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EXPRESSION OF SS AND SE GENES IN *P. japonicus* HAIR ROOTSAND ITS GINSENOSIDE RE SYNTHESIS

Lai Zhang[1,*] Min-Jie Qi[1,2] and E Liang[1,2]

[1]Anshun University/Key Lab of Function Material and Resource Chemistry GZSJYT, Anshun 561000, China; [2] School of Life science, Guizhou Normal University, Guiyang 550000, China.
***Corresponding author's e-mail: 975575681@qq.com**

In order to study the effect ofSS(squalene synthase) and SE (squalene epoxidase) genes co-over-expression in hairy roots of *Panax japonicu*s on ginsenoside Resynthesis, the callus of *P· japonicus* was used as receptor material. The double expression vector of SS and SE genes were constructed respectively and transformed the *P· japonicus* callus by *Agrobacterium* LBA4404. Finally, C58Cl was used to induce hairy roots from Callus of transgenic *P· japonicus*. The results of PCR showed that SS and SE genes could be integrated into hairy roots of *P· japonicus* individually or jointly. RP-HPLC indicated that Re content in transgenic *P·japonicus* hairy roots was 2.4 times and 2.3 times of that in non-transgenic ones (CK) when SS and SE geneswere transferred alone.The Re content in transgenic *P· japonicus* hairy roots was 5.3 times that of the non-transgenic ones when SS and SE genes co-over-expression. It can be seen that the SS and SE genes has a positive regulatory effect on Re synthesis in *P· japonicus* hairy roots and the co-over-expression of SS and SE genes canincrease Re content.
Keywords: *Panax japonicus, SS, SE,* Ginsenoside Re, Expression, Hairy Roots.

INTRODUCTION

Panax japonicus is a perennial herb belonging to the Araliaceae ginseng genus. It is mainly distributed in China, Japan and Korea, while southwest China shows the highest distribution of this species (Zhang *et al.,* 2010). *Panax. japonicus* has pharmacologically effects of promoting blood flow of *P. notoginseng* and strengthening tonics of *P. ginseng*. The saponin substances in *P. japonicus* are mostly triterpenoid saponins (TS).The study of *P. japonicus* involves biological characteristics (Lin *et al.,* 2007; Yi *et al.,* 2018), introduction and domestication(Qi, 1987), germplasm resources (You, 2007; Ding *et al*, 2019),chemical composition (Wu *et al.,* 2012; Shi *et al.,* 2013; He *et al.,* 2014; Wu *et al.,* 2016), pharmacognosy identification (Zhang and Sun, 2009), gene cloning (Zhang and Sun, 2014), hairy root cultures (Zhang, 2011) and biological activity(Li *et al.,* 2012)so on. The effects of polygene coconversion on synthesizing triterpene saponins were not reported in the hairy roots. Therefore, on the basis of the culture of *P. japonicus* hairy roots, the efficient plant expression vector for SS and SE genes was constructed, and introduced and integrated into the *P. japonicus* hairy roots genome. PCR was used to detect the expression of SS and SE genes in hairy roots, at the same time ginsenoside Re was detected to clarify the synergistic regulatory role of SS and SE genes in *P. japonicus*. It provides the theoretical and experimental basis for constructing efficient homologous expression system of triterpene saponin synthesis.

MATERIALS AND METHODS

The experimental material was*P. japonicus*. *Agrobacterium* C58Cl was provided by Professor Zhi hua Liao.The carrier is a dual expression carrier pCXSN was gift by Prof. KemingLuo. *Agrobacterium* LBA4404 is preserved in our laboratory.

Callus induction of *P. japonicus*: The *P. japonicus* explant was washed for 2 h by running water. After being sterilized by different disinfection methods, rinsed with sterile water 5 timesand inoculated on 1 mg/L 2,4-D MS solid medium. Each method treated 30 explants. After light culture 15 d, the number of explants contaminated and survived was recorded. The embryos of *P. japonicus* seeds were took out after disinfection; the stems were cut into 1 cm long small sections, and the leaves were cut into 5 mm × 5 mm small pieces. After disinfection, the explants were inoculated on MS medium, after 5 d and then on MS solid media for light culture and dark culture were carried out under the condition of (23 ± 1) °C. Each bottle was inoculated 10 explants, and each level was repeated 3 times.

Construction of SS and SE super-expression vector of *P. japonicus*: The positive monophyletic colonies of the SS and SE genes stored in the laboratory were selected, and inoculated in LB liquid medium containing 10 mL antibiotics (conc) for overnight. The Plasmid was extracted by alkaline cracking (Ref). The pCXSN-SS and pCXSN-SE carriers were introduced into the *Agrobacterium* LBA4404 receptor cells by $CaCl_2$ freezing method and cultivated on the 50 mg/L Kan

+20 mg/L Rif LB solid medium. The PCR detected whether the intended carrier was successfully transferred to LBA4404. SS and SE genes transform the callus of *P. japonicus*: The *P. japonicus* callus were cultivated 4d on 40 mg/L AS+MS and then transferred to LBA4404-pCXSN-SS and LBA4404-pCXSN-SE bacteria solution with OD600 value of 0.6, infected 18 min at 26℃ and 120 r/min. Removed the bacterial solution and used a sterile filter paper to absorb the bacterial solution of *P. japonicus* callus, then cultured 4d on 40 mg/L AS + MS, took out them and rinsed with 400 mg/L Cef aseptic water for 5 times, then placed on 400 mg/L Cef + MS to culture 10 d, finally cultured on 50 mg/L Kan +25 mg/L Hyg+ MS to screen 4 times, and each time was 20-30 d.

Induce transgenic *P. japonicus* callus to produce hairy roots : The callus of *P. japonicus* which had been transformed into SS and SE genes was infected with activated C58C1 for 0-35 min, then used sterile filter paper to absorb the bacterial solution and cultured on 100 mg/L AS +MS filter medium for 3-6d. Took out them and rinsed with sterile water and inoculated on 500 mg/L Cef + MS solid medium for screening. In the same way, the bacteria were repeatedly removed until they were sterile, and then transferred to MS solid medium to produce hairy roots. When hairy roots grew to 3 cm, a thick, white hair rootswas cut aseptically under aseptic conditions and inoculated on 1/2 MS liquid medium, and incubated at 135 rpm and (25 ± 1) ℃ for 10 d in the dark, repeat 5 to 6 times.

Detectionof the target genes in transgenic *P. japonicus* hairy roots: The CTAB method was used to extract the hair roots DNA of transgenic and non-transgenic *P. japonicus*. The primers used for PCR amplification were designed according to *rolB* in the modified C58C1 strain. The forward primers were 5'-GCTCTTGCAGTGCTAGATTT-3'and the reverse primers were 5'-GAAGGTGCAAGCTACCTCTC-3'. The forward and reverse primers of SS gene and SE gene were 5'-GCAGGACTTGTTGGATTAGGGT-3' and 5'-AACATGCGTGACTTTGGTATCTC-3';5'-TAAATAATATGAATTCATCTTCTTCTACAG-3'and5'-GCCTCGAGTTAGCGAATGAGCTCT-3'.The PCR reactions were carried out in a total 50 μL , including 5 μL 10×buffer, 3 μL 25 mmol·L^{-1} $MgCl_2$, 1 μL 10 mmol.L^{-1} dNTP, 1 μL forward and reverse primers, 0.5 μL TaKaRa EX Taq, 37.5 μL ddH_2O, 1 μL DNA template. PCR amplification conditions were94℃ for 3 min, 94℃ for 45 s, 45℃ for 1 min, 72℃ for1 min, and the last 72℃ for 10 min. a total of 35 cycles. All reactions add up to 35 cycles. The PCR amplification products were stained with 0.1% gold-view agarose-gel for electrophoresis (120 V, 100 mA, 20 min). The electrophoresis resultswere observed and photographed under the gel imaging system (UV 260 nm).

Detectionof the Re content in transgenic *P.japonicus* hairy roots:RP-HPLC instrument is Waters 2487 (USA), chromatographic condition: Symmetry-C18 column (4.6 mm ×150 mm, 5μm); the mobile phase was 20% acetonitrile. Flow rate 0.8 mL / min; column temperature 25℃; the detection wavelength was 203 nm, injection volume was 10 μL. The Re (provided by China pharmaceutical biological products testing institute, for content determination) was accurately weighed 5mg and added into 5 mL volumetric flask, then dissolved with 20% acetonitrile and fixed 5mL, finally, the 1mg·mL^{-1} control sample solution was obtained.

RESULTS

The *P. japonicus* callus: The sensitivity of young stem and leaf of *P. japonicus* to alcohol was high, proper alcohol treatment time is important to obtain sterile explants for stems and leaves. The seeds treated with 75% alcohol for 60 s and soaked in 0.1% mercury chloride for 12 min had higher survival rate and lower contamination rate. Young stems and leaves were treated with 75% alcohol for 15 s and soaked with 5% sodium hypochlorite for 5 min, which could achieve better disinfection effect. In the light conditions (Fig.1A and B), the germination of *P.japonicus* embryos began about 25 d after inoculation into the corresponding concentration of medium, the callus was produced at the base, after about 45 d the germinated seedlings began to wither and die, and the callus continued to grow. The stems were inoculated into corresponding concentration medium for 5 d, the incision at both ends of the stem began to swell, and the pale yellow-green callus grew about 10 d later, and could proliferate rapidly. The callus of leaves began to form about 20 d after inoculating into corresponding concentration medium, they grew slowly, loose and fragile, with a crystalline shape. Compared with the callus induced by embryos and tender stems, the degree of browning was less and the proliferation rate was slower. The results indicated that the callus of *P. japonicus* could be induced by 1.5 mg/L NAA+1.5 mg/L 2, 4-D +0.1 mg/L KT in embryos, 1.5 mg/L NAA+ 1.0 mg/L 2, 4-D + 0.1mg /L KT in stems, and 1.5 mg/L NAA+1.0 mg/L 2, 4-D + 0.2 mg/L KT in leaves. The callus of *P. japonicus* could be induced more easily in stems than in embryos and leaves, and the proliferation rate was faster.

In the dark conditions (Fig.1C and D), the germination of *P. japonicus* embryo was about 5 d slower than that under light conditions at the same medium, the callus of *P. japonicus* was formed at about 40 d, after a period the seedlings began to wither and die, but the growth of callus was not affected. The callus of stem began to form about 15 d after being inoculated on the corresponding medium and it was yellow or light yellow, the white somatic embryo was formed after three months. If the callus was cultivated in 1.5 mg /L GA_3 MS for 25 d by light, then it could germinate normally.When the leaves were inoculated on the corresponding concentration of medium for about 25 d, they began to appear crystalline, produced loose and fragile callus, but the growth rate was slower. From the selection of induction rate, the embryos were induced by 1.0 mg/L NAA+ 1.5 mg /L 2, 4-D + 0.2 mg/L KT, the leaves were induced by 1.5 mg/L NAA+ 1.0 mg/L 2,

4-D + 0.1mg/L, and the stems were induced by 1.5 mg/L NAA+1.5 mg/L 2, 4-D +0.1 mg/L KT.

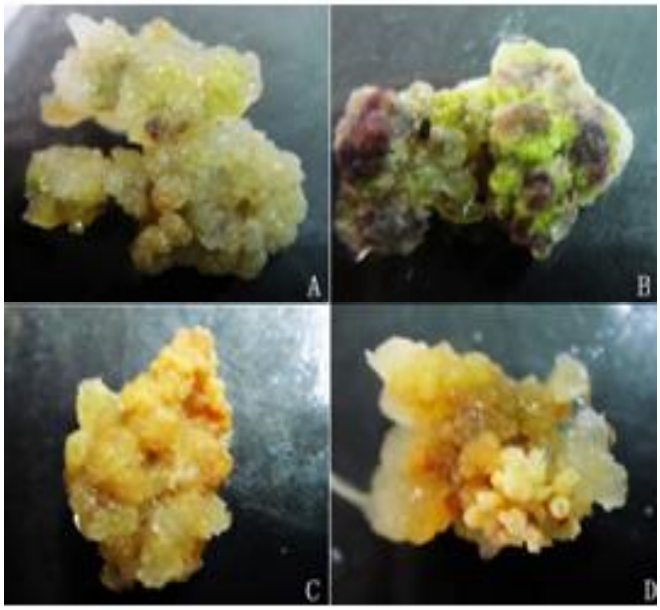
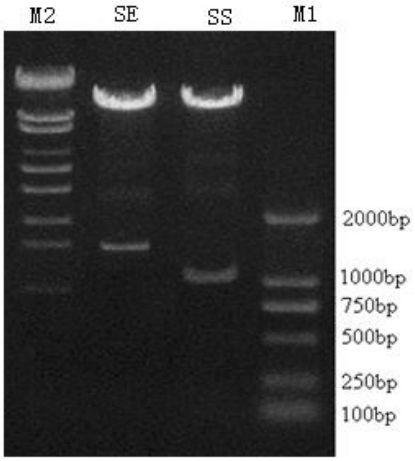


Figure 1. Callus induction. A, B. callus with different browning degree in light; C. callus in dark; D. callus producing somatic embryo in dark

Construction and transformation of expression vectors for *P. japonicus* SS and SE genes: The vector used in the experiment was a binary expression pCXSN, which could be used as either a clone vector or a plant expression vector. Therefore, on the basis of cloning of SS and SE genes, to extract full-length plasmid can become a highly efficient plant expression vector for SS and SE genes.The efficient plant expression vectors of SS and SE genes were digested with BamH I, and results (Fig.2) showed that the 1350 bp SSand 1632 bpSE were obtained, proving that SS and SE genes had been successfully inserted into plant expression vector pCXSN.



M1: DL2000 Marker ; M2: λ-EcoT14 I digest Marker

Figure 2. BamH I digest of recombinant plasmid

The pCXSN-SS and pCXSN-SE were transformed into *Agrobacterium* LBA4404 competent cell, the colony was selected for PCR identification with corresponding primers. The results showed that the 1350 bp SS and 1632 bp SE were obtained (Fig.3), indicating that the pCXSN-SS and pCXSN-SE had been transferred into *Agrobacterium* LBA4404.The engineering bacteria LBA4404-pCXSN-SS and LBA4404-pCXSN-SE could be used for transformation.

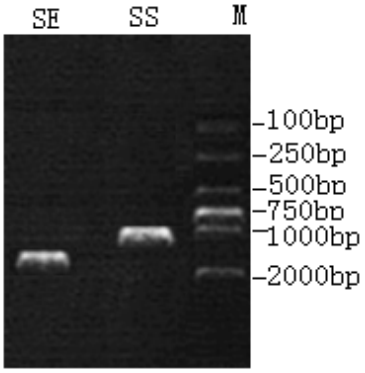


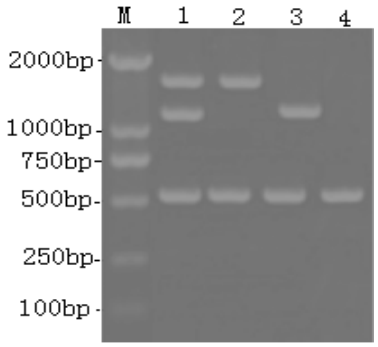
Figure 3. PCR verified the SS and SE of LBA4404

Induction and culture of hairy roots from transgenic *P. japonicus* callus: The callus of SS and SE genes were disseminated by C58C1 bacterial solution, and the hairy roots could not be induced in 0~5 min, butcould be induced in 5~35 min. However, in 5-25 min, the induction rate presented a positive correlation with the time; in 25-35 min, the induction rate presented a negative correlation with the time. It can be seen that the best induction time of C58C1 for SS and SE gene callus was 25min; the induction rate was 90 %. The *P. japonicus* callus of SS and SE genes was disseminated with C58C1 after 6-8 d and began to swell. After 8-11 d the hairy roots were generated, which were all white, with more branches, dense clumps and anisotropy (Fig.4).

Figure 4. Hairy roots induction and culture of transgenic *P. japonicus* callus. a: C58C1 induces the *P.japonicus* callus of SS and SE to produce hairy roots; b: monoclonal hairy roots; c: expanded culture of monoclonal hairy roots.

PCR detection of the target gene in the *P. japonicus* hairy roots: The PCR results (Fig.5) indicated that the *rolB* was about 550 bp, the SS and SEwere about 1350 bp and 1632 bp, which were consistent with the expected results. However,only the *rolB* band was found in the *P. japonicus*

hair roots without SS and SE genes. It can be concluded that the SS and SE genes have been integrated into the hairy roots of transgenic *P. japonicus*, and achieved our purpose.



M: DL2000 Marker ; 1: SS+SE ; 2: SE ; 3: SS ; 4: *rolB*

Figure 5. Detection of the target gene in transgenic *P. japonicus* hairy roots

Re content in transgenic *P· japonicus* hairy roots: Test results (Fig.6) indicated that the Re content in transgenic *P· japonicus* hairy roots was 2.4 times and 2.3 times of that in non-transgenic ones (CK) when SS and SE geneswere transferred separately. The Re content in transgenic *P· japonicus* hairy roots was 5.3 times that of the non-transgenic ones when SS and SE gene co-over-expression. So, it could be concluded that SS and SE geneswere expressed separately in *P· japonicus* hairy roots has made for promoting Re synthesis. Then SS and SE genes over-expression had positive regulatory function, which was advantageous to the large accumulation of Re content in *P· japonicus* hairy roots.It was a great scientific significance to increase Re content in *P· japonicus* for SS and SE expression alone and co-over-expression. The findings further deepened the understanding of triterpenoid saponins (TS) biosynthesis and regulation in *P· japonicus*, and provided valuable experimental evidence for*P· japonicus* TS biosynthesis.

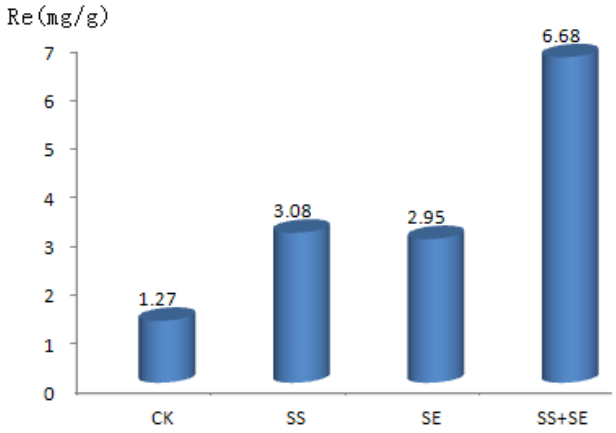


Figure 6. Re content in transgenic *P· japonicus* hairy roots

DISCUSSION

The expression of SS and SE genes in hairy roots of *P. japonicus* was studied. The callus of *P. japonicus* was induced and the expression vectors of SS and SE were constructed; then the Callus of *P. japonicus* with SS and SEgenes over-expression were cultured and infected with C58C1 activated medium;finally, the content of Re in hairy roots of transgenic *P. japonicus* was detected.The biosynthesis of TS requires the catalysis of key enzymes such as SS and SE, so either single or co-over-expression can promote the synthesis of Re.It can be seen that the SS and SE genes had a positive regulatory effect on Re synthesis in *P· japonicus* hairy roots, the effect of co-over-expression of the two genes was stronger than that of single expression.

***Conclusions**:* In order to study the co-over-expression effect of SS and SE genes for Re synthesis in *P. japonicus* hairy roots, we carried out the following work. Firstly,the *P· japonicus* callus was established and used as the recipient material and secondly the pcxsn-ss and pcxsn-se expression vectors were constructed, respectively and transformed by *agrobacterium*LBA4404. Finally, the transgenic*P· japonicus* callus was induced to come into hairy roots by C58C1. PCR showed that both SS and SE genes could be integrated into the hairy roots of *P· japonicus*individually or jointly. RP-HPLC indicated that Re contentin hairy roots of transgenic *P· japonicus* transfected with SS and SE genes was 2.4 and 2.3 times higher than that of non-transgenic ones (ck), respectively.The Re content in transgenic *P· japonicus* hairy roots was 5.3 times that of the non-transgenic ones when SS and SEgenes co-over-expression. So it could be concluded that SS and SE gene expressed alone in *P· japonicus* hairy roots has made for promoting and stimulating Re synthesis, the Re content of co-transformation in *P· japonicus* hairy roots was obviously higher than that of single transformation, therefore the co-over-expression of SS and SE genes in *P· japonicus* hairy roots has positive control function for TS synthesis.According to the study results, both SS and SEgenes play a positive regulatory role for Re biosynthesis in *P· japonicus*and the co-over-expression of SS and SEgenes can further increase the Re content. It can be seen that there may be a positive synergistic regulatory role of two or even multiple genes in Re biosynthesis. This experimental phenomenon provides a research idea for improving Re biosynthesis and positive regulation by metabolic engineering.

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