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BRIEF COMMUNICATION

Transformation of potato *via Agrobacterium* coated microparticle bombardment

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Abstract

The transformation of potato (*Solanum tuberosum* L. cv. Désirée) was extended by the *Agrobacterium*-mediated biolistic method. Using this approach transgenic shoots could be obtained at a similar frequency to that achieved through conventional biolistics. Leaves from shoot cultures were bombarded with gold particles coated in *Agrobacterium tumefaciens* cells harboring a binary plasmid encoding three genes of interest in the T-DNA. Nine shoots were obtained from 20 shots, with selection of transgenic shoots on a series of media containing progressively increasing concentrations of hygromycin from 5 to 20 mg dm^{-3}.

Additional key words: *Agrobacterium* transformation, *ipt* gene, *Solanum tuberosum,* transgenic potato.

Most transformation procedures developed for potato used *Agrobacterium tumefaciens* (Millam 2004, 2006, Börnke *et al*. 2007) and a variety of traits such as starch modification, insect and virus resistance, abiotic stress tolerance and production of pharmaceutical proteins have been the goals of potato biotechnology (Börnke *et al*. 2007, Byun *et al.* 2007). Biolistic gene transfer, a method by which foreign genes may be introduced into cells *via* high velocity microprojectiles (Klein *et al.* 1988) has been used in transforming monocots such as orchids (Suwanaketchanatit *et al*. 2007) and dicots such as soybean (El-Shemy *et al*. 2007), but has only recently been used in potato. Up to 0.5 transformants per leaf shot were achieved using particle bombardment (Craig *et al.* 2005), almost two orders of magnitude higher than the first report in potato (Romano *et al.* 2003).

In order to further develop transformation systems for potato, an *Agrobacterium*-biolistic-mediated DNA delivery method was applied to the commercial cultivar Désirée. *Agrobacterium*-biolistics is a refinement to microbombardment-mediated transformation developed to counter the frequency with which broken fragments of the transgene and superfluous plasmid DNA are integrated into plant genome (Taylor and Fauquet 2002). This strategy involves bombardment of leaf tissue with gold particles coated with *Agrobacterium* cells. The approach is attractive as large-scale plasmid preparations are not required and preparation and execution of experiments are much simpler. It may also reduce the incidence of multiple copy inserts often associated with biolistic delivery of plasmid DNA (Taylor and Fauquet 2002). However, there is only one report of the production of stable transformed strawberry plants using this method (De Mesa *et al.* 2000). We report here the application of the *Agrobacterium*-biolistic method to potato.

Two step shoot regeneration media and culture conditions for potato shoot cultures were as described previously (Nguyen *et al.* 2005). The only additional medium used here is a root induction medium (RIM) which is similar to the basal medium except 3 % (m/v) Phytagel was used instead of agar and 0.1 mg dm^{-3} IAA was included. The binary vector pVDH396 (15890 bp) (Fig. 1), supplied in *Agrobacterium tumefaciens* LBA4404 strain by Dr. K. Van Dun (Rijk Zwaan Breeding, The Netherlands), contains the *uidA* gene (reporter gene encoding β-glucuronidase) and *hpt* gene (selectable marker gene encoding hygromycin phosphotransferase)

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Abbreviations: CIM - callus induction medium; GUS - β-glucuronidase; LB - Luria and Bertani broth; PCR - polymerase chain reaction, RIM - root induction medium; SIM - shoot induction medium.
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under the control of cauliflower mosaic virus 35S promoters and terminators, and the *ipt* gene (encoding isopentenyltransferase, responsible for the synthesis of the cytokinin, isopentenyladenine) from *Agrobacterium tumefaciens*, driven by the pSAG12 promoter from senescence associated gene 12 of *Arabidopsis thaliana*, and its terminator.

Leaves from axenic potato shoot cultures (Nguyen *et al.* 2005) were used as the target tissue for bombardment experiments. Two larger leaves or 5 leaflets were placed on a plastic 9-cm Petri dish abaxial side up on callus induction medium (CIM; Nguyen *et al.* 2005) either on the day of the bombardment experiments, or one day beforehand. *Agrobacterium tumefaciens* microprojectile preparation was based on the method described by De Mesa *et al*. (2000) with some modifications. An overnight culture of *A. tumefaciens* strain LBA4404 containing the plasmid pVDH396 growing in LB medium at pH 5.8, supplemented with 50 mg dm^{-3} kanamycin and 50 μM acetosyringone, was centrifuged at 70 *g*. Cell pellets were then resuspended in sterile water to an absorbance, A_{600} = 10. Aliquots of 0.1 cm^3 of this culture were transferred to micro-centrifuge tubes and centrifuged at 6 800 *g* for 1 min. The pelleted cells were washed with 1 cm^3 of tris-EDTA (TE) buffer. Cells were centrifuged and resuspended in 0.05 cm^3 of TE by vortexing. An aliquot of 0.025 cm^3 of gold particle solution at a concentration of 60 mg cm^{-3} was centrifuged at 16 000 *g* for 1 min, resuspended in 0.04 cm^3 of the bacterial suspension and vortexed for 20 s. A 0.005 cm^3 aliquot of bacterial/Au particle mixture was spread over the centre of the each macrocarrier. After bombardment the leaf samples were incubated in dim light at 22 °C for 48 h.

Two days after bombardment, the leaf samples were washed in sterile water and blotted dry on sterile filter paper. Bombarded leaves were cut into small pieces (4 × 4 mm) and placed upside down onto CIM containing 5 mg dm^{-3} of hygromycin and 250 mg dm^{-3} cefotaxime. After 1 week, the explants were transferred onto shoot regeneration medium (SIM; Nguyen *et al.* 2005) containing 10 mg dm^{-3} of hygromycin and 250 mg dm^{-3} of cefotaxime. After a further 4 weeks, hygromycin resistant shoots were transferred to rooting medium (RIM) containing 20 mg dm^{-3} hygromycin and 250 mg dm^{-3} cefotaxime.

Transgene presence and integration in transgenic shoots was analysed by PCR and Southern blotting. For PCR analysis, total plant DNA from wild type and hygromycin resistant calli or shoots was isolated using the *DNeasy® Plant Mini Kit* (*Qiagen*, Valencia, CA, USA), following the kit instructions. The presence of *uidA* and *hpt* was confirmed by PCR with primer pairs described previously (Craig *et al*. 2005) generating 365 and 508 bp products respectively. *Ipt* was screened for with the following primer pair: ipt forward 5'-TCAACCGGAAGCGGACGACC-3', reverse 5'-GCC ATGTTGTTTGCTAGCCA-3' product size 355 bp. The thermal cycling programs for *uidA* and *hpt* were as described (Craig *et al*. 2005). The thermal cycling program for *ipt* primers was set at 94 °C for 5 min, followed by 35 cycles at 1 min at 94 °C, 30 s at 60 °C and 1 min at 72 °C with a final extension at 72 °C for 7 min.

For Southern blots, genomic DNA was extracted from leaves according to Frey (1999) with some modifications. Leaf tissue was ground in lysis buffer (20 mM Tris, pH 8.0; 20 mM of EDTA; 2 M NaCl), incubated at 85 °C for 5 min and then placed on ice for 5 min. This step was repeated 3 times. The microcentrifuge tubes were vortexed, and then centrifuged at 16 000 *g* for 10 min. The supernatant was RNase A (30 mg cm^{-3}) treated and DNA precipitated and then resuspended in sterile water to a final concentration of 1 mg cm^{-3}. Southern blots were performed according to the method of McCabe e*t al*. (1997) with *Hind*III digested DNA. PCR DIG-labelling kit (*Roche Applied Science*, Indianapolis, USA) was used to generate the probes. Bands on blots were visualised after incubation with chemiluminescent CDP-star for 5 min, sealed in a plastic bag and hybridisation signals were detected by exposing the membrane to *Kodak* X-ray film for 1 - 10 min.

Bombardment of potato leaves with gold particles coated with the large binary plasmid pVDH396 did not yield any transformed shoots (data not shown). We then tested the integration of the three genes using *Agrobacterium*-biolistic mediated transformation of potato with the strain LBA4404 carrying plasmid pVDH396 (*hpt-ipt-uidA*). Selection and transformation carried out using the same conditions for biolistic transformants (Craig *et al*. 2005), yielded 9 hygromycin resistant lines from 20 shots (Table 1). All hygromycin resistant lines were tested in numerous PCRs for the presence of *ipt, uidA* and *hpt*. Representative gels of some of these PCRs are shown (Fig. 1*B*,*C*,*D*). All lines were positive for *hpt*, but some truncated T-DNA transfer may have occurred, as two transgenic lines did not carry a *uidA* insert (lines D2 and D3) and one line did not carry an *ipt* inserted gene (line D9, not shown). Seven of these 9 hygromycin resistant shoots were GUS positive according to histochemical assays (data not shown), corresponding to the same lines which were positive by PCR for *uidA* (Table 1). Genomic DNA from the 8 lines which were positive with PCR for *ipt* was extracted and digested with *HindIII*. In a representative blot, as expected, a band about 1.8 kb (the correct size of the inserted fragment) which hybridised with the DIG-

Table 1. Summary of the molecular analysis of pVDH496 transgenic potato plants generated using *Agrobacterium*-biolistics. Values are the number of positive plants over the total number tested.

Transgenic type	GUS assay	PCR			Southern blot
		uidA	*hpt*	*ipt*	*ipt*
LBA4404/pVDH496	7/9	7/9	9/9	8/9	8/8

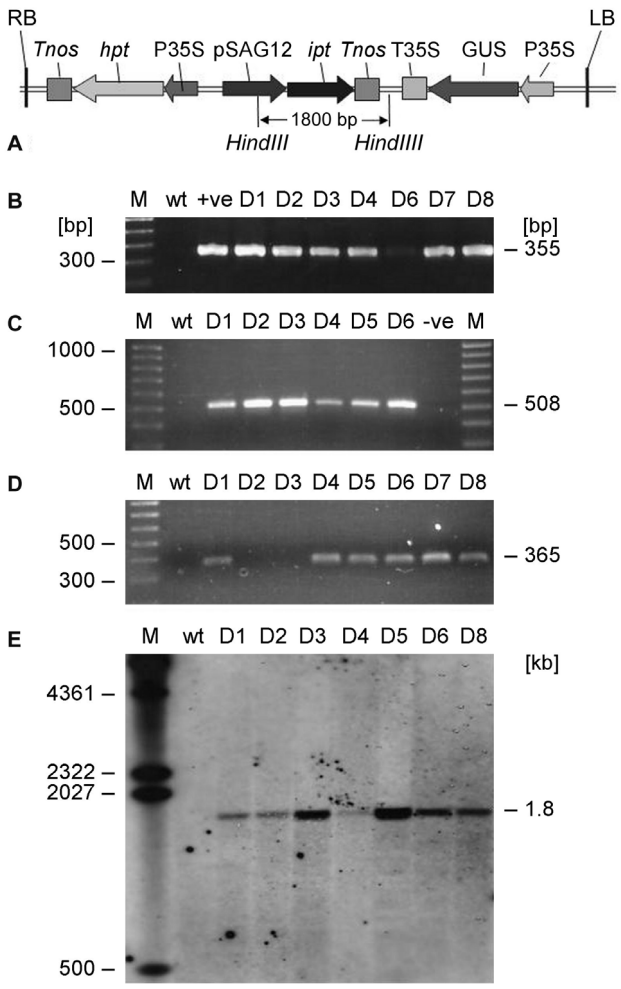

Fig. 1. Generation and PCR and Southern analysis of transgenic potato plants by *Agrobacterium*-biolistics transformation. *A*: Genetic map of T-DNA of binary vector pVDH396. GUSINT - *uidA* gene (GUS) with an intron, *hpt* - hygromycin phosphotransferase gene, *ipt* - isopentenyl transferase gene from *Agrobacterium tumefaciens*, LB - left border, pSAG-12 - promoter from senescence associated gene 12 of *Arabidopsis thaliana*, P35S - CaMV 35S promoter, RB - right border, Tnos - nopaline synthase terminator, T35S - 35S terminator. *B*: PCR using internal primers shows the presence of *ipt* in resistant plants *via* the 355 bp band. *C*: PCR using internal primers shows the presence of *hpt* in resistant plants by a 508 bp band. *D*: PCR using internal primers shows the presence of *uidA* in resistant plants by a 365 bp band. *E*: Southern blot of transformants was probed with a 355 bp *ipt* fragment. A band of approximately 1 800 bp indicates presence of T-DNA in genomic DNA. Each lane contained 10 μg of genomic DNA digested with *HindIII*. M - contains DIG-labelled DNA molecular mass marker II (*Roche*). For gels *B* - *D*: M - 100 bp ladder molecular mass marker (*Sigma*), wt - wild type *S. tuberosum* cv. Désirée DNA, D1 - D8: putative transformants, +ve - positive control (pVDH396), -ve - negative control, no DNA.

labelled *ipt* probe was observed only in the transgenic plants (Fig. 1*E*).

Whilst transgenic strawberry plants were obtained at a 2.9 times higher transformation frequency, compared with that obtained with *Agrobacterium*-mediated transformation, using this method (De Mesa *et al.* 2000), we made no direct comparison with *Agrobacterium*-mediated potato transformation, however the transformation frequency was similar to that previously reported using conventional biolistics with plasmid DNA (Craig *et al.* 2005). This suggests important time and cost savings using the *Agrobacterium*-biolistics method, especially where 3 or more genes are to be transferred. Indeed, biolistic experiments with the purified large pVDH396 plasmid failed to deliver any transformants in our hands (data not shown), further militating in favour of the *Agrobacterium*-biolistics approach. However transgenic potato plants generated by bombardment containing 3 genes from one plasmid or from 3 separate plasmids has been reported (Romano *et al*. 2003). Using a single plasmid is preferable as 83 % of transgenics expressed all three genes compared to only 6 % when the three genes were co-bombarded on separate plasmids (Romano *et al*. 2003). Using *Agrobacterium*-biolistics could be a simpler option as we have shown that three genes are present in the majority of our transgenics, but we only examined expression for two of these. There is potential therefore to co-bombard with more than one *Agrobacterium* strain containing separate binary vectors to transfer multiple genes. However the gene integration and expression patterns would need to be determined precisely in a number of transgenics before the system could be used routinely.

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