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Ri-mediated transformation of *Artemisia annua* with a recombinant farnesyl diphosphate synthase gene for artemisinin production

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Abstract

A transgenic system was developed for *Artemisia annua* L. via *Agrobacterium rhizogenes*-mediated transformation. Using this system a cDNA encoding farnesyl diphosphate synthase (FDS) placed under a CaMV 35S promoter was transferred into *Artemisia annua* using *Agrobacterium rhizogenes* strain ATCC15834. Among the 150 hairy root lines established, 16 lines showed resistance to kanamycin (20 mg l^{-1}). The intergration of FDS gene was confirmed by PCR and Southern blot analysis, and analysis of Northern blot revealed that the foreign FDS gene was expressed at the transcriptional level in three hairy root lines (F-1, F-24 and F-26 root line). F-1, F-24 and F-26 root lines grew faster than the control hairy root line. However, on the MS medium growth of F-26 root line was abnormal in that callus frequently formed. Analysis of artemisinin demonstrated that about 2–3 mg g^{-1} DW of artemisinin were then detected in the three root lines, which is about 3–4 times higher than that in the control hairy roots.

Abbreviations: Kan – kanamycin; cb – carbenicillin; CaMV – cauliflower mosaic virus; FDP – farnesyl diphosphate; FDS – FDP synthase; GA – gibberellin; NPTII – neomycin phosphotransferase II; PCR – polymerase chain reaction

Introduction

Artemisia annua L. is one of the Chinese traditional medicinal plants for reducing temperature and bringing down fever. Its active principle, artemisinin (Qinghaosu), is an endoperoxide sesquiterpene lactone (Qinghaosu Research Group, 1980). This secondary metabolite shows activity against *Plasmodium*, the causative parasite of malaria (Qinghaosu Antimalarial Coordinating Research Group 1979; Klayman 1985). Because of increasing resistance to traditional antimalarial drugs, such as chloroquine, by *Plasmodium*, artemisinin and its derivatives are considered the most potentially important and clinically useful agents in the treatment of malaria. Due to limited native resources and difficulty of total chemical synthesis, attempts at increasing the production of artemisinin in *A. annua* cells by means of biotechnology have been made (Van Geldre et al. 1997). However, production of artemisinin at significant level in *A. annua* cells and tissue cultures has not been successful. It would therefore be of interest to develop transgenic *A. annua* plants or organs with increased accumulation of artemisinin, produced by introducing key genes encoding for enzymes regulating the biosynthetic pathway leading to the formation of artemisinin.

In the isoprenoid biosynthesis pathway, farnesyl diphosphate synthase (FDS, EC2.5.1.1/EC2.5.1.10)

catalyzes two consecutive condensations of isopentenyl diphosphate with dimethylallyl diphosphate, forming geranyl diphosphate (Cane 1990). The ultimate product of these two reactions, farnesyl diphosphate (FDP), is a common precursor for the biosynthesis of sterols, dolichols, mitochondrial electron transfer chain components, prenylated proteins and a wide range of secondary sesquiterpenoids (Chappell 1995). Recently, cDNAs encoding FDS have been isolated from a number of plant species, including *Arabidopsis thaliana* (Delourme et al. 1994), *Lupinu albus* (Attucci et al. 1995), *A. annua* (Matsushita et al. 1996; Chen et al. 1999; GenBank accession No. AF 112881) and *Gossypium arboreum* (Liu et al. 1998). Since the 15-carbon FDP can be converted by sesquiterpene cyclases to form cyclic sesquiterpenes (Chappell 1995; Bohlmann et al. 1998), overexpressing FDS by introducing a foreign gene into *A. annua* holds a possibility of affecting accumulation of artemisinin and related sesquiterpenoids.

Materials and methods

Plant materials

High artemisinin yielding clones 001 and 025 of *A. annua* were collected from Sichuan Province of China. The seeds were germinated in agar aseptically and leaves from the seedlings were used as explants for genetic transformation.

Vector construction

The procedure for the construction of plasmids for plant transformation was: 1. The pFDSSK, containing a FDS cDNA insert from *G. arboreum*, was digested with Smal I and Kpn I to obtain a 1.2 kb fragment containing the complete open reading frame. This fragment was then placed between a CaMV 35*S* promoter and a NOS terminator to form an intermediate vector pB35SFDS. This vector was then digested with Xbal I and Sal I, and the fragment containing the chimeric FDS gene was inserted into a pBIlOl.2 vector which had been digested with the same enzymes, forming the binary vector pBIFDS that was then used for *A. annua* transformation.

Transformation of A. rhizogenes

After amplification in *E. coli* strain DH5a, plasmid DNA of pBIFDS was used to transform *A. rhizogenes* strain ATCC 15834, which was then selected on Kan-free YEB medium (yeast extract 1.0 g, beef extract 5.0 g, peptone 5.0 g, sucrose 5.0 g, $MgSO_4 7H_2O$ 0.49 g, pH 7.0) supplemented with 50 mg l^{-1} Kan. The wild type of this strain was cultured in YEB medium without Kan.

Induction and selection of A. annua hairy root

This was carried out as described previously (Qing et al. 1994). Briefly, leaf explants from 2–3 week old aseptic seedlings were floated in 8 ml MS (Murashige & Skoog 1962) liquid medium containing 2 ml of a late log phase *A. rhizogenes* (activated two times), in a 50 ml Erlenmeyer flask. After 20 minutes of infection, the explants were blotted with sterile filters, and subcultured on MS solid medium in an experimental greenhouse at 25 °C for two days. The explants were transferred onto fresh solid MS medium supplemented with 500 mg l^{-1} cb to eliminate *Agrobacterium*. The same medium supplemented with 0, 10, 20, 30, 50 to 100 mg l^{-1} Kan was then used to select the transformants, based on incorporation and expression of the NPTII gene. The explants were transferred to fresh medium weekly in the first month, afterwards subcultures were made every three weeks. After 10 weeks the concentration of cb was reduced to 100 mg l^{-1}, and it was completely omitted after 12 weeks. The Kan resistant hairy roots were further examined by PCR and Southern blotting analysis for the confirmation of the genetic transformation.

DNA isolation, PCR and Southern blotting analysis

DNA was isolated from hairy roots using a CTAB method (Futerer et al. 1995). PCRs were carried out by 30 cycles of 94 °C for 40 sec, 47 °C for 60 sec and 72 °C for 30 sec, followed by a final extension step of 72 °C, 5 min. DNA (50 ng) isolated from hairy roots was used as templates. A forward primer FF1 (5′-atggcggatctcaggtc-3′) and a reverse primer FR2 (5′-gtccatgatgtcaag-3′) were synthesized according to *G. arboreum* FDS cDNA sequences, and used for PCR amplification. PCR products were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide.

The hairy root DNA (20 μg) was digested with Xba I (2 μ μg^{-1} DNA) at 37 °C overnight and fractionated on a 0.8% agarose gel. The DNA was then transferred onto a NC membrane (Amersham). The DNA fragment, obtained by PCR amplification using FF1 and FR2 primers, was labeled by digoxingenin