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Short communication

Genetic transformation of *Prunus domestica* L. using the *hpt* gene coding for hygromycin resistance as the selectable marker

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ABSTRACT

The study and development of transformation technology with new selection schemes is important for various fundamental studies and for crop trait improvement via genetic engineering. Here we have shown that hygromycin resistance is an effective system for plum genetic transformation. Embryonic axes of mature seeds were co-cultivated with *Agrobacterium* strain LBA4404 containing the pC1381 plasmid carrying the hygromycin phosphotranferase gene (*hpt*) and β-glucuronidase (GUS) gene or with strain EHA105 containing the plasmid pC1301 carrying the same marker and reporter genes. The latter strain containing a pC2301 plasmid carrying the neomycin phosphotransferase gene (*nptII*) gene was used as a control. Infected explants were placed on shoot induction medium containing either 5 mg L^{-1} hygromycin or 75 mg L^{-1} kanamycin for selection. Green shoots developed from the explants under hygromycin pressure. These shoots showed continued and vigorous growth and development upon transfer onto fresh hygromycin medium. PCR using *hpt* sequence primers, and Southern blot analysis using a probe from the *hpt* gene, confirmed the presence of the transgenes and their stable integration in regenerated plants. Full transgenic plants were obtained in a greenhouse. Hygromycin selection was very effective and no escapes were observed. The study demonstrated that hygromycin resistance can be used as an effective selectable marker for plum transformation. The new system developed here is important and useful for multiple gene transformation in plum.

1. Introduction

Fruit tree breeding is a lengthy and expensive procedure due to the long breeding cycles, heterozygosity, polyploidy, and long field evaluation procedures. Genetic transformation can be an alternative and has advantages for genetic improvement of fruit tree plants (Petri and Burgos, 2005).

Transgenic research and technology have been progressing steadily and the introduction of multiple genes into known fruit cultivars via transformation has become more important for desired trait improvement. However, transformations of multiple traits into fruit varieties usually require the use of different selectable marker systems. European plum (*Prunus domestica* L.) is an important fruit crop (Okie and Ramming, 1999) and there have been a number of reports on genetic transformation of this species (Mante et al., 1991; Camara Machado et al., 1994, 1995, 1999; Korte et al., 1994; Scorza et al., 1994, 1995; Yancheva et al., 2002; Padilla et al., 2003; Petri et al., in press); however, in all of these cases *nptII* conferring resistance to kanamycin was used as the solo selectable marker. Lack of alternative selection systems can restrict the technology development for more desired trait improvement.

The *hpt* gene of *E. coli* codes for hygromycin B phosphotransferase (HPT) and confers resistance to the antibiotic hygromycin B. Hygromycin resistance has been used for plant transformation in different species (e.g., Twyman et al., 2002; Akutsu et al., 2004). Hygromycin resistance and kanamycin resistance involve very different metabolic pathways in plants and no obvious interaction is found between them. These two selection schemes can be used for sequential or double selection for diverse purposes (Jacob and Veluthambi, 2002; Wu et al., 2003). Thus, hygromycin resistance may be an interesting and useful marker system in plum transformation for different purposes. Use of hygromycin for transformation selection were described in some *Prunus* species before but the results were very preliminary (Dolgov and Firsov, 1999; Mikhailov and Dolgov, 2007).

In this report, we studied the suitability of hygromycin resistance as a selectable marker system in European plum. We report here that the *hpt* gene coding for hygromycin resistance is an effective and reliable alternative selectable marker for plum transformation. The marker system developed here is a progressive

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step towards multiple genetic transformations of plum and provides an important alternative selection system.

2. Materials and methods

Plum fruits were collected from mature trees of cv. Stanley in Vineland Station, Ontario, Canada. Explants preparation was as described previously (Tian et al., in press).

Shoot induction medium consisted of MS salts (Murashige and Skoog, 1962) supplemented with vitamins (555 μM myo-inositol, 1.2 μM thiamine HCl, 1.4 μM nicotinic acid, 2.4 μM pyridoxine HCl), 2.5 μM indolebutyric acid (IBA), 25 g L^{-1} sucrose, and 7 g L^{-1} Bactogar (pH 5.9). Thidiazuron (TDZ) was added to the induction medium at 7.5 μM after autoclaving. The regeneration procedure was as described before (Tian et al., in press).

In all experiments, pCAMBIA vectors with different constructs were used. *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) carrying plasmid pC1381 and *A. tumefaciens* strain EHA105 (Hood et al., 1993) containing plasmid pC1301 or pC2301 were used for transformation studies. The plasmid pC1381 contained a β-glucuronidase (GUS) reporter gene under control of the tCUP promoter (Foster et al., 1999) and a selectable marker gene *hpt* under the control of 35S promoter in the following order: tCUP-GUS-35S-*hpt*. The plasmid pC1301 contained a β-glucuronidase (GUS) reporter gene and selectable marker gene *hpt* under the control of 35S promoters in the following order: 35S-GUS-35S-*hpt*. The plasmid pC2301 contained a β-glucuronidase (GUS) gene and selectable marker gene *nptII* for kanamycin resistance under control of 35S promoters in the following order: 35S-GUS-35S-*nptII*.

Bacterial suspensions were grown overnight with appropriate antibiotics and resuspended to OD_{600} = 0.3 for inoculation. Slices of embryonic axes were dipped for 30 min in the bacterial suspension and cultured on shoot induction medium containing 20 mg L^{-1} acetosyringone for 3–5 days at 24 ± 1 °C. The explants were then placed on shoot induction medium containing 300 mg L^{-1} timentin for 1 week before being transferred onto shoot induction medium containing 5 mg L^{-1} hygromycin or 75 mg L^{-1} kanamycin, and 300 mg L^{-1} timentin for selection of transformants. The explants were transferred to fresh induction medium every 4 weeks. Putatively transformed shoots formed after 3–12 weeks. As seed dissection and related procedures were very time consuming, research was conducted at different times. The results are the combination of different experiments. Results from some initial experiments were used to calculate transformation efficiencies and some lines from other experiments were included for molecular analyses.

Individual shoots which reached 0.5 cm or over in length were cut from the explants and transferred to a rooting medium consisting of half-strength MS salts (Murashige and Skoog, 1962), vitamins as described above, 5 μM naphthalene acetic acid (NAA), 0.01 μM kinetin, 10 g L^{-1} sucrose, and 7 g L^{-1} Bactoagar at pH 5.9. Antibiotics were not included in the rooting medium. Rooted plants that were 4–5 cm in height were transferred to soil and recovered in a greenhouse.

Putative transformants which survived hygromycin or kanamycin selection were tested by three methods to verify the gene incorporation and expression.

Histochemical *gus* assay of putative transformants was performed as described in Jefferson et al. (1987). Explants were stained in 1.5 mM X-glucuronide (X-Gluc) overnight at 37 °C. After incubation, explants were washed using 70% ethanol to visualize blue staining.

DNA was extracted from ground leaf tissue with a cell lysis buffer (250 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), followed by precipitation with isopropanol and washing with 70% ethanol (Sambrook et al., 1989). PCR amplifications of putative transformants were carried out using *nptII* primers (forward GAG GCT ATT CGG CTA TGA CTG and reverse ATC GGG AGC GGC GAT ACC GTA), GUS primers (forward GGT GGG AAA GCG CGT TAC AAG and reverse AGG TGC GGA TTC ACC ACT TGC) and *hpt* primers (forward GTC TCC GAC CTG ATG CAG CTC TC and reverse ACT CGC CGA TAG TGG AAA CCG AC). DNA amplification reactions were performed in a thermocycler (model mastercycler gradient; Eppendorf Canada Ltd., Mississauga, Ontario) in the following cycle parameters: 95 °C for 5 min, 37 cycles of 45 s at 95 °C, 30 s at 58 °C (60 °C for *hpt*) and 1 min 72 °C, with a final extention step at 72 °C for 5 min. The amplification products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed.

For Southern blot analysis, DNA was isolated using Qiagen DNeasy Plant Maxi Kit (Qiagen Inc., Valencia, CA, USA) from plum leaves of eight randomly selected plants which recovered from hygromycin selection and were analyzed by PCR. Ten micrograms of DNA was digested with *Eco*R1 restriction enzyme, size-fractionated by agarose gel electrophoresis and transferred to a positively charged nylon membrane for hybridization (Sambrook et al., 1989). The probes for DNA blotting were made by PCR using Roche PCR DIG Probe synthesis kit (Roche Applied Science, Indianapolis, Ind.) with the same hygromycin primers used for PCR as described above. DNA blots were non-radioactively probed at 48 °C overnight with the DIG labeled hygromycin probe (Roche Applied Science, Indianapolis, IN, USA). Blots were detected using Digoxigenin detection kit from Roche with a 10-min exposure to Kodak BioMax Light film (Kodak cat# 178 8207, Rochester, NY, USA).

3. Results and discussion

Kanamycin around 50–100 mg L^{-1} was generally used in plum transformation experiments (Mante et al., 1991; Camara Machado et al., 1994, 1995, 1999; Korte et al., 1994; Scorza et al., 1994, 1995; Yancheva et al., 2002; Padilla et al., 2003). In the preliminary experiments to determine the optimal hygromycin concentration suitable for transformation selection, different concentrations (0, 2.5, 5, 10, 20, 30 mg L^{-1}) of hygromycin were tested by culturing hypocotyl segments of cv. Stanley on the antibiotic-containing medium. No shoot regeneration was observed and explants were dead in 5 weeks when hygromycin concentrations at or over 5 mg L^{-1} were used. Therefore, hygromycin at 5 mg L^{-1} was used in transformation experiments to exert selection pressure. Hygromycin at much higher concentrations were needed and used for transformation selection in some other species (e.g., Finer and McMullen, 1991; Ghareyazie et al., 1997; Park et al., 2002). The sensitive response of plum tissues to hygromycin suggested that hygromycin was a suitable and effective chemical for plum transformation selection.

Putatively transformed shoots started to form after 3 weeks on the hygromycin medium (Fig. 1A). New shoots continued to form from the explants in the following 3–4 weeks. The developed shoots from hygromycin selection were cut out from the explants and plated onto fresh hygromycin-containing medium. The shoots showed vigorous and continuous growth on the fresh hygromycin medium (Fig. 1B). On the other hand, the control plants turned brown and could not grow and survive on the same hygromycin medium (Fig. 1C). Putative transformants developed roots and plants grew vigorously when the plantlets were transferred into the Magenta boxes.

Plants regenerated on hygromycin selection showed high levels of *gus* reporter gene expression via histochemical assay (Fig. 1D), indicating that these plants contained the *gus* gene. Hygromycin-