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Short communication

Efficient production of transgenic plants using the *bar* gene for herbicide resistance in sweetpotato

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ABSTRACT

Efficient production of transgenic sweetpotato (*Ipomoea batatas* (L.) Lam.) plants using the *bar* gene for herbicide resistance was achieved through the use of embryogenic suspension cultures and *Agrobacterium tumefaciens*-mediated transformation. Cell aggregates from embryogenic suspension cultures of sweetpotato cv. Lizixiang were cocultivated with *A. tumefaciens* strain EHA 105 harboring a binary vector pCAMBIA3300 with the *bar* gene and *uidA* gene. Selection culture was conducted using 0.5 mg/l PPT. A total of 1431 plants were produced from the inoculated 870 cell aggregates via somatic embryogenesis. GUS assay and PCR analysis of the regenerated plants randomly sampled showed that 86.5% of the regenerated plants were transgenic plants. Stable integration of the *bar* gene into the genome of transgenic plants was confirmed by Southern blot analysis and transgene expression was demonstrated by Northern blot analysis. The copy number of integrated *bar* gene ranged from 1 to 3. Transgenic plants exhibited functional expression of the *bar* gene by in vivo assay for herbicide resistance. This study also provides a simple and efficient transformation system of sweetpotato based on the use of *bar* gene as a selectable marker gene, which can be combined with other agronomically important genes for the improvement of sweetpotato.

1. Introduction

Sweetpotato, *Ipomoea batatas* (L.) Lam., is an important food and industrial material crop in the world. It is also an alternative source of bio-energy as a raw material for fuel production. The improvement of this crop by conventional hybridization is limited because of its high male sterility, incompatibility and the hexaploid nature (Dhir et al., 1998). Genetic engineering offers great potential for the improvement of sweetpotato. There have been several reports on this subject in the literature. Transgenic plants expressing cowpea trypsin inhibitor (*CpTI*), snowdrop lectin, delta-endotoxin, soybean kunitz trypsin inhibitor (*SKTI*-4), oryza-cystatin-I (*OC*I), sweetpotato feathery mottle virus (SPFMV-S) coat protein, granule-bound starch synthase I (*GBSS*I), tobacco microsomal ω-3 fatty acid desaturase (*NtFAD*3) or starch branching enzyme II (*IbSBE*II) gene have been produced (Newell et al., 1995; Morán et al., 1998; Cipriani et al., 1999, 2001; Okada et al., 2001; Kimura et al., 2001; Wakita et al., 2001; Shimada et al., 2006). But, in most cases only a low transformation efficiency was obtained, which limits the successful application of genetic engineering in sweetpotato improvement.

The *bar* gene is widely used for producing herbicide-resistant plants in many crop species. The enzyme phosphinothricin (PPT) acetyltransferase encoded by the *bar* gene inactivates PPT, the active ingredient of herbicides such as Basta and Buster, by acetylating its free ammonium group, thereby rending it non-toxic (De Block et al., 1987; Strauch et al., 1988). Otani et al. (2003), Yi et al. (2007) and Choi et al. (2007) obtained only a few herbicide-resistant plants expressing the *bar* gene from sweetpotato embryogenic calluses using *Agrobacterium tumefaciens*-mediated transformation or particle bombardment. Moreover, embryogenic calluses are not readily available target tissues for most of sweetpotato cultivars due to low frequencies of embryogenic callus formation in apical meristem cultures (Al-Mazrooei et al., 1997; Liu et al., 1997; Wang et al., 1998).

The availability of protocol to achieve high-frequency plant regeneration from cultured cells or tissues is a prerequisite for the application of genetic engineering. We succeeded in developing an efficient system of embryogenic suspension cultures for a wide range of sweetpotato genotypes especially for commercial cultivars (Liu et al., 2001). Using embryogenic suspension cultures of sweetpotato and *hpt*II/hygromycin selection system, we have

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also established an efficient *A. tumefaciens*-mediated transformation system (Yu et al., 2007). This paper describes efficient production of herbicide-resistant sweetpotato plants with the *bar* gene through the use of embryogenic suspension cultures and direct *bar*/PPT selection.

2. Materials and methods

2.1. Plant material

Sweetpotato cv. Lizixiang used in this study is one of commercial cultivars planted in China. Embryogenic suspension cultures of Lizixiang were prepared according to the method of Liu et al. (2001). Sixteen weeks after initiation, cell aggregates 0.7–1.3 mm in size from embryogenic suspension cultures of 3 days after subculture were used for the transformation.

2.2. Bacterial strain and plasmid

The *A. tumefaciens* strain EHA 105 harboring a binary vector, plasmid pCAMBIA3300/*uidA* was used in the present study. This vector contains the *bar* gene driven by a CaMV 35S promoter and the fragment of *uidA* gene with the CaMV 35S promoter excited from pBI121 in the following order: 35S-*bar*-35S-*uidA*.

2.3. Sensitivity of cell aggregates to PPT

The sensitivity of the uninoculated cell aggregates to PPT was tested in order to determine the optimal concentration of PPT in the selective medium. The uninoculated cell aggregates were cultured on Murashige and Skoog (1962) (MS) medium supplemented with 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg/l carbencillin (Carb) and different concentrations of PPT (0, 0.1, 0.3, 0.5, 0.8, 1.0, 2.0, 5.0, 10.0, 15.0, 20 mg/l) in the dark at 27 ± 1 °C for 6 weeks. The growth of cell aggregates was observed. The experiments were repeated three times with 50 cell aggregates per treatment.

2.4. Transformation, selection and plant regeneration

The *Agrobaterium* single colony was cultured in 25 ml Luria–Bertani (LB) liquid medium containing 50 mg/l kanamycin and 50 mg/l rifampycin on a reciprocal shaker (200 rpm) at 28 °C for 16–18 h until $OD_{600\ nm} = 0.5$ was reached. The bacteria were collected by centrifugation at 5000 rpm for 5 min, washed with LB liquid medium and further with MS liquid medium containing 2.0 mg/l 2,4-D, and then were resuspended in 25 ml MS medium containing 2.0 mg/l 2,4-D for the inoculation. Cell aggregates were infected for 5 min in the bacteria suspensions at room temperature. Following inoculation, the cell aggregates were blotted on sterile filter paper and placed on filter paper in a Petri dish containing 25 ml solid MS medium with 2.0 mg/l 2,4-D and 30 mg/l acetosyringone (AS) and cocultivated for 3 days in the dark at 27 ± 1 °C. After cocultivation, the cell aggregates were washed twice with liquid MS medium containing 2.0 mg/l 2,4-D and 500 mg/l Carb and maintained in liquid MS medium with 2.0 mg/l 2,4-D and 100 mg/l Carb on a reciprocal shaker (100 rpm) at 27 ± 1 °C under 13 h of cool-white fluorescent light at 10 μmol/(m^2 s) for 1 week, and then were cultured at 2-week interval on MS solid medium supplemented with 2.0 mg/l 2,4-D, 100 mg/l Carb and 0.5 mg/l PPT for the selection culture in the dark at 27 ± 1 °C.

Six to eight weeks after selection, the obtained PPT-resistant embryogenic calluses were transferred to MS medium supplemented with 1.0 mg/l abscisic acid (ABA), 100 mg/l Carb and 0.5 mg/l PPT to induce the formation of somatic embryos and regeneration of plantlets at 27 ± 1 °C under 13 h of cool-white fluorescent light at 54 μmol/(m^2 s). The regenerated plantlets were further transferred to the basal medium and developed into whole plants at 27 ± 1 °C under 13 h of cool-white fluorescent light at 54 μmol/(m^2 s).

2.5. GUS assay

The PPT-resistant calluses, and leaves, stems and roots of transgenic plants were tested for GUS expression using histochemical GUS assay as described by Jefferson et al. (1987). The explants were incubated in GUS assay buffer at 37 °C for 12 h. Blue staining of the cells or tissues denoted positive reaction.

2.6. PCR and Southern blot analyses

Genomic DNA was extracted from fresh leaf tissues of in vitro-grown GUS-positive/-negative plants and untransformed control plants by the cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al., 1984). Specific primers for the *bar* gene: ATG AGC CCA GAA CGA CGC and TCT CAA ATC TCG GTG ACG were used. These primers were expected to give products of 550 bp. PCR analysis was done according to the method of Yu et al. (2007).

For Southern blot analysis, 25 μg of transgenic plants and control plants DNA was digested with *EcoR*I. The restriction fragments were size-fractionated by 0.8% (w/v) agarose gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, UK). Coding sequence of the *bar* gene was used as probe. The labeling of probe, prehybridization, hybridization and detection were performed by the protocol of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Germany).

2.7. Northern blot analysis

Total leaf RNA was extracted from in vitro-grown plants of the transgenic plants and the untransformed control plants using the plant RNAtrip kit (Applygen Technologies Inc, China). Equal amounts of total RNA (20 μg) were separated by a denaturing 1.2% (w/v) formaldehyde–agarose gel (Sambrook et al., 1989). The RNA was blotted to a Hybond-N+ nylon membrane. The labeling of probe, prehybridization, hybridization and detection were performed by the protocol of DIG High Prime DNA Labeling and Detection Starter KitII.

2.8. In vivo assay for herbicide resistance

The transgenic plants and the untransformed control plants were transplanted to pots with a mixture of soil and vermiculite (1:1) in a greenhouse for the domestication. They were propagated by cutting, and individuals from 3 cuttings per plant were evaluated for in vivo herbicide resistance at each Basta concentration under greenhouse conditions. The plants were sprayed directly to leaves with 1000 mg/l (normal field dosage) and 2000 mg/l PPT aqueous solution of commercial product Basta (Bayer Crop Science, Germany). Symptoms of the plants were continuously observed for 4 weeks.

3. Results and discussion

3.1. Sensitivity of cell aggregates to PPT

The sensitivity of cell aggregates from embryogenic suspension cultures of sweetpotato cv. Lizixiang to PPT was tested in order to establish an efficient selection system. The PPT concentrations tested ranged from 0.1 mg/l to 20 mg/l. The results showed that PPT concentrations significantly influenced the growth and