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Construction of *P.Ginseng* DS Gene RNA Interference Plant Expression Vector

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Abstract. This study is to utilize the recombinant PCR reaction and double endonuclease restriction enzyme digestion connection to construct the RNA interference plant expression vector of DS (Dammarenediol-Ⅱ synthase) gene, undergoing the heat-process to transform into *Agrobacterium rhizogenes* A4 strains, to get DS RNA interference plant expression vector engineered bacteria. 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, RNAi

1. Introduction

Ginseng (*Panax ginseng* C.A.), a perennial herb, belongs to *Panax Araliaceae* family. The main effective component of ginseng is ginsenoside. Recently, the development in the industrialization of TCM catalyzes the requirement of ginsenoside. However, its low propagation coefficients and disposable cultivation[1] inspires to improve ginsenoside content of ginseng by regulation of ginsenoside biological synthesis.

Ginsenosides extracted from ginseng root are triterpenoid saponins[2], are mainly responsible for the pharmacological activities of ginseng[3][4]. The first committed step in ginsenoside synthesis is the cyclization of 2,3-oxidosqualene. 2, 3-oxidosqualene in *P. ginseng* is flowed into three metabolic pathways, two of which lead to saponins including dammarane-type ginsenosides and oleanane-type ginsenosides, while the other leads to steroid alcohols. The pathway leading to saponin is controlled by dammarenediol-Ⅱ(DS) and β-amyrin synthase (β-AS)[5]. dammarenediol is converted from 2,3-oxidosqualene catalyzing by Dammarenediol-Ⅱ synthase firstly [5], then hydroxylased by cytochrome P450[6], and at last formed seven kinds of tetracyclic triterpenoids dammarane-type ginsenosides-including Rb1,Rb2,Rc,Re,Rf and Rg1—by glycosyltransferase[5][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. Materials and Methods

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2.1. Materials

Plant materials: 21-day-old hairy roots of *P. ginseng* cultured in MS solid medium, and these ginseng hair roots preserved in our laboratory.

Strains and Plasmid: *Agrobacterium rhizogenes* A4, plant binary expression vector pBI121, and *E. coli* JM109 were kept in our laboratory.

Reagents: Taq DNA polymerase, PrimeSTARTM HS DNA polymerase(Takara), dNTPmix, DNA gel extraction kit, DNA the Marker, T4, ligase mRNA extraction kit, plasmid mentioned kit (TaKaRa Company, Dalian), kanamycin (Dingguo biotech companies), and other domestic reagents purchased from Beijing DingGuo Biotechnology Company.

2.2. Methods

2.3. Constraction 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.

Recombinant PCR reaction to construct DS-RNAi element contains three round PCR amplification with 4 pairs of primers. The first PCR amplified the properly core spliced fragment of DS using primers P1/P2. Sense and antisense fragments with complementary ends were amlified by the second PCR using two pairs of primers P3/P4 and P5/P6 and fragments of the first PCR as templates. In the third PCR, sense fragments and antisense fragements were spliced to get recombinant fragments of DS-RNAi by using P7/P8 [9][10] (Fig.1). Specific primers were designed by Primer Premier 5.0, and synthesized by TaKaRa company.

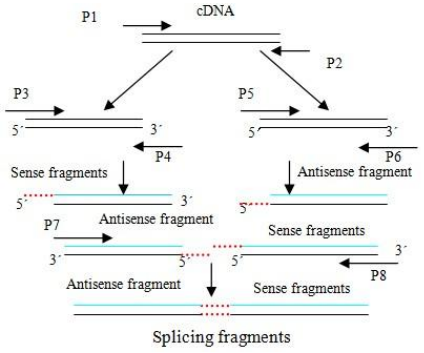

Fig. 1: Schematic diagram of using recombinant PCR technology to contract RNAi component

First PCR was carried out with P1 and P2 (1.0μl each) primers using 0.2μl EX-Taq DNA polymerase (Takara) with dNTP(1.0μl, concentration: 2.5mmol/L), 10×PCR Buffer(2.5μl) and cDNA(1.0μl) in a final volume of 25μl. PCR was carried out for 30 cycles with a program (94℃, 1min, 53.5℃, 50sec, 72℃, 1min) of denaturation at 94 ℃ for 5 minutes and final extension at 72 ℃ for 10 minutes. The system and amplification procedure of the second PCR are the same with those of the first PCR. Sense and antisense fragment obtained by primers P3, P4 and P5, P6 each in the second PCR.

Third PCR reaction mixtures (50μl) contained sense and antisense fragment (1μl each), 5μl of 10×PCR Buffer, 2.0μl of dNTP mix, 2.0μl of each primer P7 and P8, 36.5μl of ddH2O. Stand for eight hours after mixing, thereby add 0.5μl of PrimeSTARTM HS DNA 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).

2.4. Construction and Enzyme Digestion of pBI121-DS-RNAi Plant Expression Vector

DS-RNAi component was ligated into the corresponding sites of pBI121 which was digested with ClaI and PstI to obtain the recombinant plasmid pBI121-DS-RNAi. The plasmid pBI121-DS-RNAi was transformed into *E. coli* competent state cell by heat-process. The transformant was inoculated in LB liquid medium containing 50mg/L kanamycin at 25℃ for 2 days, then extracted recombinant plasmids for analysis

of restriction enzymes [11][12][13]. Selected positive single colony, and extracted recombinant plasmids, then digested with Pst1 and Cla 1 confirmed by restricting enzyme digestion [12][13].

2.5. Construction and Identification of Engineering *Agrobacteria* pBI-DS-RNAi

Recombinant plasmid pBI121-DS-RNAi was transformed to *Agrobacterium rhizogenes* A4 competent cells by heat-process, to construct DS gene RNAi plant expression vector engineering bacteria. Coat YEP plate overnight to culture the transformed cell, then selected positive colony shaking culture in 50mg/L kanamycin liquid medium, and extracted Ri plasmid from 5ml transformed A4 bacteria and identified by PCR [14][15].

3. Results and Analysis

3.1. Design of Primers Used in Recombinant PCR

Here, we obtained the conservative sequence of DS gene by homologous genes sequence alignment, and confirmed the core domain sequence of DS gene (GenBank Accession No. AB265170) by selected a extremely similar sequence after compared with these coding area sequences of CS (GenBank Accession No.AB009029),β -AS (GenBank Accession No. AB009030) and LS (GenBank Accession No. AB009031).

Primers used in Recombinant PCR were showed in Table 1. All of these 4 pairs of primers do not contain Pst I and Cla I restriction nuclease sites, which would be used in following experiment.

Table 1: PCR amplification of DS recombinant gene primer design RNAi componentsresults

Primer	Nucleotide sequence
P1	5 ´ CCGCCGTTGAGATTAGATGA 3 ´
P2	5 ´ TGACCCAATCATCGTGCTGT 3 ´
P3	5′ CTACGCCGCTATTAG/AAGCTT/CCGCCGTTGAGATTAGATGA 3′
P4	5′GAATTCGAGCTCGGGCCCGTCGAC/TGACCCAATCATCGTGCTGT 3′
P5	5′GTCGACGGGCCCGAGCTCGAATTC/TGACCCAATCATCGTGCTGT 3′
P6	5′ GACGCCCTTATTTTA/CTGCAG/CCGCCGTTGAGATTAGATGA 3′
P7	5′GCATAGTCGTCCACTAAAGCTT 3′
P8	5′AGTATGCCCGTAGTCAATCGAT 3′

3.2. Recombinant PCR Amplification of DS-RNAi

Total RNA were extracted from ginseng hairy roots, then reverse transcribed into cDNA. The cDNA was used as a template for the first round PCR amplification with primer P1/P2. This amplification produced clear corresponding target band about 300bp (Fig.1.a). The products gained from the first round PCR were used as the template for the second round PCR, with primer P3/P4 and P5/P6, which produced two overlapping complementary sequences of 346bp (Fig.1.b).

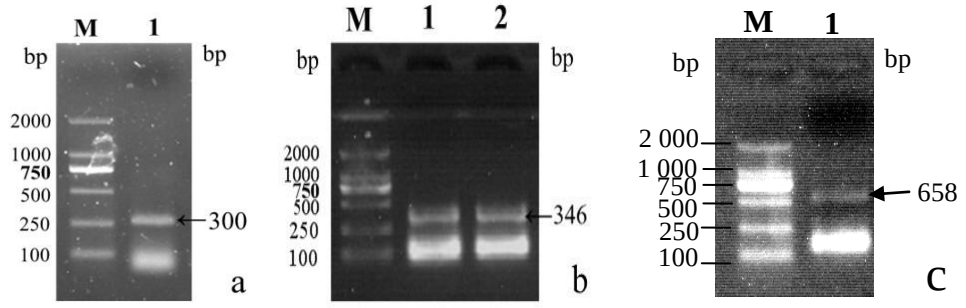

Fig. 1: Electrophoresis of the three round PCR products

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 (Primer P3/P4) ;2 :Second round PCR products sample (Primer P5/P6) ;

c: Electrophoresis of the third round PCR products

M: DNA Marker 10,000; 1 :Third round PCR products sample (Primer P7/P8) ;

The objective fragments obtained in secondary round PCR would be linked through annealing of their overlapping ends, followed by PCR amplification of the entire assembled DNA fragment with primer P7 and P8. Then plenty of DS RNAi component of 658bp were obtained (Fig.1.c), and identified by nucleotide sequencing (Sequencing was performed by Sangon Co., Ltd.). It is shown that the RNAi recombinant component of DS gene is successfully amplified, namely DS-RNAi.

3.3. DS-RNAi Expression Vector Construction and Restriction Enzyme Digestion

DS-RNAi component was connected into the pBI121 vector with double restriction enzyme digestion, and obtained DS-RNAi plant expression vector, named pBI121-DS-RNAi. These recombined plasmids were transformed into competent *E.coli* cells by heat-process. We extracted recombinant plasmids and confirmed by restricting enzyme digestion with Pst I and Cla I. The result of agarose gel electrophoresis was shown in Fig. 2. Band in lane 1 was DS-RNAi component; band in lane 2 was recombinant plasmid, whose molecular weight is the sum molecular weight of pBI121 plasmid and DS-RNAi component; Bands in lane 3 were products of double restriction enzyme digestion of pBI21-DS-RNAi. In the lane 3, the upper band was pBI121-DS-RNAi, and the lower band was DS-RNAi after double restriction enzyme digestion of pBI-DS-RNAi. These results accorded with the experimental conception that proves the feasibility to apply recombinant PCR technology for constructing plant gene RNA interference structure. The result of restriction enzyme digestion demonstrated that the recombinant pBI121-DS-RNAi plant expression vector was successfully constructed.

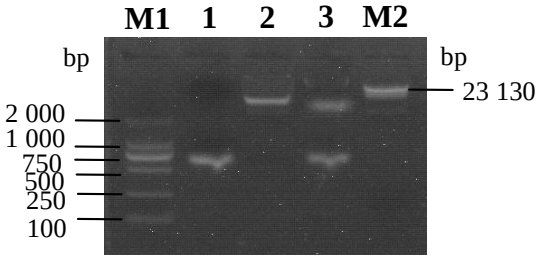

Fig. 2: Double restriction enzyme digestion of recombinant plasmid electrophoresis

M1 :DNA Marker 2000;1: RNAi component;2 :Recombinant plasmid;

3: Products of double restriction enzyme digestion of pBI121-DS-RNAi; M2 :λ/HindIII DNA Maker

3.4. Engineering Bacteria Construction Identification of DS-RNAi Plant Expression Vector

Recombinant plasmid pBI121-DS-RNAi was transformed to *Agrobacterium rhizogenes* A4 competent cells by heat-process. DS gene RNAi vector engineering bacteria was acquired. Ri Plasmid was extracted identified by PCR from 5ml of *Agrobacterium rhizogenes* A4 bacterial liquid. The PCR analysis was conducted using primer P1/P2 with Ri plasmid as template. The results of agarose gel electrophoresis were shown in Fig.3. Bands in lane 1 and 2 were products of colony PCR and products of plasmid PCR respectively. Size of bands was 300, and that was identical with expected, which 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.

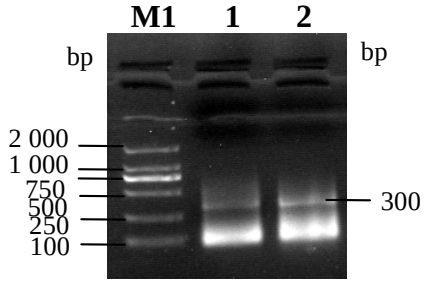

Fig. 3: Electrophoresis of PCR identification

M :DNA Marker 2000;1 :Products of colony PCR;2 :Products of plasmid PCR;

4. Discussion

Recently, RNAi technology became the highly efficient application in the plant metabolic engineering. Compared with anti sense RNA and homologous inhibition technology, it is easier to occur function loss and depressed mutation [16]. 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 was transformed into the *Agrobacterium rhizogenes* A4 to get DS-RNAi plant expression vector engineered bacteria. We showed the assumption of ginsenoside conents 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.

5. References

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