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GENETIC TRANSFORMATION AND HYBRIDIZATION

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# The isopentenyl transferase gene is effective as a selectable marker gene for plant transformation in tobacco (*Nicotiana tabacum* cv. Petite Havana SRI)

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**Abstract** A selection method for transformed cells which does not inhibit regeneration is important for the establishment and optimization of a transformation protocol. We have assessed the 35S-*ipt* gene from *Agrobacterium tumefaciens* as a selectable marker gene. The identification of *ipt*-expressing cells from nontransformed cells enabled morphological selection without the use of kanamycin and also allowed for the elimination of a high proportion of nonexpressing cells. *Ipt* selection of tobacco leaf discs (*Nicotiana tabacum* cv. Petite Havana SRI) resulted in a 2.7-fold higher transformation frequency compared to kanamycin selection. Overexpression of the *ipt* gene favored plant regeneration from transformed cells, and the transformation frequency of the *ipt* plus kanamycin selection resulted in a 1.6-fold higher transformation frequency than kanamycin selection alone. These results indicate that this procedure might provide a strategy whereby transgenic plants can be efficiently obtained and some of the problems related to the use of antibiotics diminished.

**Keywords** Transgenic plants · Selectable marker · Isopentenyl transferase

**Abbreviations** *BA* Benzylaminopurine · *ESP* Extreme shooty phenotype · *GUS* β-Glucuronidase · *ipt* Isopentenyl transferase · *MAT* Multi-auto-transformation · *MS* Murashige and Skoog medium (1962) · *NAA* Naphthaleneacetic acid

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## Introduction

In major plant transformation systems that generate a substantial number of nonchimeric primary transformants, genes conferring resistance to selective chemical agents such as antibiotics or herbicides can be used to select transformants. These resistance genes enable the transformed cells to survive on medium containing the selective agent, while nontransformed cells and tissue die. However, these selective agents have inhibitory effects on the regeneration of cells, even if they are transformed. In selection using antibiotics and herbicides, most transformed cells do not regenerate easily. Furthermore, necrotic substances, which are excreted into the medium from dying untransformed tissues and cells, may inhibit the growth and regeneration of the transformed cells. The development of a selection system which does not damage either transformed cells or nontransformed cells is important for the efficient recovery of regenerants during transformation.

The hazards mentioned above can be avoided by using positive selection systems for plant transformation. In 'positive selection' systems, such as selection using the β-glucuronidase gene, the xylose isomerase gene or oncogenes from *Agrobacterium tumefaciens* or *A. rhizogenes*, neighboring cells are not exposed to toxic secretions from dying cells (Morton and Okkels 1996; Christy et al. 1997; Haldrup et al. 1998). One advantage of using the oncogene from *Agrobacterium* as the selectable marker gene is that clonal transgenic tissue can be identified by changes in morphology without the use of antibiotics or herbicides. The major drawback of this approach is that plants are morphologically abnormal, due to the continued expression of the oncogenes in the T-DNA (reviewed in Christy 1997). As one solution to this problem, Ebinuma et al. (1997) developed the MAT vector system, which uses the *ipt* gene from *A. tumefaciens* as the selectable marker gene and the maize transposable element *Ac* for removing the *ipt* gene.

The important difference of the MAT vector system from traditional methods is that this system uses a removal system for marker-free transgenic plants and the *ipt* gene as the selectable marker gene. In previous reports, it has been demonstrated that marker-free transgenic plants can be obtained using the MAT vector system (Ebinuma et al. 1997; Sugita et al 1999). However, there have been no detailed studies comparing the *ipt* gene and antibiotic resistance genes as selectable markers.

In this paper, we examine the selection system using the *ipt* gene as the selectable marker gene. For this purpose, we used the IPT5 plasmid containing the *ipt* gene under control of the 35S CaMV promoter to analyze whether the selection system using the *ipt* gene was superior to antibiotic selection with respect to enabling the elimination of nontransformed cells from transformed tissues. We confirmed that the transformation efficiency using the *ipt* gene as the selectable marker gene was markedly higher than that of the traditional kanamycin system.

## Materials and methods

### Plasmid construction and plant transformation

The chimeric *ipt* gene under the control of the CaMV35S promoter was cloned into pNPI123 (described in Sugita et al. 1999). A *Hin*dIII fragment containing the 35S-*ipt* gene was then excised from pNPI123 and cloned into the *Hin*dIII site of pBI121 (CloneTech).

This plasmid was designated IPT5. Plasmid IPT5 was introduced into *A. tumefaciens* LBA4404 by electroporation, which was then used for transforming tobacco (*Nicotiana tabacum* cv. Petite Havana SRI).

*Nicotiana tabacum* cv. Petite Havana SRI was grown in pots in a controlled environment at 25 °C and under an 18(day)/6(night)-h photoperiod. Sterile leaf discs were cocultivated with *Agrobacterium* and explanted onto a medium without antibiotics. After incubation at 25 °C for 2 days, the leaf discs were transferred to a medium supplemented with carbenicillin.

### Tissue culture

For cultivation of the transformed tobacco tissue, four kinds of media were used: (1) MS medium without hormones (MSO); (2) MSO with 200 mg/l kanamycin (MSK); (3) MSO with 0.1 mg/l *NAA* and 1 mg/l BA (SIM); (4) SIM with 200 mg/l kanamycin-(SIMK). All of the media contained agar (8 g/l) and carbenicillin (1 g/l), and cultures were maintained at 25 °C under light. Since control tissue was transformed with the pBI121 vector only, it was cultured on the SIMK medium. Transformed tissue containing pIPT5 was cultured on MSO or MSK medium. Shoot tips were used for subculture onto MSO, MSK or SIMK medium.

### Extraction of DNA and polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from tobacco leaves and purified essentially as described by Doyle and Doyle (1989). PCR analysis was carried out under standard conditions that included 30 cycles of a 1-min denaturation at 94 °C, a 1-min annealing at 60 °C and a 2-min extension at 72 °C. The sequences of the PCR primers were as follows: IPT1, 5′-CTTGCACAGGAAAGACGTCG-3′; IPT2, 5′-AATGAAGACAGGTGTGACC-3′; NPT1, 5′-AGAGGCT-ATTCGGCTATGAC-3′; NPT2, 5′-CCATGATATTCGGCAA-GCAG-3′; GUS1, 5′-GTGGAATTGATCAGCGTTGG3′; GUS2, 5′-GCACCGAAGTTCATGCCAGT-3′.

### Histochemical assay

Histochemical assays for GUS activity were performed as described by Jefferson (1987).

## Results and discussion

### Selection of transformed shoots

In the absence of kanamycin, we examined the selection of transgenic plants using the *ipt* gene as the selectable marker gene. The IPT5 plasmid (Fig. 1) in *Agrobacterium tumefaciens* strain LBA 4404 was used to infect tobacco leaf discs, which were subsequently cultured on hormone-free MS medium in the absence of kanamycin (MSO) or in the presence of kanamycin (MSK). Initiation of adventitious shoot formation was often visible 14 days after coculture on MSO (Fig. 2A). On the other hand, no adventitious shoots were visible on transformed tobacco leaf discs which were subsequently cultivated on MSK (Fig. 2B).

One month after coculture, 1–8 adventitious shoots were produced from leaf discs infected with *Agrobacterium tumefaciens* containing the IPT5 on MSO

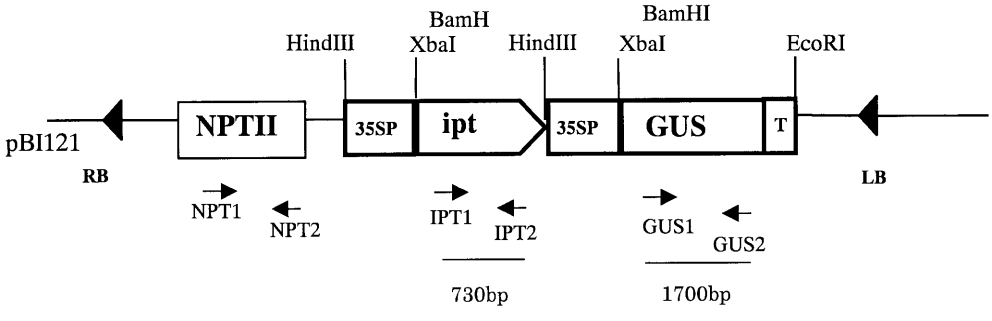


**Fig. 1** The binary vector, pIPT5, containing the isopentenyl transferase gene used for plant transformation. The order of genes in the T-DNA is: right border (*RB*), NPTII, 35SP-isopentenyl transferase gene (*ipt*), 35SP-β-glucuronidase (*GUS*), left border (*LB*). *35SP* (CaMV 35S promoter). *Bottom*: *small arrows* PCR primers, which are described in the Materials and methods, to amplify the NPTII gene, the *ipt* gene and the GUS gene

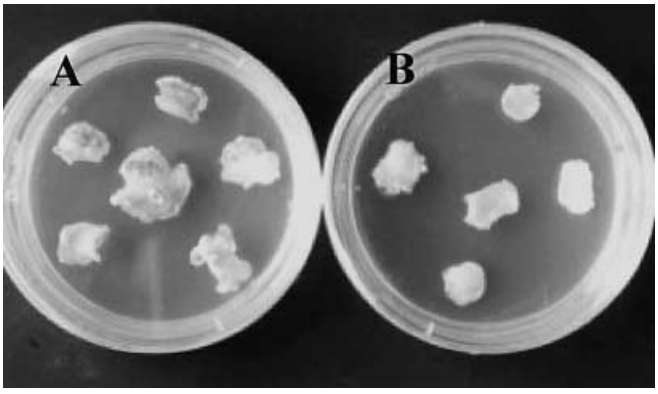


**Fig. 2** Regeneration from leaf discs transformed by IPT5 2 weeks after inoculation with *Agrobacterium* containing pIPT5 on MSO medium (**A**) and MSK medium (**B**)

medium (Fig. 3A). Seven to 8 weeks after coculture, transgenic plants could not be distinguished from nontransgenic plants. Upon subsequent transfer to hormone free MS medium (Fig. 3B), some shoots were easily distinguished from normal shoots 7–8 weeks after co-cultivation (indicated by white arrow) by a complete loss of apical dominance and the absence of roots (ESP, indicated by black arrow) (Fig. 3C, D). Two months after cocultivation, 55.4% of the regenerated shoots showed transgenic features (ESP). The *ipt* gene of pIPT5 caused this altered morphology. Overexpression of the *ipt* gene results in an increase in cytokinin content and thus an altered morophology in transformed cells (Smigocki and Owens 1989).

Some of the adventitious shoots (44.6% of the regenerated shoots), which showed apical dominance and root formation (indicated by white arrow in Fig. 3C) but did not show transgenic features, were rescued on hormone-free MS medium. By PCR analysis and GUS histochemical assays, we confirmed that these shoots had no foreign gene were nontransgenic tissue. This suggested that all transformed tissues, which have the *ipt* gene, had altered morphologies. Li et al. (1992) reported the distribution of cytokinin to other tissues. The regeneration of nontransformed shoots from leaf discs infected with *Agrobacterium* containing pIPT5 may depend on cytokinin distribution. It is hypothesized that cytokinin may be distributed from the cells expressing the *ipt* gene to other cells, thereby inducing the regeneration of normal shoots from nontransgenic cells.

## Transformation frequency

Leaf discs infected with *Agrobacterium* containing pIPT5 were transferred to the hormone free MS medium with/without kanamycin (MSO or MSK), and leaf discs infected with *Agrobacterium* containing pBI121 were transferred to shoot-inducing medium with kanamycin (SIMK). Three weeks later, the regeneration of adventitious shoots from the leaf discs, which had been transformed with pIPT5 and transferred to the medium without kanamycin, was observed, while the regeneration of shoots from the leaf discs transferred to the medium with kanamycin was not

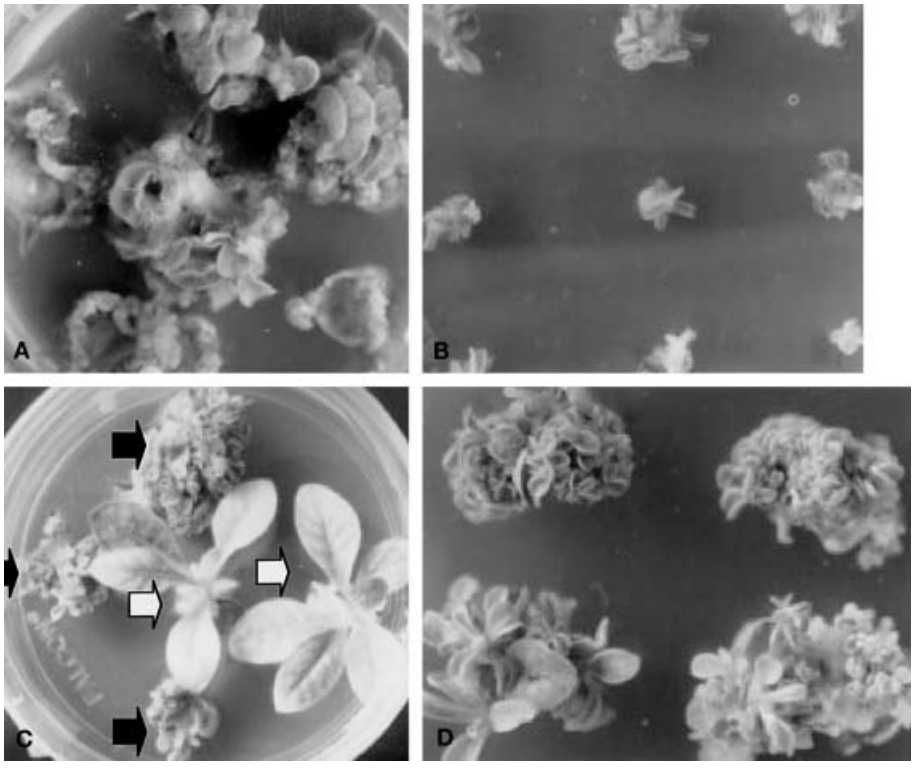


**Fig. 3** **A** Leaf discs on MSO 4 weeks after inoculation with *Agrobacterium* containing pIPT5. **B** Shoots that were regenerated from leaf discs transformed by pIPT5 and transferred to MSO medium. **C** ESP shoots (*black arrows*) and nontransgenic normal shoots (*white arrows*) 1 month after transfer to MSO medium. **D** ESP shoots

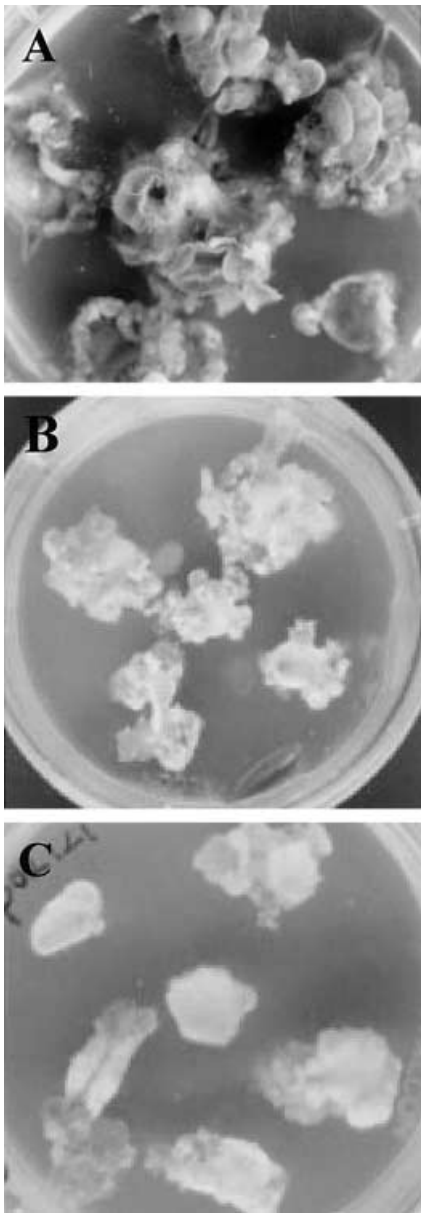


**Fig. 4** **A**, **B** Shoot regeneration from leaf discs infected with pIPT5 on MSO medium (**A**) and MSK medium (**B**) 4 weeks after inoculation. **C** Shoot regeneration from leaf discs infected with pBI121 on SIMK medium 4 weeks after inoculation

observed. Four weeks later, the regeneration of shoots from the leaf discs on the medium with kanamycin was observed. After 4 weeks of cocultivation, 83 shoots were regenerated from 20 leaf discs transformed with pIPT5 on MSO, and 28 shoots were regenerated from 20 leaf discs on MSK (Fig. 4A, B). From leaf discs transformed with pBI121, 17 shoots were regenerated from 20 leaf discs on SIMK (Fig. 4C). Two months later, 46 lines of 83 shoots, which were regenerated from the 20 leaf discs on MSO, and 28 lines, regenerated from the 20 leaf discs on MSK, exhibited a transgenic nature (Table 1). Similar results were obtained in repeat experiments.

Selection using the *ipt* gene resulted in a 2.7-higher transformation frequency than selection using only the NPTII gene (pBI121, Table 1). This increase in transformation frequency using the *ipt* gene is thought to be due to two factors. First, the stress induced from exposure to the selective chemical agent (kanamycin) is eliminated; instead selection was based on the morphological changes induced by the expression of the *ipt* gene. The number of regenerated shoots which exhibited a transgenic nature in the medium without kanamycin was about 1.5-times (46/28) higher than that with kanamycin. In addition, the appearance of regeneration from leaf discs occurred later on the medium with kanamycin than on medium without kanamycin. These results indicate that kanamycin is inhibitory to the regeneration of transformed cells.

The second factor thought responsible for the increase in transformation frequency was the increase in endogenous hormone with the introduction of the *ipt* gene. When kanamycin was present, the number of regenerated shoots from leaf discs transformed with pIPT5 increased in contrast to the situation with leaf discs transformed with pBI121. The transformation frequency of pIPT5 was 1.6-times higher (28/17) than that of pBI121 in the presence of kanamycin. With the introduction of the *ipt* gene, endogenous zeatin and zeatin riboside concentrations in transformed cells increased 100-fold compared to nontransformed cells (Smigocki and Owens 1989). The increase in endogenous cytokinin concentrations following expression of the *ipt* gene can alleviate stress due to selective chemical agents. Experiments using exogenous hormones have demonstrated the regeneration of shoots and roots from many tissues and cells in many plant species (Skoog and Miller 1957; Renaudin et al. 1990; Stable et al. 1990). Not all species respond to exogenous hormones due to a low hormone uptake, compartmentalization or the metabolism of hormones during stress situations. The ability to increase endogenous hormone levels in vivo alleviates such problems. It may be

**Table 1** Transformation efficiency of leaf discs infected with *Agrobacterium* containing pIPT5 or pBI121 under different conditions

| Construct | Medium | Number of leaf discs | Number of regenerated shoots | Number of shoots which showed a transgenic nature | Percentage of transgenic plants per regenerated shoot |
|---|---|---|---|---|---|
| pIPT5 | MSO | 20 | 83 | 46 | 55.4% |
| pIPT5 | MSK | 20 | 28 | 28 | 100% |
| pBI121 | SIMK | 20 | 17 | 17 | 100% |

[a] Regenerated shoots which showed morphologically a transgenic nature

expected that an *ipt* gene introduced by transformation would work in a similar manner in many other plant species.

## PCR analysis

To confirm the stable integration of the gene, we analyzed all regenerated shoots for the presence of the *ipt* gene, the NPTII gene and the GUS gene by PCR. In all adventitious shoots exhibiting a transgenic nature on medium without kanamycin 7–8 weeks after cocultivation, most of the adventitious shoots (90.9%) were confirmed to contain the *ipt* gene, the NPTII gene and the GUS gene by PCR analysis (Fig. 5 ; Table 2). About 10–12 weeks after co-cultivation a few adventitious shoots without the *ipt* gene exhibited altered tissue morphology – as in root formation and the recovery of the apical dominance. Adventitious shoots which were visible 12 weeks after cocultivation showed transgenic features on hormone free MS medium 6 months after cocultivation.

We examined the stable expression of a transgene in the tissue of ESP shoots using GUS expression as the marker. In approximately 27% of ESP shoots in the absence of kanamycin and 72.7% of ESP shoots in the presence of kanamycin, GUS expression was detected by histochemical assay (Table 2). In other ESP shoots, no GUS expression was detected. It is possible that the absence of GUS expression is due to the absence of the GUS gene itself. PCR analysis revealed the absence of the GUS gene. In some ESP lines, a portion of the GUS gene was detected by PCR analysis. The introduction of only a portion of the GUS gene was detected in ESP lines that showed no GUS expression in the presence or absence of kanamycin. In the *Agrobacterium* transformation system, the deletion of the near site with the left border sequence in T-DNA has been documented. In the pIPT5 plasmid, the GUS gene is located on the left side of the border sequence from the *ipt* gene used as the selectable marker gene. In addition, the presence of the NPTII gene, which is located on the right side of the border sequence from *ipt* gene, was detected in ESP lines that showed no GUS expression by PCR analysis. These results indicate the possibility that the absence or deletion of the GUS gene is due to its relocation in the T-DNA region.

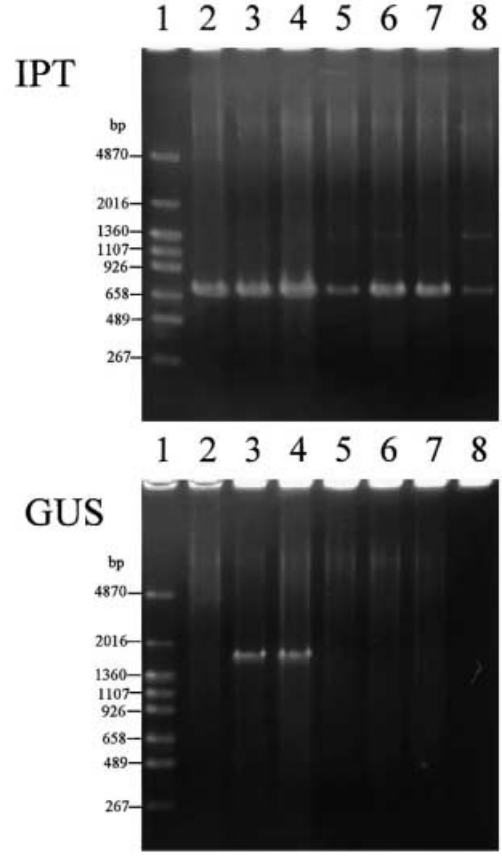


**Fig. 5A, B** PCR analysis of genomic DNA from independently ESP shoots (*lanes 2–8*). **A** Using IPT1 and IPT2 primers, **B** using GUS1 and GUS2 primers. These primers are represented in Fig. 1. *Lane* 1 Size marker (pHY marker purchased from Yakult)

**Table 2** Efficiency of obtaining stably transformed shoots from leaf discs infected with *Agrobacterium* containing pIPT5 or pBI121

| Construction | Medium | Number of explants[a] | Percentage of $G^+$ plants[b] of total plants | Percentage of PCR-positive shoots | | |
|---|---|---|---|---|---|---|
| | | | | NPT II | *ipt* | GUS |
| IPT5 | MSO | 46 | 27 | 90.9 | 90.9 | 36.1 |
| IPT5 | MSK | 28 | 72.7 | 100 | 96.4 | 72.7 |
| pBI121 | SIMK | 17 | 94.1 | 100 | – | 94.1 |

[a] Regenerated shoots which showed morphologically a transgenic nature
[b] GUS-positive shoots

## Kanamycin resistance trait of ESP

Leaf discs from ESP (no kanamycin selection) were analyzed for kanamycin tolerance and for the expression of *ipt* traits. On medium containing no hormones, either with kanamycin (MSK) or that without kanamycin (MSO), the regeneration of shoots was obtained in leaf discs of 33 ESP (no kanamycin selection) which showed a transgenic nature 12 weeks after cocultivation. Regeneration of ESP was induced on MSO in leaf discs from 23 ESP. Sixteen lines of 23 ESP, which regenerated on MSO, regenerated on both MSO and MSK (Table 3). PCR analysis revealed that all ESP contained the introduced NPTII gene and *ipt*gene (Table 2). It can be inferred from Tables 2 and 3 that transformants which were not selected by using kanamycin were selected using the *ipt* gene because 7 ESP lines in which regeneration was inhibited on MSK

**Table 3** The phenotype of shoots regenerated from leaf discs of ESP (extreme shoot phenotype) which were selected by the *ipt*gene

| Number of ESP lines (total 33) | Phenotype of regenerated shoots | | |
|---|---|---|---|
| | MSO[a] | MSK | SIM |
| 16 | ESP | ESP | ESP |
| 7 | ESP | – | ESP |
| 2 | –[b] | – | ESP |
| 3 | – | – | ESP + NS[c] |
| 5 | – | – | NS |

[a] See Materials and methods for full explanation of types of media
[b] No regenerated shoots
[c] Regenerated shoots which showed a nontransgenic nature

showed regenerated ESP on MSO. Consequently, by using the *ipt* gene as the selectable marker gene, we can obtain transformants which show a low-level expression of the marker gene. Differential gene expression between transgenic lines or tissues of transformants using a selective agent has been reported earlier (Shöpke et al. 1996). However, the inclusion of a selective agent in the regeneration and rooting media enables the selection of transgenic plants that stably express transgenes (Hiei et al. 1997). Transgenic plants that express transgenes at a low level cannot survive in a medium with a selective agent. The selection system using the *ipt* gene is a positive selection that does not stress the transformants with a low level of expression of the transgene. This is another advantage in using the *ipt* gene as a selectable marker.

## Regeneration analysis of ESP

The transgenic shoot that has been regenerated from infected tissue has the problem of being contaminated with nontransgenic cells. To analyze the contamination of nontransgenic cells in ESP, we induced shoot regeneration from leaf discs of ESP. On shoot regeneration medium containing hormones but not kanamycin (SIM), shoot regeneration was induced from leaf discs of all 33 ESP (no kanamycin selection) that showed a transgenic nature 12 weeks after cocultivation. In media containing hormone but without kanamycin, nontransgenic cells may be able to regenerate normal shoots. Twenty-eight ESP lines produced shoots which showed transgenic nature. However, 3 ESP lines produced both a normal shoot and ESP (Table 3). These results indicate that 24% of the ESP lines (8/33) were a mixture of transgenic cells and nontransgenic cells. We suggest that selection using the *ipt* gene as the selectable marker gene rescues the chimera, which includes both transgenic cells and nontransgenic cells (Table 3). However, 76% of the ESP lines were composed of only transgenic cells. These results indicate that nontransformed cells, which are present in ESP, can be removed by inducing regeneration from leaf discs on MSO.

In summary, this study demonstrates that the *ipt* gene from *A. tumefaciens* can be used for primary selection of transformed tissues and that the transformation efficiency using the *ipt* gene as the selectable marker gene is markedly higher than that of the traditional kanamycin-based system.

This selectable system was used in the MAT vector system for primary selection of transformed tissue. The MAT vector system provides a technique whereby morphologically normal transgenic plants can be efficiently obtained from morphologically abnormal tissue by inducing the expression of the *ipt* gene. This paper describes a procedure using the *ipt* gene as a selectable marker gene and compares it to that using an antibiotic selection marker. This experimental system provides a strategy whereby transgenic plants can be efficiently obtained without the use of an antibiotic resistance gene. We believe that the employment of this selection system may diminish the concerns often raised in relation to the use of antibiotic or herbicide resistance marker genes and their presence in transgenic plants. The *ipt* gene has been observed to induce abnormal shoots and cell proliferation in the medium without a supplementary hormone in potato (Ooms et al.1983), cucumber (Smigocki and Owens 1988) and several *Nicotiana* species (Smigocki and Owens 1989). Therefore, we believe that this selectable system may be applied to other plant species.

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