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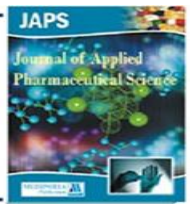

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Enhancement of camptothecin production in *Camptotheca acuminata* hairy roots by overexpressing *ORCA3 g*ene

Xiaoning Ni, Shiqiu Wen, Wei Wang, Xiaoyun Wang, Hui Xu, Guoyin Kai

Wei Wang, Hui Xu, Guoyin Kai
Laboratory of Plant Biotechnology, College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, P. R. China

Xiaoning Ni
[a]*Department of General Surgery, Zhongshan Hospital, Fudan University, Shanghai 200032, PR China.*
[b] *Laboratory of Plant Biotechnology, College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, P. R. China*

Shiqiu Wen, Xiaoyun Wang
Department of Gynaecology and Obstetrics, Kunshan Hospital of Traditional Chinese Medicine, Kunshan, Jiangsu Province 215300, People's Republic of China.

For Correspondence
Dr. Guoyin Kai
Laboratory of Plant Biotechnology, College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, China. Tel/Fax: +86-21-64321291

ABSTRACT

Being a good anti-tumor drug, camptothecin (CPT) is a kind of modified monoterpene indole alkaloid firstly isolated from the deciduous Chinese happy tree with rapidly increasing clinical demand. Due to the great importance and low content of this compound, it is very important to improve CPT production by modern biotechnology. ORCA3 is a jasmonate-responsive APETALA2-domain transcript factor isolated from *Catharanthus roseus*, with strong ability to up-regulate expression of serveral key genes involved in TIA biosynthetic pathway. To investigate physiological function of ORCA3 gene in *Camptotheca acuminata*, the *ORCA3* gene was transformed using *Agrobacterium*-mediated gene transfer technology. PCR analysis confirmed that the *ORCA3* gene was integrated into the plant genome. HPLC showed that overexpression of *ORCA3* in transgenic hairy root lines can effectively enhance the production of camptothecin with 1.5-fold compared with the control (1.12 mg/g dw). The results revealed that *ORCA3* is an effective regulatory gene for improving metabolic flux in camptothecin biosynthetic pathway at the first time.

Key words: *Camptotheca acuminata*; *ORCA3*; camptothecin; transformation; hairy root cultures

INTRODUCTION

Camptotheca acuminata, a well-known traditional Chinese medicinal plant, can produce camptothecin (CPT) (Wall *et al*., 1966). CPT is a kind of modified monoterpenoid indole alkaloids and shows an anti-tumor action due to its inhibitory activity to DNA topoisomerase I (Hsiang *et al*., 1985). Irinothecan and topothecan are CPT derivatives and have been widely used for the treatment of cancer from all over the world. About 1000 million market size of camptothecin derivatives would be needed for the manufacture (Lorence *et al*., 2004). However, this anticancer compound is produced at a very low level in *C. acuminata*, leading to the high price in the market. CPT was mainly obtained from original CPT-producing plants such as *C. acuminata* and *Nothapodytes foetida* and camptothecin derivatives were still synthesized from natural CPT (Lu *et al*., 2009). However, *C. acuminata* is a woody plant and grow very slowly. Due to the expansion of clinical trials and cancer treatments, the demand for CPT was increasing rapidly. So it is necessary to develop sustainable resources that can produce amounts of camptothecin. The development of plant metabolic engineering promises an alternative way for improvement of camptothecin content by transferring key genes into *C. acuminata* (Kai *et al*., 2008). This, however, significantly relies upon the detailed understanding of the pathway for camptothecin biosynthesis. The gene for ORCA3, a jasmonate-responsive APETALA2 (AP2)-domain, is a

transcription factor from *Catharanthus roseus*. Previous study showed that *ORCA3* over-expression can result in enhanced expression of several metabolite biosynthetic genes (such as *CPR, TDC* and *STR etc*) and in increased accumulation of terpenoid indole alkaloids in *C. roseus* (Vander and Memelink, 2000). As we known, *C. roseus* and *C. acuminata* have similar TIA biosynthetic pathway. So, in this work, *ORCA3* gene was introduced into *C. acuminata* hairy root in order to enhance the production of camptothecin. In this study, a construct harboring the *ORCA3* gene, driven by the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter, was introduced into *C. acuminata* hairy roots. The accumulation of camptothecin was investigated in the engineered hairy roots.

MATERIALS AND METHODS

Construction of plant expression vectors

The complete *ORCA3* cDNA was cloned from the sterile seedlings of *C. roseus* in our laboratory. The vectors pBI121 and pCAMBIA1304 were double-digested with *HindIII* and *EcoRI*. The smaller DNA fragment purified containing a GUS expression cassette from pBI121 was cloned into the large pCAMBIA1304 fragment to generate the recombinant plasmid pCAMBIA1304^{+}(Kai et al, 2009; 2011). The full-length *ORCA3* cDNA was inserted into the pCAMBIA1304^{+} in place of the mGFP5 and GUSA genes originally from pCAMBIA1304 to generate the expression plasmid pCAMBIA1304^{+}-*ORCA3* containing *ORCA3* gene (Fig 1). The disarmed *A. tumefaciens* strain C58C1 harbouring both the *A. rhizogenes* Ri plasmid pRiA4 and each of the plasmids constructed above, were used for plant genetic transformation.

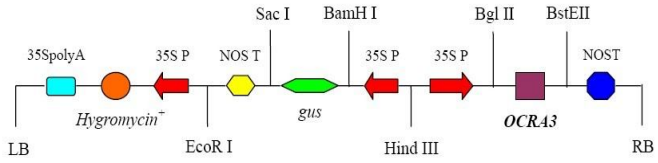

Fig. 1 Schematic representation of transformation plasmid pCAMBIA1304^{+}-*ORCA3*. The restriction enzyme sites *Bgl II* and *BstE II* were marked.

Plant transformation and hairy root cultivation

Plant materials were grown in the greenhouse under a 16h light/8h dark photoperiod. The temperatures were set to 25˚C all time. In order to induce hairy roots with disarmed *A. tumefaciens* strain C58C1 (pRi A4), the sterile hypocotyl sections, were submerged in the bacterial suspension for 10 min, blot-dried on sterile filter paper and then placed in B5 medium supplemented with 30 % sucrose, 0.8% agar (pH 5.8), at 22 °C in darkness. After co-cultivation for 2 days, the cultures were transferred to B5 medium supplemented with 30% sucrose, 0.8% agar (pH 5.8), and 500 mg/l of cefotaxime. After 2-3 weeks, hairy roots were derived from sterile hypocotyl and rapidly growing hairy roots (above 2 centimeters) were excised and cultivated individually on solid B5 medium supplemented with 30% sucrose, 0.8% agar (pH 5.8) and 500 mg/l cefotaxime, at 25 °C in darkness. Normally growing hairy roots were inoculated in 250-mL Erlenmeyer flasks each containing 200 mL 1/2MS liquid medium on an orbital shaker controlled at 100 rpm in darkness at 25 °C. The hairy roots clones were routinely subcultured every 30 days and harvested after 60 days.

DNA isolation and PCR analysis

The genomic DNA was isolated from hairy root samples by using the acetyl trimethyl ammonium bromide (CTAB) method （Doyle and Doyle, 1990）. DNA was washed and re-suspended in double distilled water. The DNA was then used in PCR analysis for detecting the presence of *Agrobacterium rol B* and *ORCA3* genes in transgenic hairy roots. For PCR analysis, the primer pair for the *ORCA3* gene coding sequence as follows: forward primer *ORCA3*F1: 5'- ATGTCCGAAGAAATCATTTCCGTCTC -3' and reverse primer *ORCA3*R1: 5'- TTAATATCGTCTCTTCTTCCTTCCTCC -3' were used. The PCR reaction consisted of an initial denaturation step of 5 min at 94 ˚C followed by 35 amplification cycles of denaturation at 94 ˚C for 45 s, primers annealing at 58 ˚C for 1 min and primers elongation at 72 ˚C for 1 min and finally 10 min at 72 ˚C. The plasmid pCAMBIAl304^{+}-*CrORCA3* was used as positive control and untransformed genomic DNA was taken as negative control, respectively for PCR analysis. PCR amplified products were loaded in 0.8% (w/v) agarose gel and photographs of amplified bands were recorded in Bio-Rad gel doc system.

Determination of camptothecin by HPLC

The dried hairy roots were powdered and extracted with 16 mL of ethanol/H_20 (95:100, v:v), and then kept at room temperature for 24 h after sonicating for 1 h. The extract was evaporated under vacuum and the deposition was re-dissolved with 1 mL of ethanol/H_20 (95:100, v:v). The solution was filtered through a 0.22 μm filter and subjected to HPLC. HPLC analysis was performed on a HITACHI L2000 apparatus equipped with a photodiode array detector. Separation was carried out on a Waters reversed-phase C18 symmetry column (5 μm, 150 mm × 4.6 mm i.d.). The temperature of column was 30 °C. The mobile phase consisted of acetonitrile-buffer salts (1000ml H_20+700ml duality+300ml phosphoric acid) (65:35, V/V). The detection wavelength was 254 nm and the flow rate was 1.00 mL/min (Shi et al., 2004). The injection volume is 20 μL. Camptothecin were detected and quantified by comparison with authentic standard curves and retention times.

Statistical analysis

All the experiments were repeated for three times. Results were presented as mean values ± S.D. The statistical significance of different camptothecin was analysed by one sample *t* test and one-way analysis of variance (ANOVA) using SPSS 11.5 software (SPSS, Inc.).

RESULTS AND DISCUSSION

Generation of transgenic hairy root lines

The *ORCA3* gene alone was introduced into *C. acuminata* and the procedures are showed in Fig 2. In total, 25 *ORCA3* hairy

root lines were generated, of which 11 lines survived during the successive subculture process. The hairy roots of *C. acuminata* grow directly from the wound and with no geotropism. The colors of the hairy roots are white and have many cilia. *C. acuminata* hairy roots grow on hormone-free medium slowly with few branches, which was mainly realted to its species (*C. acuminata* is a kind of woody plant). The hairy root lines were subculture for 3-4 weeks in hormone-free B5 solid medium with cefotaxime, the concentration of which declining gradually. Some transgenic hairy root lines with abnormal phenotypic characteristics such as brown and aged in grown stagnation were discarded. Six lines, which showed fast growth, were chosen for further examinations. As control, wild-type hairy root lines were also generated following inoculation.

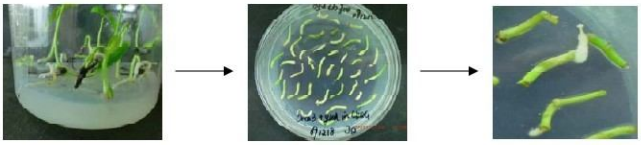

Fig. 2 The process of transgenic *C. acuminata* hairy roots.

PCR analysis of genetically engineered hairy roots

The genomic DNAs were isolated from the transgenic plants as well as from the untransformed control hairy root. All of the hairy roots contained the *rol B* gene, which was revealed by PCR analysis (Fig 3). DNA of wild type root was used as a negative control (NC). The PCR-positive hairy root lines amounted to 23.33% for *ORCA3* line, which indicated that the *ORCA3* gene was integrated into the genome of the transgenic hairy roots. As a positive control (PC), the fragment was also amplified from the 1304^{+}-*ORCA3*. None of the checked DNA bands was detected from NC line (Fig 4). 3 PCR-positive lines were selected for the further experiments.

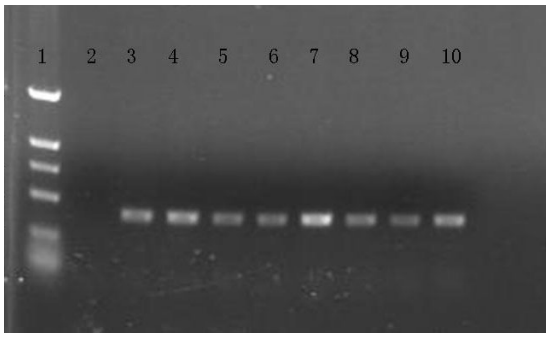

Fig. 3 PCR analysis of *rolB* gene for transgenic hairy root 1 : Marker DL2000; 2: Wild plant root as blank control; 3: plasmid as positive control.

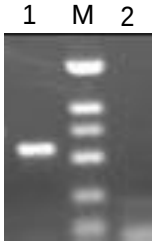

Fig. 4 Identification of transgenic hairy root by PCR M : Marker DL2000; 1: transgenic hairy root; 2: non-transgenic hairy root as blank control.

Camptothecin production analysis of transgenic hairy roots

To determine the camptothecin yield in *C. acuminata* hairy root lines, HPLC analysis was conducted. The purity of the peaks was determined by the Agilent ChemStation A09.01 software to make sure that a peak contained only one compound. Our results showed that camptothecin was detected in transgenic root lines. One sample *t* test by SPSS 11.5 software was used to analyze significant difference of camptothecin content between every single line and the control. The capacities of transgenic hairy root lines to biosynthesize camptothecin are shown in Fig 5. Over-expression of *ORCA3* consistently led to increased transcript levels in transgenic hairy roots, with great enhancement of camptothecin accumulation by 1.5-fold compared with the control (1.12 mg/g dw) (Fig 5), implying that overexpression of *ORCA3* can efficiently promote the accumulation of camptothecin in *C. acuminata*. So our current study obviously indicated the enhancement effects of camptothecin production in *ORCA3*-overexpressing *C. acuminata* hairy roots, suggesting that *ORCA3* gene was a desirable target for improved camptothecin production by metabolic engineering.

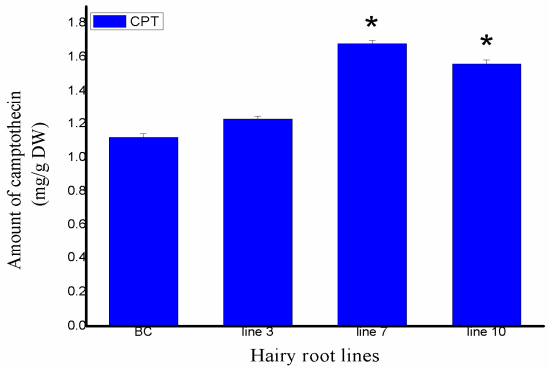

Fig. 5 Camptothecin production analyzed by HPLC from transgenic hairy root lines BC: control hairy root cultures. The values are means±S.D of triplicate analyses. *: Significant difference at $P<0.05$.

The production of camptothecin could be significantly increased by various approaches such as over-expressing genes encoding the key enzymes in the biosynthetic pathway. Recently several genes such as *G10H* and *Str* in the TIA biosynthesis pathways have been cloned (Wang *et al*., 2010; Lu *et al*., 2010), providing possibilities to enhance the yield of camptothecin by metabolic engineering. *G10H* gene encodes a cytochrome P450 monooxygenase, which hydroxylates geraniol to form 10-hydroxy-geraniol, which controls the first committed step in the biosynthesis of TIAs (Collu *et al*., 2001). A recent study demonstrated that over-expression *G10H* gene increased the catharanthine production in *C. roseus* and implies it plays an important role in TIA biosynthesis (Wang *et al*., 2010).

An earlier study has demonstrated that over-expressing *ORCA3* can increase the production of tryptamine and tryptophan in *C. roseus* and increase production of TIAs only when loganin

was supplemented in the medium (Vander and Memelink, 2000). The reason of the fact was that *G10H* was not up-regulated under *ORCA3* over-expression. A study showed that over-expressing multiple genes is crucial for improved production of useful plant secondary metabolites (Wang *et al*., 2010; Zhang *et al*., 2004). To enhance the levels of the camptothecin, co-expression of multiple genes such as *ORCA3* and *G10H* in *C. acuminata* is a promising strategy in the near future. This co-expression of *ORCA3* and *G10H* would boost the accumulation of camptothecin in engineering *C. acuminata* hairy roots in the future.

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