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

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 pNP123 (described in Sugita et al. 1999). A HindIII fragment containing the 35S-ipt gene was then excised from pNP123 and cloned into the HindIII 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.

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.