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Generation of homoplasmic plastid transformants of a commercial cultivar of potato (*Solanum tuberosum* L.)

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Abstract

This report describes the integration and expression of foreign genes into the plastid genome of a commercial cultivar of potato. Plastid transformation of potato was achieved using two tobacco specific plastid transformation vectors, pZS197 (P*rrn*/*aadA*/ *psbA3′*) and pMSK18 (*trc*/*gfp*/P*rrn*/*aadA*/ *psbA3′*). Selection was for spectinomycin resistance after biolistic delivery of plasmid DNA into leaf cells of *Solanum tuberosum* cv. Desiree. Ten transplastomic lines were obtained from 179 bombarded samples with vector pZS197 and four transplastomic lines selected out of 103 bombarded samples with vector pMSK18. Southern blot and PCR analyses confirmed homoplasmy in the primary regenerants, and incorporation of the *aadA* and *gfp* genes into the potato plastid genome by two homologous recombination events via the flanking plastid DNA sequences. Fluorometric measurements confirmed GFP expression in leaves and tubers of pMSK18 lines. No transformants were obtained with a third tobacco vector, pNtcZ7 (P*rrn*/*gfp*/ *psbA3′*/*trc*/*aadA*/*rrnB-ter*) in which the selectable marker gene is driven by a bacterial (*trc*) promoter, which does permit selection of plastid transformants in tobacco, and allows low level expression of the reporter gene, *gfp*, in potato.

Keywords: Plastid transformation; Chimeric *aadA* gene; *gfp* gene; Particle bombardment; Transplastomic potato

1. Introduction

Plastid transformation in higher plants offers some advantages over nuclear transformation, including maternal inheritance of transgenes, no position effects, as the genes of interest are introduced into the plastome via homologous recombination, and high level of foreign protein expression [1–3].

Plastid transformation in higher plants was first successfully carried out in tobacco using a mutant plastid 16S ribosomal RNA gene for selection [4], but it is most commonly reported with vectors containing a chimeric bacterial *aadA* gene, which confers resistance to spectinomycin and streptomycin [5]. Chloroplast transformation is a routine procedure only for tobacco, and extending it to other species is most important if the potential of the plastid as a production platform for large amounts of recombinant protein is to be realised. Plastid transformation has now been reported in *Arabidopsis thaliana* [6], potato [7], rice [8], tomato [9], *Brassica napus* [10] and *Lesquerella fendleri* [11]. Biolistics have been used for DNA delivery in all these studies. However, transformation efficiencies are at least 10 times lower than reported for tobacco. It can also be difficult to achieve homoplasmic transformants, where all plastid genome copies contain the introduced genes [8].

Plastid transformation in potato was first described by Sidorov et al. [7] using a breeding line, and this remains the sole report. In the current report we describe the reproducible plastid transformation of an important commercial

Abbreviations: CIM, callus induction medium; GA_3, gibberelic acid; IAA, indole-3-acetic acid; MS, Murashige and Skoog (1962); SIM, shoot induction medium

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potato cultivar, *Solanum tuberosum* cv. Desiree, and demonstrate the importance of a strong plastid promoter to obtain transformants.

2. Materials and methods

2.1. Plastid transformation vectors

Plasmid pZS197 (P*rrn*/*aadA*/T *psbA3′*) was constructed for high frequency plastid transformation in tobacco [5]. The chimeric *aadA* gene is under the control of the ribosomal RNA operon promoter (P*rrn*) and the 3′ untranslated region (UTR) of the plastid psbA gene and was cloned between the plastid *rbcL* and *accD* genes for targeting to the large single copy (LSC) region of chloroplast genome. Plasmids pMSK18 (*trc*/*gfp*/P*rrn*/*aadA*/ *psbA3′*-UTR) and pNtcZ7 (P*rrn*/*gfp*/ *psbA3′*-UTR/*trc*/*aadA*/*rrnB-ter*) were also designed for chloroplast transformation in tobacco [12]. They both insert between the coding regions for 16S rRNA and orf70B in the inverted repeat region. In pMSK18, the *gfp* coding region is under the control of the bacterial *trc* promoter, while P*rrn* drives expression of the selectable marker gene *aadA*. In pNtcZ7 this situation is reversed.

2.2. Plastid transformation procedure

Solanum tuberosum cv. Desiree was obtained from the Department of Agriculture, Food and Rural Development, Ireland. Stock shoot cultures were grown in vitro in Magenta G7 (Sigma) containers on growth regulator free Murashige and Skoog [13] (MS) medium with Gamborg's B5 vitamins [14] containing 30 g/L sucrose, and solidified with 0.8% (w/v) agar. Media were adjusted to pH 5.7 and autoclaved for 20 min at 121 °C. Shoot cultures were grown at 22 °C under a 16 h light/8 h dark light regime. Three to four weeks after each subculture, four to five dark green leaves per rooted plant were harvested and the upper nodes were subcultured on MS medium as described above. Bombardment: two to four leaves were placed adaxial side up onto callus induction medium (CIM) containing MS salts with B5 vitamins (Duchefa), 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.9 mg/L zeatin riboside (ZR), 16 g/L glucose [15] for 24 h before bombardment. Leaves were bombarded with gold particles (0.6 μm diameter) coated with plasmid DNA using a PDS 1000/He Biolistic gene gun (BioRad). A rupture disc pressure of 1100 psi, partial vacuum pressure between 25 and 28 in Hg and a target distance of 6 cm was used for bombardment. Two to three days after bombardment the leaves were cut into 3 mm × 3 mm pieces and placed on CIM containing 300 mg/L spectinomycin and incubated under dim light in a 16 h light/8 h dark regime for 4 weeks. The leaf explants were transferred to shoot induction medium (SIM) containing MS salts with B5 vitamins, 3.0 mg/L ZR, 2.0 mg/L indole acetic acid (IAA), 1.0 mg/L gibberellic acid (GA_3), 16 g/L glucose and 300 mg/L spectinomycin and subcultured to the same selective medium at 3-weekly intervals. Spectinomycin resistant shoots obtained in 8–10 weeks were identified as green shoots on bleached leaf explants. Shoots were subcultured to growth regulator-free MS medium (RM) with 400 mg/L spectinomycin, for root formation. Rooted plants were transferred to soil in pots and grown to maturity in a growth room with the same temperature and daylength settings as used for the in vitro culture. Leaf explants and tuber pieces from putative transformants were tested on SIM medium for resistance to both spectinomycin (300 mg/L) and streptomycin (300 mg/L).

2.3. PCR and Southern blot analyses of total cellular DNA

Total leaf cellular DNA was extracted from plants using a method described previously [16]. Polymerase chain reactions (PCRs) were carried out using primers specific for the chimeric *aadA* or *gfp* genes and analysis for homoplasmy used a pair of primers (RBCL/ACCD) or (pSSH-rev/trnV-*Solanum*) flanking the transgene insertion site in the potato chloroplast genome. Primers used for screening transformants and probe synthesis are as follows:

Oligo name	Sequence (5′–3′)
RBCL	CAGAGACTAAAGCAAGTGTTG
ACCD	CATGTCTTCATCCATAGGA
pSSH-rev	TCTTGATCAATCCCTTTGCCCTC
trnV *Solanum*	CATGTCTTCCATCCATAGGA
RB197F	GTCTACTTCTTCACATCCACC
RB197R	TCCATACTTCACAAGCAGC

PCR was performed with the AccuTaq kit (Sigma). The PCRs were run as follows: denaturing 94 °C, 30 s, annealing 51–63 °C (depending on the Tm) 15 s and extension 68 °C 4–6 min; for 30 cycles.

For Southern blot analysis, 5–7 μg of total DNA were digested with *Eco*RI and *Eco*RV, separated in a 0.8% (w/v) agarose gel (16 h, 25–30 V) and transferred to a nylon membrane (Hybond N^+; Amersham, Dublin, Ireland). Signal detection was performed using a non-radioactive DNA labeling [17] and detection protocol (DIG Probe Synthesis Kit, Roche, Mannheim, Germany). Plasmid pZS197 was used as template for synthesis of the probe using RB197F and RB197R primers. Digoxigenin-labeled PCR product was used for membrane hybridization. Immunological detection was performed using a chemiluminescent substrate (CDP-star, Roche). Signals were visualized by exposure to Kodak X-ray film for 1–5 min.

2.4. Detection of GFP in transplastomic potato

Total soluble protein was extracted from transplastomic lines in a buffer containing 0.1 M Na_2CO_3, pH 9.6 [18]. The protein concentration was determined using a BioRad protein assay kit. Potato leaf or tuber samples were