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Functional Expression of DS Gene in P.ginseng by Recombinant PCR

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Abstract. Being through the recombinant PCR reaction and restriction enzyme digestion connection to construct ginseng DS RNA interference plant expression vector was transformed into the *Agrobacterium rhizogenes* A4 to get DS-RNAi plant expression vector engineered bacteria. The result of transgenic *Panax ginseng* ginsenosides of PCR analysis and high performance liquid chromatography(HPLC) analysis showed that the DS gene and the final products of protopanaxadiol (Rb1) and protopanaxatriol (Rg1 and Re) activity assays revealed DS gene is a key gene that produces ginsenosides from 2,3—oxidosqualene. By artificial modification DS gene, the crucial regulation sites of metabolic pathway could be determined, and promote the production of ginsenosides, to lay a strong foundation for perfecting complete the ginsenoside metabolic pathways.

Keywords: Ginsenoside, Dammarenediol-Ⅱ synthase, Recombinant PCR

1. Introduction

Panax ginseng, is traditionally recognized for its notable pharmacological effects and is considered to be one of the most potent medicinal plants that provide resistance to stress, and disease in East Asia [1]. Ginsenosides extracted from ginseng root are triterpenoid saponins[2], are mainly responsible for the pharmacological activities of ginseng [3],[4].

Ginsenosides are known to be synthesized via the mevalonic acid (MVA) pathway, followed by the modification of their triterpene skeletons. The primary components of triterpenoid saponins are oleanane (β-amyrin), dammarene-type triterpenoid or phytosterol (Fig.1) [5]. From this pathway, triterpene and sterol share the same precursor, 2, 3-oxidosqualene, which is synthesised through the isoprenoid pathway. Dammarane-type ginsenosides is the cyclization of 2,3-oxidosqualene to dammarenediol-II catalyzed by a dammarenediol synthase(DS,) in the ginsenoside biosynthesis[6]. Dammarenediol-II is thought to be converted to protopanaxadiol (tetracyclic triterpenoids dammarane-type ginsenosides-including Rb1，Rb2，Rc，Re，Rf and Rg1) [7],[8].

This study applied the recombinant PCR reaction and double endonuclease restriction enzyme digestion connection to construct the DS RNAi plant expression vector. In this study, utilizing RNA interference (RNAi) technique to inhibit expression of DS gene, can detect changes of the products, to understand the flowing of 2,3-oxidosqualene without DS gene. It is better to know how to make more precursors flowing into ways to generate ginsenoside. Meanwhile, RNAi technology could be used for the identification function of the DS gene in secondary metabolic, to lay a strong foundation for perfecting complete the ginsenoside metabolic pathways.

2. Methods

2.1. Construction of RNAi Component Using Recombinant PCR

Total RNA was extracted from 21 days old hairy roots with RNAiso Reagent kit (TaKaRa Dalian Biotechnology Co., Ltd.)，then detected by using 1% agarose gel electrophoresis.

We selected recombinant PCR to constructed DS RNAi component. Recombinant PCR contains three round PCR amplification with 4 pairs of primers.

First and second PCR was carried out with template(1.0μl) and a set of (1.0μl each) primers (First PCR: sence fragment: CCGCCGTTGAGATTAGATGA; antisense fragment: TGACCCAATCATCGTGCTGT; Second PCR: 1. sence fragment: CTACGCCGCTATTAG/AAGCTT/CCGCCGTTGAGATTAGATGA; antisense fragment: GAATTCGAGCTCGGGCCCGTCGAC/TGACCCAATCATCGTGCTGT; 2. Sence fragment: GTCGACGGGCCCGAGCTCGAATTC/TGACCCAATCATCGTGCTGT; antisense fragment: GACGCCCTTATTTTA/CTGCAG/CCGCCGTTGAGATTAGATGA) which were synthesized based on the DS gene sequences, using 0.2μl Taq DNA polymerase (Takara) with dNTP(1.0μl, concentration: 2.5mmol/L), and 10×PCR Buffer(2.5μl) in a final volume of 25μl, and performed as follows: 94℃ for 5 min, followed by 30 cycles of 94℃ for 1 min, 53.5℃ for 50 sec, and 72℃ for 1 min, with a final 10-min extension at 72℃.

Third PCR reaction mixtures (50ul) contained 2μl mixture of the two PCR fragments produced in the second round (each of them 1μl) as template, 2.0ul of each primer which we designed a modified sence primer with a *Pst*I adaptor(GCATAGTCGTCCACTAAAGCTT) and a antisence primer with a ClaI adaptor(AGTATGCCCGTAGTCAATCGAT), 10×PCR Buffer(5μl), dNTP mix(2.0μl), ddH2O(36.5μl). Stand for eight hours after mixing, thereby add 0.5μl of EX-Taq polymerase (Takara) into the mixtures. The suitable PCR procedure is one preliminary denaturation at 94℃ for 5 min; 35 cycles each involved denaturation at 94℃ for 1min, anneal at 54℃for 50s, extended at 72℃ for 1 min, and final extension at 72 ℃ for 10 minutes.

PCR products were detected by 1% agarose gel electrophoresis, and DS-RNAi obtained by gel extraction purifications which were performed using DNA Gel Extraction Kit (Takara).

2.2. Construction pBI121-DS-RNAi Plant Expression Vector and Engineering Bacteria

DS- RNAi component was recombined into a plant expression vector pBI121 using *Cla*I and *Pst*I double digestion to obtain the recombinant plasmid pBI121-DS-RNAi. The obtained recombined plasmid pBI121-DS-RNAi was transformed into E. coli competent state cell by heat-process [9],[10]. Recombinant plasmid pBI121-DS-RNAi was subcloned into *Agrobacterium rhizogenes* A4 competent cells by heat-process, to yield DS gene RNAi plant expression vector engineering bacteria, and identified by PCR [11].

2.3. Genetic Transformation of Panax ginseng and Induction of Hairy Roots

The 4-year-old *Panax ginseng* was sterilized in 0.1%aqueous mercuric chloride for 10 min. Root slices with 0.7cm thickness excised from sterilized plants and maintained on MS medium with 3% sucrose at 25℃. After about 2 days grown, the root slices were dropped with *Agrobacterium rhizogenes* A4 strain, and cultured on MS medium supplemented with 20 mg/l kanamycin, 3% sucrose at 25℃. After cultivation for 4-5 weeks, the transgenic hairy roots who appeared at the side of the root slices, were picked off and placed in new MS medium. The transgenic hairy roots who grew to 1cm length, were cut and transferred on to MS medium without kanamycin at 25℃. After 4 months of subculturing the roots every two weeks on fresh solid medium, the hairy root lines were transferred to MS liquid medium and kept in a rotary shaker at 100 rpm, 25℃. In order to study the capacity of transgenic hairy roots to produce ginsenosides, more than 50 transgenic lines were generated, from which line T4, T16, and T39 were selected for further analysis. Each phenotype considered were selected and cultured for 4 weeks in the MS liquid medium above mentioned medium and conditions for further analysis.

2.4. PCR Analysis of Transgenic Hairy Roots

Total RNA was isolated from the transgenic hairy roots and control group of hairy root. They were then converted into cDNA. Primers included GCA TAG TCG TCC ACT AAA GCT T and AGT ATG CCC GTA GTC AAT CGA T, PCR performed as the first round PCR.

2.5. Dammarendiol-II and Ginsenoside Contents in DS-RNAi Transgenic Lines

Dammarendiol-II and ginsenosides were extracted by the method of Samukawa[12]. Ginsenosides accurately weighed, were dissolved in methanol and diluted to volume with methanol, which was taken as

the sample solution. Analysis of ginsenosides by HPLC was carried out using analysis conditions: acetonitrile (A) and 0.05% phosphoric acid aqueous solution (B) as mobile phases, 0.8mL/min of flow rate, 203nm detection wavelength, 30 °C column temperature, and 10μl of the sample volume.

3. Results and Analysis

3.1. Recombinant PCR Amplification of DS-RNAi

By the three round PCR, we obtained a recombinant DS RNAi component as 658bp (Fig1), and identified by nucleotide sequencing. It is shown that the RNAi recombinant component of DS gene is successfully amplified, namely DS-RNAi.

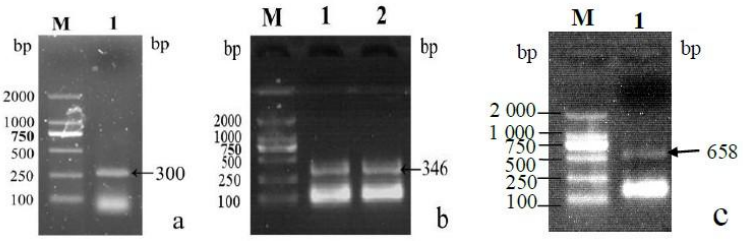

Fig.1: Electrophoresis of the three round PCR products

a: Electrophoresis of the first round PCR products M：DNA Marker 2000；1： First round PCR products sample

b: Electrophoresis of the second round PCR products

M：DNA Marker 2000；1：Second round PCR products sample 1；2：Second round PCR products sample 2；

c: Electrophoresis of the third round PCR productsM：DNA Marker 10,000；1：Third round PCR products sample

3.2. Construction of DS-RNAi Plant Expression Vector and Engineering bacteria

DS-RNAi component was connected into the pBI121 vector using double restriction enzyme digestion with *Pst*I and *Cla*I, and obtained DS-RNAi plant expression vector, named pBI121-DS-RNAi (Fig. 2).

The result of agarose gel electrophoresis (Fig.3) indicated that the engineering bacteria construction of recombinant pBI121-DS-RNAi plant expression vector was successfully. The transition truly acquired expression vector pBI121-DS-RNAi engineering bacteria.

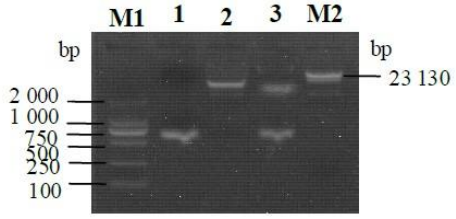

Fig.2 Double restriction enzyme digestion of recombinant plasmid electrophoresis

M1：DNA Marker 2000；M2：λ/HindIII DNA Maker

1: RNAi component；2：Recombinant plasmid；

3: Products of restriction enzyme digestion of pBI121-DS-RNAi;

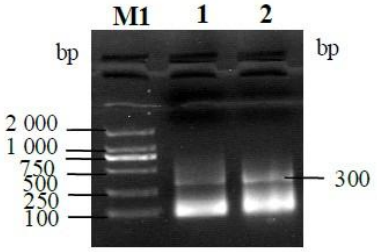

Fig.3 Electrophoresis of PCR identification

M：DNA Marker 2000；1：Products of colony PCR；

2：Products of plasmid PCR；

3.3. Genetic Transformation of *Panax ginseng* and Induction of Hairy Roots

Four-year-old *Panax ginseng* root slices were cultured on MS medium with 50mg/l kanamycing. After 5 weeks, transformed plants were positively regenerated the hairy root at the side of the root slices, no signal was observed in the wild-type plants (Fig.4). When the germination branch grew about 1cm length, we cut the branch as a new hairy root and put them into one new MS medium with 50mg/l kanamycing to measured the level of hairy roots growth (Fig.4B). Meanwhile, the wild-type hairy root was used for control. In the present study, hairy roots were obtained within 60 days of culture, indicating a rapid production of hairy roots.

3.4. PCR Analysis of Transgenic *Panax ginseng*

The accumulation of DS-RNAi and DS were analyzed in DS-RNAi transgenic plants by PCR. RNAi in *P.ginseng* caused DS expression deletion in transgenic hairy roots compared with the wild type. Densitometric analysis indicates that silencing of DS in ginsenoside biosythase depressed production of triterpenes intransgenic *P. ginseng* hairy roots.

3.5. Dammarendiol-II and Ginsenoside Contents in DS-RNAi Transgenic Lines

HPLC analysis revealed that the Dammarendiol-II ginsenoside content in RNAi transgenic roots was markedly reduced in all transgenic lines, with a corresponding decrease in DS transcript level (Fig.5). Accumulation of ginsenosides Rg1, Re and Rb1 were somewhat suppressed in all RNAi lines to these of wild-type.

The HPLC chromatogram revealed that all the ginsenosides were decreased in the T3 RNAi line (Fig. 9A). These results provided direct evidence for the expression of DDS in P. ginseng, and suggested its vital role in determining the biosynthesis of ginsenosides in *P. ginseng*. Overexpression of DS will be useful for the enhanced production of ginsenosides in *P. ginseng* or other plant species by transgenosis.

This study utilized the recombinant PCR and double endonuclease restriction and link technology to succeed in the construction of DS gene RNAi plant expression vector pBI121-DS-RNAi, which induced into Panax ginseng hairy roots to get transgenic hairy roots. We showed the assumption of ginsenoside improvement can be applied by RNAi method to regulate the ginsenoside biosynthetic pathway. This study suggested a convenient and low cost method to research in construction of RNAi expression vector, and laid the foundation for further research on regulating gisenoside biosynthesis.

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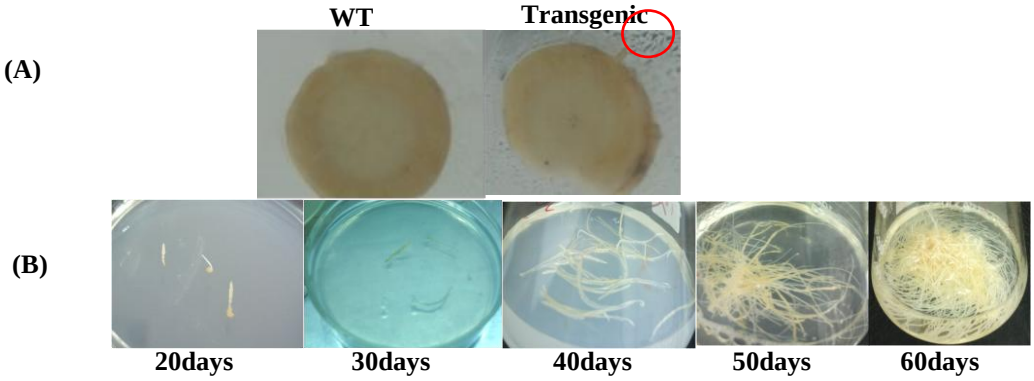

Fig.4: The expression of transgenic *Panax ginseng*
(A)The hairy root germinated on the side of the transgenic root slices after 5 weeks culture.
(B)The transgenic hairy roots grown at different time

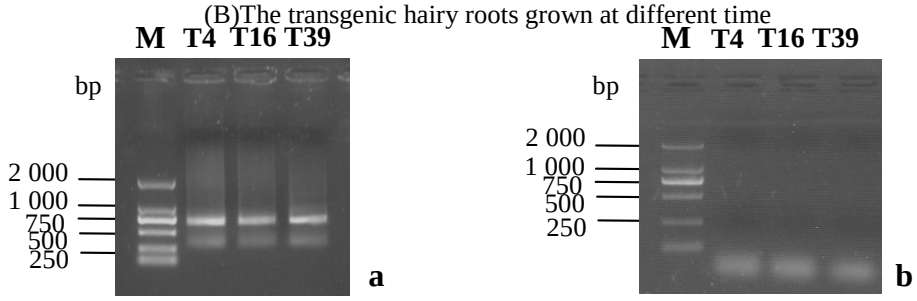

Fig.5: Analysis of the DS-RNAi and DS gene expression in the transgenic P.ginseng hairy roots
a: Analysis of the DS-RNAi gene expression. M：DNA Marker 2000；T4,T16,T39：PCR products of random sample
b: Analysis of the DS-RNAi gene expression. M：DNA Marker 2000；T4,T16,T39：PCR products of random sample

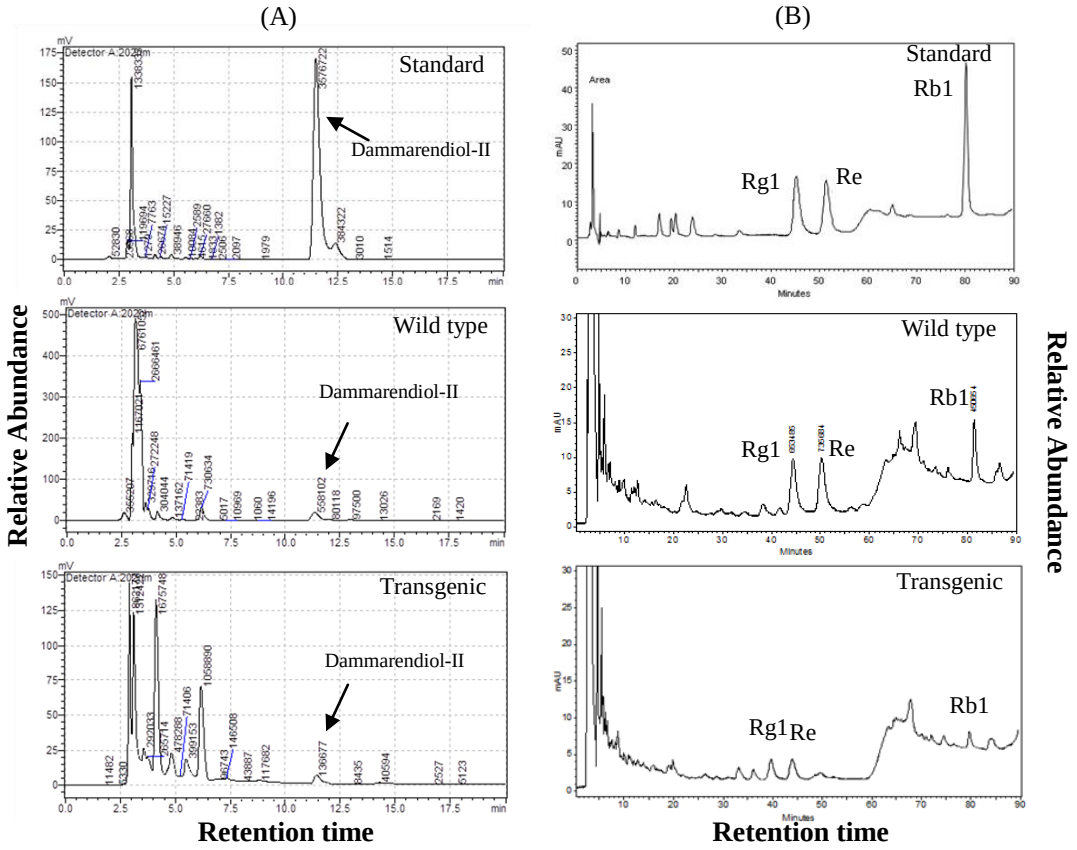

Fig.6: HPLC analysis of Dammarendiol-II and ginsenosides of transgenic DS-RNAi hairy roots.
Standard Dammarendiol-II (top) and Dammarendiol-II in the wild-type (middle) and DS-RNAi transgenic hairy roots (bottom). (B) Standard ginsenosides (top) and ginsenoside contents in the wild-type (middle) and DS-RNAi transgenic roots (bottom).