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Enhancement of artemisinin biosynthesis by overexpressing *dxr*, *cyp71av1* and *cpr* in the plants of *Artemisia annua* L.

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Abstract

Artemisinin is extracted from a traditional Chinese medicinal herb *Artemisia annua* L., which is regarded as the most efficient drug against malaria in the world. In recent years, attention has been paid to increase the artemisinin content through transgenic methods because of the low content of artemisinin in wild plants. In this article, three functional artemisinin-related genes namely *dxr*, *cyp71av1* and *cpr*, were used to genetically modify the artemisinin biosynthesis pathway in *A. annua*. Four independent transgenic lines of *A. annua* plants with overexpression of *dxr* and five independent transgenic *A. annua* lines with overexpression of both *cyp71av1* and *cpr* were obtained and confirmed through genomic PCR. All the transgenic *A. annua* plants with overexpression of the *dxr* gene showed higher levels of artemisinin than the wild type. The content of artemisinin in the Line D24 with overexpression of *dxr* (1.21±0.01 mg·g^{-1} DW) was more than two times compared with that in the wild type (0.52±0.01 mg·g^{-1} DW). All the five lines of co-overexpressing *cyp71av1* and *cpr* had an increase of artemisinin production compared with the wild-type *A. annua*. Line No. 16 with overexpression of *cyp71av1* and *cpr* had the highest content of artemisinin (2.44±0.13 mg·g^{-1} DW) at nearly three times of the wild-type *A. annua* (0.91±0.02 mg·g^{-1} DW). Thus, the present study demonstrated that genetic modification of the upstream 2-C-methyl-D-erythritol 4-phosphate pathway or metabolic engineering of the artemisinin-specific pathway could, respectively, enhance artemisinin biosynthesis. These strategies could be applied to develop transgenic *A. annua* with higher levels of artemisinin.

Keywords: artemisinin, *Artemisia annua* L., *cyp71av1*, *cpr*, *dxr*, transgene.
Abbreviations: ADS: Amorpha-4,11-diene synthase gene; CPR: Cytochrome P450 reductase; CYP71AV1: Amorpha-4,11-diene hydroxylase; DMAPP: Dimethylallyl diphosphate; DW: Dry weight; DXP: 1-deoxy-D-xylulose-5-phosphate; DXR: 1-Deoxy-D-xyluolse 5-phosphate reductase; ELSD: Evaporative Light Scattering Detector; FPS: Farnesyl pyrophosphate synthase; HMGR: 3-Hydroxy-3-methyl-glutaryl-CoA reductase; HPT: Hygromycin phosphotransferase; IPP: Isopentenyl diphosphate; MEP: 2-C-methyl-D-erythritol 4-phosphate; MS: Murashige and Skoog; MVA: Mevalonic acid; NOS: Nopaline opine synthase; *npt*II: Neomycin phosphotransferase II gene; SDS: Sodium dodecyl sulfate; WHO: World health organization.

Introduction

Malaria is a serious disease especially in the underdeveloped countries. It is caused by *Plasmodium falciparum* infection through mosquito bite. Every year, more than one million deaths are caused by malaria infection, which brings great pain and economic loss, especially in Africa, where the environment is suitable for malaria spreading, and the medical service is very limited (Greenwood and Mutabingwa, 2002). Fortunately, artemisinin-combined treatment is the most promising method to cure malaria with little side effect and low toxicity recognized by WHO (Graham et al., 2010). Artemisinin is a sesquiterpene with an endoperoxide bridge which is the active group contributing to its antimalarial function. It is an active drug against both chloroquine-resistant and chloroquine-sensitive *Plasmodium falciparum*. Besides, it is also effective against other diseases, such as breast cancer and leukemia (Romero et al., 2005; Sen et al., 2007). Due to its high medical values, many research groups have been focusing on this kind of Chinese medicine, *Artemisia annua* L. Artemisinin is mainly extracted from the aerial part (leaves, flowers, stems) of *A. annua*, which mainly grows in Sichuan, Guizhou, Chongqing, Yunnan, Guangxi and Hunan province of West China. However, the artemisinin content is so limited in the wide-type *A. annua* plants, which can not meet the urgent demands of the world market (van Agtmael et al., 1999). Recently, with the development of molecular biology and biochemistry of artemisinin biosynthesis, it has become possible to improve the production of artemisinin by genetic engineering.

The 5-carbon precursors such as IPP and DMAPP are provided by the cytosolic MVA pathway and the plastidial MEP pathway. Classic research demonstrated that only the MVA pathway provided the IPP and DMAPP for artemisinin biosynthesis (Akhila et al., 1987). Further, transgenic studies

showed that genetic modification of the MVA pathway could enhance artemisinin biosynthesis by overexpressing the committed-step enzyme HMGR involved in the MVA pathway (Nafis et al., 2011). In the recent years, the MEP pathway was found to provide IPP and DMAPP for artemisinin biosynthesis (Towler and Weathers. 2007).

1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is the committed-step enzyme involved in the MEP pathway, which catalyzes the conversion of DXP to form MEP (Hasunuma et al., 2008). The gene encoding DXR has been successfully employed to enhance terpenoid biosynthesis such as monoterpenoids in peppermint by overexpressing *dxr*. According to the genetic map of *A. annua* constructed by Graham et al (2010), the *dxr* gene was tightly linked with high levels of artemisinin.

Accordingly, overexpression of *dxr* was used to test the effects of genetic modification of the MEP pathway on artemisinin biosynthesis in the present study. Amorpha-4,11-diene hydroxylase (CYP71AV1) is a multifunctional enzyme belonging to the cytochrome P450 family, which catalyzes multiple oxidations of the sesquiterpene intermediate amorpha-4, 11-diene to form artemisinic acid (Covello et al., 2007; Ro et al., 2005). The expression analysis exhibited that *cyp71av1* specifically expressed in glandular secretory trichomes (GSTs) which was regarded as the biological factory of artemisinin biosynthesis, and might be a candidate gene for metabolic engineering of artemisinin biosynthesis (Teoh et al., 2006; Olsson et al., 2009). Additionally, Cytochrome P450 reductase (CPR) also belongs to the cytochrome P450 family, which is a functional assistant gene of *cyp71av1* (Teoh et al., 2006). In the present study, the three genes including *dxr*, *cyp71av1* and *cpr* were also used to genetically modify the biosynthetic pathway of artemisinin by the gene-overexpression strategy and to develop transgenic *A. annua* with higher levels of artemisinin.

Results

Establishment of transgenic Artemisia annua

The leaf explants were cultured on shoot induction medium supplemented with 20 $mg{\cdot}L^{1}$ kanamycin (overexpression of *dxr*) or 10 $mg{\cdot}L^{-1}$ hygromycin (overexpression of *cyp71av1* and *cpr*). The kanamycin-resistant or hygromycin-resistant explants generated shoot clusters. Instead, the non-resistant explants turned yellow and ultimately died (Fig. 1). The putative transgenic resistant shoots were sub-cultured for 3 times to get rid of *Agrobacterium* and were further elongated during one month culture on shoot elongation medium devoid of kanamycin and hygromycin. Roots grew within 1 week after the shoots were transferred onto rooting medium (Fig. 1). A 295-bp product was amplified from *Agrobacterium* with fvirD1 and rvirD1 as primers. This product could not be amplified from all *A. annua* non-transformed lines under the same amplification condition. The 783-bp *nptII* and the 607-bp *dxr* fragments were amplified from transgenic *A. annua* overexpressing *dxr* and the plasmid p2301^{+}-*dxr*, while not amplified from nontransgenic *A. annua* (Fig. 2A). The 794-bp *hpt*, the 502-bp *cyp71av1* fragment and the 376-bp *cpr* fragment, were simultaneously amplified from transgenic *A. annua* with overexpression of *cyp71av1* and *cpr* and the positive control (p1304^{+}-*cyp71av1*-*cpr*), but were not amplified from nontransgenic *A. annua* (Fig. 2B). All the PCR results showed that the genes of interest including *dxr*, *cyp71av1* and *cpr* integrated into the genome of *A. annua.*

HPLC analysis of artemisinin

Artemisinin content in leaves of transgenic and non-transgenic plants was analyzed by HPLC. Retention time of standard artemisinin was 11.81 min (Fig. 3A), which was identical with that of artemisinin in plant samples (Fig. 3B). Artemisinin was detected in both transgenic and non-transgenic *A. annua* materials but varied in the different lines. The results indicated that all the transgenic plants had an increase of artemisinin levels compared with non-transgenics. In all the four lines of *dxr*-transformed *A. annua* plants, the contents of artemisinin were 0.62±0.01 $mg{\cdot}g^{-1}$ DW to 1.21±0.01 $mg{\cdot}g^{-1}$ DW (Fig. 4A), which were 1.21 to 2.35 folds higher, respectively, compared to wild type (0.52±0.01 $mg{\cdot}g^{-1}$ DW). Transgenic line D18 and D47 only exhibited a slight increase of artemisinin (about 20% increase), while line D36 (75% increase) and especially D24 exhibited a dramatic increase of artemisinin (135% increase). Co-overexpression of *cyp71av1* and *cpr* showed remarkable increase of artemisinin in all the five transgenic *A. annua* plants. The contents of artemisinin were lowest 1.46±0.03 $mg{\cdot}g^{-1}$ DW to highest 2.44±0.13 $mg{\cdot}g^{-1}$ DW (Fig. 4B), which were 1.61 to 2.69 folds, respectively, compared with those of wild type (0.91±0.02 $mg{\cdot}g^{-1}$ DW). The results of HPLC analysis of artemisinin demonstrated that genetic engineering of both the upstream MEP pathway or the downstream artemisinin-specific pathway could enhance biosynthesis of artemisinin.

Discussion

Metabolic engineering is an efficient method to genetically modify the targeted plant metabolites, and it is also a hot spot of genetic engineering of medicinal plants. Previously, the strategy of metabolic engineering to break the rate-limiting reactions was applied to enhance biosynthesis of artemisinin. Both *hmgr* and *ads* were simultaneously overexpressed in *A. annua*, where the results demonstrated that co-transformation of *hmgr* and *ads* was a promising approach to improve the biosynthetic ability of artemisinin in *A. annua* (Alam and Abdin, 2011); overexpression of both *hmgr* and *fps* also led to enhancement of artemisinin accumulation in transgenic *A. annua* (Wang et al., 2011).

These reports demonstrated that breaking the rate-limiting reaction by overexpressing the rate-limiting enzymes was a suitable strategy to enhance biosynthesis of artemisinin. However, all the published literatures did not confirm the genetically modification of the MEP pathway that might also be important in artemisinin biosynthesis. DXR has been deemed to be the rate-limiting enzyme involved in the MEP pathway and successfully used to enhance biosynthesis of monoterpenoids such as peppermint essential oils (Hasunuma et al., 2008). Furthermore, the genetic map of *Artemisia annua* showed that *dxr* was tightly linked with artemisinin production (Graham et al., 2010). The transgenic results in the present study indicated that metabolic engineering of the MEP pathway by overexpression of *dxr* in *A. annua* could lead to higher production of artemisinin and this provided direct proofs that the MEP pathway anticipated in artemisinin biosynthesis.

Amorpha-4,11-diene hydroxylase (CYP71AV1) assisted by CPR oxidizes amorpha-4,11-diene to artemisinic alcohol, turns to artemisinic aldehyde, and artemisinic acid sequentially by three steps of oxidation processes. Afterwards, artemisinic acid or its hydrogenated product, dihydroartemisinic acid, is transformed to artemisinin (Covello et al., 2007). CYP71AV1 plays a very important role in artemisinin biosynthesis. The present transgenic results exhibited that all the transgenic *A.*

Table 1. The primers used to construct the plant expression vectors. The restriction sites were underlined.

Gene	Sequences	Restriction site
dxr	Forward: 5′-CCGGATCCatggctttgaattcgctgtc-3′	*Bam*H I
	Reverse: 5′-CCGAGCTCtcatacaagggcagggct-3′	*Sac* I
cyp71av1	Forward:5′-GCTCTAGAATGAAGAGTATACTAAAAGCA-3′	*Xbar* I
	Reverse: 5′-CCGGATCCCTAGAAACTTGGAACGAGTA-3′	*Bam*H I
cpr	Forward: 5′-CCACTAGTATGCAATCAACAACTTCCG-3′	*Spe* I
	Reverse: 5′- CCGGTTACCTTACCATACATCACGGAGATA -3′	*BstP* I

Table 2. The primers used to detect the genes of interest.

Primer	Sequence
fnptII	5′-tgggtggagaggctattcg-3′
rnptII	5′-tgagcctggcgaacagttc-3′
f35S	5′-catcgttgaagatgcctctgc-3
rdxr	5′- tcaacaacaccttgttcgccag -3′
rcyp71av1	5′-gatttggaacgagtagcgaacttg-3′
rcpr	5′-ctcatcctccgtcactttcttc-3′
fhpt	5′-agtacttctacacagccatcgg-3′
rhpt	5′-tccggaagtgcttgacattgg-3′
fvirD1	5′-gagtatgaaaccttttcctatca-3′
rvirD1	5′-gggatacggacaaaatggag-3′

annua plants (overexpression of cyp71av1 and cpr) had significantly higher contents of artemisinin than the non-transgenic plants.

These results indicated that CYP71AV1 assisted by CPR might be the bottleneck reaction involved in the artemisinin biosynthetic pathway and overexpression of *cyp71av1* and *cpr* could greatly enhance biosynthesis of artemisinin. Since engineering the upstream MEP pathway or the downstream artemisinin-specific pathway could promote artemisinin biosynthesis, a possible better strategy to enhance artemisinin biosynthesis might be engineering both the upstream pathway and the downstream pathway.

Materials and methods

Plant materials and culture conditions

The seeds of *A. annua* (harvested in the Medicinal Plant Garden of Southwest University, Chongqing, China) were surface-sterilized with 75 % alcohol for 30 sec, then followed by washing with sterile distilled water for three times. The washed seeds were sterilized with 0.1% mercuric chloride for 10 min again before being washed with sterile distilled water three times. Finally the bacteria-free seeds were put on the sterile filter papers and transferred to MS solid medium in flasks. The plants were cultured at 25 ± 1 °C in 16 h photoperiods.

Plant expression vector construction

The coding sequence of *dxr* (AF112881) was amplified from *Catharanthus roseus* (periwinkle) with a pair of primes, fdxr with *Bam*H I and rdxr with *Sac* I. The coding sequence of *dxr* was introduced in to the plant expressing vector p2301^{+} (Liao, 2004) by the restriction sites of *Bam*H I and rdxr with *Sac* I to generate the plant expression vector p2301^{+}-*dxr* (Fig. 5A). The coding sequence of *cyp71av1* (DQ268763) was cloned from *A. annua* by the forward primer fcyp71av1 with *Xba* I and the reverse primer with *Bam*H I, *cpr* (DQ318192) by the forward primer fcpr with *Spe* I and the reverse primer rcpr with *Bst*P I. The plant expressing vector p1304^{+} (Liao, 2004) was used to carry both *cyp71av1* and *cpr* to generate the plant expression vector p1304^{+}-*cyp71av1-cpr* (Fig. 5B). Finally, *Agrobacterium tumefaciens* strain LBA4404 was employed to harbor p2301^{+}-*dxr* and p1304^{+}-*cyp71av1-cpr*. The sequences of

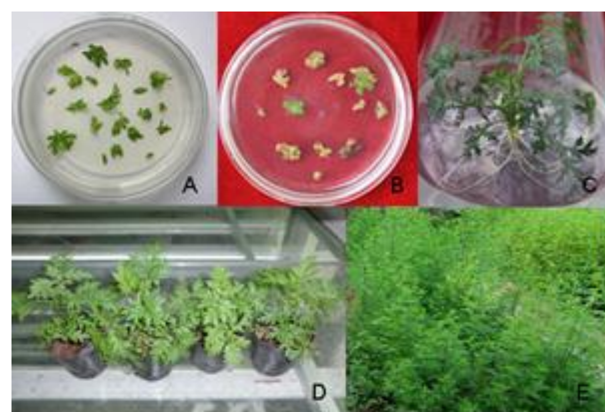

Fig 1. Establishment of transgenic *A. annua* plants. A: obtainment of antibiotics-resistant shoots. B: propagation of transgenic *A. annua*. C: rooting of transgenic *A. annua*; D: transplantation of transgenic *A. annua* into pot; E: transgenic *A. annua* growing in the field.

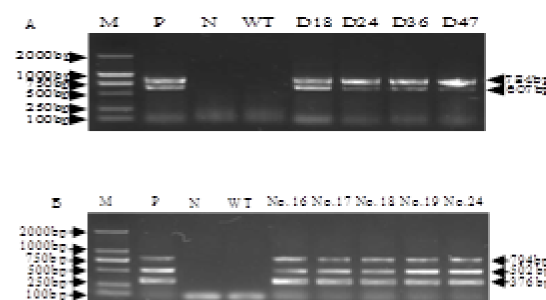

Fig 2. Detection of the genes of interest by genomic PCR. A: detection of *nptII* (784 bp) and *dxr* (607 bp); B: detection of *hpt* (794 bp), *cyp71av1* (502 bp) and *cpr* (376 bp). M: DL200marker; P: positive control; N: negative control; WT: wild-type *A. annua*; D18-D47: transgenic *A. annua* with overexpression of *dxr*; No.16-No.24 transgenic *A. annua* plants with overexpression of *cyp71av1* and *cpr*. The arrows indicated the related DNA fragments.

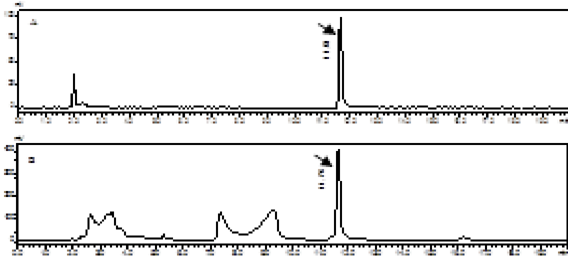

Fig 3. HPLC analysis of artemisinin. A: analysis of authentic artemisinin (retention time: 12.99 min); B: analysis of artemisinin in plant samples (retention time: 13.15 min). The peak of artemisinin was marked with an arrow.

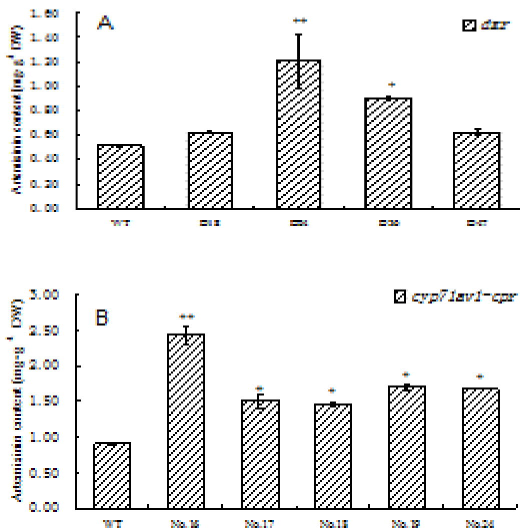

Fig 4. The artemisinin contents in plants. A: the contents of artemisinin in *dxr*-overexpressed *A. annua* and the wile-type; B: the contents of artemisinin in *cyp71av1-cpr*-overexpressed *A. annua* and the wild type (**P<0.01, *P<0.05).

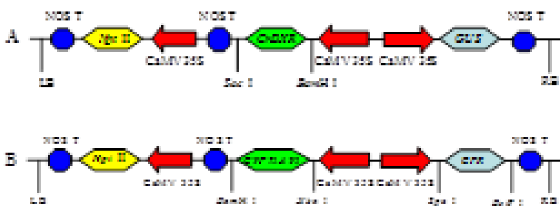

Fig 5. The sketch maps of A: p2301+-*dxr* and B: p1304+-*cyp71av1-cpr*.

primers used to construct plant expression vectors were listed in Table 1.

Establishment of transgenic A. annua plants

The genetic transformation of *A. annua* explants were performed according to Wang et al. 2011 with some modification as follows: the transformed explants of *A. annua* were put on filter papers on MS medium instead of on MS medium directly. After a 2-day coculture on basic MS medium added with 100 μM·L^{-1} acetosyringone in dark, infected explants were transferred to shoot induction medium with 1 mg·L^{-1} 6-benzyladenine (6-BA), 0.1 mg·L^{-1} α-naphthaleneacetic acid (NAA), 400 mg·L^{-1} cefotaxime and 10 mg·L^{-1} hygromycin (for screening transformants of *cyp71av1* and *cpr*) or 20 mg·L^{-1} kanamycin (for screening transformants of *dxr*). The 2cm long buds were cut and sub-cultured on solid MS medium with 0.1 mg·L^{-1} NAA, 250 mg·L^{-1} cefotaxime as root induction medium. All plant cultures were maintained at 25 °C under 16 hour photoperiod. When the antibiotics-resistant plantlets grew to 5-7 cm high, they were transferred in to pot with Pindstrup substrates and fertilized with MS liquid medium and finally grew in the field under natural sunlight. The dxr-transformed plants were harvested in May of 2011 when they grew to 0.5 m height and the plants with co-transformation of *cyp71av1* and *cpr* were collected in August of 2011 when they grew to about 1 m.

DNA isolation and PCR analysis

Genomic DNAs of *A. annua* were isolated by SDS method (Dellaporta et al., 1983). Integration of the transformed gene into the plant genome was confirmed by genomic PCR using primers (Table 2). For amplification of *dxr*, *cyp71av1* and *cpr*, a DNA fragment of 35S promoter (f35S) was used as the forward primer. The gene-specific reverse primers were rdxr, rcyp71av1 and rcpr for the corresponding genes, respectively. p2301+-*dxr* and p1304+-*cyp71av1-cpr* were used as the positive control. The wild-type *A. annua* plants were used as the negative control. The standard genomic PCR was performed to amplify the gene fragments of interest. Meanwhile, to avoid the interference of *Agrobacterium* to PCR results, a pair of primers, fvirD1 and rvirD1, were designed according to our previous study (Wang et al., 2011).

Measurements of Artemisinin

The leaves of *A. annua* were collected and dried at 50 °C for 48 h. A 0.2 g of the dry powder were extracted with 20 mL of petroleum ether in an ultrasonic bath for 30 min then filtered and evaporated to dryness in vacuum at 50° C. Finally the residue was re-dissolved in 5 mL of methanol. One milliliter of the solution was filtered through a 0.45 μm nylon membrane filter. The filtered solutions were analyzed by Shimadzu LC-6AD HPLC system (Shimadzu ELSD-LTII detector). The HPLC conditions were Phenomenex C18 column using acetonitrile: water (60:40 v/v) mixture as mobile phase at a flow rate of 1 mL·min^{-1}. ELSD conditions were optimized at nebulizer-gas pressure of 325Kpa and drift tube temperature of 60 °C and the gain was set at 8. The artemisinin purchased from Sigma was used as the authentic control in the measurement. For each sample, the injection volume was 20 μL, and the results were analyzed with Empower data system. At least three replications (n≥3) were performed and the statistical significance of artemisinin contents was analyzed by T-test using SPSS.

Conclusion

Engineering the MEP pathway in *A. annua* by overexpressing *dxr* enhanced biosynthesis of artemisinin. This suggested that the MEP pathway participated artemisinin biosynthesis. Overexpression of both *cyp71av1* and *cpr* also promoted accumulation of artemisinin in transgenic *A. annua*. These transgenic results indicated that the gene-overexpression strategy of metabolic engineering was a suitable approach to develop transgenic *A. annua* plants with higher levels of artemisinin.

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