BIOLOGIA PLANTARUM 54 (2): 265-271, 2010

Production and selection of marker-free transgenic plants of *Petunia hybrida* using site-specific recombination

R.S. KHAN, I. NAKAMURA and M. MII*

Laboratory of Plant Cell Technology, Graduate School of Horticulture, Chiba University, 648 Matsudo, Chiba 271-8510, Japan

Abstract

MAT (multi-auto-transformation) vector system has been one of the strategies to excise the selection marker gene from transgenic plants. *Agrobacterium tumefaciens* strain EHA105 harboring an *ipt*-type MAT vector, *pNPI*132, was used to produce morphologically normal transgenic *Petunia hybrida* 'Dainty Lady' employing isopentenyl transferase (*ipt*) gene as the selection marker gene. β-glucuronidase (*GUS*) gene was used as model gene of interest. Infected explants were cultured on Murashige and Skoog (MS) medium without plant growth regulators (PGR) and antibiotics. Shoots showing extreme shooty phenotype (ESP) were produced from the adventitious shoots separated from the explants. Visual selection was carried out until production of morphologically normal shoots (approximately 4 months after infection). Histochemical *GUS* assay detected *GUS* gene in both ESP and normal shoots. PCR analysis confirmed the presence of model gene (*GUS* gene) and excision of the selection marker (*ipt*) gene in the normal transgenic plants. The insertion sites (1 - 3 for *ipt* gene and 1 - 2 for *GUS* gene) were detected by Southern blot analysis using DIG-labeled probes of both genes. These results show that *ipt*-type MAT vector can be used successfully to produce marker-free transgenic *Petunia hybrida* plants on PGR- and antibiotic-free MS medium.

Additional key words: extreme shooty phenotype, *GUS* gene, *ipt* gene, MAT vector, PCR, Southern blot, transformation.

Introduction

In plant genetic transformation system, selection marker is used to recover transgenic plants from the rare transformed cells. However, once transformation is accomplished, the presence of the marker gene in transgenic plants is no longer needed and becomes undesirable. Genes conferring resistance to selective chemical agents, such as antibiotics or herbicides, have been widely used as selection markers. The presence of such selection marker genes in GM crops has caused public concerns on the medical implications of using GM food and on the environmental implications of growing GM crops. Strategies to produce marker gene-free transgenic plants can minimize the public concerns and save the time-consuming and expensive safety evaluations.

One of the strategies to avoid using antibiotics for selecting transgenes and produce marker-free transgenic plants is the MAT (multi-auto-transformation) vector system (Ebinuma *et al*. 1997) which uses oncogenes of *Agrobacterium* (*ipt* gene or *rol* gene) for selection of transgenic tissues leading to the production of marker-free transgenic plants. The *ipt* gene encodes the enzyme isopentenyltransferase, which catalyzes the rate-limiting step in cytokinin biosynthesis (Akiyoshi *et al*. 1984). Expression of the *ipt* gene causes abnormal shoot morphology called extreme shooty phenotype (ESP), since the transgenic plants that overproduce cytokinins show reduced stature, release of apical dominance, changes in vascular development and inhibited root growth (Klee *et al*. 1987, Ainley *et al*. 1993). ESP shoots subsequently reverts into normal shoots with the desired transgenes due to the excision of *ipt* gene by the function of "hit and run" cassette system (Ebinuma and Komamine 2001). By using this MAT vector system, marker-free transgenic plants have been produced in tobacco (Ebinuma *et al*. 1997, Sugita *et al*. 2000), hybrid aspens (Matsunaga *et al*. 2002), rice (Endo *et al*. 2002),

Received 7 May 2008, *accepted* 5 January 2009.

Abbreviations: GUS - β-glucuronidase; ipt - isopentenyltransferase; MAT - multi-auto-transformation; PCR - polymerase chain reaction; PGR - plant growth regulator.

Acknowledgement: The authors are grateful to Dr. H. Ebinuma, Pulp and Paper Research, Nippon Paper Industries, Tokyo, for providing the MAT vector constructs.

* Corresponding author; fax: +81-47-308-8852, e-mail: miim@faculty.chiba-u.jp

Antirrhinum majus (Minlong *et al*. 2000), *Nierembergia* (Khan *et al*. 2006) and *Citrus* (Ballester *et al*. 2007).

In this study, we tried to produce marker-free transgenic *Petunia hybrida*, an important model plant for biotechnological and biochemical studies, using *ipt*-type of MAT vector.

Materials and methods

***Agrobacterium* strain and binary vector:** *Escherichia coli* strain DH5-α containing the MAT binary vector plasmid, *pNPI*132, used in this study was constructed by Sugita *et al*. (1999), and kindly provided by Nippon Paper Industries, Japan. The plasmid containing kanamycin resistance gene (*nptII*), *GUS* gene and the removable cassette in the T-DNA region (Fig. 1) was transferred into *A. tumefaciens* strain EHA105 by triparental mating and used for transformation. *A. tumefaciens* was grown overnight at 28 ºC in liquid LB medium (pH 7.2) containing 50 mg dm^{-3} kanamycin and 25 mg dm^{-3} chloramphenicol. The bacterial suspension was centrifuged (3 000 *g*) for 10 min, the supernatant was decanted and the bacterial pellet resuspended in 1:2 volume of MS hormone-free medium containing 100 μM acetosyringone. The inoculum prepared in this way was used for transformation.

Composition of the MAT vector: The T-DNA region of the MAT vector, *pNPI*132, has been designed (Sugita *et al*. 1999) as shown in Fig. 1. The β-glucuronidase (*GUS*) gene, located outside the removable cassette, was used as model gene of interest. The *ipt* gene in the MAT vector causes morphological changes which allow visual selection of *ipt* transgenic plants. Overexpression of the *ipt* gene under the CaMV 35S promoter in tobacco resulted in an approximately 100-fold increase in endogenous cytokinin content and induced expression of the ESP (Smigocki and Owens 1988).

Plants and transformation: *Petunia hybrida* 'Dainty Lady', cultured under *in vitro* condition, were used as the experimental material. *In vitro* plants were multiplied from the apical and nodal cuttings on 70 cm^3 hormone-free ½ MS medium in glass tubes (20 × 100 mm).

For transformation of *Petunia hybrida* by *A. tumefaciens*, leaf explants from *in vitro* grown plants were incubated in the overnight-grown bacterial suspension (inoculum A_{600} = 0.6) for about 10 min, blotted dry with sterilized filter paper to remove excess bacteria, and cultivated on Murashige and Skoog (1962; MS) medium without plant growth regulators (PGR) and supplemented with 30 g dm^{-3} sucrose, 0.25 % gellan gum (*Gelrite*, *Kelco*, *Division of Merck*, San Diego, CA, USA) and 100 μM acetosyringone, for 3 d under the dark condition. Thereafter, the explants were washed in liquid PGR-free MS medium containing 10 mg dm^{-3} meropenem (*Sumitomo Pharmaceuticals*, Osaka, Japan) and transferred to PGR- and selective antibiotic-free MS medium containing 20 mg dm^{-3} meropenem to remove the bacteria. Acetosyringone and meropenem were filter sterilized and added to the autoclaved medium when needed. The explants and the regenerated shoots were subcultured on the same medium every 2 - 3 weeks.

Histochemical *GUS* assay: Leaves from ESP shoots and normal transgenic *Petunia* plants were tested for histochemical *GUS* expression by soaking in X-Gluc solution (Jefferson *et al*. 1987). After overnight (15 - 16 h) staining, chlorophyll was excluded by soaking the tissues for several hours in 70 % ethanol.

PCR analysis: Genomic DNAs were extracted from ESP and normal shoots, and control *Petunia* plants following cetyl trimethyl ammonium bromide (CTAB) method (Rogers and Bendich 1988). PCR was performed using genomic DNA of each plant as a target and the oligonucleotide primers (*Bex Company*, Tokyo, Japan) for *GUS* and *ipt* genes, and also a pair of primers for amplification of excision cassette (3.4 kb) (Fig. 1).

The PCR products expected to be amplified by these primers were 1.2 kb fragment for the *GUS* gene, 0.8 kb for the *ipt* gene, and 3.4 kb for the excision fragment, respectively. DNA amplification was carried out in 0.02 cm^3 of total volume containing 0.001 cm^3 of 10 μg cm^{-3} genomic DNA, 0.001 cm^3 of 20 μM each of the primers, 1.25 mM dNTP (*Pharmacia,* Tokyo, Japan), 0.0025 cm^3 of PCR buffer (800 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 5 g dm^{-3} bovine serum albumin, 1 % sodium cholate and 1 % Triton X-100) and 1 unit of DNA Taq Polymerase (*Takara Shuzo Company*, Kyoto, Japan) per tube. Amplification was carried out in a thermal cycler (*PTC-200 Peltier Thermal Cycler,* USA) with the optimized cycling parameters for each pair of primers used. After PCR, the amplification products (0.005 cm^3) were mixed with loading buffer and run on 0.9 % (m/v) agarose gel (*FMC Bio Products*, Rockland, USA) at 100 V for 30 - 40 min with Tris-EDTA as a running buffer. Then the gel was stained with ethidium bromide solution for 20 min, visualized and imaged using *Nighthawk*™ gel documentation system (*PDI Inc.*, USA). The sequences of the oligonucleotide PCR primers were as follow: GUS1, 5′-GGTGGGAAAGCGCGTTACAAG-3′; GUS2, 5′- TTT ACGCGTTGCTTCCGCCA-3′; IPT1, 5′-CTTGCACAGGAAAGACGTCG-3′; IPT2, 5′-AAT GAAGACAGGTGTGACGC-3′; EXC1, 5′-TTGTCAAG ACCGACCTGTCC-3′; EXC2, 5′-TGCATCGGCGAA CTGATC GT-3′.

Southern blot hybridization: 10 μg of *Hin*dIII-digested genomic DNA samples from *ipt* and normal shoots and non-transformed control *Petunia hybrida* plants were fractionated on a 0.8 % (m/v) agarose gel, blotted to

nylon membrane (*Hybond-N*$^+$, *Amersham Pharmacia Biotech,* Tokyo, Japan) and hybridized with a DIG-labeled probes of the *GUS* and *ipt* genes separately. The probe DNA fragments, corresponding to a part of the *GUS* and *ipt* genes, were labeled by PCR using DIG-dUTP, following the supplier`s instructions (*Boehringer*, Mannheim, Germany). Hybridization, washing and detection were performed using *DIG Easy Hyb* (hybridization solution) and *DIG Wash* and *Block* buffer set following the supplier's instructions (*Boehringer-Mannheim*). Hybridization with the DIG-labeled probes was performed for 16 h at 42 °C for both *ipt* and *GUS* genes probes (McCabe *et al*. 1997). Hybridization patterns were detected with the chemiluminescent substrate CDP-Star (*Roche Molecular Biochemicals*, USA) and anti-DIG AP. The hybridized blot was exposed to *Hyperfilm TM-MP* X-ray film (*Amersham*) for 15 - 20 min at room temperature.

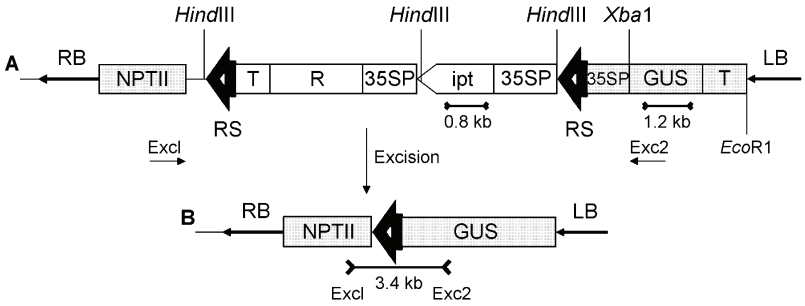

Fig. 1. Schematic representation of the T-DNA region of *ipt*-type MAT vector, *pPNI*132. *A* - The MAT vector with a "hit and run" cassette in which the chimeric *ipt* gene is inserted into the *R/RS* system as a selectable marker. *RB* - right border sequence of a T-DNA; *LB* - left border sequence of a T-DNA. The *CaMV* 35S promoter drives the *GUS, ipt* (isopentenyl transferase) and *R* (recombinase) genes. The *nptII* is driven by the nopaline synthase promoter. The terminators of *GUS, R* and *nptII* genes are derived from the nopaline synthase and that of the *ipt* gene is its native terminator. *GUS* and *nptII* genes are located outside the "hit and run" cassette. *B* - T-DNA region after excision of the "hit and run" cassette. Primer positions and length of PCR products are indicated by *double arrows*. Recognition sites of restriction enzymes are also indicated.

Results and discussion

Production of *ipt* and marker-free transgenic plants: Leaf explants of *Petunia hybrida* were infected with the *Agrobacterium tumefaciens* harboring *pNPI*132, an *ipt*-type MAT vector containing *ipt* and *R* (recombinase) genes in the removable cassette flanked by directly oriented recombination sites (RS) and cultured on PGR- and antibiotic-free MS medium. Uninfected control explants were cultured in line with the infected ones for evaluating organogenesis potential of the *ipt* gene. The *ipt* gene is one of the tumor inducing genes from *Agrobacterium tumefaciens*, which codes for isopentenyl-transferase to catalyze the condensation of isopentenyl pyrophosphate with AMP to produce isopentenyl AMP, a precursor of several cytokinins. Cytokinins stimulate organogenesis in many cultured plant tissues and are widely used to regenerate transgenic plants after transformation. Following gene transfer, over-expression of the *ipt* gene results in an increase in endogenous cytokinins (Kunkel *et al*. 1999) and the production of an extreme shooty phenotype, which exhibits the loss of apical dominance and root formation. Therefore, it is easy to visually detect transgenic plants that carry a functional *ipt* gene.

Two weeks after infection, cut ends of leaf explants started formation of nodular compact calli with shoot primordia (Fig. 2*B*,*C*). The uninfected control explants did not produce calli or shoots and subsequently died. Ninety four adventitious shoot lines (ASLs) were separated from 45 leaf explants one month after infection and transferred to PGR- and selective antibiotic-free MS medium supplemented with 20 mg dm^{-3} meropenem. These ASLs were differentiated into normal looking shoots as well as *ipt* shoots. On the basis of their morphology we could distinguish these types of shoots, and selection of only *ipt* shoots was carried out in every subculture (Fig. 2*D*,*E*). At this stage, we continued selection and transfer of 32 ASLs. A number of ESP shoots were produced from each ASL and these shoots were separated in every subculture, since a mass of ESP shoots might consisted of transgenic as well as non-transgenic tissues, and transferred to the same medium. During this study, we observed that the ESP shoots could be distinguished from other shoots by their profuse proliferation, morphology, deep green colour and abnormal appearance. In addition, ESP shoots are characterized by the loss of apical dominance and inability to root presumably because of elevated cytokinin content (Smigocki and Owens 1988).

Expression of the recombinase *R* in transgenic plants excises the fragment between the two recognition RS sites, thus removing the selectable marker genes that are also integrated in the transgenic plant genome (Ebinuma *et al*. 1997, Sugita *et al*. 1999). Excision of the *ipt* genes from the transgenic tissues leads to decrease in cytokinin content and subsequently formation of morphologically normal shoots. Approximately 4 months after infection,

29 normal looking shoots (Fig. 2*F*) were obtained from the separation of *ipt* shoots of different ASLs. Eight of the total normal shoots (29) and ESP shoots from 6 of 12 ASLs were subjected to histochemical GUS assay (Fig. 2*H*,*I*) and all of the tested shoots showed *GUS* expression. These GUS^+ shoots were further confirmed by molecular analysis.

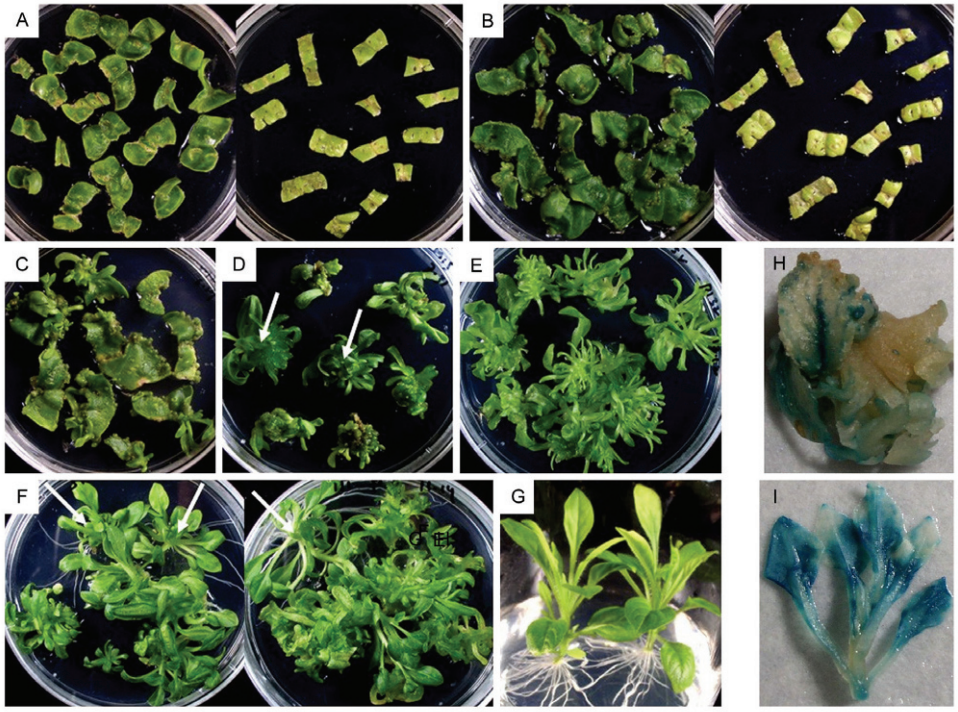

Fig. 2. Regeneration of normal shoots from leaf explants of *Petunia hybrida* cv. Dainty Lady through *ipt* shoot formation after infection with *Agrobacterium tumefaciens* harboring *ipt*-type MAT vector. *A* - Comparison of the response between infected (*left*) and non-infected (*right*) leaf explants. Note increased size of the explants because of cell division on hormone- and antibiotic-free MS medium 10 d after infection. *B* - Callus formation by the infected (*left*) and non-infected (*right*) leaf explants 20 d after infection. *C* - adventitious shoot lines (ASLs) formation by the infected explants one month after infection. *D* - Differentiation of ASLs into ESP shoots (*arrows*) 3 weeks after separation. *E* - Mass of ESP shoots produced from an ASLs 3 months after infection. *F* - Morphologically normal shoots with root formation (indicated by *arrows*) produced from ESP shoots approximately 4 months after infection. *G* - Morphologically normal rooted plant of *Petunia hybrida* free from selection marker. *H*, *I* - Histochemical GUS assay of ESP and normal shoots. ESP shoot (*H*) and a normal transgenic shoot (*I*) were tested for histochemical *GUS* expression by soaking in X-Gluc solution. After 15 - 16 h staining, chlorophyll was excluded by soaking the tissues for several hours in 70 % ethanol.

Molecular analyses: For PCR analysis three oligonucleotide primers that could amplify *GUS* and *ipt* genes, and the DNA fragment with excision event were used. The predicted 1.2 kb *GUS* fragment was amplified in all *ipt* and morphologically normal shoots (Fig. 3*C*). PCR analysis amplified the *ipt* gene in 5 *ipt* shoots and not in 7 of the 8 normal shoots (Fig. 3*B*). However, interestingly, in one of the normal well-rooted shoots, the *ipt* fragment (Fig. 3*B*, *lane* 11) was amplified. Presence of *ipt* gene in this plant was also confirmed by Southern blot analysis (Fig. 4*B*), suggesting its chimerism, in which both the excised and not-excised T-DNA fragments might coexist in the same plant or tissue. These results are in agreement with our previously reported ones in *Nierembergia caerulea* (Khan *et al*. 2006) where two of the morphologically normal rooted transgenic plants had *ipt* gene. Chimerism has also been previously reported in tobacco (Sugita *et al*. 1999) and sweet orange (Ballester *et al*. 2007) using *ipt* gene as the selectable marker. Problems of chimerism were also reported by Schaart *et al*. (2004) in strawberry transformation using a Cre/*lox* based site-specific recombination system for the recovery of marker-free plants. Chimerism is one of the pitfalls of recombination-based marker-free systems. However, as the recombinase gene in the MAT vector is controlled by the strong constitutively expressed 35S promoter, it could be expected that excision would be produced sooner or later in all transformed cells, finally resulting in non-chimeric *ipt*-free plants. Nevertheless, control of recombination and excision by inducible promoters directing recombinase expression could provide a good alternative to avoid problems of chimerism and inefficient excision (Endo *et al*. 2002, Matsunaga *et al*. 2002).

Methylation of a promoter triggered by double-stranded RNA has been reported to inactivate the gene (Mette *et al*. 1999, 2000). DNA methylation of the *ipt* gene also as reported by John and Amasino (1989) might inactivate the *ipt* gene resulting in production of

morphologically normal shoots**.** Therefore, careful screening of normal shoots with molecular analyses will be needed to exclude such chimeric transgenic plants.

The *ipt*-type MAT vector, *pNPI*132, used in this study contains the *R/RS* fragment called "hit and run" cassette and was used for selection of transgenic cells. In this cassette the chimerical *ipt* and *R* (recombinase) genes fused with a 35*S* promoter are located between two directly oriented *RS* (recombination sites) sequences (Fig. 1). The site-specific recombination *R/RS* system had been isolated from a circular plasmid, *pSR*1 of *Zygosaccharomyces rouxii* by Araki *et al*. (1987). This recombination system allows excision of a DNA fragment with an *R* gene product (recombinase) from a plant genome between two directly oriented recombination sites (Onouchi *et al*. 1991). In the present study, results of the PCR analysis revealed that the normal shoots as well as *ipt* shoots had the 3.4 kb fragment (Fig. 3*A*), which showed the evidence of expected excision event as shown in Fig. 1. Moreover, both faint and strong bands

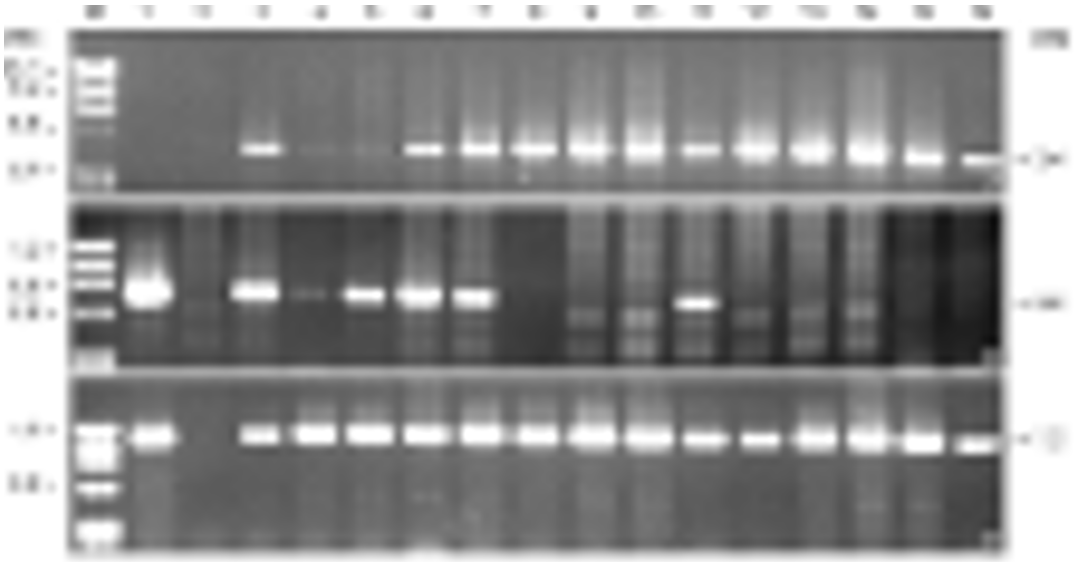

Fig. 3. PCR analysis of *ipt* and normal shoots of *Petunia hybrida* obtained through ESP shoot formation after infection with *A. tumefaciens* harboring *ipt*-type MAT vector. *A* - Amplification of PCR fragment (3.4 kb) from an excision event using EXC1 and EXC2 primers. *B* - Amplification of *ipt* gene (0.8 kb) in *ipt* (*lanes* 3 - 8) and normal shoots (*lanes* 9 - 16). *C* - Amplification of *GUS* gene (1.2 kb) in *ipt* (*lanes* 3-8) and normal shoots (*lanes* 9 - 16). M - size markers, *λ*/*Hind*III digests (*A*), *øX174/Hae*III digests (*B* and *C*), *lane* 1 - positive control (plasmid DNA), *lane* 2 - negative control (DNA from non-transformed plant). ESP (*lanes* 3 - 8) and morphologically normal (*lanes* 9 - 16) shoots.

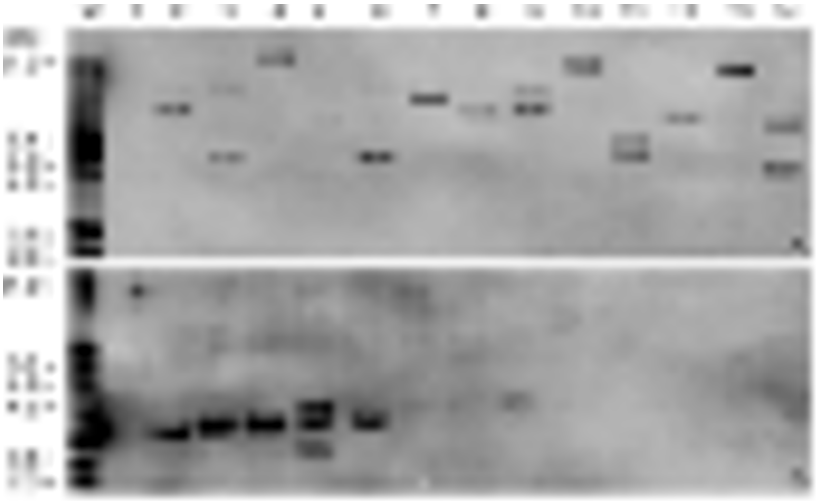

Fig. 4. Southern blot analysis of the *GUS* and *ipt* genes in ESP and normal shoots of *Petunia hybrida*. Genomic DNA from ESP shoots and morphologically normal transgenic plants was digested with *Hin*dIII and hybridized with the probes of DIG-labeled *GUS* (*A*) and *ipt* (*B*) genes. M - DIG-labeled DNA molecular mass marker III, *lane* 1 - untransformed control, *lanes* 2 - 6 - ESP shoots, lanes 7 -14 - marker-free transgenic normal shoots produced from ESP shoots except *lane* 9, which was morphologically normal transgenic plant but still containing the *ipt* gene.

of 3.4 kb were detected in *ipt* shoots (Fig. 3*A*), which might indicate that excision of the *ipt* gene had started more or less in all the *ipt* shoots while the shoots were in the *ipt* shoot stage and complete excision might take longer time for the production of marker-free normal plants.

Southern blot analysis of *ipt* and normal marker-free transgenic plants was carried out to know the number of insertion sites. Genomic DNA from *ipt* and normal transgenic and non-transformed control plants was digested with *Hin*dIII and hybridized with DIG-labeled probes of *GUS* and *ipt* genes. Results of hybridization (Fig. 4), indicate that the transgenic plants had 1 - 3 (*ipt* gene probe, Fig 4*B*) and 1 - 2 (*GUS* gene probe, Fig. 4*A*) of T-DNA insertions. Most of the marker-free transgenic plants had one site of transgene integration. Low number of T-DNA insertion sites is advantageous to stable gene expression as it has been found that multiple copies of the transgene may lead to gene inactivation (Hobbs *et al*. 1993). Hybridization was not detected in untransformed plant DNA (Fig. 4*A*,*B*).

This study demonstrated that the *ipt* overexpression was sufficient to promote organogenesis and that the *ipt* gene can be used as a positive selection marker in genetic transformation of *Petunia hybrida.* The overexpression of *ipt* gene in *Petunia* has been described previously to delay leaf senescence (Dervinis *et al*. 1999), its use as a selection marker in transformation of *Petunia* has not been reported. Our data demonstrated that the yeast recombinase system R/RS was active in removal of *ipt* marker gene from transgenic *Petunia* cells and produced the intended results supporting our previous research work (Khan *et al*. 2006). Similar observations have been recently reported by Ballester *et al*. (2007) and Zelasco *et al*. (2007) who produced *ipt* marker-free transgenic *Citrus sinensis* and *Populus alba*, respectively.

It could be concluded that the production of marker-free normal transgenic plants is possible without using selective chemical agents on PGR-free MS medium employing *ipt*-type MAT vector. Our next goal is to introduce disease resistance gene in *Petunia hybrida*, an important model plant for biotechnological studies and is susceptible to a number of devastating diseases, especially fungal diseases, using our established protocol for MAT vector system.

References

Ainley, W.M., McNeil, K.J., Hill, J.W., Lingle, W.L., Simpson, R.B., Brenner, M.L., Nagao, R.T., Key, J.L.: Regulatable endogenous production of cytokinins up to toxic levels in transgenic plants and plant tissues. - Plant mol. Biol. **22**: 13-23, 1993.

Akiyoshi, D.E., Klee. H., Amasino, R.M., Nester, E.W., Gordon, M.P.: T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. - Proc. nat. Acad. Sci. USA. **81**: 5994-5998, 1984.

Araki, H., Jearnpipatkul, A., Tatsumi, H., Sakurai, T., Ushino, K., Muta, T., Oshima, Y.: Molecular and functional organization of yeast plasmid pSR1. - J. mol. Biol. **182**: 191-203, 1987.

Ballester, R., Cervera, M., Peña, L.: Efficient production of transgenic citrus plants using isopentenyl transferase positive selection and removal of the marker gene by site-specific recombination. - Plant Cell Rep. **26**: 39-45, 2007.

Dervinis, C., Clark, D.G., Klee, H.I., Barrett, J.E., Nell, T.A.: Prevention of leaf senescence via genetic transformation with sag-IPT. - Proc. Florida State hort. Soc. annu. Meetings **111**: 12-15, 1999.

Ebinuma, H., Sugita, K., Matsunaga, E., Yamakado, M.: Selection of marker-free transgenic plants using the isopentenyl transferase gene. - Proc. nat. Acad. Sci. USA **94**: 2117-2121, 1997.

Ebinuma, H., Komamine, A.: MAT (multi-auto-transformation) vector system. The oncogenes of *Agrobacterium* as positive markers for regeneration and selection of marker-free transgenic plants. - *In Vitro* cell. dev. Biol. Plant **37**: 103-113, 2001.

Endo, S., Kasahara, T., Sugita, K., Ebinuma, H.: A new GSTMAT vector containing both *ipt* and *iaaM/H* genes can produce marker-free transgenic tobacco plants with higher frequency. - Plant Cell Rep. **20**: 923-928, 2002.

Hobbs, S.L.A., Warkentin, T.D., Delong, C.M.O.: Transgene copy number can be positively or negatively associated with transgene expression. - Plant mol. Biol. **21**: 17-26, 1993.

Jefferson, R.A.: Assaying chimeric genes in plants: the GUS gene fusion system. - Plant mol. Biol. Rep. **5**: 387-405, 1987.

John, M.C., Amasino, R.M.: Extensive change in DNA methylation patterns accompanies activation of a silent T-DNA *ipt*-gene in *Agrobacterium tumefaciens*-transformed plant cells. - Mol. cell. Biol. **9**: 4298-4303, 1989.

Khan, R.S., Chin, D.P., Nakamura, I., Mii, M.: Production of marker-free *Nierembergia caerulea* using MAT vector system. - Plant Cell Rep. **25**: 914-919, 2006.

Klee, H., Horsch, R., Rogers, S.: *Agrobacterium*-mediated plant transformation and its further applications to plant biology. - Annu. Rev. Plant Physiol. **38**: 467-486, 1987.

Kunkel, T., Niu, Q.W., Chan, Y.S., Chua, N.H.: Inducible isopentenyl transferase as a high-efficiency marker for plant transformation. - Nat. Biotechnol. **17**: 916-919, 1999.

Matsunaga, E., Sugita, K., Ebinuma, H.: Asexual production of selectable-marker free transgenic woody plants, vegetatively propagated species. - Mol. Breed. **10**: 95-106, 2002.

McCabe, M.M., Power, J.B., de Laat, A.M.M., Davey, M.R.: Detection of single copy genes in DNA from transgenic plants by non-radioactive Southern blot analysis. - Mol. Biotechnol. **7**: 79-84, 1997.

Mette, M.F., Aufsat, Z.W., van derWinden, J., Matzke, M.A., Matzke, A.J.M.: Transcriptional silencing and promoter methylation triggered by double-stranded RNA. - EMBO J. **19**: 5194-5201, 2000.

Mette, M.F., Van der Winden, J., Matzke, M.A., Matzke, A.J.M.: Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters *in trans*. - EMBO J. **18**: 241-248, 1999.

Minlong, C., Takayanagi, K., Kamada, H., Nishimura, S., Handa, T.: Transformation of *Antirrhinum majus* L. by a *rol*-type multi-auto-transformation (MAT) vector system. - Plant Sci. **159**: 273-280, 2000.

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

Onouchi, H., Yokoi, K., Machida, C., Matsuzaki, H., Oshima, Y., Matsuoka, K., Nakamura, K., Machida, Y.: Operation of an efficient site-specific recombination system of *Zygosaccharomyces rouxii* in tobacco cells. - Nucl. Acids Res. **19**: 6373-6378, 1991.

Rogers, O.S., Bendich, J.A.: Extraction of DNA from plant tissues. - In: Gelvin, S.B., Schiliperoort, R.A., Verma, D.P.S. (ed): Plant Molecular Biology Manual. A6: 1-10. Kluwer Academic Publishers, Dordrecht - Boston - London 1988.

Schaart, J.G., Krens, F.A., Pelgrom, K.T.B., Mendes, O., Rouwendal, G.J.A.: Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. - Plant Biotech. J. **2**: 233-240, 2004.

Smigocki, A.C., Owens, L.D.: Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. - Proc. nat. Acad. Sci. USA **85**: 5131-5135, 1988.

Sugita, K., Matsunaga, E., Ebinuma, H.: Effective selection system for generating marker-free transgenic plants independent of sexual crossing. - Plant Cell Rep. **18**: 941-947, 1999.

Sugita, K., Matsunaga, E., Kasahara, T., Ebinuma, H.: Transgene stacking in plants in the absence of sexual crossing. - Mol. Breed. **6**: 529-536, 2000.

Zelasco, S., Ressegotti, V., Confalonieri, M., Carbonera, D., Calligari, P., Bonadei, M., Bisoffi, S., Yamada, K., Balestrazzi, A.: Evaluation of MAT-vector system in white poplar (*Populus alba* L.) and production of *ipt* marker-free transgenic plants by ‘single-step transformation’. - Plant Cell Tissue Organ Cult. **91**: 61-72, 2007.