Overexpression of artemisinic aldehyde Δ11 (13) reductase gene–enhanced artemisinin and its relative metabolite biosynthesis in transgenic *Artemisia annua* L.

Yuan Yuan[1†]
Wanhong Liu[2†]
Qiaozhuo Zhang[1]
Lien Xiang[1]
Xiaoqiang Liu[1]
Min Chen[3]
Zhi Lin[1]
Qiang Wang[4]
Zhihua Liao[1*]

[1] *Key Laboratory of Eco-Environments in Three Gorges Reservoir Region (Ministry of Education), Chongqing Engineering and Technology Research Center for Sweetpotato, School of Life Sciences, Southwest University, Chongqing 400715, People's Republic of China*

[2] *School of Chemistry and Chemical Engineering, Chongqing University of Science and Technology, Chongqing 401331, People's Republic of China*

[3] *College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, People's Republic of China*

[4] *China Rural Technology Development Center, Ministry of Science and Technology, Beijing 100045, People's Republic of China*

Abstract

Artemisinic aldehyde Δ11 (13) reductase (DBR2) is the checkpoint enzyme catalyzing artemisinic aldehyde to form dihydroartemisinic aldehyde directly involved in artemisinin biosynthetic pathway. In the present study, DBR2 was employed to engineer the biosynthetic pathway of artemisinin in transgenic plants of *Artemisia annua* L. Seven independent transgenic plants of *A. annua* with DBR2 overexpression driven by the cauliflower mosaic virus 35S promoter were obtained by *Agrobacterium*-mediated genetic transformation and confirmed by genomic PCR. The results of real-time qPCR analysis showed that the expression levels of *DBR2* gene in all the seven transgenic lines were significantly higher than in nontransgenic control. The high-performance liquid chromatography analysis of artemisinin and its relative metabolites demonstrated that the contents of artemisinin and its direct precursor dihydroartemisinic acid were remarkably increased in the transgenic plants of *A. annua* with DBR2 overexpression. Interestingly, it was also found that the contents of arteannuin B and its direct precursor artemisinic acid in the branch pathway competing against artemisinin biosynthesis were also improved in DBR2-overexpressed *A. annua* plants. The transgenic results in the present study indicated that *DBR2* is a useful structural gene in engineering the artemisinin biosynthetic pathway to develop genetically modified *A. annua* with the higher yield of artemisinin. Volume 62, Number 1, Pages 17–23, 2015

Keywords: *Artemisia annua L., DBR2, overexpression, artemisinin biosynthesis*

1. Introduction

As is known, death caused by malaria is a terrible shadow over developing countries, especially in Africa [1]. It is bad news for malarial patients that the two

Abbreviations: *ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; CPR, cytochrome P450 reductase; CYP71AV1, cytochrome P450 monooxygenase; DBR2, artemisinic aldehyde Δ11 (13) reductase; DMAPP, dimethylallyl diphosphate; DW, dry weight; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; GSTs, glandular secretory trichomes; HPLC, high-performance liquid chromatography; HPT, hygromycin phosphotransferase; MEP, methylerythritol phosphate; MS, Murashige and Skoog; MVA, mevalonate; NAA, α-naphthaleneacetic acid; IPP, isopentenyl diphosphate.*

**Address for correspondence: Zhihua Liao, PhD, Professor of Plant Genetics and Biotechnology, School of Life Sciences, Southwest University, People's Republic of China. Tel.: +86 23 68367146; Fax: +86 23 68367146; e-mail: zhliao@swu.edu.cn, zhihualiao@163.com.*

†These authors contributed equally to the paper.

Received 3 March 2014; accepted 10 April 2014

DOI: 10.1002/bab.1234

Published online 7 July 2014 in Wiley Online Library (wileyonlinelibrary.com)

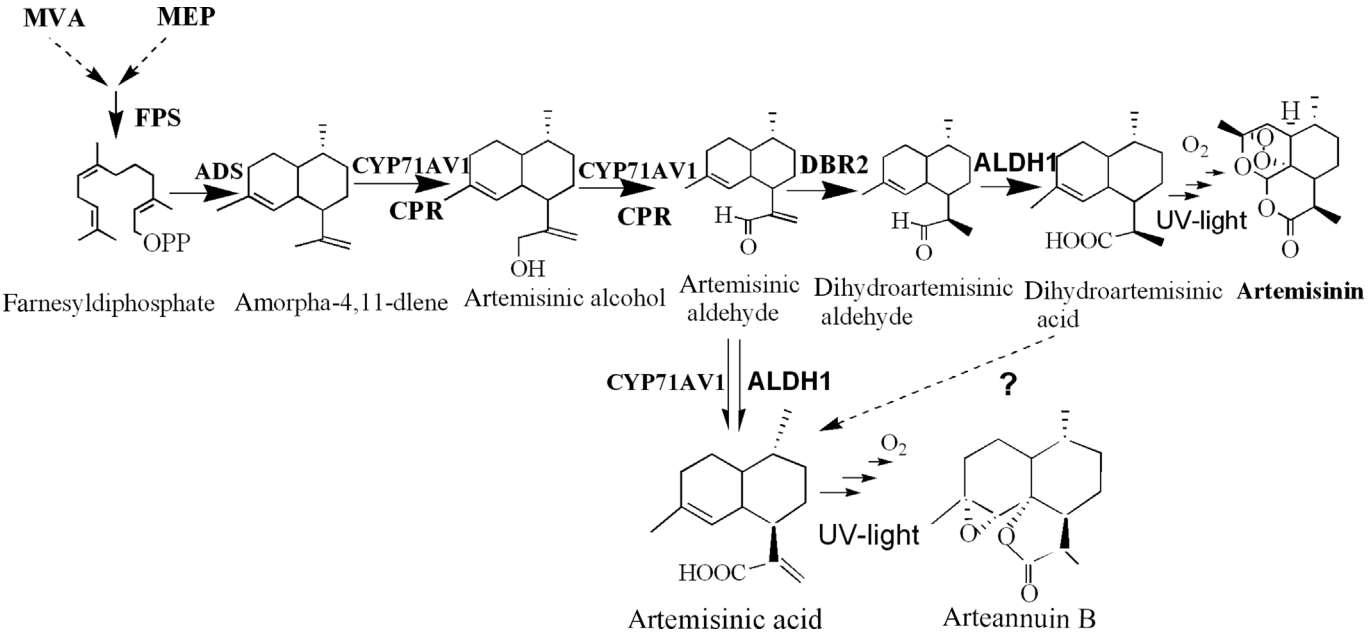

FIG. 1 *The biosynthetic pathway of artemisinin modified from Olofsson et al. [19]. MVA, mevalonate; MEP, methylerythritol phosphate; FPS, farnesyl diphosphate synthase; ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase; DBR2, artemisinic aldehyde △11 (13) reductase; ALDH1, aldehyde dehydrogenase 1. The solid arrows represent the confirmed steps, and the dashed arrows represent the putative step.*

first-line drugs, chloroquine and fansidar, are no longer effective in many parts of East Africa where chloroquine resistance is rampant [2]. Fortunately, artemisinin-based combination therapies have been recommended as the preferred treatment method of drug-resistant malaria by the Word Health Organization for its high efficacy, fast action, and no serious side effects [3, 4]. However, the artemisinin content in the plants of *Artemisia annua* L. is very low, ranging from 0.01% to 0.5% of dry weight [5], and this has been strongly limiting the artemisinin supply. As a result, the production of artemisinin cannot meet the increasing demands. Chemical synthesis of artemisinin is academically successful, but its cost is extremely high for poor malarial victims. So, the plants of *A. annua* are the main source of artemisinin extraction. It is urgent to develop *A. annua* with higher content of artemisinin.

The biosynthetic pathway of artemisinin has been unveiled at the molecular and biochemical levels, in which all the structural genes involved were functionally identified [6]. Artemisinin belongs to sesquiterpenes, and both the classic cytosolic mevalonate (MVA) pathway [7] and the plastidial methylerythritol phosphate pathway (MEP) pathway [8] provide the universal five-carbon precursors including isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) for artemisinin. Two molecules of IPP and one molecule of DMAPP are condensed into 15-carbon acyclic farnesyl diphosphate (FPP), which is the general precursor for all sesquiterpenes including artemisinin [9]. Amorpha-4,11-diene synthase catalyzes the cyclization of FPP to form amorpha-4,11-diene that is a unique 15-carbon cyclic precursor for artemisinin and its relative metabolites [10]. After a series of sequential enzymatic reactions in which cytochrome P450 monooxygenase (CYP71AV1) [11] combined with cytochrome P450 reductase (CPR) [12], DBR2 [13], and aldehyde dehydrogenase 1 (ALDH1) [14, 15] are involved, the product of interest artemisinin is produced (Fig. 1). The structural genes involved in artemisinin biosynthetic pathway have been used to genetically modify artemisinin biosynthetic pathway in transgenic *A. annua* plants. Overexpression of amorpha-4,11-diene synthase (ADS) [16], CYP71AV1 [17], and the combination of ADS and CYP71AV1 led to the enhancement of artemisinin production [18].

Artemisinic aldehyde is reduced to form dihydroartemisinic aldehyde under DBR2 catalysis [13] and dihydroartemisinic aldehyde is used to produce artemisinin [19]; at the same time, artemisinic aldehyde can also become artemisinic acid under catalysis of CYP71AV1 and ALDH1 and finally artemisinic acid is oxidized to form arteannuin B [19]. So, DBR2 is the checkpoint enzyme directing artemisinic aldehyde to artemisinin biosynthetic pathway. The gene expression analysis showed that *DBR2* had much higher expression level in glandular secretory trichomes (GSTs) of *A. annua* in which the artemisinin is biosynthesized [20]; the transgenic results demonstrated that the promoter of *DBR2* was GSTs specific in *A. annua* [21]. All the researches on *A. annua* DBR2 suggested that it might play a key role in artemisinin biosynthetic pathway and a useful candidate gene improving artemisinin production. However, there is no report on *DBR2*-transgenic plants. In the present study,

TABLE 1 *Primers used in this; the underlined letters show the restriction sites*

Primer	*Sequence (5′ – 3′)*	*Restriction site*	*Purpose*
Aa.Fdbr2	GA AGATCT CTGATCCTGATCAACATC	*Bgl*II	Vector construction
Aa.Rdbr2	G GGTCACC TCCTATATACTTTTACAGTC	*Bst*EII	
F35S	TCCATTGCCCAGCTATCTGTC		Genomic PCR detection
Rdbr2-jc	TGGTCAAGTAGATAACCATGTGCTC		
FHygr	AGTACTTCTACACAGCCATCGG		
Rhygr	TCCGGAAGTGCTTGACATTGG		
FvirD1	GAGTATGAAACCTTTTCCTATCA		
RvirD1	GGGATACGGACAAAATGGAG		
Fq-dbr2	CCCAAATCGACCCATTCCTAAAC		Gene expression analysis by qPCR
Rq-dbr2	GCAGCATAGTAATGTTCTCACCAG		
Fq-RPII	TGGCATCAATCCTCATACC		
Rq-RPII	AAACTGTCCTCTTTGGCATA		

the *DBR2* gene of *A. annua* was overexpressed in transgenic *A. annua* plants to investigate its biological effects on artemisinin and its relative metabolite biosynthesis.

2. Materials and Methods

2.1. Plant expression vector construction

The coding sequence of the *A. annua DBR2* gene was amplified using a pair of primers, AaFdbr2 (the forward primer) with the restriction site of *Bgl*II and AaRdbr2 (the reverse primer) with the restriction site of *Bst*EII (Table 1). The coding sequence of DBR2 harboring *Bgl*II and *Bst*EII restriction sites was inserted into pGEM T-easy vector (Promega, WI, USA) and sequenced. After confirmation by sequencing, DBR2 was inserted into pCAMBIA1305 and DBR2 was driven by the Cauliflower Mosaic virus 35S promoter. The constructed plant expression vector harboring *A. annua* DBR2 was named to be pCAMBIA1305–DBR2. Then, pCAMBIA1305–DBR2 was introduced into *Agrobacterium tumefaciens* strain LBA4404 to generate engineered bacteria, which was used for genetic transformation of *A. annua*.

2.2. Establishment of transgenic *A. annua*

The transgenic method was modified according to the previous study [17]. The sterile seeds of *A. annua* were generated and the bacteria-free seedlings with seven leaves were used as initial materials for genetic transformation. The fresh leaves of *A. annua* were excised from seedlings for *Agrobacterium*-mediated leaf disc transformation. The infected leaves of *A. annua* were put on the Murashige and Skoog (MS) medium containing 20 mg L^{-1} acetosyringone for 2 days in the dark at 25 °C. Then, infected leaves were transferred into shooting MS medium added with 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA), 1 mg L^{-1} 6-benzyladenine, 400 mg L^{-1} cefotaxime, and 8 mg L^{-1} hygromycin, and cultivated at 25 °C under the 16-H lighting condition. The 2-cm-high regenerated shoots with hygromycin resistance were cut and transferred into rooting MS medium with 0.1 mg L^{-1} NAA and 250 mg L^{-1} cefotaxime to get hygromycin-resistant plantlets. When the hygromycin-resistant plantlets grew to 5–8 cm height, they were used for 2-week acclimation in PinderStrap medium. The growing-well plants were planted in the filed. Before flowering, the plants were harvested for molecular analysis and metabolite detection.

2.3. Molecular detection

Genomic PCR was used to confirm the authentic transgenic plants of *A. annua*. A DNA fragment of DBR2 was amplified by PCR, with F35S as forward primer (a chimeric primer containing a fragment of 35S promoter and DBR2) and specific primer Rdbr2-jc as reverse primer. Simultaneously, the plasmid of pCAMBIA1305–DBR2 was used as the positive control. Wilde-type *A. annua* was used as the negative control. Additionally, the *virD1* gene was detected through the PCR analysis to avoid the contamination of *Agrobacterium* to PCR results by using primers named FvirD1 and RvirD1.

qPCR was used to detect the expression level of *DBR2*. Total RNAs were extracted from the leaves of *A. annua* and then reversely transcribed into cDNAs using SYBR PrimerScript™ RT-PCR Kit (Takara, Kyoto, Japan) for real-time qPCR analysis on iQ™5 (Bio-Rad, CA, USA). The gene of RPII encoding DNA-directed RNA polymerase II subunit in cell was used as the

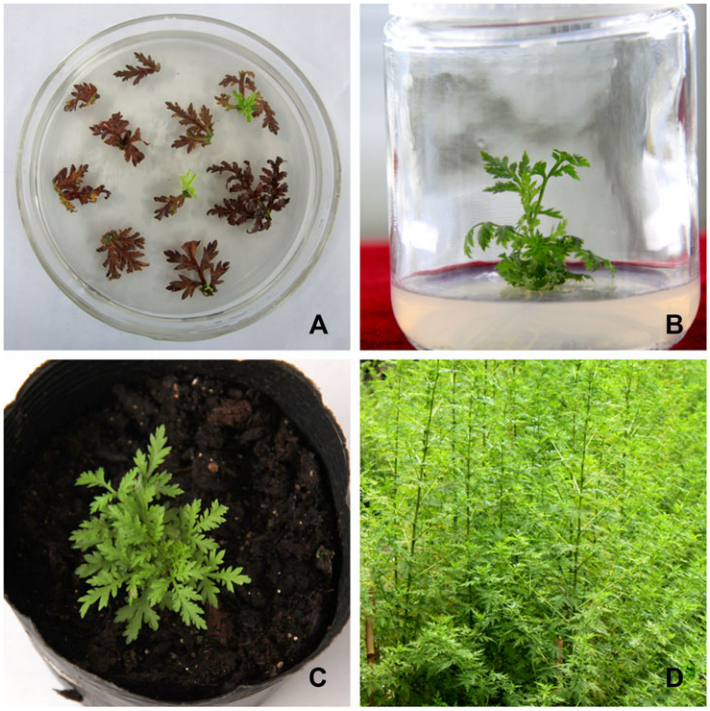

FIG. 2 *Establishment of transgenic A. annua. A: Hygromycin-resistant shoots generated from leaves; B: rooted hygromycin-resistant plantlets; C: acclimated transgenic plants of A. annua; D: transgenic plants of A. annua in field.*

reference gene. The expression level of *DBR2* was detected by primers named Fq-dbr2 and Rq-dbr2, the expression level of RPII was detected by primers of Fq-RPII and Rq-RPII. The experiments were repeated for three times on independently isolated mRNA preparation.

2.4. Analysis of artemisinin and its relative metabolites

The extraction method, according to Xiang et al. [17], was modified and the procedures were described as follows: the leaves from transgenic and nontransgenic *A. annua* plants were dried at 45 °C and grounded into very fine powder; then, the samples (0.1 g) were extracted with 20 mL petroleum and treated in an ultrasonic bath for 1 H; the debris of plant materials were get rid of and the petroleum solvent was collected; the petroleum solvent was dried in rotavapor at 50 °C; finally, the residue was redissolved in 1 mL of methanol and filtered through nylon filter (0.22 μm); the filtered solutions were used for high-performance liquid chromatography (HPLC) analysis by Shimadzu LC-6AD HPLC system (SPD-M20A PDA detector) and the injection volume was 20 μL for each sample. The column of HPLC was C18 column (Phenomenex, CA, USA) and the detection wavelength was 209 nm. The mobile phase was isocratic and composed of 50% acetonitrile and 50% of a 0.1% acetic acid aqueous solution (pH 3.2) with a flow rate of 1.0 mL Min^{-1} at room temperature 30 °C [22, 23]. The authentic

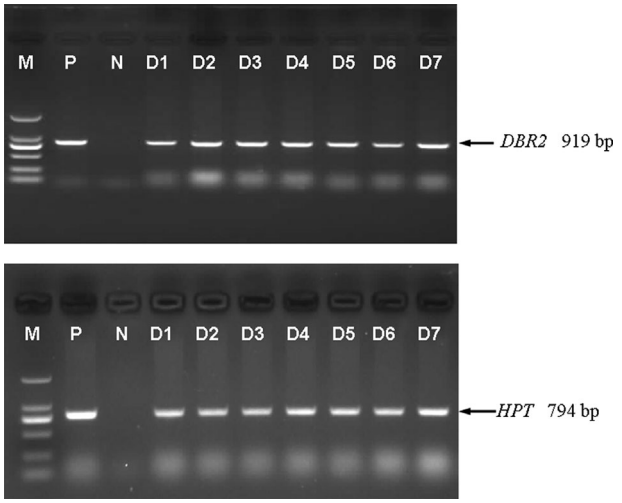

FIG. 3 *Detection of the genes of interest in transgenic A. annua plants. A: Detection of DBR2 (919 bp); B: detection of HPT (794 bp). M, DNA marker; P, positive control; N, negative control; D1–D7: transgenic A. annua with overexpression of DBR2.*

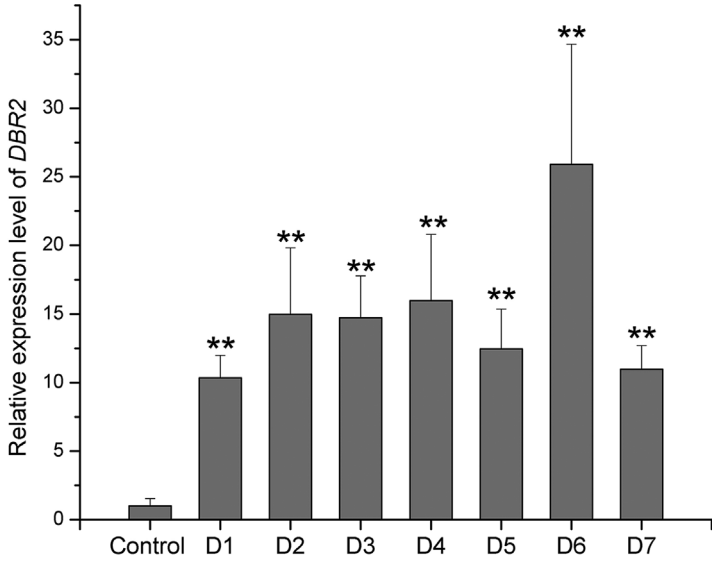

FIG. 4 *Relative expression level of DBR2 gene in A. annua plants. Control: nontransgenic A. annua; D1–D7: different transgenic A. annua plant lines. **Significant difference at the level of $P < 0.01$.*

artemisinin, dihydroartemisinic acid, artemisinic acid, and arteannuin B were purchased from Sigma (Sigma–Aldrich, IL, USA). There were three biological replicates in the experiments.

3. Results and Discussion

3.1. Establishment of *DBR2*-overexpressed *A. annua* plants

The infected leaf explants were cultured on shoot induction medium supplemented with 400 mg L^{-1} cefotaxime to kill the

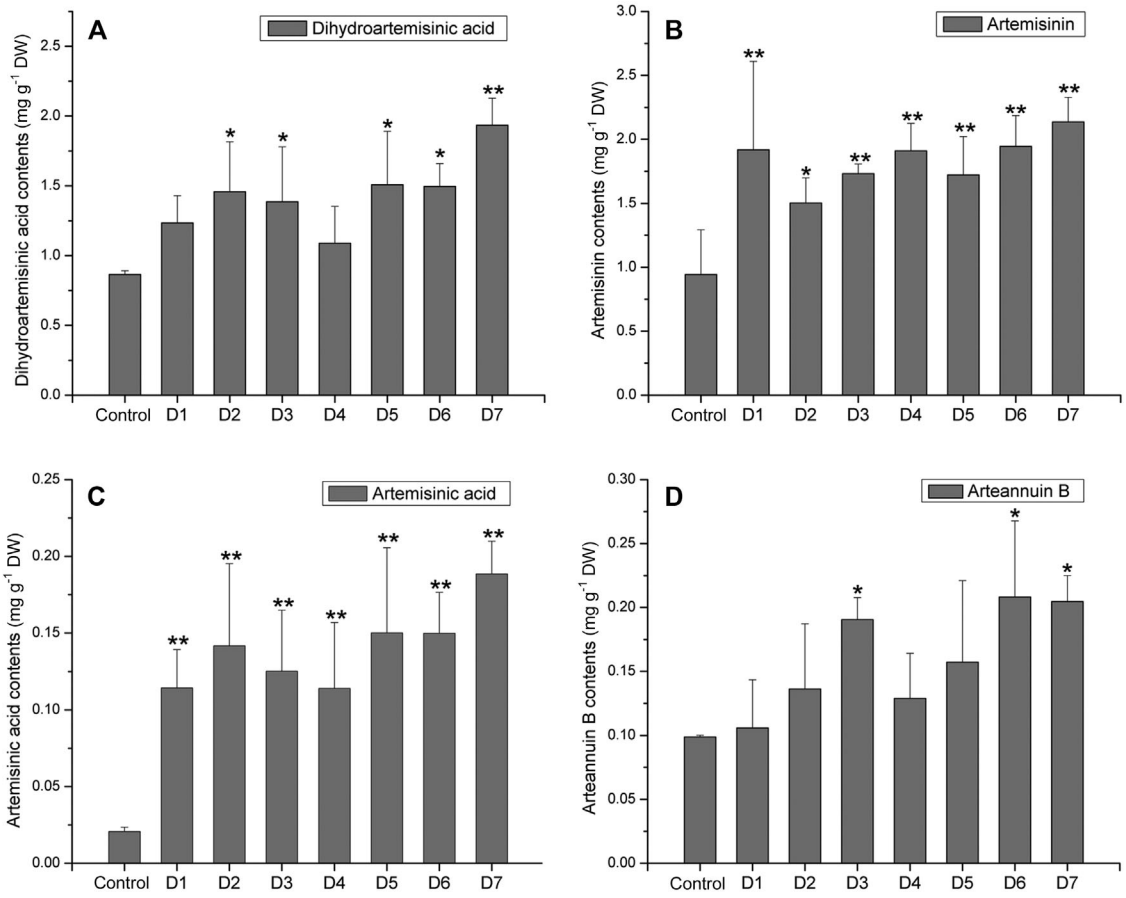

FIG. 5 *The contents of artemisinin and its relative metabolites in A. annua. A: the content of dihydroartemisinic acid; B: the content of artemisinin; C: the content of artemisinic acid; D: the content of arteannuin B. Control: nontransgenic A. annua; D1–D7: different transgenic A. annua plant lines. **Significant difference at the level of $P < 0.01$; *significant difference at the level of $P < 0.05$.*

bacteria and 8 mg L^{-1} hygromycin B to screen the transformants. Only a few hygromycin-resistant shoots were generated from the wounded sites of levels because the wounded plant cells secreted some low-molecular phenolic signals that facilitated the plasmids to enter cells [24]; while most of the infected leaves became brown and finally died because of lack of hygromycin resistance (Fig. 2A). The hygromycin-resistant shoots were subcultured three times to get rid of *A. tumefaciens* strain LBA4404 and were further elongated during 1 month culture on shoot elongation medium without antibiotics. Roots grew within 1 week after the shoots were transferred onto rooting medium (Fig. 2B). The strong rooted plantlets were transferred into PinderStrap medium for acclimation (Fig. 2C) and then grown in the field (Fig. 2D). The putative transgenic plants with *DBR2* overexpression showed no significantly morphological differences compared with the nontransgenic controls (Fig. 2).

3.2. Molecular analysis of transgenic plants

To confirm the integration of the *DBR2* gene into the genome of *A. annua*, genomic PCR was used to detect hygromycin-resistant gene (*HPT*) and *DBR2* gene. The 794-bp fragments of *HPT* and the 919-bp fragments of *DBR2* fused with 35S promoter were simultaneously amplified from transgenic *A. annua* with *DBR2* overexpression and the plasmids of pCAMBIA1305–DBR2 as positive control (Fig. 3); but these corresponding fragments did not occur in nontransgenic *A. annua* (Fig. 3). To avoid a false positive result from the contamination of engineered *A. tumefaciens* strain LBA4404, a 295-bp fragment was amplified from *Agrobacterium* with FvirD1 and RvirD1 as primers, but was not amplified from all transgenic/nontransgenic lines in the same amplification condition. The genomic PCR analysis confirmed that *DBR2* was integrated into the genome of *A. annua*, and the hygromycin-resistant plants were transgenic plants of *DBR2*.

Real-time qPCR was used to analyze the relative expression level of the *DBR2* gene in plants of *A. annua* at the transcriptional level. The result showed that the gene expression level of *DBR2* was dramatically increased in the transgenic plants of *A. annua*, about 10.3- to 25.9-fold compared with the

nontransgenic control (Fig. 4). These results suggested that the 35S promoter caused overexpression of the gene of interest (*DBR2*) in transgenic plants, and the enzymatic step controlled by DBR2 involved in artemisinin biosynthesis was successfully genetically modified. Consequently, this might lead to more accumulation of artemisinin and its relative metabolites. As expected, the following analysis of metabolites supported this.

3.3. Analysis of artemisinin and its relative metabolites

DBR2 is the checkpoint enzyme catalyzing artemisinic aldehyde to form dihydroartemisinic aldehyde, which is directly involved in artemisinin biosynthesis [13, 19]. Overexpression of key enzymes usually leads to more accumulation of target compounds [25–27]. According to the previously reported results, it was reasonable to predict that overexpression of *DBR2* might facilitate metabolic flux forward to artemisinin biosynthesis. The HPLC results demonstrated that artemisinin contents were increased significantly in all seven *DBR2*-overexpressed transgenic lines, ranging from 1.50 to 2.14 mg g^{-1} DW, 1.59–2.26 times compared with the content of 0.94 mg g^{-1} DW in control (Fig. 5B). Dihydroartemisinic acid as the direct precursor for artemisinin was also detected. The HPLC analysis results showed that all the transgenic lines with *DBR2* overexpression had higher contents of dihydroartemisinic acid (Fig. 5A). The formation of artemisinin from dihydroartemisinic acid is not dependent on enzymes but on natural light oxidation [28]. So, more accumulation of dihydroartemisinic acid will definitely cause more production of artemisinin.

Artemisinic aldehyde is also the precursor for artemisinic acid and arteannuin B [19, 29]. In fact, biosynthesis of artemisinic acid and arteannuin B is a competitive pathway against dihydroartemisinic acid and artemisinin biosynthesis. As expected, the transgenic results above indicated that overexpression of *DBR2* enhanced biosynthesis of dihydroartemisinic acid and artemisinin biosynthesis. Surprisingly, it was also found that all seven *DBR2*-overexpressed transgenic lines had much higher contents of artemisinic acid (0.11–0.19 mg g^{-1} DW), about 5.48–9.06-fold, respectively, compared with nontransgenic control (Fig. 5C). Furthermore, the contents of arteannuin B in some *DBR2*-overexpressed transgenic lines had increased (Fig. 5D). Because DBR2 was not involved in biosynthesis of artemisinic acid and arteannuin B, the probably reasonable explanation for *DBR2* overexpression leading to the enhancement of artemisinic acid biosynthesis was that dihydroartemisinic acid was also the precursor for artemisinic acid (Fig. 1). Actually, the previous reported studies indicated that artemisinic acid came from dihydroartemisinic acid by an oxidative selenation procedure *in vitro* [30]. However, the conversion from dihydroartemisinic acid to artemisinic acid *in planta* needed more evidence. The enhancement of artemisinic acid by overexpressing *DBR2* might suggest that dihydroartemisinic acid was converted into artemisinic acid *in planta*.

4. Conclusions

In this study, the checkpoint enzyme DBR2 was used to genetically engineer the artemisinin biosynthetic pathway for the first time. The transgenic results indicated that overexpression of *DBR2* led to enhancement of artemisinin and its direct precursor dihydroartemisinic acid accumulation. It can be concluded that *DBR2* is a useful structural gene to develop transgenic *A. annua* plants with higher content of artemisinin. At the same time, biosynthesis of the metabolites, especially artemisinic acid, in the competitively branched pathway of artemisinin biosynthesis was also improved, and this suggested that dihydroartemisinic acid might be converted to artemisinic acid *in planta*.

5. Acknowledgment

This work was financially supported by the NSFC projects (31070266 and 31200223), the National Hi-Tech Project (2011AA100605), the Program for New Century Excellent Talents in University (NCET-12-0930), the Fundamental Research Funds for the Central Universities (XDJK2013A024 and SWU111012), and the Scientific and Technological Research Program of Chongqing Municipal Education Commission (KJ131404). The authors declare no conflicts of interest.

6. References

[1] Miller, L. H., and Greenwood, B. (2002) Science 298, 121–122.
[2] Greenwood, B., and Mutabingwa, T. (2002) Nature 415, 670–672.
[3] Graham, I. A., Besser, K., Blumer, S., Branigan, C. A., Czechowski, T., Elias, L., Guterman, I., Harvey, D., Isaac, P. G., Khan, A. M., Larson, T. R., Li, Y., Pawson, T., Penfield, T., Rae, A. M., Rathbone, D. A., Reid, S., Ross, J., Smallwood, M. F., Segura, V., Townsend, T., Vyas, D., Winzer, T., and Bowles, D. (2010) Science 327, 328–331.
[4] Zhang, L., Jing, F. Y., Li, F. P., Li, M. Y., Wang, Y. L., Wang, G. F., Sun, X. F., and Tang, K. X. (2009) Biotechnol. Appl. Biochem. 52, 199–207.
[5] Wallaart, T. E., Pras, N., and Quax, W. J. (1999) Planta Med. 65, 723–728.
[6] Zeng, L. X., Chen, M., Lan, X. Z., and Liao, Z. H. (2012) J. Med. Plants Res. 6, 7–13.
[7] Akhila, A., Thakur, R. S., and Popli, S. P. (1987) Phytochemistry 26, 1927–1930.
[8] Towler, M. J., and Weathers, P. J. (2007) Plant Cell Rep. 26, 2129–2136.
[9] Paiva, N. L. (2000) J. Plant Growth Regul. 19, 131–143.
[10] Wallaart, T. E., Bouwmeester, H. J., Hille, J., Poppinga, L., and Maijers, N. C. A. (2001) Planta 212, 460–465.
[11] Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006) Nature 440, 940–943.
[12] Teoh, K. H., Polichuk, D. R., Reed, D. W., Nowak, G., and Covello, P. S. (2006) FEBS Lett. 580, 1411–1416.
[13] Zhang, Y. S., Teoh, K. H., Reed, D. W., Maes, L., Goossens, A., Olson, D. J., Ross, A. R., and Covello, P. S. (2008) J. Biol. Chem. 283, 21501–21508.
[14] Teoh, K. H., Polichuk, D. R., Reed, D. W., and Covello, P. S. (2009) Botany 87, 635–642.
[15] Lin, X. Y., Zhou, Y., Zhang, J. J., Lu, X., Zhang, F. Y., Shen, Q., Wu, S. Y., Chen, Y. F., Wang, T., and Tang, K. X. (2011) Biotechnol. Appl. Biochem. 58, 50–57.
[16] Ma, C. F., Wang, H. H., Lu, X., Wang, H., Xu, G. W., and Liu, B. Y. (2009) Metabolomics 5, 497–506.
[17] Xiang, L. E., Zeng, L. X., Yuan, Y., Chen, M., Wang, F., Liu, X. Q., Zeng, L. J., Lan, X. Z., and Liao, Z. H. (2012) POJ 5, 503–507.

[18] Lu, X., Shen, Q., Zhang, L., Zhang, F. Y., Jiang, W. M., Lv, Z. Y., Yan, T. X., Fu, X. Q., Wang, G. F., and Tang, K. X. (2013) Ind. Crops Prod. 49, 380–385.
[19] Olofsson, L., Engstrom, A., Lundgren, A., and Brodelius, P. E. (2011) BMC Plant Biol. 11, 45.
[20] Soetaert, S. S. A., Van Neste, C. M. F., Vandewoestyne, M. L., Head, S. R., Goossens, A., Van Nieuwerburgh, F. C. W., and Deforce, D. L. D. (2013) BMC Plant Biol. 13, 220.
[21] Jiang, W. M., Lu, Xu., Qiu, Bo., Zhang, F. Y., Shen, Q., Lv, Z. Y., Fu, X. Q., Yan, T. X., Gao, E. D., Zhu, M. M., Chen, L. X., Zhang, L., Wang, G. F., Sun, X. F., and Tang, K. X. (2013) Plant Mol. Biol. Rep. 32, 82–91.
[22] Ferreira, J. F. S., and Gonzalez, J. M. (2009) Phytochem. Anal. 20, 91–97.
[23] Briars, R., and Paniwnyk, L. (2013) Ind. Crop Prod. 42, 595–600.
[24] Lan, X. Z., and Quan, H. (2010) J. Med. Plant. Res. 4, 1477–1481.
[25] Kai, G. Y., Zhang, A., Guo, Y. Y., Li, L., Cui, L. J., Luo, X. Q., Liu, C., and Xiao, J. B. (2012) Mol. BioSyst. 8, 2883–2890.
[26] Kai, G. Y., Xu, H., Zhou, C. C., Liao, P., Xiao, J. B., Luo, X. Q., You, L. J., and Zhang, L. (2011) Metab. Eng. 13, 319–327.
[27] Kai, G. Y., Yang, S., Luo, X. Q., Zhou, W. T., Fu, X. Q., Zhang, A., Zhang, Y., and Xiao, J. B. (2011) BMC Biotechnol. 11, 43.
[28] Ferreira, J. F. S. (2007) J. Agric. Food Chem. 55, 1686–1694.
[29] Brown, G. D. (2010) Molecules 15, 7603–7698.
[30] Brown, G. D., and Sy, L. K. (2007) Tetrahedron 63, 9548–9566.