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Dicistronic expression of the green fluorescent protein and antibiotic resistance genes in the plastid for selection and tracking of plastid-transformed cells in tobacco

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Abstract A plastid transformation vector was constructed for dicistronic expression of the aminoglycoside 3'-adenyltransferase (*aadA*) and green fluorescent protein (*gfp*) genes under the control of the plastid *rrn* promoter. Gold particles coated with the vector DNA were bombarded onto tobacco leaf explants using a particle delivery system. Leaf explants produced adventitious shoots when cultured on shoot-inducing medium containing 500 mg l⁻¹ spectinomycin. Shoots that exhibited green fluorescence under UV light were selected. Southern blot analysis detected the presence of the *aadA* and *gfp* genes between *trnA* and *trnI* in the plastid genome. Northern blot analysis revealed that the *aadA* and *gfp* genes were both properly transcribed into a dicistronic transcriptional unit. The expression of the *gfp* gene in the plastid enabled separation of transformed chloroplasts from wild-type chloroplasts in the protoplast under a fluorescent microscope. The overall results indicate that dicistronic expression of the *aadA* and *gfp* genes in the plastid simplifies gene manipulation, facilitating selection and tracking of plastid-transformed cells.

Keywords *Nicotiana tabacum* L. · Operon · Plastid transformation

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Abbreviations *aadA*: Aminoglycoside 3'-adenyltransferase · *FITC*: Fluorescein isothiocyanate · *gfp*: Green fluorescent protein · *MS*: Murashige and Skoog

Introduction

Plastid transformation in higher plants provides advantages over nuclear transformation. One of the most prominent advantages is expression of multiple heterologous genes under the control of a single promoter in the plastid, which simplifies gene manipulation (De Cosa et al. 2001).

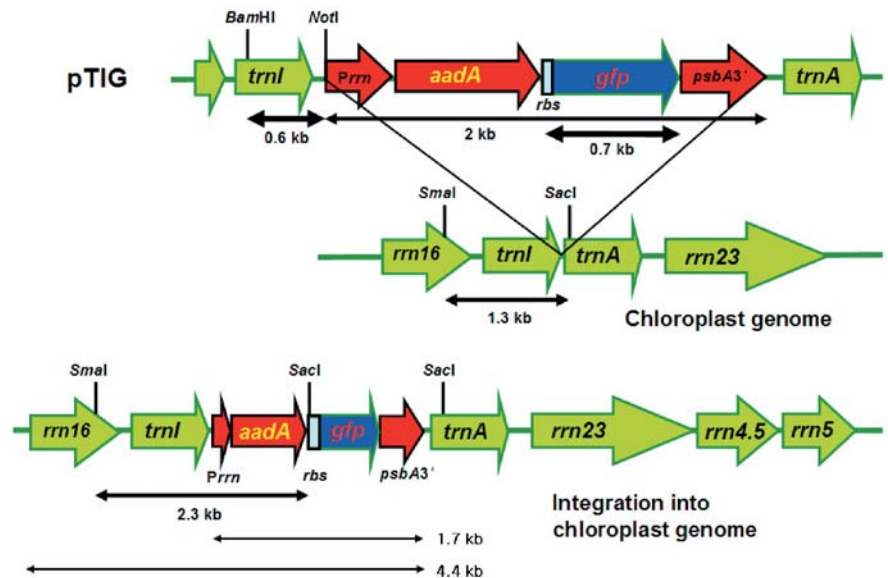
The aminoglycoside 3'-adenyltransferase (*aadA*) gene, which confers spectinomycin resistance, has been used for selection of cells with transformed plastids. The green fluorescent protein (*gfp*) gene from *Aequorea victoria* has been widely used for molecular tracking in cell and molecular biology. The *aadA* and *gfp* genes have been introduced into the plastid genome of potato and expressed under the control of *psbA* and *rrn* promoters, respectively (Sidorov et al. 1999). A chimeric gene comprising the *aadA* and *gfp* genes was constructed and incorporated into the plastid genome of tobacco (Khan and Maliga 1999). However, the capacity of the plastid for dicistronic expression of the *aadA* and *gfp* genes has not been tested. We constructed a dicistronic expression vector for the *aadA* and *gfp* genes under the control of a single promoter and incorporated the genes into the plastid genome of tobacco.

Materials and methods

Plant material

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were maintained in flasks containing Murashige and Skoog (MS) (1962) basal medium. Leaf blades (approximately 3 cm long) were excised and were subjected to particle bombardment.

Fig. 1 Plastid transformation vector (*pTIG*) containing the aminoglycoside 3'-adenyltransferase (*aadA*) and green fluorescent protein (*gfp*) genes under the control of the *rrn* promoter (*Prrn*) that targets integration of the *aadA* and *gfp* genes into the inverted repeat region of the tobacco plastid genome. Probes used for Southern (0.6 kb) and northern blot analyses (0.7 kb) are marked with *thick lines*. Possible dicistronic (1.7 kb) and polycistronic transcription units (4.4 kb) are marked with *thin lines*



Construction of a dicistronic expression vector in the plastid

A plastid transformation vector was constructed by referring to pSBL-ctV2 (Guda et al. 2000) for dicistronic expression of the *aadA* and *gfp* genes under the control of the plastid *rrn* promoter and was named pTIG (Fig. 1). The antibiotic resistance gene *aadA* from the *Shigella sonnei aadA* gene (GenBank accession no. U57972) was cloned by PCR. The *gfp* gene from the plasmid pSMGFP (Davis and Vierstra 1998) was cloned by PCR using primers based on the *gfp* sequence (GenBank accession no. U70495). The *aadA* gene was connected by a linker sequence (aggaggtataaca) to the 5' end of the *gfp* gene. The *aadA* and *gfp* genes were targeted to integrate into the inverted repeat region of the tobacco plastid genome (Guda et al. 2000). *Escherichia coli* exhibited green fluorescence under UV light when transformed with the vector pTIG, confirming that the vector was correctly constructed.

Biolistic DNA delivery of the vector on leaf explants

Leaf blades were placed abaxial side up onto filter paper discs (70 mm in diameter; Advantec Toyo, Tokyo Japan) on MS medium supplemented with 4.44 μM 6-benzylaminopurine and 0.54 μM α -naphthaleneacetic acid (shoot-inducing medium) in plastic Petri dishes (87 \times 15 mm). Leaf blades were subjected to bombardment by gold particles (0.6 μm) coated with vector DNA using a particle delivery system (PDS 1000/He, Bio-Rad, Hercules, Calif.) at 28 inch (711 mm) Hg using 1,100 psi (7,580 MPa) rupture disks. Bombarded leaf blades were placed in Petri dishes and maintained in the dark for 48 h. Leaf blades were then cut into 5 \times 5 mm explants before being placed adaxial side up onto shoot-inducing medium (15 leaf explants per dish) and cultured. Unless mentioned otherwise, all cultures were maintained under light (approximately 3 W m⁻² from cool-white fluorescent lamps with 16-h photoperiods) at 25°C.

Selection of putative plastid-transformed adventitious shoots

Adventitious shoots formed on leaf explants were illuminated with UV light (365 nm, model UVL-56, UVP). Shoots emitting green fluorescent light were selected as putative plastid-transformed shoots. To remove chimeric shoots, leaves of the selected shoots were cut into 3 \times 3 mm explants and placed onto shoot-inducing medium (15 leaf explants per dish). Putative plastid-transformed

shoots were selected after repeating the same procedure, and then transferred onto MS basal medium supplemented with 500 mg l⁻¹ spectinomycin to induce rooting. Putative plastid-transformed plantlets were acclimated, transplanted to potting soil, and then maintained in a growth chamber (27°C; approximately 15 W m⁻² from cool-white fluorescent lamps with a 16-h photoperiod).

Observation of GFP expression at the cellular level

Protoplasts were isolated from leaves of putative plastid-transformed plants and wild-type plants using an enzyme solution containing 2% cellulase Onozuka R-10 (Yakult, Tokyo, Japan) and 0.5% Macerozyme R-10 (Yakult) by incubation at 25°C for 4–8 h. Isolated protoplasts were observed under a fluorescent microscope (Axioskop, Zeiss, Oberkochen, Germany) using a fluorescein isothiocyanate (FITC) set (excitation 450–490 nm, dichroic mirror 510 nm) and a bandpass filter (515–561 nm) to determine whether transformed chloroplasts with the *gfp* gene can be distinguished from wild-type chloroplasts in protoplasts.

Southern blot analysis

Total genomic DNA was extracted from leaves of one wild-type plant and two independent transgenic plants at the same developmental stage and maintained in the same growth chamber using a DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was digested with *SmaI* and *SacI*. Approximately 4 μg digested DNA was electrophoresed on a 1% agarose gel and blotted onto Zeta-Probe GT Blotting Membrane (Bio-Rad). The 0.6 kb *BamHI-NotI* DNA fragment containing the *trnI* gene was labeled with [α -³²P] dCTP and used as a probe to detect insertion of the *aadA* and *gfp* genes into the plastid genome. Prehybridization and hybridization were performed in 0.25 M sodium phosphate (pH 7.2) buffer and 7% (w/v) SDS at 58°C overnight. The membranes were washed twice in 20 mM sodium phosphate (pH 7.2) buffer and 5% (w/v) SDS at 58°C for 10 min.

Northern blot analysis

Total RNA was extracted from leaves of plants described in Southern blot analysis using TRIzol Reagent (Invitrogen, Carlsbad, Calif.) following the manufacturer's instructions. Approximately 20 μg total RNA was subjected to electrophoresis using an agarose

gel containing 5.1% (v/v) formaldehyde and blotted onto a Zeta-Probe GT Blotting Membrane (Bio-Rad). The 0.7 kb *SacI* DNA fragment containing the *gfp* gene was labeled with [α - 32 P] dCTP and used as a probe to detect a dicistronic transcript unit. Prehybridization and hybridization was performed in 0.25 M sodium phosphate (pH 7.2) buffer and 7% (w/v) SDS at 58°C overnight. The membranes were washed twice in 20 mM sodium phosphate (pH 7.2) buffer and 5% (w/v) SDS at 58°C for 10 min.

Results and discussion

Production of plastid-transformed plants

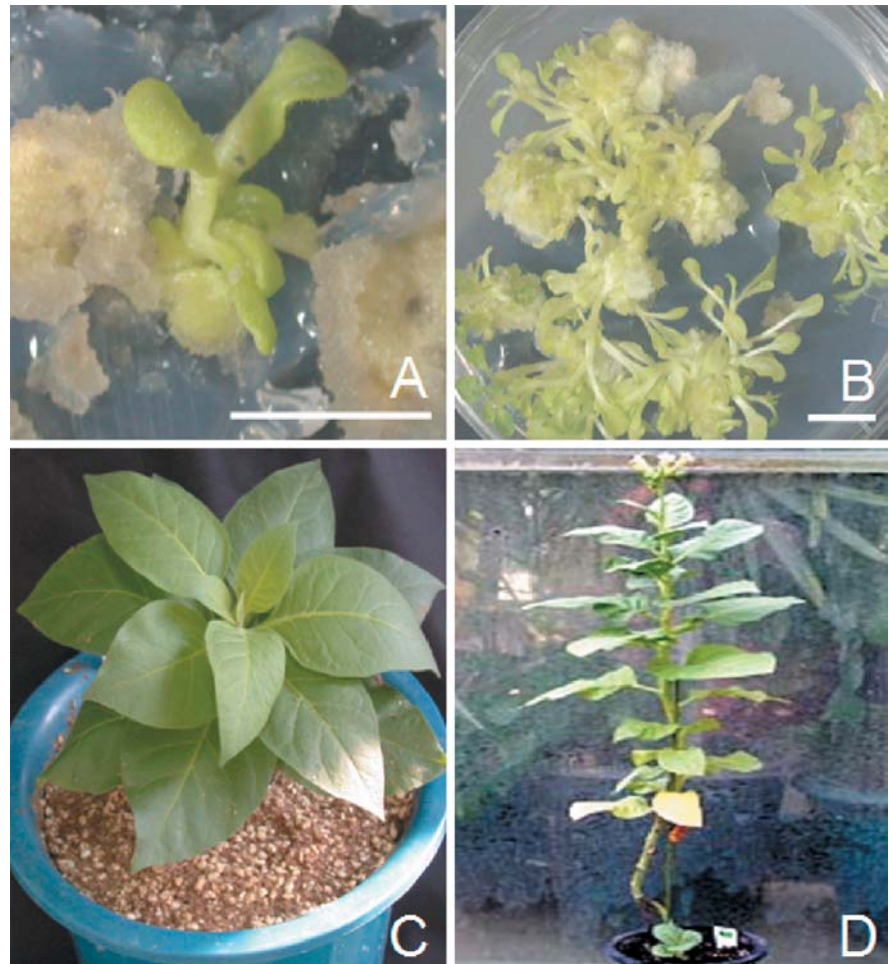
Adventitious shoots were formed on leaf explants in 30–40% of Petri dishes after 4–5 weeks of culture (Fig. 2A). Among Petri dishes that contained leaf explants with adventitious shoots, 5–10 shoots were produced per dish. Approximately 60% of shoots emitted green fluorescence light after exposure to UV light, indicating expression of the *gfp* gene. Shoots that did not exhibit green apparently developed spectinomycin resistance via spontaneous mutation in the plastid genome. Numerous adventitious shoots formed on leaf explants were selected after 4–5 weeks of culture in a second round selection (Fig. 2B). The selected shoots were rooted at a frequency of 80–90%

after 3 weeks of culture. Plantlets transplanted to potting soil (Fig. 2C) were grown to maturity (Fig. 2D). Mature plants appeared morphologically normal.

Tracking of cells

Chloroplasts in protoplasts isolated from putative plastid-transformed plant leaves (Fig. 3A) appeared red, green, or yellow under UV light, whereas chloroplasts from wild-type plant leaves appeared red (Fig. 3B). Green chloroplasts expressed the *gfp* gene and yellow chloroplasts appeared to be due to a mixture of green fluorescence due to *gfp* gene expression and red fluorescence due to chlorophyll (Fig. 3B). Chloroplasts from putative plastid-transformed plant leaves were green under fluorescent light with an FITC filter, whereas chloroplasts from wild-type plant leaves appeared dark (Fig. 3C). These results indicate that the *gfp* gene was properly expressed in the plastid-transformed plant and that expression of the *gfp* gene facilitates tracking of cells.

Fig. 2A–D Production of plastid-transformed tobacco plants. **A** Adventitious shoots formed on leaf explant in first round selection. **B** Adventitious shoots formed on leaf explants in second round selection. **C** A plastid-transformed plant transplanted to potting soil. **D** A plastid-transformed plant grown to maturity. Bars 1 cm



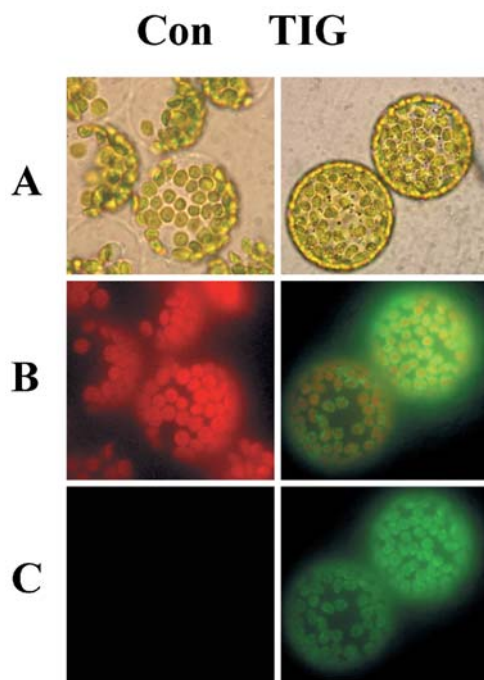


Fig. 3A–C GFP expression in chloroplasts of protoplasts isolated from tobacco tissue. *Con* Protoplasts isolated from leaf tissues of a wild-type plant, *TIG* protoplasts isolated from leaf tissues of plastid-transformed plants. **A** Bright field, **B** UV light without filter, **C** UV light with a fluorescein isothiocyanate (FITC) filter set

Confirmation of plastid-transformed plants

The *aadA* gene was detected by PCR in DNA extracts from 12 of 13 plants tested (data not shown). Southern blot analysis using a *trnI* DNA fragment as a probe detected a 1.3 kb band from a wild-type plant and a 2.3 kb band from two independent plastid-transformed plants, indicating insertion of the *aadA* and *gfp* genes between *trnI* and *trnA* in the plastid genome (Fig. 4A). Southern blot analysis revealed that these plastid-transformed plants were homoplasmic for the transgenes (Fig. 4A). Northern blot analysis using a *gfp* DNA fragment as a probe detected no band from a wild-type plant, but detected more than three bands, including 1.7, 2.5, and 4.4 kb bands, from two independent plastid-transformed plants (Fig. 4B). These results indicate that the *aadA* and *gfp* genes were both properly transcribed into the 1.7 kb dicistronic transcription unit (Fig. 1) as well as two polycistronic units (2.5 and 4.4 kb) thought to be connected to intrinsic rRNA transcripts. The 4.4 kb polycistronic unit can be ascribed to the plastid DNA region containing the integrated *aadA* and *gfp* genes (Fig. 1). However, the 2.5 kb polycistronic unit is not readily defined, not least because many plastid genes organized into polycistronic transcriptional units in higher plants give rise to complex sets of overlapping mRNAs (Sugita and Sugiura 1996).

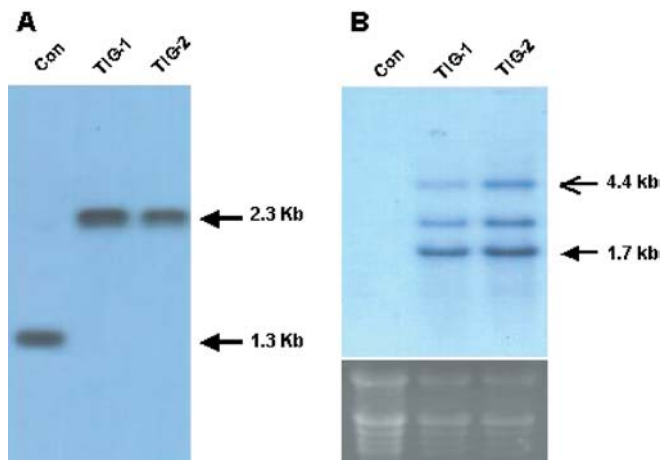


Fig. 4A, B Southern and northern blot analyses of putative plastid-transformed tobacco plants. **A** Southern blot analysis using the 0.6 kb DNA fragment of the *trnA* gene as a probe. **B** Northern blot analysis using the 0.7 kb DNA fragment of the *gfp* gene as a probe. The two probes are marked on the physical map in Fig. 1. *Con* Wild-type plant; *TIG-1*, *TIG-2* two independent plastid-transformed plants. Bands below the northern blot show ethidium bromide staining of rRNA to indicate equal loading of RNA in all lanes

Dicistronic expression of the *aadA* and *gfp* genes in the plastid

Because plastid transformation is accomplished through a gradual process, simultaneous expression of the *aadA* and *gfp* genes in the plastid facilitates selection and tracking of cells during the process. Sidorov et al. (1999) introduced the *aadA* and *gfp* genes under the control of the *psbA* and *rrn* promoters, respectively, into the plastid genome of potato. Khan and Maliga (1999) transformed the plastid of tobacco with a chimeric gene of the *aadA* and *gfp* genes. The chimeric gene comprising the *aadA* and *gfp* genes has the advantage of preventing physical separation of the two genes and simplifying gene manipulation once the chimeric gene is constructed. However, the protein encoded by the chimeric gene is a new artifact for which environmental safety has not been proven. Therefore, use of the chimeric gene requires more caution in extension of studies to plants grown outdoors. Furthermore, the approaches of Sidorov et al. (1999) and Khan and Maliga (1999) require more complicated gene manipulation for construction of vector and chimeric gene, respectively, than the approach in which we expressed the *aadA* and *gfp* genes under the control of a single promoter (*Prrn*). The dicistronic expression employed in this study simplified gene manipulation without producing a new artifact. It has reported that dicistronic or polycistronic expression of transgenes in the plastid enables the coordinated expression of such genes. The *aadA* gene has been expressed together with the petunia 5-enol-pyruvate shikimate-3-phosphate synthase gene (Daniell et al. 1998), the *Bacillus thuringiensis* *Cry2Aa2* operon (De Cosa et al. 2001), or the yeast trehalose

phosphate synthase gene (Lee et al. 2003) under the control of a single promoter in the tobacco plastid.

The number of adventitious shoots formed on explants was not reduced by dicistronic expression of the *aadA* and *gfp* genes in the plastid when compared with the number of shoots obtained by expression of the *aadA* gene alone (data not shown). In addition, dicistronic expression of the *aadA* and *gfp* genes did not lead to morphological aberrations of the plants, indicating that the *gfp* gene product is not toxic to plant cells. Non-toxicity of *gfp* gene expression in the plastid is in agreement with the findings of Sidorov et al. (1999). Overall, the results indicate that dicistronic expression of the *aadA* and *gfp* genes in the plastid simplifies gene manipulation, facilitating selection and tracking of cells.

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