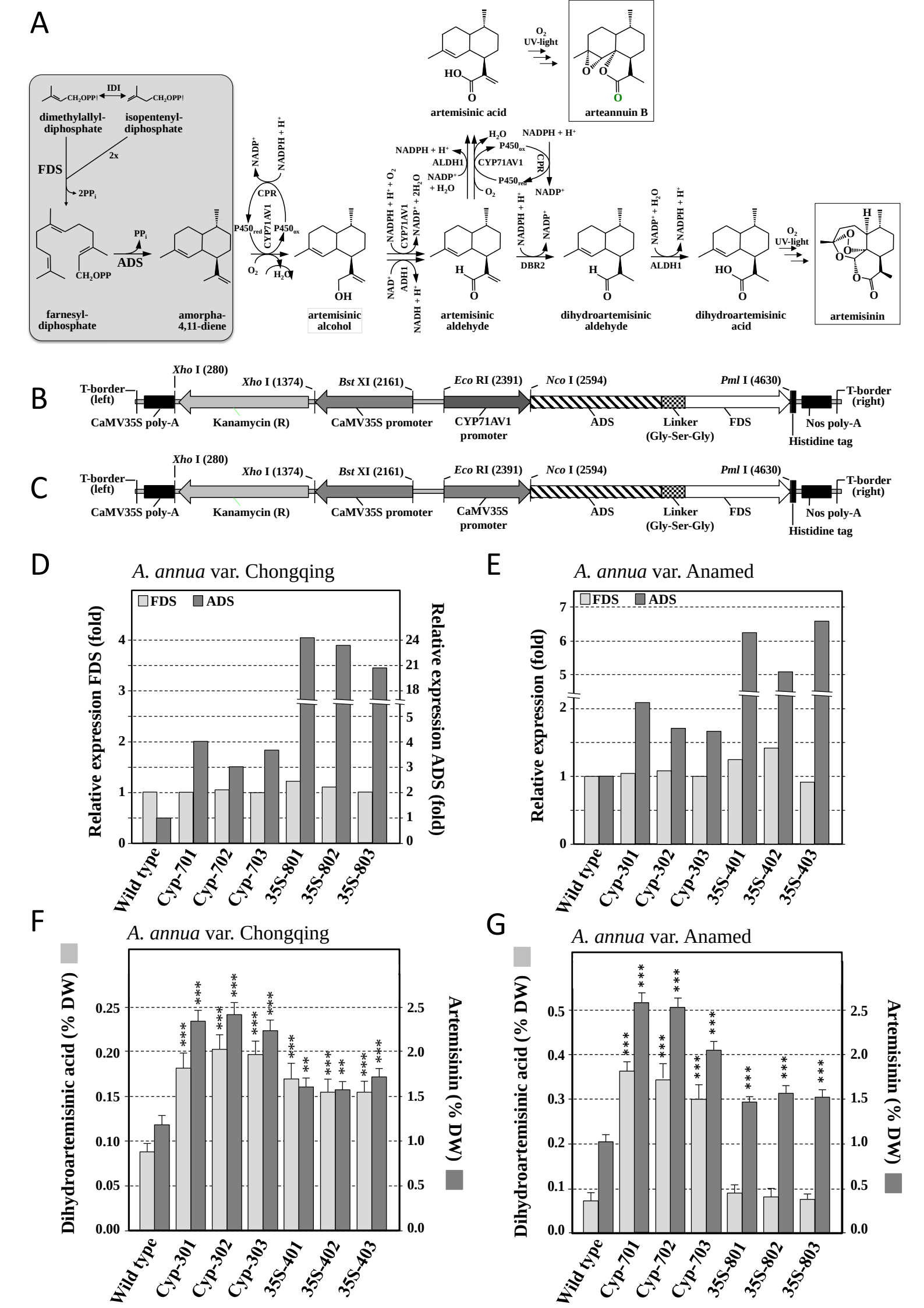
Overexpression of fused genes in *Artemisia annua.*

**(A)** Artemisinin biosynthetic pathway in *A. annua*. FDS: farnesyl diphosphate synthase; ADS: amorph-4,11-diene synthase; CYP71AV1: amorpha-4,11-diene 12-hydroxylase; ADH1: alcohol dehydrogenase 1; DBR2: artemisinic aldehyde Δ11(13) reductase; ALDH1: aldehyde dehydrogenase 1

**(B, C)** Schematic representation of the T-DNA region of the *pCAMBIA-1381Z-M::pCYP71AV1:: [ADS-FDS]* (B) and *pCAMBIA-1381Z-M::pCaMV35::[ADS-FDS]* (C) constructs for stable transformation of *A. annua*.

**(D, E)** Relative expression of *ADS* and *FDS* in young leaves of trangenic *A. annua* var. Chongqing (D) and var. Anamed (E). In transgenic lines CYP-301, CYP-302, CYP-303, CYP-701, CYP-702 and CYP-703 the fusion gene *[ADS-FPS]* was expressed using the CYP71AV1 promoter; In transgenic lines 35S-401, 35S-402, 35S-403, 35S-801, 35S-802, 35S-803 the fusion gene *[ADS-FPS]* was expressed using the CaMV35S promoter.

**(F, G)** The content of dihydroartemisinic acid and artemisinin in transgenic *A. annua* var. Chongqing (F) and var. Anamed (G). In transgenic lines CYP-301, CYP-302, CYP-303, CYP-701, CYP-702 and CYP-703 the fusion gene *[ADS-FPS]* was expressed using the CYP71AV1 promoter; In transgenic lines 35S-401, 35S-402, 35S-403, 35S-801, 35S-802, 35S-803 the fusion gene *[ADS-FPS]* was expressed using the CaMV35S promoter.


A
IDI
dimethylallyl-diphosphate
isopentenyl-diphosphate
FDS
2x
2PPi
PPi
ADS
CH2OPP
farnesyl-diphosphate
amorpha-4,11-diene
CPR
CYP71AV1
artemisinic alcohol
ALDH1
artemisinic aldehyde
DBR2
dihydroartemisinic aldehyde
dihydroartemisinic acid
artemisinic acid
UV-light
arteannuin B
artemisinin
B
T-border (left)
Xho I (280)
Xho I (1374)
Bst XI (2161)
Eco RI (2391)
Nco I (2594)
Pml I (4630)
T-border (right)
CaMV35S poly-A
Kanamycin (R)
CaMV35S promoter
CYP71AV1 promoter
ADS
Linker (Gly-Ser-Gly)
FDS
Nos poly-A
Histidine tag
C
CaMV35S promoter
D
A. annua var. Chongqing
FDS
ADS
Relative expression FDS (fold)
Relative expression ADS (fold)
Wild type
Cyp-701
Cyp-702
Cyp-703
35S-801
35S-802
35S-803
E
A. annua var. Anamed
Relative expression (fold)
Cyp-301
Cyp-302
Cyp-303
35S-401
35S-402
35S-403
F
Dihydroartemisinic acid (% DW)
Artemisinin (% DW)
G