BIOLOGIA PLANTARUM 53 (4): 637-642, 2009

Production of transgenic *Podophyllum peltatum* *via Agrobacterium tumefaciens*-mediated transformation

V.R. ANBAZHAGAN[1], Y.-S. KIM[2] and Y.-E. CHOI[1]*

Division of Forest Resources, College of Forest Sciences, Kangwon National University, Chunchon 200-701, Kangwon-do, South Korea[1]
Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Nara 630-0192, Japan[2]

Abstract

Transgenic *Podophyllum peltatum* plants were successfully produced by *Agrobacterium tumefaciens*-mediated transformation. Embryogenic callus was co-cultivated with *Agrobacterium tumefaciens* harboring a binary vector pBI 121 carrying β-glucuronidase (*GUS*) and neomycinphosphotransferase (*NPT II*) gene. GUS-histochemical analysis revealed that, 50 μM acetosyringone treatments during *Agrobacterium* infection and 3 d co-cultivation with *Agrobacterium* showed enhanced transformation efficiency. Percentage of GUS positive callus increased rapidly as the subculture time proceeded on selection medium containing 100 mg dm^{-3} kanamycin. Kanamycin resistant somatic embryos were formed from embryogenic callus after cultivation with 11.35 μM abscisic acid (ABA) for 3 weeks and then on hormone-free selection medium. Somatic embryos were germinated and converted into plantlets on medium containing 2.89 μM gibberellic acid (GA_3). The integration of *GUS* and *NPT II* gene into transgenic plants was confirmed by polymerase chain reaction and Southern analysis.

Additional key words: abscisic acid, embryogenic callus, gibberellic acid, *GUS* gene, kanamycin resistance, *NPT II* gene.

Introduction

Podophyllum peltatum (common name mayapple) belongs to the family *Berberidaceae*. It is perennial and grows in patches as branched rhizome system in Southern Canada and Eastern USA. The rhizome contains podophyllotoxin (PTOX), and the semi-synthetic derivatives of this compound are used as anti-cancer drugs (Damayanthi and Lown 1998). Both *P. peltatum* and *P. hexandrum* have been extensively used. *Podophyllum* species has long growth period and produce single solitary flower, which develops into single berry. Under natural conditions these plants flower only after attaining the age of 5 to 6 years (Nadeem *et al.* 2000). Due to long life span and low reproductive value, conventional breeding of these plant species is very difficult and time consuming. Consequently, genetic transformation techniques could be valuable tools to improve the genetic traits.

Genetic transformation of *P. peltatum* is an attractive way to improve its medicinal values by controlling the PTOX biosynthesis. To produce transgenic plants, regeneration protocol is an important step. In our previous studies, we established plant regeneration system *via* somatic embryogenesis (Kim *et al.* 2007). However, there is no report on the production of transgenic plant *via Agrobacterium tumefaciens*-mediated transformation in *Podophyllum* species. Only transgenic callus formation *via A. rhizogenesis* transformation was reported in *P. hexandrum* (Giri *et al.* 2001). The present study was aimed to develop *Agrobacterium*-mediated transformation of *P. peltatum*.

Received 1 October 2007, *accepted* 16 May 2008.
Abbreviations: ABA - abscisic acid; CaMV - cauliflower mosaic virus; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; GUS - β-glucuronidase; MS - Murashige and Skoog; NOS - nopaline synthase; NPT II - neomycinphosphotransferase II; PCR - polymerase chain reaction; PTOX - podophyllotoxin.
Acknowledgements: This work was supported by 2007 Research Grant from Kangwon National University, and from the WCU project of the Ministry of Education, Science & Technology (MEST), Republic of Korea.
* Corresponding author; fax: (+82) 33 2528310, e-mail: yechoi@kangwon.ac.kr

Materials and methods

The protocol for embryogenic callus induction from the culture of zygotic embryos and plant regeneration *via* somatic embryogenesis of *P. peltatum* developed by Kim *et al.* (2007) was followed for our transformation studies. To investigate the effective selection of transformed callus, embryogenic callus was cultured on Murashige and Skoog (1962; MS) basal medium containing various concentrations of kanamycin (0, 25, 50, 100, and 200 mg dm^{-3}). The resistant threshold of non-transformed embryogenic callus was evaluated based on tissue browning and lethality.

The binary vectors pBI 121 carrying *NPT II* regulated by NOS promoter, *GUS* gene regulated by CaMV 35S promoter and both the genes terminated by NOS terminator was introduced into *Agrobacterium* strain GV 3101. *Agrobacterium* was grown at 28 °C in liquid LB medium (10 g dm^{-3} bacto-tryptone, 5 g dm^{-3} bacto-yeast extract, 10 g dm^{-3} NaCl) containing 100 mg dm^{-3} kanamycin with shaking at 120 rpm over night till the culture reaches absorbance 1.0 (Chilton *et al.* 1974). *Agrobacterium* culture was centrifuged at 2 400 *g* for 15 min, and the bacterial pellets were suspended in ½ MS liquid medium with 3 % sucrose. Later the embryogenic callus was infected with *Agrobacterium* suspension for 20 min. In the similar manner, in order to study the effect of acetosyringone on transformation, different concentrations of acetosyringone (0, 25, 50 and 75 μM) were treated during *Agrobacterium* infection. After infection of embryogenic calluses with *Agrobacterium,* they were blotted using the sterilized filter paper (*Whatman No. 1*) for 30 min. The blotted embryogenic callus was cultivated for 2, 3, and 5 d on ½ MS solid medium containing sucrose (3 %) and *Gelrite* (0.25 %), thereafter was transferred onto selection medium (MS basal medium with 3 % sucrose, 0.25 % *Gelrite,* 100 mg dm^{-3} kanamycin, and 300 mg dm^{-3} cefotaxime).

To induce somatic embryos, embryogenic callus was precultured on selection medium containing 11.35 μM ABA for 3 weeks and transferred to same medium lacking ABA. Embryogenic calluses were sub-cultured 4 times at 2 weeks intervals until somatic embryos were induced. Kanamycin resistant somatic embryos were transferred to medium with 2.89 μM gibberellic acid (GA_3) to stimulate germination. The germinated plantlets were maintained on the same medium for 4-weeks to attain well-developed shoot and roots and finally acclimatized in greenhouse.

Histochemical assay of *GUS* activity was carried by X-Gluc reaction following the procedure of Xiao *et al.* (2005). The embryogenic callus, somatic embryos and germinated plantlets were submerged in a substrate solution containing 100 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3 % (m/v) *X-Gluc* and 0.1 % (v/v) *Triton X-100* and incubated overnight at 37 °C. Expression of *GUS* gene was visualized after the tissues were destained by soaking and washing in 70 % ethanol for several times.

Genomic DNA was isolated from transformed and non-transformed embryogenic callus, somatic embryos and germinated plants following the CTAB procedure (Lichtenstein and Draper 1985). Polymerase chain reaction (PCR) was carried out on the samples by amplifying the coding regions of transgenes using the following sets of *NPT II* oligonucleotide primers: 5'ATC GGG AGC GGC GAT ACC GTA3' (forward) and 5'GAG GCT ATT CGG CTA TGA CTG 3' (reverse) and *GUS* primers 5'-CTG TAG AAA CCC CAA CCC GTG-3' (forward) and 5'-CAT TAC GCT GCG ATG GAT CCC-3' (reverse). The expected PCR products of *NPT II* and *GUS* gene were 750 bp and 514 bp, respectively. The total volume of the reaction was 0.025 cm^3, including 500 ng genomic DNA, 20 pmol of each forward and reverse primers, 0.0125 cm^3 of PCR master mix and 0.0065 cm^3 of nuclease-free water. Cycling parameters began with an initial hot start at 94 °C, 5 min, then 35 cycles of denaturation (94 °C, 1.0 min), annealing (55 °C, 1.0 min), extension (72 °C, 1.0 min), followed by final extension of 7 min at 72 °C. PCR amplified products were analyzed by electrophoresis in 1 % agarose/ethidium bromide gels.

To confirm the insertion and copy number of transgene, genomic DNAs from untransformed control plantlets and GUS positive plantlets of *P. peltatum* were isolated with a DNeasy Plant mini kit (*Qiagene*, Hilden, Germany) according to the procedure specified by the manufacturer. The genomic DNA was digested with *Eco*RI (*TaKaRa*, Kyoto, Japan) that were only one restriction site reside in the plasmid DNA. The restriction fragments were then separated by electrophoresis on a 0.8 % agarose gel and blotted onto *Hybond-N*$^+$ nylon membrane (*Amersham*, Buckinghamshire, UK). The 514-bp GUS gene fragment as probe was amplified by PCR and labeled with non-radioactive *AlkPhos* direct system (*Amersham*). Hybridization, washing, and detection were performed according to the instruction manuals provided by manufacturer. Hybridization signals were detected using *Hyper film-ECL* (*Amersham*).

The data were statistically analyzed using Duncan's multiple range test (DMRT) with the significance being determined at the $P \leq 0.05$.

Results and discussions

To effectively select transformed embryogenic callus, resistance of embryogenic callus to different concentrations of kanamycin was tested preliminary. Among the different concentrations (0, 25, 50, 100, and 200 mg dm^{-3}), 100 mg dm^{-3} kanamycin showed clear browning and lethality of embryogenic callus after 8 weeks of culture.

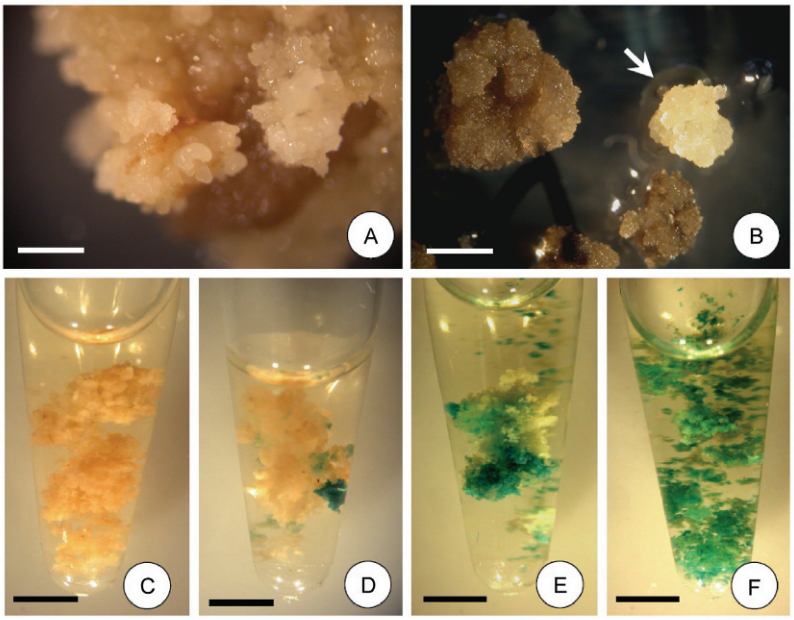

Fig. 1. Induction of embryogenic callus and GUS expression during different subculture periods on selection medium. *A* - Embryogenic callus induction from cotyledonary somatic embryos on MS medium fortified with 6.78 μM 2,4-D. *B* - Kanamycin resistant embryogenic callus formation (*arrow*) on selection medium with 300 mg dm^{-3} cefotaxime + 100 mg dm^{-3} kanamycin. *C* - *X-Gluc* reaction of non-transformed embryogenic callus. *D* - *X-Gluc* reaction of *Agrobacterium* co-cultivated embryogenic callus after first 3 weeks of culture on selection medium. *E* - GUS expression of embryogenic callus after first subculture on selection medium. *F* - GUS expression of embryogenic callus after second subculture on selection medium (*bar* = 1.0 cm in all figures).

To suppress the bacterial growth, 300 mg dm^{-3} cefotaxime was appropriate and used for suppression of bacterial growth. In this study, 100 mg dm^{-3} kanamycin and 300 mg dm^{-3} cefotaxime were adopted for selection of transgenic callus and suppression of *Agrobacterium* growth, respectively. In monocotyledonous plants like rice and wheat, kanamycin is found to be less effective than hygromycin for the selection of transgenic plants (Ayres and Park 1994; Hiei *et al.* 1997). In contrast, kanamycin has been widely used as effective selection agent for the production of transformed plants in dicotyledonous plants (Schroeder *et al.* 1993, Saini *et al.* 2005).

Embryogenic callus (Fig. 1*A*) of *P. peltatum* was co-cultivated with *Agrobacterium* at different periods (2, 3, and 5 d) and thereafter transferred to the selection medium (MS solid medium containing 100 mg dm^{-3} kanamycin and 300 mg dm^{-3} cefotaxime) containing various levels of acetosyringone. Kanamycin resistant callus with bright yellow colour (Fig. 1*B*) was obtained among the browned callus. After 3 weeks of culture X-gluc reaction revealed that 3-d co-cultivation showed maximum efficiency of transformation (Table 1). The non-co-cultivated embryogenic callus did not stain by X-Gluc (Fig. 1*C*). A co-cultivation period longer than 3 d led to a reduction in transformation frequency due to the bacterial overgrowth. Similar results were reported in rice (Li *et al.* 1992), *Citroncirus webberi* (Cervera *et al.* 1998), and blackgram (Saini and Jaiwal 2007). At the same time, 50 μM acetosyringone treatments during 3-d co-cultivation with *Agrobacterium* resulted in the further increase in the number of *GUS*-positive embryogenic calli (Table 1, Fig 2). Stachel *et al.* (1985) reported that, expression of virulence gene in *Agrobacterium tumefaciens* was activated specifically by the aceto-syringone and α-hydroxyacetosyringone. Van Wordragen and Dons (1992) reported that the addition of aceto-syringone is very effective for transformation of recalcitrant crops. However, Godwin *et al.* (1991) stated that acetosyringone was not effective for transformation in some plant species.

In *P. peltatum*, the frequency of transgenic callus was increased rapidly as the subculture time on selection medium was proceeded (Figs. 1, 2), indicating effective selection of transgenic callus by kanamycin. It was reported that subsequent subculture of transformed tissues

Table 1. Effect of acetosyringone (AS) and co-cultivation period on efficiency of transformation of embryogenic callus of *P. peltatum* after 3 weeks of culture on selection medium. Twenty five embryogenic calluses were cultured per Petri dish. Means ± SE of three independent replicates. The data were statistically analyzed using DMRT with the significance being determined at the $P \leq 0.05$ and indicated by different letters.

AS [μM]	Number of GUS positive calli 2 d	3 d	5 d	Efficiency [%]
0	2.3 ± 0.33^{bc}	3.3 ± 0.33^{c}	1.3 ± 0.33^{b}	13.2^{c}
25	2.6 ± 0.67^{b}	7.6 ± 0.67^{ab}	2.3 ± 0.33^{ab}	30.4^{ab}
50	3.7 ± 0.67^{a}	8.7 ± 0.33^{a}	3.3 ± 0.33^{a}	34.4^{a}
75	1.3 ± 0.33^{d}	2.3 ± 0.33^{cd}	0	9.2^{cd}

on selection medium is important to eliminate non-transformed tissues (May *et al.* 1995, Howe *et al.* 2006).

In our previous report, ABA pretreatment of embryogenic callus in *P. peltatum* for three weeks was effective for induction of somatic embryos from embryogenic callus (Kim *et al.* 2007). Thus, to induce somatic embryos, kanamycin resistant embryogenic callus was pre-cultured for 3 weeks on medium with 11.35 µM ABA, and followed by subculturing to same medium lacking ABA. High frequency of somatic embryo formation (Fig. 3*B*) was obtained from kanamycin resistant embryogenic callus after 4 subculture at 2-week intervals (Fig 3*A*). Acetosyringone treatments enhanced the production of transgenic somatic embryos. The numbers of transgenic somatic embryos per embryogenic callus (200 mg f.m.) was 6.3, 9.7, 13.5, and 3.8 in 0, 25, 50, and 75 µM acetosyringone, respectively, indicating that 50 µM acetosyringone produced maximum number of transgenic somatic embryos (Tiwari *et al*. 2008).

Along with the transformed embryos, non-transformed embryos were also developed. In order to remove the non-transformed embryos, somatic embryos were manually selected on the surfaces of embryogenic callus and subcultured on the new selection medium for complete selection and maturation of transgenic

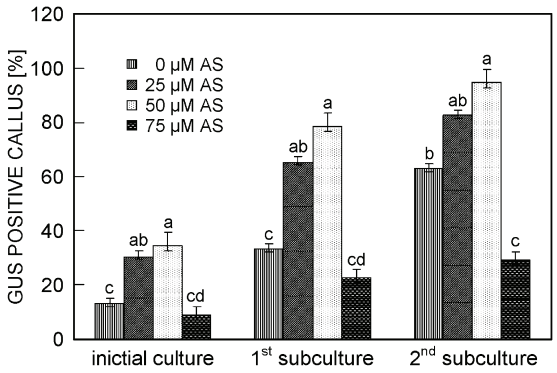

Fig. 2. Effect of acetosyringone concentration on the percentage of GUS-positive embryogenic callus as the subculture proceeded at three-week intervals on selection medium. Means ± SE of three independent replicates. The data were statistically analyzed using DMRT at the $P \leq 0.05$ and results indicated by different letters. AS - acetosyringone.

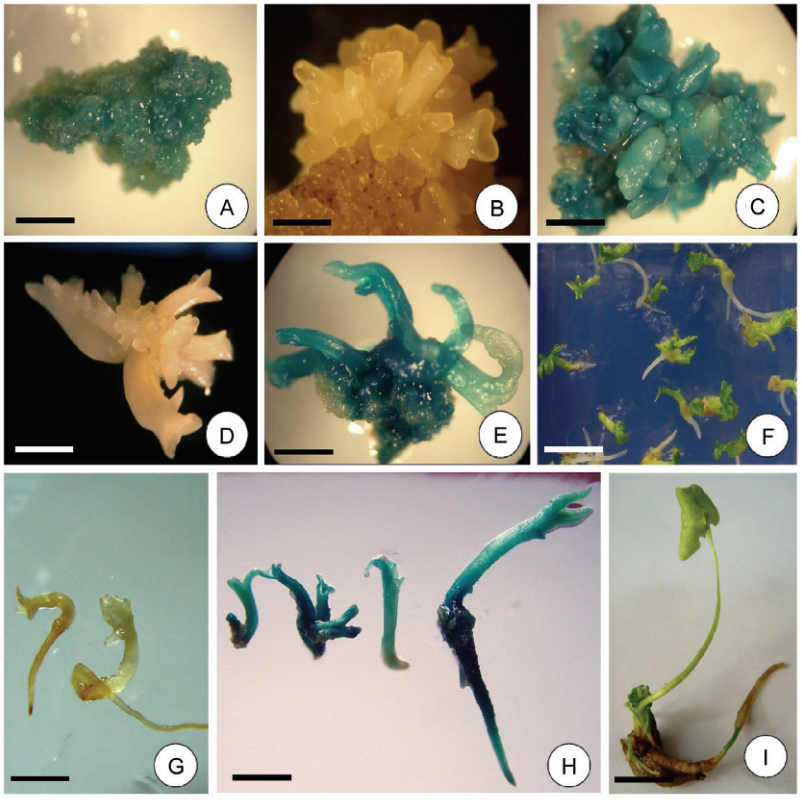

Fig. 3. Production of transgenic plants *via* somatic embryogenesis from embryogenic callus. *A* - Selection of pure GUS-positive embryogenic callus. *B* - Development of kanamycin resistant somatic embryos on selection medium. *C* - GUS expression of transgenic somatic embryos. *D* - Kanamycin resistant cotyledonary somatic embryos. *E* - GUS expression of cotyledonary somatic embryos. *F* - Germination of kanamycin resistant somatic embryos on selection medium with 2.89 µM GA_3. *G* - Non-transformed germinated somatic embryos without GUS staining. *H* - Transgenic somatic embryos and plantlet showing GUS expression. *I* - A transgenic plantlet. *Bars* in *A* - *E* = 0.5 cm, in *F* = 3.0 cm, in *G* - *I* = 1.0 cm.

embryos. After mature to cotyledonary stage, survived somatic embryos were subcultured to germination medium containing 2.89 μM GA_3. The germinated somatic embryos (Fig. 3*F*) were developed into plantlets with shoot and roots (Fig. 3*I*).

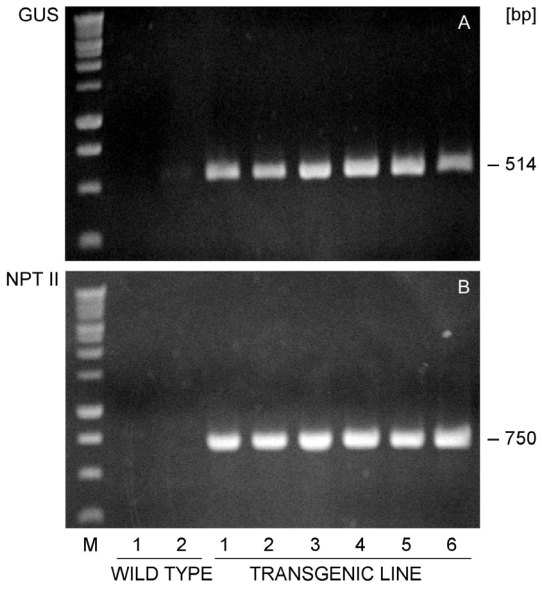

Fig. 4. Confirmation of transgenic plants by PCR analysis. *A* - Integration of *GUS* gene (514 bp of amplified product) in the genome of transgenic *P. peltatum* plants. *B* - Integration of *NPT II* gene (750 bp of amplified product) in the genome of transgenic *P. peltatum* plants. *Lane* M - 1 kb molecular size marker (*Hind*III digest of λ phage DNA), *lanes* 1,2 - wild-type, *lanes* 1 to 6 - transgenic plants.

The appropriate amount of kanamycin for the selection of callus or organs varied depending on the type of tissues, developmental stages and plant species (Han *et al.* 2000, Franklin and Lakshmi Sita 2003, Manickavasagam *et al.* 2004). In the present study, 100 mg dm^{-3} kanamycin was effective for the selection of both transgenic callus and somatic embryos. Although high concentrations of selection agent is effective for the selection of transformed callus and organs, sometimes these concentrations tend to suppress the development of somatic embryos or shoot formation from explants (Yu *et al.* 2003). In *P. peltatum* transformation, somatic embryo-genesis was not severely affected on selection medium containing 100 mg dm^{-3} kanamycin.

The *X-Gluc* reaction revealed that, transformed somatic embryos (Fig. 3*E*) and plantlets (Fig. 3*H*) were all GUS-positive. Whereas, the non-transformed somatic embryos and plantlets (Fig. 3*G*) did not stain.

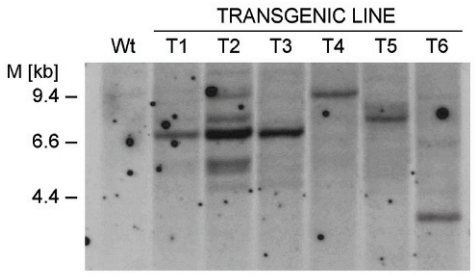

Fig. 5. Southern blot analysis of transgenic plants of *P. peltatum*. Genomic DNA was digested with *Eco*RI and hybridized with the *GUS* probe: Wt - wild type, T1 to T6 - independent transgenic plants. Molecular mass marker is indicated on left.

The GUS-positive transgenic *P. peltatum* plants were analyzed by PCR reactions. The integration of the *GUS* and *NPT* II genes in the genome of *P. peltatum* was confirmed by the presence of an amplified fragment of 514 bp (Fig. 4*A*) and 750 bp, respectively (Fig. 4*B*). Amplification of this fragment was not observed in non-transformed wild plants.

Six transgenic plantlets from independent transformation events were analyzed by Southern hybridization (Fig 5). No hybridization signal was detected in non-transformed control plants. In *GUS*-positive 6 transgenic lines, 5 lines had one copy of the *GUS* gene and the remaining one line had two or more copies of *GUS* gene signals. This confirms the foreign genes integrated into the *P. peltatum* genome.

In conclusions, the present study is the first report on *Agrobacterium tumefaciens*-mediated transformation in *P. peltatum*.

References

Ayres, N.M., Park, W.D.: Genetic transformation of rice. - Crit. Rev. Plant Sci. **13**: 219-239, 1994.

Cervera, M., Pina, J.A., Juarez, J., Navarvo, L., Pena, L.: *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. - Plant Cell Rep. **18**: 271-278, 1998.

Chilton, M.D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P., Nester, E.W.: *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. - Proc. nat. Acad. Sci. USA **71**: 3672-3676, 1974.

Damayanthi, Y., Lown, J.W.: Podophyllotoxins: current status and recent developments. - Curr. Med. Chem. **5**: 205-52, 1998.

Franklin, G., Lakshmi Sita, G.: *Agrobacterium tumefaciens*-mediated transformation of egg plant (*Solanum melongena* L.) using root explants. - Plant Cell Rep. **21**: 549-554, 2003.

Giri, A., Giri, C.C., Dhingra, V., Narasu, M.L.: Enhanced podophyllotoxin production from *Agrobacterium rhizogenes* transformed cultures of *P. hexandrum*. - Nat. Prod. Lett. **15**: 229-235, 2001.

Godwin, I., Todd, G., Ford-Lloyd, B., Newbury, H.J.: The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. - Plant Cell Rep. **9**: 671-675, 1991.

Han, K.H., Meilan, R., Ma, C., Strauss, S.H.: An

Agrobacterium tumefaciens transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*). - Plant Cell Rep. **19**: 315-320, 2000.

Hiei, Y., Komari, T., Kubo, T.: Transformation of rice mediated by *Agrobacterium tumefaciens*. - Plant mol. Biol. **35**: 205-218, 1997.

Howe, A., Sato, S., Dweikat, I., Fromm, M., Clemente, T.: Rapid and reproducible *Agrobacterium*-mediated transformation of *Sorghum*. - Plant Cell Rep. **25**: 784-791, 2006.

Kim, Y.S., Lim, S., Choi, Y.E., Anbazhagan, V.R.: High frequency plant regeneration via somatic embryogenesis in *Podophyllum peltatum* L., an important medicinal plant for source of anticancer drug. - Curr. Sci. **92**: 662-666, 2007.

Li, X.Q., Liu, C.N., Ritchie, S.W., Peng, J., Gelvin, S.B., Hodges, T.K.: Factors influencing *Agrobacterium*-mediated transient expression of *gus*A in rice. - Plant mol. Biol. **20**: 1037-1048, 1992.

Lichtenstein, C., Draper, J.: Genetic engineering of plants. - In: Glover, D.M. (ed.): DNA Cloning. Pp. 67-119. IRL Press, Oxford 1985.

Manickavasagam, M., Ganapathi, A., Anbazhagan, V.R., Sudhakar, B., Selvaraj, N., Vasudevan, A., Kasthurirengan, S.: *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharam* species hybrids) using axillary buds. - Plant Cell Rep. **23**: 134-143, 2004.

May, G.D., Afza, R., Mason, H.A., Wiecko, A., Novak, F.J., Arntzen, C.J.: Generations of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. - Biotechnology **13**: 486-492, 1995.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nadeem, M., Palni, L.M.S., Purohit, A.N., Pandey, H., Nandi, S.K.: Propagation and conservation of *Podophyllum hexandrum* Royle: an important medicinal herb. - Biol. Conserv. **92**: 121-129, 2000.

Saini, R., Jaiwal, P.K.: Efficient transformation of a recalcitrant grain legumes *Vigna mungo* L. Hepper via *Agrobacterium*-mediated gene transfer into shoot apical meristem cultures. - Plant Cell Rep. **24**: 164-171, 2005.

Saini, R., Jaiwal, P.K.: *Agrobacterium tumefaciens*-mediated transformation of blackgram: an assessment of factors influencing the efficiency of *uidA* gene transfer. - Biol. Plant. **51**: 69-74, 2007.

Schroeder, H.E., Schotz, A.H., Wardley-Richardson, T., Spencer, D., Higgins, T.J.V. Transformation and regeneration of two pea (*Pisum sativum*) cultivars. - Plant Physiol. **101**: 751-757, 1993.

Stachel, S.E., Messens, E., Montagu, M.V., Zambryski, P.: Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. - Nature **318**: 624-629, 1985.

Tiwari, R.K., Trivedi, M., Guang, Z.-C., Guo, G.-O., Zheng, G.-C.: *Agrobacterium rhizogenes* mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots. - Biol. Plant. **52**: 26-35, 2008.

Van Wordragen, M.F., Dons, H.J.M.: *Agrobacterium tumefaciens* mediated transformation of recalcitrant crops. - Plant mol. Biol. Rep. **10**: 12-36, 1992.

Xiao, K., Zhang, C., Harrison, M., Wang, Z.Y.: Isolation and characterization of a novel plant promoter that directs strong constitutive expression of transgenes in plants. - Mol. Breed. **15**: 221-231, 2005.

Yu, T.A., Yeh, S.D., Yang, J.S.: Comparison of the effects of kanamycin and geneticin on regeneration of papaya from root tissue. - Plant Cell Tissue Organ Cult. **74**: 169-178, 2003.