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# Construction of *P.Ginseng* LS 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 LS (Lansosterl sythase) gene, undergoing the heat-process to transform into *Agrobacterium rhizogenes* A4 strains, to get LS RNA interference plant expression vector engineered bacteria. By inhibiting lanosterol synthase gene expression, detemine the major precursor of phytosterols in ginseng, to lay a strong foundation for perecting complete the ginsenoside metabolic pathways.

**Keywords:** Ginsenoside, Lansosterl sythase, RNAi

## 1. Introduction

Ginsenoside, which are steroid compounds (tetracyclic triterpenenoid saponins), are regarded as mainly active ingredients which present in *P.ginseng*[1]. The first committed step in ginsenoside synthesis is the cyclization of 2,3-oxidosqualene.2,3-oxidosqualene is cyclized to generate two types of products. One parts are various triterpene products (ginsenosides) which catalyzed by the β -amyrin synthase (β -AS) and Dammarenediol-Ⅱ synthase (DS): the others are the sterol materials, catalyzed by the cycloartenol synthase (CS) and Lansosterl sythase (LS) [2].

Utilizing RNA interference (RNAi) technique to inhibit expression of LS gene, can make more precursors flowing into ways to generate ginsenoside. Meanwhile, RNAi technology could be used for the identification function of the LS gene in secondary metabolic, which would determine the major precursor of phytosterols in ginseng, to lay a strong foundation for perfecting complete the ginsenoside metabolic pathways.

This study applied the recombinant PCR reaction and double endonuclease restriction enzyme digestion connection to construct the LS RNAi plant expression, and laid the foundation for further study on inducing transformation of ginseng explants, and the identification of LS gene.

## 2. Materials and Methods

### 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: pfu, DNA polymerase (Shanghai Sangon), Taq DNA polymerase, dNTPmix, DNA gel extraction kit, DNA the Marker, T4, ligase mRNA extraction kit, plasmid mentioned kit (TaKaRa Company,

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Dalian), kanamycin (Dingguo biotech companies), and other domestic reagents purchased from Beijing Ding Guo Biotechnology Company.

## 2.2. Methods

### 2.2.1. 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 LS-RNAi element contains three round PCR amplification with 4 pairs of primers.

The first and second PCRs were performed with buffer(2.5μl), 10×dNTP(1μl)， sense primer(1ul), antisense primer(1μl), DNA templates(1μl),Pfu DNA polymerase(0.2μl), and DDH2O(18.3μl), ina final volume of 25ul, and was carried out for 35 cycles with a program (94℃, 1 min, 55℃, 50 s, and 72℃, 1 min, and final 8-min extension at 72℃).

For the third round PCR, we used a final volume of 25μl, which contained mixture of the two PCR fragments produced in the second round (each of them 0.5μl), buffer(2.5μl), 10×dNTP(1μl), primer P7(1ul), primer P8 (1ul), Taq DNA polymerase(0.2μl), and DDH2O，(18.3μl). No DNA polymerase was added to the overlap extension mixture. After 4 hours standing at 20℃, added 0.2μl Taq DNA polymerase into mixture, then performed as follows: 94℃ for 5 min, followed by 35 cycles of 94℃ for 1 min, 58℃ for 50 s, and 72℃ for 1 min, with a final 8-min extension at 72℃.

PCR products were detected by using 1% agarose gel electrophoresis. Isolation and purification target fragments with Gel extraction kit.

### 2.2.2. LS-RNAi Expression Vector Construction and Enzyme Digestion

LS-RNAi component was connected with a plant expression vector pBI121 by Pst I and Cla I double enzyme digestion. The obtained recombined plasmid was transformed into competent *E.coli* cells by heat-process. Selected positive single colony, and extracted recombinant plasmids, then digested with Pst1 and Cla 1 confirmed by restricting enzyme digestion [3][4].

### 2.2.3. Engineering Bacteria Construction and Identification of LS Gene RNAi Plant Expression Vector

Construction and Identification of LS Gene RNAi Plant Expression Vector Engineering Bacteria

Recombinant plasmid pBI-LS-RNAi was transformed into *Agrobacterium rhizogenes* A4 competent cells by heat-process, to construct LS gene RNAi plant expression vector engineering bacteria. Coat YEP plate overnight to culture the transformed cell, then select positive colony for shaking culture in 50mg/L kanamycin liquid medium, and extracted Ri plasmid from 5ml transformed A4 bacteria and identified by PCR [5][6].

# 3. Results and Analysis

## 3.1. Design of Primers Used in Recombinant PCR

Here, we obtained the conservative sequence of LS gene by homologous genes sequence alignment, and confirmed the core domain sequence of LS (GenBank Accession No.AB009031)gene 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 DS (GenBank Accession No. AB265170).

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.

Primer P1 and P2 are used in the first round PCR reaction for amplification of LS gene core area, with length 444bp fragment. Primer P3 and P6 includes three parts: template specific combined area, restriction site area and amplified primer combined area of third round PCR: Primer P4 and P5 include two parts: template combined area and overlapped area. 5' end of primer P3 has restriction site of Pst I: 5' end of P4 and P5 have 24bp complementary overlapping area, the overlapping parts have restriction sites for EcoRⅠ, Sac

Ⅰ, Apa I and Sal I. 5'end of P6 primer have Cla I restriction site, the length with overlapping area (overlapped area, secondary PCR specific combined area and restriction site area) is 490 bp. Primer P 7 and P8 are amplified primer specific combined area and restriction site area of third round PCR for P3 and P6 respectively. Primer 5.0 is used to assist design: primer is synthesized by TaKaRa Co., Ltd.

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

| Primer | Nucleotide sequence |
|---|---|
| P1 | 5 ´ GCAACAACCCTCTACCACCA 3 ´ |
| P2 | 5 ´ CCACCCAACAACAGACCATG 3 ´ |
| P3 | 5′GCATAGTCGTCCACTAAAGCTT/GCAACAACCCTCTACCACCA 3′ |
| P4 | 5′GAATTCGAGCTCGGGCCCGTCGAC/CCACCCAACAACAGACCATG 3′ |
| P5 | 5′GTCGACGGGCCCGAGCTCGAATTC/CCACCCAACAACAGACCATG 3′ |
| P6 | 5′AGTATGCCCGTAGTCA/ATCGAT/GCAACAACCCTCTACCACCA 3′ |
| P7 | 5′GCATAGTCGTCCACTAAAGCTT 3′ |
| P8 | 5′AGTATGCCCGTAGTCAATCGAT 3′ |

## 3.2. Recombinant PCR Amplification of LS-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 444bp (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 490bp (Fig.1.b).

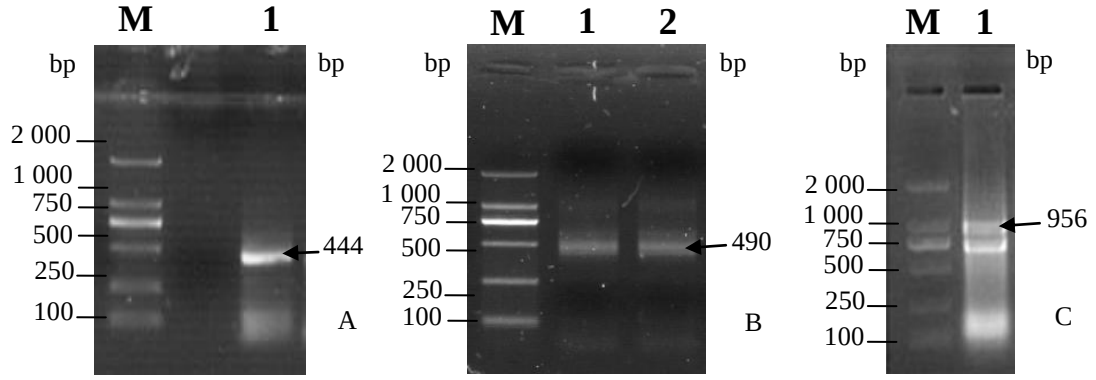


Fig.1 Electrophoresis of the three round PCR products

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 LS RNAi component of 956bp were obtained (Fig.1.c). Retrieve target bands on 956 bp and identified by nucleotide sequencing (Sequencing was performed by Sangon Co., Ltd.). It is shown that the RNAi recombinant component of LS gene is successfully amplified, namely LS-RNAi.

## 3.3. LS-RNAi Expression Vector Construction and Restriction Enzyme Digestion

LS-RNAi component (the tertiarky PCR products) was connected into the pBI121 vector with double restriction enzyme digestion, and obtained LS-RNAi plant expression vector, named pBI121-LS-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 the lane 1 was LS-RNAi component: band in the

lane 2 was pBI121-LS-RNAi recombinant plasmid, whose molecular weight was approximately 15,000: bands in the lane 3 were products of double restriction enzyme digestion of pBI121-LS-RNAi. The upper band was pBI121-LS-RNAi, and the lower band was LS-RNAi after double restriction enzyme digestion of pBI121-LS-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-LS-RNAi plant expression vector was successfully constructed.

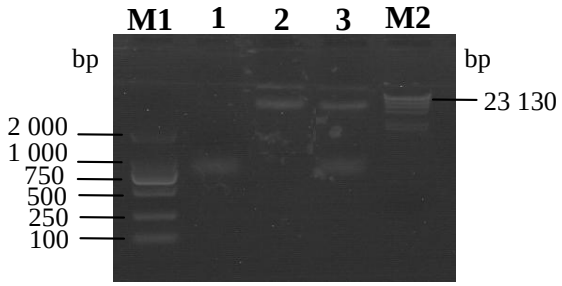


Fig.2 Double restriction enzyme digestion of recombinant plasmid electrophoresis
M1: DNA Marker 2000:1: RNAi component: 2: Recombinant plasmid:
3: Products of double restriction enzyme digestion of pBI-CS: M2: λ/HindIII DNA Maker

### 3.4. Engineering Bacteria Construction Identification of LS Gene RNAi Plant Expression Vector

Recombinant plasmid pBI121-LAS-RNAi was transformed to *Agrobacterium rhizogenes* A4 competent cells by heat-process. LAS 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 490, and that was identical with expected, which indicated that the engineering bacteria construction of recombinant pBI121-LS-RNAi plant expression vector was successfully. The transition truly acquired expression vector engineering bacteria A4-pBI121-LAS-RNAi.

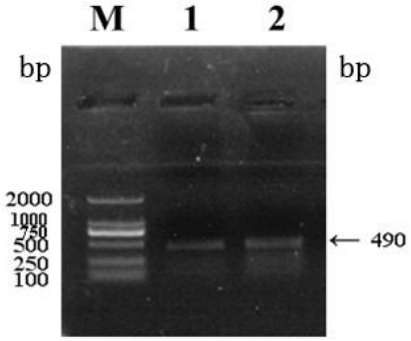


Fig.3 Electrophoresis of PCR identification
M: DNA Marker 2000:1: Products of colony PCR: 2: Products of plasmid PCR:

## 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[7]. The development of RNAi technology provided a new method for crop genetic improvement, and this technique has been successfully applied in rice, wheat and barley planting [8].In this experiment, the LS- RNAi was constructed through the recombinant PCR reaction, and connected to pBI121 plant expression vector, transformed into *Agrobacterium rhizogenes* A4 strains by the heat-process to, to obtain LS RNAi plant expression vector engineered bacteria.

In this study, we utilized the recombinant PCR and double endonuclease restriction and link technology to succeed in the construction of LS gene RNAi plant expression vector pBI121-LS-RNAi. We showed the assumption of ginsenoside conents improvement can be applied by RNAi method to regulate the ginsenoside biosynthetic pathway.

## 5. References


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