In vitro regeneration and Agrobacterium tumefaciens-mediated genetic transformation in asakura-sanshoo (Zanthoxylum piperitum (L.) DC. F. inerme Makino) an important medicinal plant

Xiaofang Zeng¹,², Degang Zhao¹,²
¹Key Laboratory of Plant Resources Conservation and Germplasm Innovation in Mountainous Region, Ministry of Education, Institute of Agro-Bioengineering, ²The State Key Lab of Green Pesticide and Agricultural Biological Engineering, Center for Research and Development of Fine Chemicals, Guizhou University, Guiyang 550025, Guizhou Province, P. R. China

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Context: Asakura-sanshoo (Zanthoxylum piperitum [L.] DC. f. inerme Makino) is an important medicinal plant in East Asia. Transgenic technique could be applied to improve plant traits and analyze gene function. However, there is no report on regeneration and genetic transformation in Asakura-sanshoo.

Aims: To establish a regeneration and Agrobacterium tumefaciens-mediated genetic transformation system in Asakura-sanshoo, which could be used for cultivar improvement and gene function analysis.

Settings and Design: The various combinations of indole-3-butyric acid (IBA), 6-benzylaminopurine (BA) and naphthalene acetic acid (NAA) were explored for the optimal plant regeneration from petiole and stem of Asakura-sanshoo. The half-strength woody plant medium (WPM) with different concentrations of NAA and IBA was used to induce root. For genetic transformation, A. tumefaciens strain EHA-105 harboring the plasmid pBin-Ex-H-ipt which carries the isopentenyl transferase (ipt) gene, β-glucuronidase (GUS) gene and kanamycin resistance gene neomycin phosphotransferase II (NPTII) were used. The transformation efficiency was detected by the kanamycin resistant frequency.

Materials and Methods: Petioles and stems were obtained from the in vitro cultured Asakura-sanshoo. The petiole and stem segments were precultured for 3 days, and then inflected using the bacterium at the concentration of OD₆₀₀ 0.5–0.8 for 10 min, followed by 3 days co-cultivation. Selection of the transgenic plants was carried out after 7 days the regeneration using gradient kanamycin at 30 mg/L and 50 mg/L, respectively. Successful transformed plants were confirmed by GUS histochemical assays, polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), and Southern blotting analysis. Results: The highest shoots regeneration was obtained on WPM supplement with 0.5 mg/L BA and 0.2 mg/L NAA. The optimal rooting medium was half strength macro-element WPM. The kanamycin resistant frequency of petiole and stem was 24.66% and 25.93%, respectively. Thirty-five shoots in thousands adventitious buds were confirmed through GUS histochemical assays, PCR, RT-PCR, and Southern blotting. The regeneration shoot per explants elevated 5.85 fold compared with the wild-type plants. Conclusions: Individual transgenic Asakura-sanshoo lines were obtained. In this paper, it first revealed the expression of ipt gene significantly promoted the adventitious buds induction in Asakura-sanshoo as the same action as in other plants.

Key words: Agrobacterium tumefaciens, asakura-sanshoo, isopentenyl transferase gene, regeneration, transformation

INTRODUCTION

Zanthoxylum piperitum DC., an aromatic plant of Rutaceae family, has been used as an important food condiment for centuries to impart fresh flavor and a traditional medicinal plant to treat against cold, vomiting, toothache, diarrhea, and hypotension in China, Japan and Korea.[1-4] For the existence of essential oils and alkaloids, Z. piperitum has a wide spectrum of biological activities showed strong resistance to fungi and insects.[5-8] The extractives from fruits and leaves have been used in the field of human cosmetic and medicine for their antioxidant and anticancer activities.[9,10] The young sprout which possessing the same
antioxidant and anticancer activities has also been used as a traditional vegetable and a supplement to proper drugs. Due to the complex chemical structure, some of these phytochemicals are fairly difficult to artificial synthesize. To maintain these valuable characters, one of the main breeding goals of this plant is to cultivate and select highly productive and essential oil content individuals. However, as a woody plant (WP), the long generation time of Z. piperitum was one of the main obstacles to traditional breeding. Genetic engineering has been utilized to integrate valuable genes into the genome of WPs as well as overcome the limitations of traditional breeding. It can also be a powerful tool for analyzing the function of genes that related in the secondary metabolites biosynthesis pathway in medicinal plants. In order to obtain successful transgenic plants via Agrobacterium tumefaciens, a major problem is to establish an efficient organ regeneration and transformation system in plants. However, compared with the biological activities and the pharmacological effects of chemical constituents, there is no report on A. tumefaciens-mediated genetic transformation and regeneration by petiole and stem in Z. piperitum except one earlier report on the in vitro propagation from stems by Hwang (1995).

To establish a regeneration and Agrobacterium tumefaciens-mediated genetic transformation system in Asakura-sanshoo, which could be used for cultivar improvement and gene function analysis. Therefore, as a first breakthrough of genetic engineering in Asakura-sanshoo (Z. piperitum [L.] DC. f. inerme Makino), a thornless variant of Z. piperitum, here we report the in vitro plant regeneration from petioles and stems in this plant. Subsequently, the isopentenyl transferase (ipt) gene and the selection marker gene β-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) all under the control of Cauliflower Mosaic Virus 35S (CaMV 35S) promoter, and the flippase (FLP) site-specific recombinase protein-coding gene FLP under the control of Arabidopsis thaliana heat shock inducible promoter heat shock protein 18.2. All the constructs were flanked by two locus of crossover (x) in P1/FLP recognition target fusion sites. A single clone of A. tumefaciens strain (EHA-105) containing pBin-Ex-Ipt plasmid was grown in 5 ml liquid yeast extract peptone (YEP) medium containing 50 mg/L kanamycin and 20 mg/L rifampicin at 28°C overnight on a shaker at 200 rpm. Then, taken out 2 ml bacterial suspension added to 50 ml same YEP medium and cultured at 28°C with continuous shaking (200 rpm). The bacterial cells were collected by centrifugation at 6,000 rpm for 5 min and then re-suspended in liquid WPM containing 100 μM acetosyringone (AS) to maintain the desired OD 600 (0.5–0.8). Petiole and stem segments were precultured on WPM containing 0.5 mg/L BA and 0.2 mg/L IBA in dark at 28°C for 3 d; subsequently, they were soaked in the bacterial re-suspension for 10 min. The explants were dried on sterile paper and incubated on WPM containing 0.5 mg/L BA, 0.2 mg/L IBA and 100 μM AS at 28°C in the dark for 3 d. And then explants were transferred into the resting medium (WPM supplemented with 0.5 mg/L BA, 0.2 mg/L IBA, and 100 mg/L Timentin) for recovering.

 Selection and regeneration After 7 d of resting cultivation, explants were transferred into the selection medium I (WPM supplemented with 0.5 mg/L BA, 0.2 mg/L IBA, 100 mg/L Timentin and 30 mg/L Kanamycin) for 20 d, then transferred into the selection medium II (WPM supplemented with 0.5 mg/L BA, 0.2 mg/L IBA, 100 mg/L Timentin, and 50 mg/L Kanamycin). For adventitious bud induction, the selection medium II was replaced with fresh medium.
every 3 weeks. The surviving shoots which elongated to 2–3 cm in length were transferred into the rooting medium (half-strength WPM containing 50 mg/L kanamycin and 100 mg/L timentin) for root induction. Well-rooted plantlets were transferred into pots, and then grown in the greenhouse.

**Histochemical assay for glucuronidase**

Histochemical assay of GUS was performed according to Jefferson *et al.*, (1987). Stem cross sections, shoots, and leaves were incubated at 37°C in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid overnight. Stained tissues were washed with 75% ethanol to remove the chlorophyll.

**Polymerase chain reaction, reverse transcription-polymerase chain reaction, and Southern blot analysis**

Total genomic DNA was extracted from fresh leaves of putatively transgenic and wild-type Asakura-sanshoo plants according to the protocol of DNA secure Plant Kit (Tiangen Corp. China). Polymerase chain reaction (PCR) amplification was performed under the conditions: 94°C for 5 min; (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) for 35 cycles; and then 72°C for 10 min, using the *ipt* gene-specific primers (Forward primer: 5’-TGCTTAACTCTGGCCTTGGC-3’; Reverse primer: 5’-ATCGGGTCCAATGCTGTCCTC-3’). A 325 bp fragment of the *ipt* gene would be amplified. The PCR products were examined by electrophoresis on 1% (w/v) agarose gel under ultraviolet (UV) light.

The total RNA was isolated by using the RNA-prep pure Plant Kit (Tiangen Corp, China). 5 μg RNA was used as a template for reverse transcription (RT) according to the Primer Script® RT-PCR Kit protocol (Takara Corp, China). *Actin* gene was used as an internal reference. RT-PCR reactions were carried out as genomic DNA PCR reactions. The PCR products were examined by electrophoresis on 1% agarose gel under UV light.

Southern blot analysis was according to the protocol of Southern (2006). 20 μg genomic DNA from leaves of the positive plants by PCR detection was digested with *Eco*RI and *Kpn*I restriction enzymes (Takara Corp, China). The digested DNA was separated by 1.0% agarose gel electrophoresis and transferred to a Hybond N+ membrane. The filter was hybridized at 38°C with a 325 bp digoxigenin-labeled probe obtained by PCR amplification of the *ipt* gene from pBin-Ex-Hipt plasmid according to the instruction of Digoxigenin High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Sciences, Mannheim, Germany).

**RESULTS**

**Establishment of in vitro asakura-sanshoo regeneration protocol**

Axillary buds sprouted after 3–5 d cultured on WPM supplemented with 1.0 mg/L BA and 0.2 mg/L IBA, and within 4 weeks, 2–3 cm sterile axillary shoots were obtained [Figure 2a]. Petiole and stem explants were excised from these axillary shoots grown *in-vitro*. Callus induction and differentiation were detected under different concentrations of plant growth regulators (PGRs) [Table 1]. Green callus were produced at the cutting section of the explants after 10–15 d cultured on the WPM containing BA, NAA, or IBA [Figure 2b-d]. Among all the treatments, 1.0 mg/L BA in combination with 0.1 mg/L IBA achieved maximum callus induction rate (petiole 98.18% and stem 98.81%). However, most of this callus ceased growing, and turned to brown and finally died. Only 13.64% of petiole and 29.76% of stem callus could be initiated the regeneration shoots. WPM containing 0.5 mg/L BA and 0.2 mg/L NAA was the optimal medium for shoot regeneration from petiole and stem callus, the measurements were 60.00% and 60.95%, respectively [Figure 2e-h]. After cultured on the same medium for 30 d, most of the shoots elongated to 2–3 cm [Figure 2i].

Roots were successfully induced after the regeneration shoots cultivated on rooting medium 12–13 d. In the present study, the half-strength macro-element WPM without any PGRs was optimal for Asakura-sanshoo rooting [Table 2]. Within 40 d of being cultured in this medium, the rooting rate of regeneration shoots could reach 94.4% [Table 2] [Figure 2j]. In this study, the existence of exogenous auxin suppressed the root initiation. The addition of NAA and IBA facilitated callus formation and then inducted the stubby roots from this callus [Figure 2k and l]. Well-developed root plantlets were planted into pots, and then grown in the greenhouse [Figure 2m].

**Regeneration and confirmation of transgenic plants**

Petioles and stems were used as explants for genetic transformation. Explants preculture, *Agrobacterium* infection, co-culture, kanamycin-resistance selection
and regeneration were carried out according to “subjects and methods”. After 60 d on selection medium II, kanamycin resistant shoots were produced from petiole and stem explants at frequencies of 24.66% and 25.93%, respectively [Table 3], [Figure 3a]. More than 1000 kanamycin‑resistant regenerated shoots were obtained in our study [Figure 3b]. However, typical cytokinin‑overproducing response such as restraining apical dominance, increasing branching, emerging epiphyllous shoots, and reducing root formation was observed in most of these resistant shoots [Figure 3c‑e]. All analyzed 35 cytokinin‑syndrome shoots were tested GUS positive [Figure 3g‑i]. However, the root initiation was extremely restrained in these shoots, it was fairly difficult to induce root from these shoots, only few shoots could form adventitious roots and grow to plantlets [Figure 3f]. The well rooted, kanamycin resistance, and GUS positive plantlets were transplanted into pots [Figure 3j].

To verify the transgenic plant, genomic DNA was isolated from the GUS positive plantlets. PCR analysis was carried out using specific primers for ipt gene sequence. An expected 325 bp fragment was amplified in all tested plants as well as in the plasmid and was not detected in wild‑type plant [Figure 4a]. The results of RT‑PCR analysis shown the expression of the ipt gene in six transgenic lines and plasmid, but not detected in wild‑type plant [Figure 4b]. Southern blot analysis results present that only one copy of ipt gene was present in the genome of transgenic line 1 and 2, and more than one copy was detected in the genome of transgenic line 3 and 4. No hybridization signal was detected in wild‑type plant [Figure 4c]. This result indicated that Asakura‑sanshoo wild‑type does not have a sequence homologous to the ipt gene.

Expression of isopentenyl transferase gene promoted the shoots induction in asakura‑sanshoo

In this research, we also found that ipt gene expressing effectively promoted the adventitious shoots induction of in vitro cultured callus in Asakura‑sanshoo [Table 4]. The regeneration frequency of transgenic shoots increased 21.7% compared with the wild‑type. The number of regeneration shoot per transgenic explants was 25.57 in average, which elevated 5.85 fold compared with the wild‑type plants. However, transformation of ipt gene into Asakura‑sanshoo resulted in typical cytokinin‑overproducing morphological changes in transgenic plants grown in the greenhouse. Stem elongation and apical dominance were severely restrained in the transgenic Asakura‑sanshoo lines [Figure 3j].

DISCUSSION

Plant growth regulators play a key role in vitro regeneration of plant. The combination of BA and NAA has been
In our study, the combination of BA and NAA was also found to be suitable for shoot differentiation in Asakura-sanshoo. The optimal PGRs combination and concentration for shoots regeneration were 0.5 mg/L BA with 0.2 mg/L NAA. BA in combination with IBA resulted in the higher axillary bud count, but lower frequency shoots regeneration. In our previous study, there was a 5 years juvenile phase in plantlets which regenerated from callus. Thus, for in vitro propagation, the optimal PGRs combination is 1.0 mg/L BA in combination with 0.1 mg/L or 0.2 mg/L IBA.

Genetic engineering holds potential applications to improve the trait and can overcome the long breeding time for WPs. But its usage is often limited in some WPs because of the low regeneration and transformation frequencies. Several earlier reports demonstrated that the \textit{ipt} gene can promote shoot regeneration and enhance transformation efficiency in tobacco. Our results indicated that the \textit{ipt} gene expression could significantly improve the callus induction and shoot regeneration in Asakura-sanshoo. It confirmed the observation in several of our laboratory reports and others. However, the expression of \textit{ipt} gene controlled by CaMV 35S promoter leads to extreme overproduction of cytokinins in transgenic plants and then causes a range of morphological changes. The typical detrimental phenotypes caused by cytokinin-overproducing were also founded in transgenic Asakura-sanshoo shoots widely used for shoots regeneration from callus in various species of plants. In our study, the combination of BA and NAA was also found to be suitable for shoot differentiation in Asakura-sanshoo. The optimal PGRs combination and concentration for shoots regeneration were 0.5 mg/L BA with 0.2 mg/L NAA. BA in combination with IBA resulted in the higher axillary bud count, but lower frequency shoots regeneration. In our previous study, there was a 5 years juvenile phase in plantlets which regenerated from callus. Thus, for in vitro propagation, the optimal PGRs combination is 1.0 mg/L BA in combination with 0.1 mg/L or 0.2 mg/L IBA.

Table 1: Effects of PGRs on callus and shoot induction from petioles and stems

| PGRs (mg/L) | Number of explants cultured | Percentage of callus induction from different explants (%) | Percentage of shoot induction from different explants (%) |
|------------|-----------------------------|----------------------------------------------------------|-----------------------------------------------------------|
| BA: 0.5    | 128 Petiole 65 Stem         | 93.75          Stem 93.85                                   | 27.34 Stem 29.23                                         |
|            |                             |               |                                                          |
|            |                             | 97.75 Stem 97.67                                     | 16.85 Stem 23.26                                         |
|            |                             | 91.75 Stem 92.59                                     | 21.65 Stem 33.33                                         |
| 1.0       | 0.1 NAA 110 Stem           | 98.18 Stem 98.81                                     | 13.64 Stem 29.76                                         |
| 1.0       | 0.2 NAA 135 Stem           | 88.89 Stem 97.78                                     | 14.81 Stem 25.93                                         |
| 1.0       | 0.5 NAA 128 Stem           | 93.75 Stem 95.71                                     | 13.28 Stem 21.42                                         |
| 2.0       | 0.1 NAA 116 Stem           | 87.93 Stem 92.64                                     | 10.34 Stem 47.06                                         |
| 2.0       | 0.2 NAA 100 Stem           | 82.00 Stem 97.14                                     | 18.00 Stem 42.86                                         |
| 2.0       | 0.5 NAA 110 Stem           | 80.00 Stem 96.92                                     | 19.09 Stem 38.46                                         |
| 0.5       | 0.1 BA 103 Stem           | 93.20 Stem 95.92                                     | 29.13 Stem 40.82                                         |
| 0.5       | 0.2 BA 105 Stem           | 95.23 Stem 98.01                                     | 60.00 Stem 60.95                                         |
| 0.5       | 0.5 BA 118 Stem           | 92.37 Stem 94.03                                     | 47.46 Stem 43.28                                         |
| 1.0       | 0.1 IB 120 Stem           | 90.83 Stem 97.81                                     | 29.17 Stem 26.28                                         |
| 1.0       | 0.2 IB 154 Stem           | 88.31 Stem 96.19                                     | 25.97 Stem 31.43                                         |
| 1.0       | 0.5 IB 118 Stem           | 81.36 Stem 96.67                                     | 27.50 Stem 35.00                                         |
| 2.0       | 0.1 IB 112 Stem           | 87.5 Stem 95.38                                     | 41.96 Stem 52.31                                         |
| 2.0       | 0.2 IB 142 Stem           | 80.28 Stem 96.67                                     | 40.85 Stem 53.33                                         |
| 2        | 0.5 IB 128 Stem           | 92.19 Stem 98.28                                     | 44.53 Stem 58.62                                         |

BA: 6-benzylaminopurine; NAA: Naphthalene acetic acid; IBA: Indole-3-butyric acid; PGRs: Plant growth regulators

Table 2: The effect of different auxin concentration on roots formation of Asakura-sanshoo

| Medium               | Number of shoots cultured | Number of roots/shoot explants (mean±SE)* | Rooting rate (%) |
|----------------------|---------------------------|--------------------------------------------|-----------------|
| 1/2 WPM              | 36                        | 3.54±0.11a                                 | 94.4            |
| 1/2 WPM+0.3 mg/L NAA | 40                        | 3.67±7.16a                                 | 30.0            |
| 1/2 WPM+1.0 mg/L IBA | 32                        | 3.83±0.13a                                 | 25.0            |
| 1/2 WPM+1.0 mg/L IBA | 32                        | 4.40±0.08a                                 | 50.0            |

*Mean (±) followed the same letter(s) in each column were not significantly different at P<0.05 using Tukey’s test. WPM: Woody plant medium; SE: Standard error; NAA: Naphthalene acetic acid; IBA: Indole-3-butyric acid, a,b: P<0.05

Table 3: Transformation efficiency of petiole and stem

| Explant | Number of explants | Number of explants resistant to kanamycin | Kanamycin resistant frequency (%) |
|---------|--------------------|------------------------------------------|----------------------------------|
| Petiole | 156                | 37                                       | 23.72                            |
|         | 205                | 51                                       | 24.88                            |
|         | 134                | 34                                       | 25.37                            |
| Total   | 495                | 122                                      | 24.66±0.85*                      |
| Stem    | 114                | 28                                       | 24.56                            |
|         | 60                 | 16                                       | 26.67                            |
|         | 113                | 30                                       | 26.55                            |
| Total   | 287                | 74                                       | 25.93±1.19*                      |

*Indicated the mean value

Genetic engineering holds potential applications to improve the trait and can overcome the long breeding time for WPs. But its usage is often limited in some WPs because of the low regeneration and transformation frequencies. Several earlier reports demonstrated that the \textit{ipt} gene can promote shoot regeneration and enhance transformation efficiency in tobacco. Our results indicated that the \textit{ipt} gene expression could significantly improve the callus induction and shoot regeneration in Asakura-sanshoo. It confirmed the observation in several of our laboratory reports and others. However, the expression of \textit{ipt} gene controlled by CaMV 35S promoter leads to extreme overproduction of cytokinins in transgenic plants and then causes a range of morphological changes. The typical detrimental phenotypes caused by cytokinin-overproducing were also founded in transgenic Asakura-sanshoo shoots widely used for shoots regeneration from callus in various species of plants.
cultured in vitro. Root initiation and shoot elongation were significantly restrained. Most of the GUS positive shoots could not be initiated root in the rooting medium. To solve this problem, one approach is fusing the ipt gene under the control of a tissue-specific or inducible promoter.\[26,31-33\] Another is to remove or restrain the ipt gene expression in transgenic plants.\[34,35\] Therefore, in the future studies, we can control the expression ofipt gene using tissue-specific or inducible promoter to eliminate the undesirable abnormalities in transgenic plants. Moreover, for some regenerated difficult WPs, we can use the ipt gene to promote shoot induction and then take advantage of the genetically modified “gene-deletor” system to excise the ipt gene after the initiation of shoots.\[28,35,36\]

CONCLUSIONS

In this paper, we first reported an A. tumefaciens-mediated transformation and regeneration system in Asakura-sanshoo. The ipt transgenic Asakura-sanshoo plants were successfully obtained for the first time. It first revealed the expression of ipt gene significantly promoted the adventitious buds induction in Asakura-sanshoo as the same action as ipt in other plants. Our protocol will be applied to analyze gene function and improve trait in Asakura-sanshoo in future.

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