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Construction and transformation of expression vector containing *Panax japonicus SS* gene

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ABSTRACT. *Panax japonicus* C.A. Meyer, a perennial herb belonging to the Araliaceae ginseng genus, is one of the seven rare and endangered Chinese medical herbs. By cloning the *SS* segment, the expression vectors pCXSN-*PjSS* and pCXSN-anti*PjSS* were constructed and introduced into *Agrobactria* LBA4404, which is used for engineering bacteria. Polymerase chain reaction (PCR) showed that the *PjSS*, anti*PjSS*, and *Hyg* were integrated in *Nicotiana tabacum*. Reverse transcription-PCR indicated that the *PjSS* was transcribed into mRNA in *N. tabacum* and was highly expressed, while the anti*PjSS* was not expressed. Detection of Ginsenoside Re content showed that transgenetic *N. tabacum* can increase the content of Ginsenoside Re and that anti-transgenetic *N. tabacum* decreased the content of Ginsenoside Re.

Key words: *Panax japonicus*; *SS*; Expression vector; Transformation; *Nicotiana tabacum*

INTRODUCTION

Panax japonicus C.A. Meyer, a perennial herb belonging to Araliaceae, is one of seven rare and endangered Chinese medical herbs and is known as the King of the Herbs. It has medicinal effects such as promotion of blood circulation to limit blood stasis by *Panax notoginseng* and nourishing tonic by *Panax ginseng* (Committee of Pharmacopeia, 2008). Ginsenoside, the main pharmaceutical ingredient of *P. japonicus*, has many pharmacological effects, such as anti-inflammation, analgesia, anti-fatigue, anti-diabetic, anti-aging, immune regulating, and anti-cancer (Qian et al., 2008; Zhang et al., 2011; Liu et al., 2013). Studies on *P. japonicus* have mainly focused on resource distribution, biological characteristics, tissue culture, active ingredients, pharmacognostic identification, and clinical application (Zhang et al., 2010; Luo et al., 2011; He et al., 2012; Zhang, 2012; Zhang and Sun, 2014). The saponin substances in *P. japonicus* are mostly triterpenoid saponins (TS), and more than 20 components have been reported with important pharmacological activities. However, TS has not been produced on the industrial level in *P. japonicus*. The squalene synthase (SS) expression vector for the biosynthesis of TS was developed by genetic engineering technology; its transformation was examined, providing a foundation for molecular regulation.

MATERIAL AND METHODS

Construction of PjSS expression vector

Cloned SS positive single colonies were selected and placed in 10 mL LB nutrient medium with the appropriate antibiotics overnight. Next, the plasmid DNA was extracted using the alkaline cleavage method and got sense and antisense cloned vectors:pCXSN-*PjSS* and pCXSN-anti*PjSS*. The plasmids were transformed into *Agrobactria* LBA4404.

Genetic transformation

A soft leaf of a *Nicotiana tabacum* aseptic seedling was removed and cut into 1-cm^2 blades and inoculated into MS containing hygromycin. The antimicrobial nutrient medium used was MS+300 mg/L Cef. *N. tabacum* transformation was observed by leaf transformation by tissue culture and cultivation.

Molecular detection

N. tabacum DNA was extracted with cetyltrimethylammonium bromide (CATB) method; non-transformed *N. tabacum* was used as a negative control. Polymerase chain reaction (PCR) amplification was conducted using *Pjss* primers (Full-F-*SS* and Full-R-*SS*). The plant expression vector *Hyg* of pCXSN was used for PCR amplification to detect *Hyg* resistance. After extracting RNA from trans-genetic SS and non-transgenetic *N. tabacum* roots, stems, and leaves, the RNA was reverse-transcribed into cDNA. For PCR amplification, cDNA was used as a template and 18S as a reference gene, the ant*iPjSS* primers (f-SS and r-SS) and *PjSS* primers (Full-F-SS and Full-R-SS) were used to detect the transcription level. The primer sequences were as follows: Full-F-*SS*: 5'-TAGAGAGAAAATGGGAAGTTTGGGG-3', Full-R-*SS*: 5'-GAACTGGGGTTCTCACTGTTTGTTC-3', f*SS*: 5'-GGCCTCGCCAGATTTGGAG

TAAA-3', r*SS*: 5'-GCAATCAGGGCTGAATTGTGTCC-3', FHyg: 5'-CGATTTGTGTACGCC C GACAGTC-3', RHyg: 5'-CGATGTAGGAGGGCGTGGATATG-3'.

Ginsenoside Re detection

Ginsenoside Re was extracted and detected from trans-genetic SS *N. tabacum* as described by He et al. (2012).

RESULTS

Construction of *PjSS* and anti*PjSS* expression vector

pCXSN is a binary expression vector and can be used as both a cloning vector and plant expression vector. *SS* was cloned to extract the full-length and core segment plasmid and to obtain the *PjSS* and anti*PjSS* plant expression vectors (Figures 1 and 2).

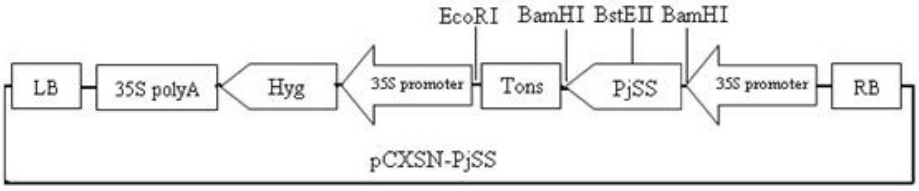

Figure 1. pCXSN-*PjSS* plant expression vector.

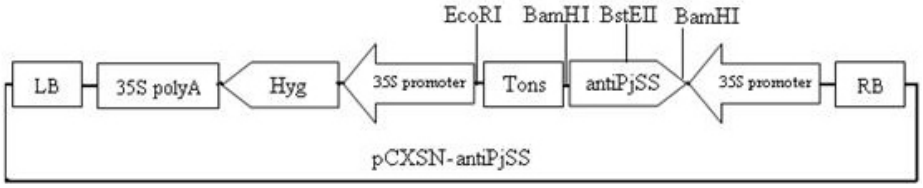

Figure 2. pCXSN-anti*PjSS* plant expression vector.

For the *PjSS* and anti*PjSS* plant expression vector, a 1350-base pair (bp) *PjSS* segment and 500-bp anti*PjSS* core segment were obtained by *Bam*HI digestion (Figure 3), demonstrating that the *PjSS* and anti*PjSS* gene segments were incorporated into the expression vector pCXSN.

The pCXSN-*Pjss* and pCXSN-anti*Pjss* vectors were transformed into *Agrobactria* LBA4404, and the segments were detected in the colonies using corresponding primers. PCR showed that *Pjss*, anti*PjSS*, and *Hyg* were of 1350, 500, and 800 bp (Figure 4), demonstrating that pCXSN-*PjSS* and pCXSN-anti*PjSS* were transformed into *Agrobactria* LBA4404. LBA4404-pCXSN-*PjSS* and LBA4404-pCXSN-anti*Pjss* were used for transformation.

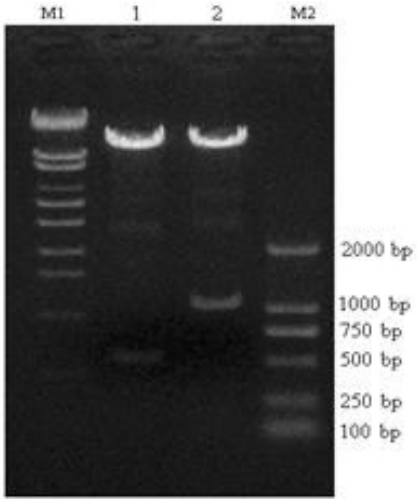

Figure 3. Electrophoresis of plasmid gene digested with *Bam*HI.

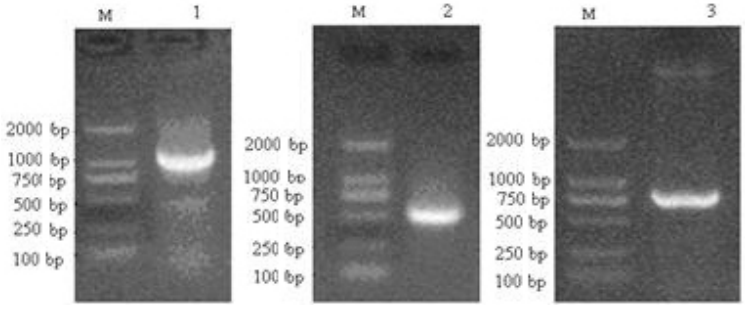

Figure 4. Electrophoresis of *PjSS*, anti*PjSS*, and *Hyg* transformed into LBA4404.

Determination of lethal hygromycin (Hyg) concentration

The *N. tabacum* cells showed greater cell death as Hyg concentration increased. All inoculated *N. tabacum* cells died when the Hyg concentration reached 50 mg/L; therefore, a concentration of 40 mg/L was selected. The lethal rates were 0, 10, 30, 50, 75, and 100% at concentrations from 0-60 mg/L (Figure 5).

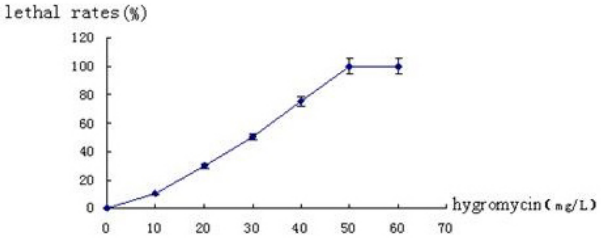

Figure 5. Concentration of hygromycin in selection medium.

Engineering bacterium transformed *N. tabacum* and its growth

N. tabacum was transformed with LBA4404-pCXSN-*PjSS* and LBA4404-pCXSN-anti*Pjss* using the leaf dish method; controls were also constructed. The *N. tabacum* plates were cultured in the dark for 2 days and then placed in MS_B for differentiation. A callus was formed after 8-10 days; it formed buds, which were placed in MS_C to induce root formation. The edges of the leaves in the control group turned yellow after 7 days, gradually died, and completely died after 14 days (Figure 6).

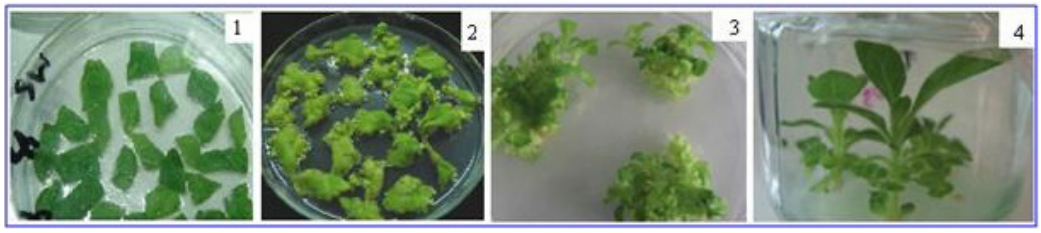

Figure 6. Engineering and regeneration of bacteria transformed in *Nicotiana tabacum*.

Molecular detection for transgenetic *N. tabacum*

The results of target gene detection (Figure 7) showed that *PjSS* was 1300 bp long and anti*PjSS* was 500 bp long, but non-transgenetic *N. tabacum* showed no amplified band, 7indicating that *PjSS* and anti*PjSS* were integrated into *N. tabacum*.

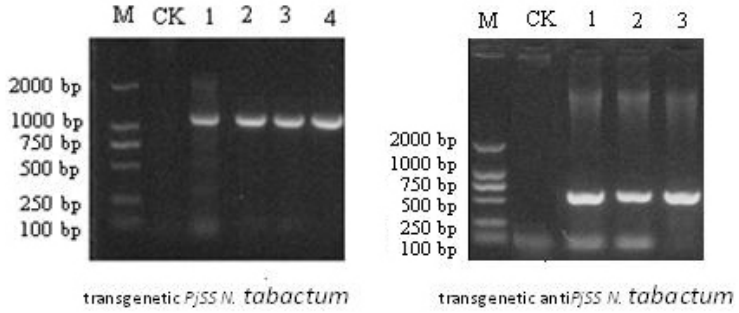

Figure 7. PCR detection results of *PjSS* and anti*PjSS* in *Nicotiana tabacum*.

DNA from positive transgenetic *N. tabacum* and non-transgenetic *N. tabacum* was extracted using CTAB. The PCR result showed that transgenetic and anti-transgenetic *N. tabacum* were not contaminated with *Agrobactria* by using kanamycin gene primers. *Hyg* was amplified by PCR, showing transgenetic and anti-transgenetic *N. tabacum* amplification products of the 800-bp *Hyg* in resistant plants. Thus, *Hyg* was integrated into transgenetic *N. tabacum* (Figure 8).

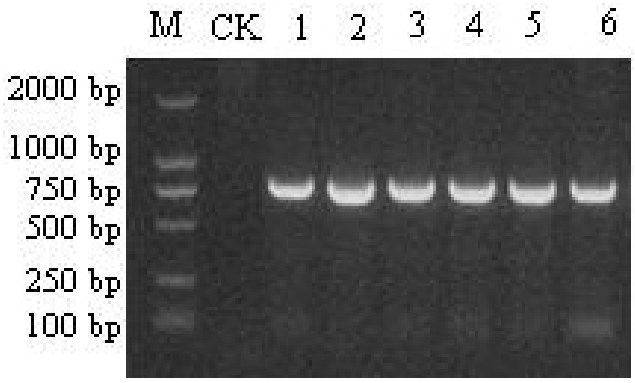

Figure 8. *Hyg* regenerated *Nicotiana tabacum*.

DNA was extracted from the roots, stems, and leaves from transgenetic and anti-transgenetic *N. tabacum* and was reverse-transcribed into cDNA. Using the 18S gene as a reference gene, the *SS* gene specific primers were amplified by RT-PCR. The result showed that a 1350-bp band was amplified from transgenetic *N. tabacum*, demonstrating that *PjSS* was transcribed into mRNA and expressed in *N. tabacum*. Anti-transgenetic *N. tabacum* was not amplified. Because the homology of the cloned antisense segment and *N. tabacum* was over 80%, anti*PjSS* may prevent the expression of the *N. tabacum* SS gene (Figure 9).

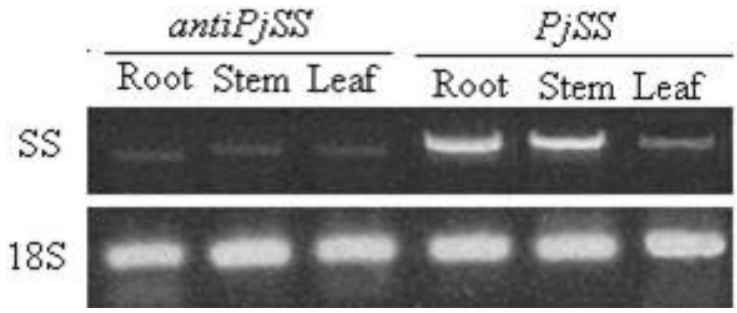

Figure 9. *PjSS* and anti*PjSS* regenerated *Nicotiana tabacum*.

Content of ginsenoside Re in transgenetic *N. tabacum*

Ginsenoside Re was detected in the root, stem, and leaf of *Pj*SS in *N. tabactum*. The highest content was 56.48 mg/g in the root, followed by 41.57 mg/g in the stem, and 36.54 mg/g in the leaf. These values were higher than those in non-transformed control plants. For transgenetic anti*PjSS* in *N.tabacum*, ginsenoside Re in the root (27.31 mg/g), stem (24.12 mg/g), and leaf (19.82 mg/g) was lower than in controls. The results showed that *Pj*SS in *N. tabactum* increased the content of ginsenoside Re, while anti*PjSS* in *N. tabacum* decreased the content of ginsenoside Re. The expression vector containing *SS* was transcribed into mRNA in *N. tabacum* and showed high-level transcription and over-expression; however, the anti-sense SS gene expression vector showed opposite results (Figure 10).

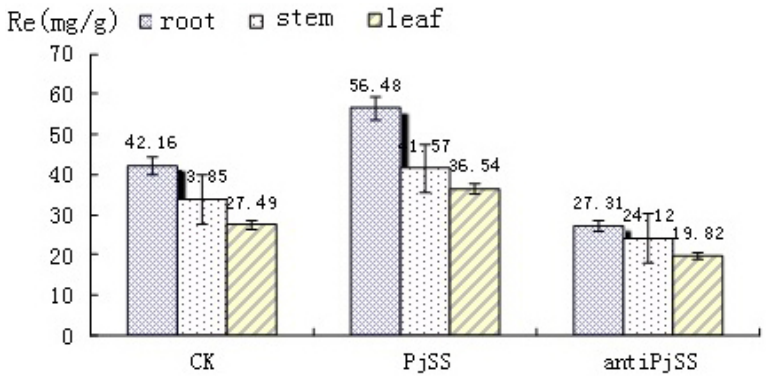

Figure 10. Ginsenoside Re content of transgenetic *Nicotiana tabacum*.

DISCUSSION AND CONCLUSION

By full-length and core conserved segment cloning of the SS gene, the plant expression vectors pCXSN-*PjSS* and pCXSN-anti*PjSS* were constructed and transformed into *Agrobactria* LBA4404. PCR showed that there was a 1350-bp amplified band in the agarose gel electrophoresis of *PjSS*, while anti*PjSS* showed a band at 500 bp, indicating that *PjSS* and anti*PjSS* were integrated into *N. tabacum*. The transgenetic and anti-transgenetic *N. tabacum* amplified the 800-bp Hyg gene in resistant plants. Thus, *Hyg* can be integrated into transgenetic *N. tabacum* as well.

Using the 18S gene as a reference, the *SS*-specific primers were used for RT-PCR. The transgenetic *N. tabacum* showed a band at 1350 bp, indicating that *PjSS* was transcribed and expressed in mRNA in *N. tabacum*. Anti-transgenetic *N. tabacum* did not show this band. The homology between the cloned antisense segment and *N. tabacum* was over 80%, suggesting that anti*PjSS* prevents the expression of the *N. tabacum* SS gene.

Ginsenoside Re was detected in the root, stem, and leaf of *Pj*SS *N. tabactum*. The highest content was 56.48 mg/g in the root, followed by 41.57 mg/g in the stem and 36.54 mg/g in the leaf; these values were higher than those in non-transformed controls. For transgenetic anti*PjSS* in *N. tabacum*, the levels of ginsenoside Re in the root (27.31 mg/g), stem (24.12 mg/g), and leaf (19.82 mg/g) were lower than in controls.

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