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

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Submitted: 10-04-2014 Revised: 27-05-2014 Published: 12-03-2015

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.153092

Quick Response Code:

ABSTRACT

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 OD_{600} 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

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antioxidant and anticancer activities has also been used as a traditional vegetable and a supplement to proper drugs.[11,12] Due to the complex chemical structure, some of these phytochemicals are fairly difficult to artificial synthesize.[13] 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.[2] 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.[14] It can also be a powerful tool for analyzing the function of genes that related in the secondary metabolites biosynthesis pathway in medicinal plants.[15] 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.[16-20] 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).[2]

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 were successfully introduced into Asakura-sanshoo via *A. tumefaciens* for the first time. The efficient *A. tumefaciens*-mediated transformation system will be used for gene functional analysis and trait improvement in Asakura-sanshoo through genetic engineering in future.

SUBJECTS AND METHODS

Plant materials

The stem segments of Asakura-sanshoo excised from growing plants were sterilized with 75% alcohol for 45 s, washed with sterile water. The surface-sterilized stem segments were dipped in 0.1% (w/v) $HgCl_2$ containing tween 80® for 7 min, and washed with sterile water for five times, and dried on sterile paper. The stem segments were transferred on the WP medium (WPM)[21] supplemented with 1.0 mg/L 6-benzylaminopurine (BA) and 0.2 mg/L indole-3-butyric acid (IBA). Segments were grown on medium for axillary shoots inducing for 4 weeks at 25°C under 16 h light/8 h dark cycles. and then stem and petiole segments excised from these aseptic shoots were transplanted and incubated on WPM with different combinations of IBA, naphthalene acetic acid (NAA) and BA for callus and shoots induction. All the mediums were supplemented with 0.7% (w/v) agar and 3% (w/v) sucrose, and adjusted the pH to 5.8. The explants were subcultured into the same composition fresh medium every 20 d. The 2–3 cm shoots separated from the adventitious bud were transferred into the rooting medium. Well-developed root plantlets were transferred into pots, and then grown in the greenhouse.

Plant genetically transformation

Plasmid, pBin-Ex-Hipt [Figure 1], contained the fusion gene *GUS: NPTII* and *ipt* gene under the control of the CaMV 35S promoter, and the flippase (FLP) site-specific recombinase protein-coding gene *FLP* under the control of an *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-Hipt 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

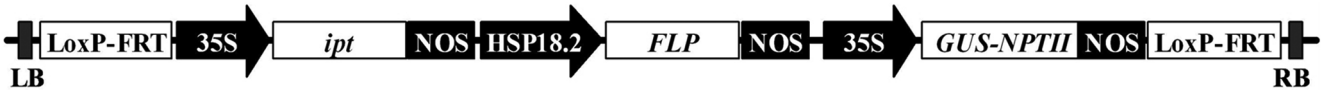

Figure 1: Plant expression vectors pBin-Ex-Hipt

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).[22] 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).[23] 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

Figure 2: Plant regeneration of Asakura-sanshoo. (a) Axillary shoots. (b-d) Callus induction from stems and petioles. (e-i) Regeneration shoots. (j-l) Roots induced from different medium. (m) Rooted plantlets transferred to pots

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

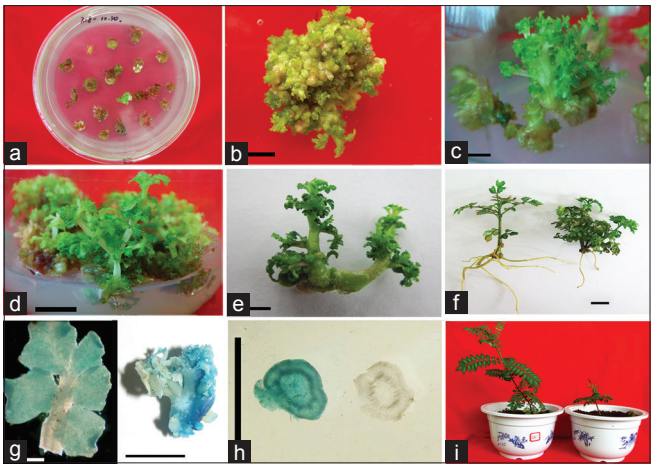

Figure 3: Genetic transformation of Asakura-sanshoo by *Agrobacterium tumefaciens*. (a and b) Kanamycin-resistant callus and shoots. (c-e) Transgenic malformed shoots. (f) Rooted wild-type (left) and transgenic plantlet (right). (g-i) Glucuronidase histochemical expression. (j) Rooted wild-type (left) and transgenic plantlet (right) grown in the soil

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

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	IBA	NAA	Petiole	Stem	Petiole	Stem	Petiole	Stem
0.5	0.1		128	65	93.75	93.85	27.34	29.23
0.5	0.2		89	43	97.75	97.67	16.85	23.26
0.5	0.5		97	54	91.75	92.59	21.65	33.33
1.0	0.1		110	84	98.18	98.81	13.64	29.76
1.0	0.2		135	67	88.89	97.78	14.81	25.93
1.0	0.5		128	70	93.75	95.71	13.28	21.42
2.0	0.1		116	68	87.93	92.64	10.34	47.06
2.0	0.2		100	70	82.00	97.14	18.00	42.86
2.0	0.5		110	65	80.00	96.92	19.09	38.46
0.5		0.1	103	49	93.20	95.92	29.13	40.82
0.5		0.2	105	52	95.23	98.01	60.00	60.95
0.5		0.5	118	67	92.37	94.03	47.46	43.28
1.0		0.1	120	68	90.83	97.81	29.17	26.28
1.0		0.2	154	52	88.31	96.19	25.97	31.43
1.0		0.5	118	60	81.36	96.67	27.50	35.00
2.0		0.1	112	65	87.5	95.38	41.96	52.31
2.0		0.2	142	60	80.28	96.67	40.85	53.33
2		0.5	128	58	92.19	98.28	44.53	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±0.11[a]	94.4
1/2 WPM+0.3 mg/L NAA	40	3.67±7.16[a]	30.0
1/2 WPM+1.0 mg/L IBA	32	3.83±0.13[a]	25.0
1/2 WPM+1.0 mg/L IBA	32	4.40±0.08[b]	50.0

*Mean (±) followed the same letter (s) in each column were not significantly different at $P \leq 0.05$ 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			
1	156	37	23.72
2	205	51	24.88
3	134	34	25.37
Total	495	122	24.66±0.85*
Stem			
1	114	28	24.56
2	60	16	26.67
3	113	30	26.55
Total	287	74	25.93±1.19*

*Indicated the mean value

widely used for shoots regeneration from callus in various species of plants.[24] 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.[14] But its usage is often limited in some WPs because of the low regeneration and transformation frequencies.[25] Several earlier reports demonstrated that the *ipt* gene can promote shoot regeneration and enhance transformation efficiency in tobacco.[26-28] Our results indicated that the *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 *ipt* gene controlled by CaMV 35S promoter leads to extreme overproduction of cytokinins in transgenic plants and then causes a range of morphological changes.[27,29,30] The typical detrimental phenotypes caused by cytokinin-overproducing were also founded in transgenic Asakura-sanshoo shoots

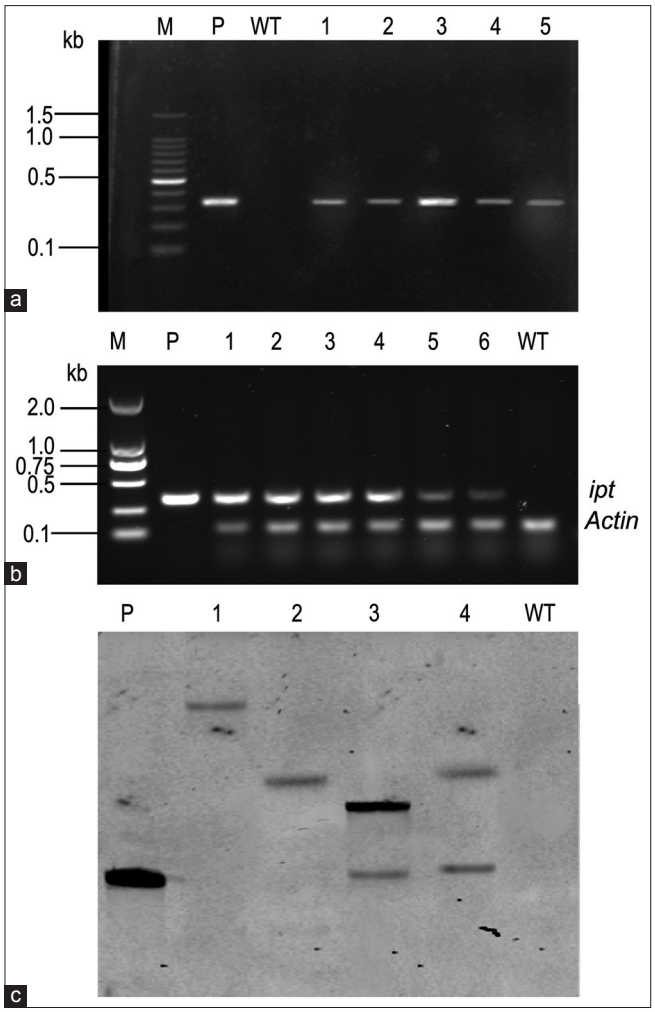

Figure 4: Polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR) and Southern blotting analysis of transgenic Asakura-sanshoo plants. (a) PCR analysis. (b) RT-PCR analysis. (c) Southern blotting analysis. M, Marker; WT, wild-type; P, plasmid; lane 1-5, 1–6 and 1–4, Transgenic lines

Table 4: Effects of *ipt* gene on shoot regeneration from petiole

Explants cultured	Number of cultured explants	Percentage of regenerated shoots induction (%)	Number of regenerated shoots per explants (mean±SE)
WT	60	60.0	4.37±1.23
Transgenic	60	81.7	25.57±3.12**

**Indicated values significantly different from control callus ($P \leq 0.01$). SE: Standard error; WT: Wild-type

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 of *ipt* 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.

ACKNOWLEDGMENT

This work was supported by the National Transgenic Major Project of China (2014ZX08010-003) and the Province Science Project in Guizhou of China (Z[2012] 4008). Authors are also grateful to Yi Li (Plant Science Department, University of Connecticut) for the gift of the plant expression vector pBin-Ex-Hipt.

REFERENCES

1. Epple G, Bryant BP, Mezine I, Lewis S. *Zanthoxylum piperitum*, an Asian spice, inhibits food intake in rats. J Chem Ecol 2001;27:1627-40.
2. Hwang SJ. An efficient *in vitro* propagation of *Zanthoxylum piperitum*. Acta Hortic 2005;676:89-94.
3. Sun YL, Park WG, Kwon OW, Hong SK. The internal transcribed spacer rDNA specific markers for identification of *Zanthoxylum piperitum*. Afr J Biotechnol 2010;37:6027-39.
4. Yanase E, Ohno M, Harakawa H, Nakatsuka S. Isolation of N, N-dimethyl and N-methylserotonin 5-O-ß-glucosides from immature *Zanthoxylum piperitum* seeds. Biosci Biotechnol Biochem 2010;74:1951-2.
5. Hieu TT, Kim SI, Ahn YJ. Toxicity of *Zanthoxylum piperitum* and *Zanthoxylum armatum* oil constituents and related compounds to *Stomoxys calcitrans* (Diptera: Muscidae). J Med Entomol 2012;49:1084-91.
6. Kamsuk K, Choochote W, Chaithong U, Jitpakdi A, Tippawangkosol P, Riyong D, *et al*. Effectiveness of *Zanthoxylum piperitum*-derived essential oil as an alternative repellent under laboratory and field applications. Parasitol Res 2007;100:339-45.
7. Kim S, Yeon RJ, Hyoung KD, Seung LH, Joon AY. Insecticidal activities of aromatic plant extracts and essential oils against *Sitophilus oryzae* and *Callosobruchus chinensis*. J Stored Prod Res 2003;39:293-303.
8. Negi JS, Bisht VK, Bhandari AK, Singh P, Sundriyal RC. Chemical constituents and biological activities of the genus *Zanthoxylum*. Afr J Pure Appl Chem 2011;12:412-6.

9. Lee KT, Kim BJ, Kim JH, Heo MY, Kim HP. Biological screening of 100 plant extracts for cosmetic use (I): Inhibitory activities of tyrosinase and DOPA auto-oxidation. Int J Cosmet Sci 1997;19:291-8.
10. Lee SJ, Lim KT. Glycoprotein of *Zanthoxylum piperitum* DC has a hepatoprotective effect via anti-oxidative character *in vivo* and *in vitro*. Toxicol In Vitro 2008;22:376-85.
11. Chon SU, Heo BG, Park YS, Kim DK, Gorinstein S. Total phenolics level, antioxidant activities and cytotoxicity of young sprouts of some traditional Korean salad plants. Plant Foods Hum Nutr 2009;64:25-31.
12. Heo BG, Chon SU, Park YJ, Bae JH, Park SM, Park YS, *et al.* Antiproliferative activity of Korean wild vegetables on different human tumor cell lines. Plant Foods Hum Nutr 2009;64:257-63.
13. Rout GR, Samantaray S, Das P. *In vitro* manipulation and propagation of medicinal plants. Biotechnol Adv 2000;18:91-120.
14. Osakabe Y, Kajita S, Osakabe K. Genetic engineering of woody plants: Current and future targets in a stressful environment. Physiol Plant 2011;142:105-17.
15. Abou-Alaiwi WA, Potlakayala SD, Goldman SL, Josekutty PC, Karelia DN, Rudrabhatla SV. *Agrobacterium*-mediated transformation of the medicinal plant *Centaurea montana*. Plant Cell Tissue Organ 2011;109:1-8.
16. Mahender A, Mallesham B, Srinivas K, Kumar GK, Rao KV, Rajesh Y, *et al*. A rapid and efficient method for *in vitro* shoot organogenesis and production of transgenic *Bacopa monnieri* L. mediated by *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol Plant 2012;48:153-9.
17. Pushyami B, Beena MR, Sinha MK, Kirti PB. *In vitro* regeneration and optimization of conditions for *Agrobacterium* mediated transformation in jute, *Corchorus capsularis*. J Plant Biochem Biotechnol 2011;20:39-46.
18. Thangjam R, Sahoo L. *In vitro* regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation of *Parkia timoriana* (DC.) Merr.: A multipurpose tree legume. Acta Physiol Plant 2012;34:1207-15.
19. Tsai YT, Chen PY, To KY. Plant regeneration and stable transformation in the floricultural plant *Cleome spinosa*, a C_3 plant closely related to the C_4 plant C. gynandra. Plant Cell Rep 2012;31:1189-98.
20. Wang H, Wang C, Liu H, Tang R, Zhang H. An efficient *Agrobacterium*-mediated transformation and regeneration system for leaf explants of two elite aspen hybrid clones *Populus alba×P. berolinensis* and *Populus davidiana×P. bolleana*. Plant Cell Rep 2011;30:2037-44.
21. Lloyd G, McCown B. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combust Proc Int Plant Prop Soc 1980;30:21-427.
22. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 1987;6:3901-7.
23. Southern E. Southern blotting. Nat Protoc 2006;1:518-25.
24. Dhar U, Joshi M. Efficient plant regeneration protocol through callus for *Saussurea obvallata* (DC.) Edgew. (Asteraceae): Effect of explant type, age and plant growth regulators. Plant Cell Rep 2005;24:195-200.
25. Matsunaga E, Nanto K, Oishi M, Ebinuma H, Morishita Y, Sakurai N, *et al. Agrobacterium*-mediated transformation of *Eucalyptus globulus* using explants with shoot apex with introduction of bacterial choline oxidase gene to enhance salt tolerance. Plant Cell Rep 2012;31:225-35.
26. Sa G, Mi M, He-chun Y, Ben-ye L, Guo-feng L, Kang C. Effects of *ipt* gene expression on the physiological and chemical characteristics of *Artemisia annua* L. Plant Sci 2001;160:691-98.
27. Li Y, Hagen G, Guilfoyle TJ. Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Dev Biol 1992;153:386-95.
28. Lu LT, Zhu YY, Liu Y, Zhao DG. Ethanol inducible isopentenyl transferase as a high efficiency marker for tobacco transformation. Afr J Biotechnol 2010;9:8139-45.
29. Smigocki A, Neal JW Jr, McCanna I, Douglass L. Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene. Plant Mol Biol 1993;23:325-35.
30. Hewelt A, Prinsen E, Schell J, Van Onckelen H, Schmülling T. Promoter tagging with a promoterless *ipt* gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: Implications of gene dosage effects. Plant J 1994;6:879-91.
31. Gan S, Amasino RM. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 1995;270:1986-8.
32. Khodakovskaya M, Li Y, Li J, Vanková R, Malbeck J, McAvoy R. Effects of *cor15a-IPT* gene expression on leaf senescence in transgenic *Petunia* × *hybrida* and *Dendranthema* × *grandiflorum*. J Exp Bot 2005;56:1165-75.
33. Khodakovskaya M, Vanková R, Malbeck J, Li A, Li Y, McAvoy R. Enhancement of flowering and branching phenotype in chrysanthemum by expression of *ipt* under the control of a 0.821 kb fragment of the *LEACO1* gene promoter. Plant Cell Rep 2009;28:1351-62.
34. Khan RS, Ntui VO, Chin DP, Nakamura I, Mii M. Production of marker-free disease-resistant potato using isopentenyl transferase gene as a positive selection marker. Plant Cell Rep 2011;30:587-97.
35. Luo K, Sun M, Deng W, Xu S. Excision of selectable marker gene from transgenic tobacco using the GM-gene-deletor system regulated by a heat-inducible promoter. Biotechnol Lett 2008;30:1295-302.
36. Luo K, Duan H, Zhao D, Zheng X, Deng W, Chen Y, *et al.* 'GM-gene-deletor': Fused loxP-FRT recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants. Plant Biotechnol J 2007;5:263-74.

Cite this article as: Zeng X, Zhao D. *In vitro* regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation in asakura-sanshoo (*Zanthoxylum piperitum* (L.) DC. F. *inerme* Makino) an important medicinal plant. Phcog Mag 2015;11:374-80.

Source of Support: The National Transgenic Major Project of China [2014ZX08010-003] and the Province Science Project in Guizhou of China [Z (2012) 4008], **Conflict of Interest:** None declared.