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Stable transformation of petunia plastids

Mikhajlo K. Zubko[†], Elena I. Zubko[†], Karen van Zuilen[1], Peter Meyer[2] & Anil Day[1,*]
[1]*School of Biological Sciences, The University of Manchester, 3.614 Stopford Building, Oxford Road, Manchester M13 9PT, UK*
[2]*Leeds Institute for Plant Biotechnology and Agriculture (LIBA), Centre for Plant Sciences, The University of Leeds LS2 9JT, UK*

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Abstract

Plastid transformation results in stably expressed foreign genes, which for most Angiosperms are largely excluded from sperm cells, thereby greatly reducing the risk of foreign gene spread through pollen. Prior to this work, fertile plastid transformants were restricted to tobacco, tomato and *Lesquerella*. Application of plastid engineering in the important floriculture industry requires the development of stable plastid transformation in a major ornamental plant species such as *Petunia hybrida*. Here we describe the successful isolation of fertile and stable plastid transformants in a commercial cultivar of *P. hybrida* (var. Pink Wave). Plastid targeting regions from tobacco were used to integrate *aad*A and *gusA* between the *acc*D and *rbc*L genes of *P. hybrida* plastid DNA following particle bombardment of leaves. For three spectinomycin and streptomycin resistant lines, DNA blot analysis confirmed transgene integration into plastid DNA and homoplasmy. Maternal inheritance and homoplasmy resulted in 100% transmission of spectinomycin resistance to progeny after selfing. Plastid transformants expressed the *gusA* gene uniformly within leaves and to comparable levels in all three lines. Insertion of trait genes in place of *gusA* coding sequences enables immediate applications of our plastid transformation vector. Establishment of plastid transformation in *P. hybrida* facilitates a safe and reliable use of this important ornamental plant for research and plant biotechnology.

Introduction

The insertion of genes into plastids (Bock, 2001; Maliga, 2003) is an attractive alternative to conventional nuclear engineering for constructing transgenic plants with value added traits such as herbicide (Daniell et al., 1998; Iamtham & Day, 2000; Lutz et al., 2001) and insect resistance (McBride et al., 1995; Kota et al., 1999; De Cosa et al., 2001). Plastid engineering is a clean gene transformation process allowing the precise integration of desirable foreign trait genes, or site-directed modification of native plastid genes, without marker genes or vector sequences. Homologous recombination (Iamtham & Day, 2000; Klaus et al., 2004) or site-specific recombinases (Corneille et al., 2001; Hajdukiewicz et al., 2001) allow efficient excision of marker genes. In many crops, plastids are inherited maternally preventing the pollen mediated spread of plastid-localised transgenes. Silencing resulting in variable and unpredictable nuclear gene expression is not associated with plastid transgenes. Plastid transformants exhibit uniform foreign

[*]Author for correspondence (E-mail: anil.day@man.ac.uk)
[†]These two authors contributed equally to this work.

gene expression and foreign gene products can accumulate to extraordinarily high levels, 5–47% of total soluble protein (Staub et al., 2000; De Cosa et al., 2001; Tregoning et al., 2003).

Stable plastid transformation of land plants was first established in *Nicotiana tabacum* (Svab et al., 1990; Svab & Maliga, 1993) and *N. plumbaginifolia* (Oneill et al., 1993). More recently, stable plastid transformation has been demonstrated in *Arabidopsis thaliana* (Sikdar et al., 1998), potato (Sidorov et al., 1999), tomato (Ruf et al., 2001) and *Lesquerella fendleri* (Skarjinskaia et al., 2003). Fertile plastid transformants are limited to tobacco, tomato and *L. fendleri*. Rice (Khan & Maliga, 1999) and oilseed rape (Hou et al., 2003) appears to be responsive to plastid transformation but as yet homoplasmic plants have not been obtained. The small number of species amenable to plastid transformation has limited the widespread application of plastid engineering in transgenic research related to plant biotechnology.

Ornamental plants, used in the floriculture industry, are attractive targets for genetic modification with performance-enhancing transgenes such as those conferring herbicide and pest resistance. Whilst food safety aspects do not arise there is still a need for measures to minimise transgene escape into the environment, by for example, introducing foreign genes into plastids. Here we describe for the first time plastid transformation of an ornamental plant species. We have isolated stable and fertile plastid transformants in a commercial cultivar of *Petunia hybrida*. The work will facilitate new applications of plastid transformation in the floriculture industry and allow fundamental studies on the plastid genetic system of *P. hybrida*, a species that is tractable to genetic analysis.

Materials and methods

Vector construction

Plastid transformation vector pUM73(AD) was constructed by standard cloning techniques in *Escherichia coli* Top10 (Invitrogen). A linker containing *Not*I and *Apa*I sites, made by annealing oligonucleotides NotApa-F (5′ TTAGGGC CCGGGAAAGCGGCCGC-3′) and NotApa-R (5′TAAGCGGCCGCTTTCCCGGGCCC-3′), was inserted into the unique *Aoc*I site of pTB27 (Sugiura et al., 1986). Expression cassettes containing the *aad*A and *gusA* genes were cloned into the *Apa*I site of pTB27-link in inverted orientation. The promoter regions were comprised of the plastid 16S rrn promoter from *N. tabacum* or *Brassica napus* fused to the ribosome-binding site of the *N. tabacum* plastid *rbc*L gene. The 16Srrn promoter regions were amplified with primers SAR5F (5′CC CGCATGCCTTAGGTTTTCTAGTTGGATTTG C-3′) and XR3R (5′ GGAGCCCGGGAGTTC GCTCCCAGAAAT-3′) using *B. napus* or *N. tabacum* plastid DNA as template. These were ligated via a *Sma*I site to a modified tobacco *rbc*L 5′ UTR containing the ribosome binding site (RBS) flanked by a *Sma*I and *Nco*I site (5′CC CGGGCGAATACGAAGCGCTTGGATACAGT TGTAGGG-AGGGATCCATGG 3′). The 5′ regulatory region comprising the *B. napus* 16Srrn promoter fused to the RBS from the *N. tabacum rbc*L gene was designated rrnBn (EMBL accession number AJ276677). The *N. tabacum* 16Srrn promoter fused to the RBS of the *N. tabacum rbc*L gene was named rrnNt. The 3′ regulatory elements were from the *N. tabacum psb*A (NtPsbA) and *B. napus psb*C (BnPsbC) genes. The NtPsbA element has been described (Iamtham & Day, 2000). The BnPsbC 3′ element (EMBL accession number AJ578474) was amplified from *B. napus* plastid DNA using primers BnpsbC-F 5′-GGCCGCGGC-TGCAGCATGCA TGACCCCTCTTAA-3′ and BnpsbC-R 5′-GGG GATCCTAAGGCTCGAGAATCCCTCTCTC-TC C-3′ and cloned into the *Eco*RV site of pBluescript (Short et al., 1988). The BnPsbC 3′ regulatory element can be excised with *Not*I and *Pst*I as a 247 bp fragment. An *aad*A expression cassette was constructed by inserting a 0.8 kbp *Nco*I-*Pst*I fragment containing *aad*A coding sequences from pUC-atpX-AAD (Goldschmidt-Clermont, 1991) between 5′rrnBn and 3′ BnPsbC regulatory elements. A plastid *gusA* expression cassette was made by inserting a 1.8 -kbp *Nco*I–*Hin*dIII *gusA* fragment between 5′rrnNt and 3′ NtPsbA regulatory elements. The *aad*A and *gusA* expression cassettes were excised with *Not*I and *Apa*I and cloned into the *Apa*I site of pTB27-link in inverted orientation to create pUM73(AD).

Plastid transformation

Petunia hybrida var. Pink Wave (Thompson and Morgan UK) was chosen because of its high regeneration efficiency from leaf explants (Zubko et al., 2002). Leaf pieces were placed abaxial side up within a 4 cm wide circle in the centre of a 9 cm petri-dish containing MS medium (Murashige & Skoog, 1962) supplemented with 1 mg/l 6-benzylaminopurine, 0.1 mg/l indole-3-acetic acid, 30 g/l sucrose and solidified with 0.8% (W/V) agar (MSB30 medium). Leaves were bombarded using a Bio-Rad PDS 1000/He device. Operating conditions included a 6-cm target distance (stopping screen to target plate distance), 1100 psi rupture disks and 1.0 μm gold particles coated with pUM73(AD). After bombardment plates were stored in dim light for 48 h at 25 °C. Leaf explants were cut into 2–5 mm wide pieces and placed on MSB30 medium supplemented with 200 mg/l of streptomycin sulphate (Sigma) and 200 mg/l of spectinomycin dihydrochloride pentahydrate (Duchefa). Leaf explants were transferred to new plates with fresh medium every 3–4 weeks. Resistant shoots first appeared after 8 weeks.

DNA blot analysis

Southern blot analysis of DNA from aseptic plants was carried out as described (Zubko & Day, 2002). [α-^{32}P]dCTP hybridisation probes prepared with High Prime (Roche Applied Science) were comprised of a 0.80 kbp *Nco*I–*Pst*I *aad*A fragment from pUC-atpX (Goldschmidt-Clermont, 1991) and 1.4 kbp PCR product of the tobacco *rbc*L coding region (map co-ordinates 57595–59028) prepared with primers 5′-ATGTCACCACAAACAGAGACTA-3′ and 5′-TTACTTATCCAAAACGTCCACT-3′.

Measurement of GUS activity

GUS assays using 5-Bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc, Biosynth AG) or 4-methylumbelliferyl β-D-glucuronide (MUG, Sigma) were carried out according to Jefferson (1987). Fluorescence was measured in a Hitachi F-2000 fluorescence spectrophotometer. Protein concentration in extracts were estimated using the Lowry assay (Bio-Rad D_C Protein assay). Plants were grown in a walk-in growth room at light intensities of 80–100 μE at 25°C in a 16-h day/8-h night cycle. Leaves were excised from the top and middle of mature bushy plants. For each soil grown plant we averaged the values of four separate measurements on different green leaves. The significance of differences in mean GUS activities between plants was evaluated by the analysis of variance using JMP5 statistical software (SAS institute). Analysis of variance provided an F ratio of 1.12 (9 degrees of freedom) with a 0.37 probability of higher values. Furthermore, a Turkey–Kramer HSD test on the means demonstrated no significant difference.

Inheritance of spectinomycin resistance

Seeds from selfed plants were sterilised by placing them in 70% (V/V) ethanol for 1 min, then 10 min in diluted sodium hypochlorite (5% active chlorine). After four washes in sterile water seeds were germinated on MS medium (Murashige & Skoog, 1962) containing 500 mg/l spectinomycin.

Results

Construction of plastid transformation vector pUM73(AD)

We used the *aad*A selectable marker gene, which confers spectinomycin and streptomycin resistance, to select *P. hybrida* plastid transformants and also included the *gusA* reporter gene to facilitate the identification of transformants. The vector pUM73(AD) is comprised of inverted plastid expression cassettes containing the *aad*A and *gusA* reporter genes integrated into the *Aoc*I site between the plastid *rbc*L and *acc*D genes (Figure 1). The *aad*A gene is flanked by 5′ rrnBn and 3′ BnpsbC regulatory elements and the *gusA* gene by 5′ rrnNt and 3′ *psb*A elements. The 5′ regulatory elements contain a 16S rrn promoter from *N. tabacum* (rrnNt) or *B. napus* (rrnBn) fused to the ribosome-binding site of the *N. tabacum rbc*L gene. The 3′ elements were from the 3′ non-coding regions of the *psb*A (NtpsbA) and *psb*C (BnpsbC) genes of *N. tabacum* and *B. napus*, respectively. Following delivery of pUM73 into plastids by particle bombardment, flanking

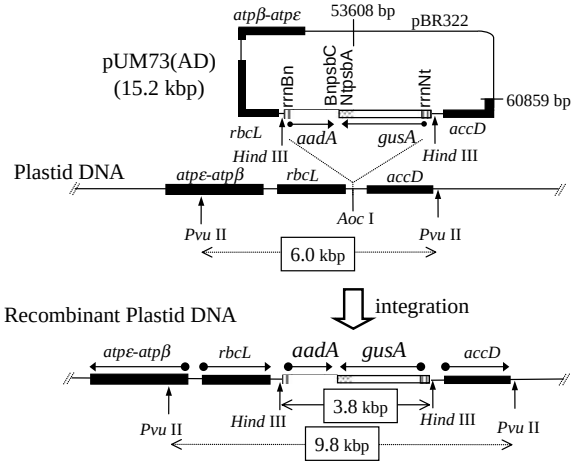

Figure 1. Integration of pUM73(AD) into the *Petunia hybrida* plastid genome. Vector pUM73(AD) contains converging *aad*A and *gusA* expression cassettes with 5′ rrnBn and rrnNt, and 3′ NtpsbA or BnpsbC regulatory elements cloned into the *Aoc*I site of a 7.25 kbp tobacco plastid DNA sequence. Map co-ordinates refer to locations, in basepairs (bp), on the 156 kbp tobacco plastid genome (Wakasugi et al., 1998). Insertion of the 3.8 kbp *aad*A–*gusA* cassettes results in the replacement of a 6.0 kbp *Pvu*II fragment with a larger 9.8 kbp *Pvu*II fragment. The *aad*A–*gusA* cassettes are excised with *Hin*dIII.

tobacco plastid DNA sequences of 5.7 and 1.5 kbp target integration of the *aad*A and *gusA* genes into the *P. hybrida* plastid genome by homologous recombination (Figure 1). The use of tobacco rather than *P. hybrida* plastid targeting DNA was possible because plastid DNA is highly conserved between these species. This approach of using heterologous plastid DNA to target integration was successful in tobacco (Daniell et al., 1998; Kavanagh et al., 1999), potato (Sidorov et al., 1999) and tomato (Ruf et al., 2001) plastid transformation. In a reversal of the origin of the donor and target recombination sites used here, *Petunia* plastid DNA has previously been used to target integration into the inverted repeat region of the tobacco plastid genome (Daniell et al., 1998).

Plastid transformation of P. hybrida

Plasmid pUM73(AD) was delivered into the plastids of green leaves by particle bombardment. Transformants were selected by placing leaf explants on media containing spectinomycin and streptomycin. Selection on two antibiotics overcomes the problem of spontaneous resistant mutants associated with using spectinomycin on its own (Eibl et al., 1999) because the majority of spectinomycin resistant mutants are sensitive to streptomycin. Transformants were visible as green resistant shoots growing amongst the bleached leaf explants. Thirty-one bombarded plates produced three resistant shoots after 8–12 weeks of propagation of leaf explants on selection media. Clones T16, T22 and T71 were isolated from different bombarded plates and are derived from independent transformation events. Plastid transformants would be expected to be spectinomycin and streptomycin resistant and also to express β-D-glucuronidase (GUS) due to the concomitant integration and expression of the *aad*A and *gusA* genes in plastids. After a second cycle of regeneration, spectinomycin plus streptomycin resistant shoots were tested for GUS activity. Immersion of leaves, from all three resistant plants grown *in vitro*, into a solution containing X-gluc produced a strong and uniform blue coloration indicative of GUS activity throughout the leaf. Blue staining in leaves from resistant clone T16 (Figure 2A) is shown in Figure 2B. No blue staining was visible in leaves from wildtype (WT) untransformed plants (not shown).

Molecular analyses of plastid transformants

After two cycles of regeneration on MSB30 medium with antibiotics plants were rooted on MS medium (Murashige & Skoog, 1962) containing 200 mg/l of spectinomycin. Total DNA was extracted from leaves of rooted plants grown *in vitro* and subjected to molecular studies to verify integration of foreign genes within the plastid genome. Because plastid DNA is present in multiple copies per cell, plastid transformation involves the gradual replacement of WT plastid genomes by transgenic plastid genomes. Stable plastid transformation is achieved once homoplasmic plants containing a uniform population of transgenic plastid genomes are obtained. DNA blot analysis was used to confirm integration of the *aad*A and *gusA* genes into the region between the plastid *rbc*L and *acc*D genes by homologous recombination. The 3.8 kbp foreign insert of *aad*A and *gusA* results in the replacement of a WT 6 kbp *Pvu*II fragment with a larger 9.8 kbp

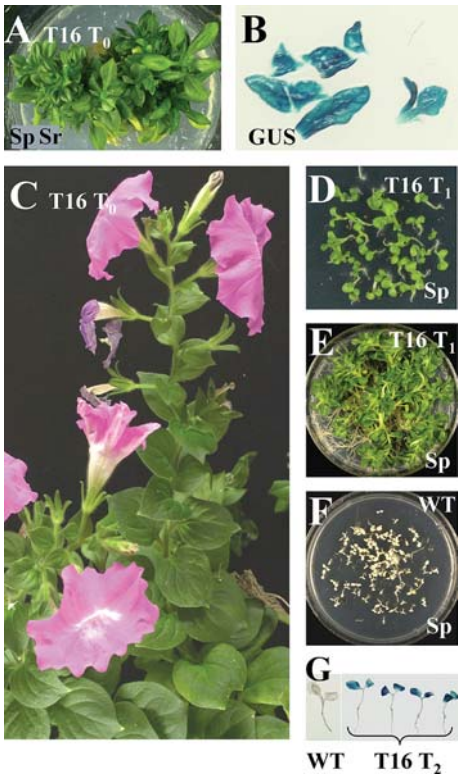

Figure 2. Isolation of *Petunia hybrida* plastid transformants. (A) Resistant T16 shoots regenerating on MSB30 media with spectinomycin (Sp) and streptomycin (Sr) each at 200 mg/l. (B) GUS staining of resistant T16 shoots. (C) Mature transplastomic T16 plant flowering in soil. (D) T16 transplastomic seedlings (T1) growing on MS medium with 500 mg/l spectinomycin. (E) Six week old T16 transplastomic plants growing on MS medium with 500 mg/l spectinomycin. (F) Bleached wildtype (WT) seedlings arrested in growth on MS medium with 500 mg/l spectinomycin. (G) GUS staining of WT and T16(T2) seedlings. T_0 is the first generation of transformed plants regenerated from leaves and T_1 and T_2 the following seed-derived generations.

recombinant band (Figure 1). The right *Pvu*II site of this 9.8 kbp fragment lies beyond the partial *acc*D gene cloned in the transformation vector pUM73(AD) and cannot be produced by unintegrated plasmid or insertion events at other sites. This integration pattern was confirmed using a tobacco *rbc*L probe. The probe hybridises strongly to a 6.0 kbp band and weakly to a 8.7 kbp band in *Pvu*II digests of WT DNA (Figure 3A). Figure 3A clearly shows the presence of the expected 9.8 kbp recombinant *Pvu*II band in all three plastid transformants (Figure 3A, lanes T16, T22, T71), which replaces the 6.0 kbp WT band. The absence of a detectable

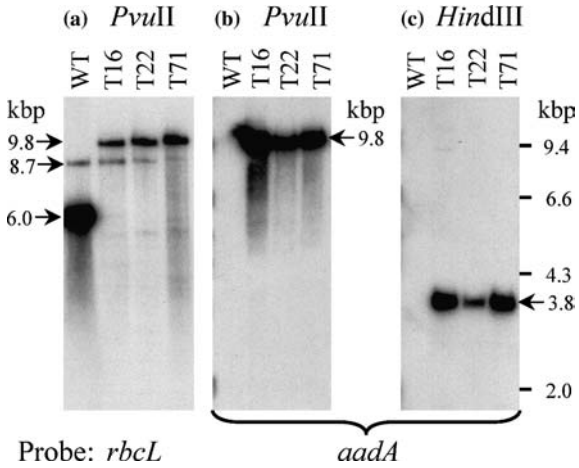

Figure 3. DNA blot analysis of *Pentunia hybrida* plastid transformants. *Pvu*II (a, b) or *Hin*dIII (c) digests of total DNA from wild type (WT) and plastid transformants (T16, T22, T71) hybridised with a tobacco *rbc*L probe (a) or *aad*A probe (b, c). Arrows indicate the sizes of hybridising bands. The positions of DNA size standards in kbp are shown on the right of blot C. Blots were washed in 0.1 × SSC at 60°C.

6 kbp WT band in lanes loaded with digests of DNA from plastid transformants is indicative of a homoplasmic population of transgenic plastid genomes. The 9.8 kbp recombinant band hybridises strongly to an *aad*A probe consistent with the presence of *aad*A in this band (Figure 3B). *Hin*dIII releases the foreign genes as a 3.8 kbp fragment (Figure 1). *Hin*dIII digests show a 3.8 kbp band that hybridises to *aad*A in all three plastid transformants (Figure 3C, lanes T16, T22, T71). These results confirm integration of the 3.8 kbp foreign insert into the plastid genome. The faint 8.7 kbp *Pvu*II band visible in WT DNA is also visible in digests of DNA from plastid transformants. Its origin is unclear. It might represent a cross-hybridising sequence in mitochondrial or nuclear DNA.

Inheritance of aadA in transplastomic plants

Rooted plastid transformants were transferred to soil. The phenotype of plastid transformants was indistinguishable from WT untransformed plants. All three plastid transformants flowered (Figure 2C) and after selfing set seed. Seeds from individual plants were sterilised and germinated on MS medium (Murashige & Skoog, 1962) containing 500 mg/l of spectinomycin. In this assay sensitive seedlings bleach white while resistant

seedlings remain green. Seedlings from all three plastid transformants were green and resistant to spectinomycin. The results with T16 seedlings are shown in Figure 2D and E. All T16 seedling cotyledons were green (Figure 2D) and these continued to grow and produce green true leaves on MS medium containing 500 mg/l spectinomycin (Figure 2E). In contrast WT seedlings germinated to produce only white sensitive seedlings that were arrested in growth at the cotyledon stage.

Wild type *P. hybrida* resembles WT *N. tabacum* in its growth arrest response to spectinomycin and is unlike WT *B. napus*, which bleaches but continues to grow on spectinomycin medium (Zubko & Day, 1998). Plastids are inherited maternally in *P. hybrida* (Nagata et al., 1999). A 100% transmission of spectinomycin resistance to progeny after selfing is consistent with *aad*A being located in plastids. Nuclear located *aad*A genes would be expected to produce some sensitive progeny at a frequency dependent on the number of chromosomal integration sites. The inheritance of the plastid *aad*A and *gusA* transgenes was followed to the T2 generation. All T16 (T_2) seedlings from selfed T16 (T_1) plants were resistant to spectinomycin (not shown) and GUS positive (Figure 2G). GUS activity was restricted to green leaves and not detectable in the roots of transplastomic seedlings. Blue staining was not observed in WT seedlings (Figure 2G). The results confirm stable inheritance and expression of the plastid localised *aad*A and *gusA* genes.

GUS activities in mature leaves of soil grown plants

The activity of the plastid-localised *gusA* gene was measured in expanded leaves of mature bushy plants growing in soil just prior to flowering. GUS activities were measured fluorimetrically using MUG (Jefferson, 1987). WT untransformed plants did not contain any detectable GUS activity. Plastid transgenes are not silenced and all three plastid transformants would be expected to give similar GUS activities. Mean GUS activities in leaves of mature T16, T22 and T71 plants were 1210 (SE 161), 1330 (SE 137) and 940 (SE 250) pmoles 4-MU/(min mg protein), respectively. Up to two-fold variation in GUS activities were found between leaves in an individual plant probably representing differences in leaf developmental stage. Analysis of variance and statistical tests on the means showed that the variation in mean GUS activities between plants was not significant.

Discussion

We have used *aad*A-based spectinomycin plus streptomycin selection to isolate stable plastid transformants in a commercial cultivar of *P. hybrida*. All three transplastomic plants isolated were homoplasmic for transgenic plastid genomes and fertile. Inclusion of a *gusA* reporter gene facilitated screening and demonstrated similar transgene expression levels in plastid transformants. To enhance the performance of *P. hybrida* by plastid engineering simply involves replacement of the *gusA* sequences in pUM73(AD) with suitable trait genes. The efficiency of plastid transformation in *P. hybrida* of one plant per ten bombarded plates is much lower than the frequency of one to five plants per bombardment obtained in *N. tabacum* (Svab & Maliga, 1993; Iamtham & Day, 2000) but comparable to plastid transformation efficiencies obtained in potato (Sidorov et al., 1999) and tomato (Ruf et al., 2001). Relatively little is known on the relationship between recombination rates and sequence divergence in plastids and whether our transformation frequencies might have been improved by using homologous *P. hybrida* plastid DNA to target integration rather than the tobacco plastid DNA sequences used here. The use of heterologous *Solanum nigrum* targeting DNA in tobacco had little effect on transformation frequency in tobacco (Kavanagh et al., 1999). The time taken for the appearance of resistant *P. hybrida* clones on selection media with two antibiotics following bombardment, about 8 weeks, is similar to tobacco plastid transformation (Iamtham & Day, 2000) and shorter than the 12 weeks reported in tomato plastid transformation using spectinomycin selection alone.

Petunia hybrida is an attractive model species in which to study plastid biology (Sutton et al., 1995; Kohler et al., 1997). The development of plastid transformation in *P. hybrida*, a species tractable to genetic analysis, provides an alterna-

tive model to *N. tabacum* to study fundamental aspects of the plastid genetic system. *Petunia hybrida*, which is diploid, is suitable for mutation screening to identify nuclear loci affecting the maintenance and expression of plastid transgenes. Neither *N. tabacum,* which is an amphidiploid and a poor genetic model, nor *A. thaliana*, which does not give rise to fertile plastid transformants (Sikdar et al., 1998), are suitable for such studies. Conservation of processes relating to the plastid genetic system such as plastid to nuclear transposition (Huang et al., 2003; Stegemann et al., 2003), which might be affected by ploidy levels, gene redundancy and amounts of non-coding DNA in the nucleus can be studied in *Petunia* and the results compared with *N. tabacum*. Uniform gene expression in plastids contrasts with the epigenetic silencing effects associated with nuclear transgenes (Kooter et al., 1999) and will enable comparative studies of transgene expression in the two genomes.

Whilst most *P. hybrida* cultivars exhibit strict maternal inheritance of plastids (Nagata et al., 1999), rare paternal transmission of chloroplasts, found in 2% of progeny, has been reported for one of 19 paternal genotypes tested (Cornu & Dulieu, 1988). The marker used, a chlorophyll deficient mutation, is inherited maternally for most genotypes indicating a cytoplasmic location. However, the molecular basis and precise location of the chlorophyll deficient mutation is not known. Spectinomycin resistance resulting from a plastid-localised *aad*A gene provides an alternative and fully characterised plastid marker for studying plastid inheritance in a range of *P. hybrida* genotypes. *Petunia* is an important ornamental bedding plant (Mol et al., 1999). For example in the United States of America, farm gate values are in the region of tens of millions of dollars. Establishment of stable plastid transformation in *P. hybrida* brings the benefits of plastid engineering, such as stable gene expression and maternal inheritance, to improve the performance of this important decorative plant.

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