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Stable plastid transformation in lettuce (*Lactuca sativa* L.)

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Abstract

Although plastid transformation in higher plants was first demonstrated in the early 1990s it is only recently that the technology is being extended to a broader range of species. To date, the production of fertile transplastomic plants has been reported for tobacco, tomato, petunia, soybean, cotton and *Lesquerella fendleri* (Brassicaceae). In this study we demonstrate a polyethylene glycol-mediated plastid transformation system for lettuce that generates fertile, homoplasmic, plastid-transformed lines. Transformation was achieved using a vector that targets genes to the *trnA*/*trnI* intergenic region of the lettuce plastid genome employing the *aadA* gene as a selectable marker against spectinomycin. Spectinomycin resistance and heterologous gene transcription were shown in T_1 plants derived from self-pollinated primary regenerants demonstrating transmission of the plastid-encoded transgene to the first seed generation. Crossing with male sterile wild-type lettuce showed that spectinomycin resistance was not transmitted *via* pollen. Constructs containing the *gfp* gene showed plastid-based expression of green fluorescent protein. The lettuce plastid could have potential both as a production and a delivery system for edible human therapeutic proteins.

Introduction

Plastid transformation was first reported in the green alga *Chlamydomonas* (Boynton *et al.*, 1988) and the technology was soon applied to the higher plant species tobacco (*Nicotiana tabacum*) (Svab *et al.*, 1990). Plastid transformation offers significant advantages over nuclear transformation technology including the potential for very high levels of expression of heterologous protein (De Cosa *et al.*, 2001; Tregoning *et al.*, 2003), transgene containment due to materal inheritance of cytoplasmic genomes in most higher plants (Svab and Maliga 1993; Birky 2001; Ruf *et al.*, 2001; Skarjinskaia *et al.*, 2003), lack of position effects due to targeted integration events (Staub and Maliga 1992) lack of gene silencing, and co-expression of multiple genes because the plastid translational machinery has the capacity to translate polycistronic transcripts (Staub and Maliga 1995; De Cosa *et al.*, 2001). However, despite these advantages it has taken nearly a decade to extend plastid transformation technology to higher plants other than tobacco.

Both biolistic bombardment of leaves (Svab *et al.*, 1990) and polyethylene glycol (PEG) treatment of protoplasts (Golds *et al.*, 1993; O'Neill *et al.*, 1993) have been used for DNA delivery into plant plastids. Plastid transformation involves targeted integration of a foreign gene, or genes, into the plastid genome *via* homologous recombination. Plastid genomes are highly polyploid (Bendich, 1987) with each plastid containing between 10 and 100 genome equivalents. The number of plastids per cell varies considerably (Pyke, 1999) and cells that are specialized for photosynthesis (e.g. mesophyll leaf cells) can have hundreds of these organelles. Stable transformation requires that all copies of the plastid genome are transformed (homoplasmic state) and that all wild-type copies of the plastid genome are eliminated by selection.

Tobacco plastid transformation is now routine in many labs and is the benchmark for assessing plastid transformation protocols developed for other plant species. Plastid transformation has also been achieved in the related solanaceous species, tomato, potato and petunia using tobacco vectors (Sidorov *et al.*, 1999; Ruf *et al.*, 2001; Zubko *et al.*, 2004), albeit at a significantly lower transformation efficiency than in the tobacco system, in the case of potato the transformed plants were sterile (Sidorov *et al.*, 1999). Plastid transformation in non-solanaceous species has been more difficult to obtain. Transformation of *Arabidopsis thaliana* plastids has been achieved but the transformation frequency was low and the transformed plants were sterile (Sikdar *et al.*, 1998). *Lesquerella fendleri* (Brassicaceae) was transformed using a chimeric *Arabidopsis*/tobacco vector and resulted in a low frequency of fertile, transformed plants (Skarjinskaia *et al.*, 2003). Recently, fertile transplastomic plants were generated in cotton (Kumar *et al.*, 2004a) and soybean (Dufourmantel *et al.*, 2004b) using species-specific vectors. Plastid transformation of carrot cell cultures was achieved using a carrot vector but there is no indication that the transformed plants were fertile (Kumar *et al.*, 2004b). A *Brassica napus* (oilseed rape) vector was used to transform oilseed rape but only heteroplasmic plastid transformed lines were obtained (Hou *et al.*, 2003). Likewise, plastid transformation of embryonic rice suspension culture cells, using a rice chloroplast DNA vector, resulted in plants that were heteroplasmic for the chloroplast insertion (Khan and Maliga, 1999).

There is significant interest in plastid-based recombinant protein production in non-toxic, edible plant species not only to minimize downstream protein processing costs but also to develop a combined production and delivery system for 'edible' protein therapies. Lettuce (*Lactuca sativa* L.) is a commercially important crop belonging to the Asteraceae. The leaves of this crop are consumed raw by humans and the time from sowing seed to edible biomass is only weeks compared to months for crops such as tomato or potato. Here we report production of fertile, homoplasmic, plastid-transformed lines in lettuce using a transformation vector that targets the *trnI*/*trnA* region of the lettuce plastid genome. In addition, we show detectable expression of recombinant green fluorescent protein in lettuce plastids. The successful transformation of lettuce plastids offers the potential to develop lettuce both as a production and a delivery system for human therapeutic proteins such as subunit vaccines.

Materials and methods

DNA sequencing

Lettuce chloroplast DNA *Sac*I clones 6 and 15 (Jansen and Palmer, 1987) were obtained from Prof. Bob Jansen, University of Texas, Austin, USA and partially sequenced. DNA sequencing was carried out using the ABI Prism BigDye terminator cycle sequencing ready reaction kit according to the manufacturers instructions (Applied Biosystems) and an ABI Prism 3310 genetic analyzer (Applied Biosystems). The lettuce sequence has been deposited in GenBank under the accession number DQ013044.

Vector and expression cassette construction

Plasmid LCV2 contains a 2253 bp lettuce chloroplast DNA target sequence cloned into a pCR2.1 backbone vector (Invitrogen). The target sequence extends from the *rrn16*/*trnI* intergenic region to the *trnA*/*rrn23* intergenic region and corresponds to nucleotide (nt) positions 104366–106260 in the tobacco chloroplast genome sequence (Wakasugi *et al.*, 1998). The target sequence was amplified in

two parts to introduce unique *Pac*I and *Asc*I restriction sites in the *trnI*/*trnA* intergenic region at a position corresponding to nt 105370 in the tobacco chloroplast genome sequence (Wakasugi *et al.*, 1998). The *trnI* gene region was PCR amplified from lettuce chloroplast DNA using the PCR primers 5′-TCGACAGTGAAGTAAGACCAAG-3′ and 5′-GGCGCGCCTTAATTAAGGAGTCA GACGCTTCTTCTATTC-3′ (*Pac*I and *Asc*I restriction sites underlined) and cloned into pCR2.1 to create pCR2.1-*trnI*. The *trnA* gene region was PCR amplified using the primer combination 5′-TTAATTAAGGCGCGCCCATGCATGCTC-CACTTGGCTCGG-3′ (*Pac*I and *Asc*I restriction sites underlined) and 5′-CATGAATGATAAAT-CATAGATCGAAC-3′. The amplified *trnA* gene region was cloned into pCR2.1 to create pCR2.1-*trnA*. The *trnA* gene region was excised from pCR2.1-*trnA*, using *Pac*I and *Xba*I, and was introduced into the *Pac*I and *Xba*I sites in pCR2.1-*trnI* to create pLCV2. The *trnI*/*trnA* insert was sequenced in pLCV2 to confirm that the target sequence was identical to the corresponding lettuce plastid genome sequence except for the inclusion of *Pac*I and *Asc*I restriction sites. The pLCV2 insert sequence has been deposited in GenBank under the accession number AY943927.

Plasmid LCV2-GFP/aadA containing *gfp* and *aadA* expression cassettes was produced by first inserting the *aadA* expression cassette from pZS197 (Svab and Maliga, 1993) into pLCV2 to yield pLCV2-aadA. The expression cassette included the tobacco *rrn* promoter (with an 18 bp synthetic leader and ribosome-binding site, Svab and Maliga, 1993), the *aadA* coding region, and the tobacco *psbA* 3′ region amplified by PCR using forward (5′-GATCGAGGCGCGCCGGAGCCCGGAACTA-GCTGCTGC) and reverse (5′-ATTTTCGGCG CGCCCCCACACCTGCGTTCGAACTCCTTC) primers both of which contained flanking *Asc*I sites (underlined). The *Asc*I-digested PCR product was inserted into dephosphorylated *Asc*I-digested pLCV2 and recombinant *E. coli* clones were selected on spectinomycin. pLCV2-GFP/aadA was then produced by inserting a *gfp* expression cassette into the *Pac*I site of pLCV2-aadA. The *gfp* expression cassette contained the tobacco *rrn* promoter (with *rbcL* 5′ UTR and ribosome-binding site), the sm*gfp*-coding region, and the *E. coli rrnB* terminator (Newell *et al.*, 2003) and was amplified by PCR from a pZS197-based plasmid containing P*rrn*-*gfp* (Newell *et al.*, 2003) using forward (5′-GAT-CTTAATTAAGCTCCCCCGCCGTCGTTCAA-TG) and reverse (5′-GATCTTAATTAAGTCG ACAACACGGGATAATACCGCGC) primers containing flanking *Pac*I sites (underlined). The *Pac*I-digested PCR product was inserted into dephosphorylated *Pac*I-digested pLCV2-aadA to yield pLCV2-GFP/aadA.

The LEC1 expression cassette contains the *aadA* gene and the influenza virus haemagglutinin gene (*HA*) under the control of the lettuce *rrn* operon promoter (P*Ls*-*rrn*) and lettuce *psbA* 3′UTR (3′*Ls*-*psbA*). A chloroplast ribosome-binding site (GGAGG) precedes both genes. The lettuce ribosomal RNA operon promoter (P*Ls*-*rrn*) was amplified from the lettuce chloroplast DNA *Sac*I clone 6 using the PCR primer pair 5′-TCGAGCTCTTAATTAAGCTACCCCGCCG-TGATTGAATGAGAAT-3′ and 5′-AAATCCC TCCCTACAACTGTATCCAAGCGCTTCGTA-TTCGC-3′ (*Sac*I and *Pac*I restriction sites underlined). The *aadA* gene, including synthetic leader and ribosome-binding site was amplified from the tobacco chloroplast transformation vector pZS197 (Svab and Maliga, 1993) using the PCR primer pair 5′-GTTGTAGGGAGGGA-TTTATGGCAGAAGCGGTGATCGCCGAA-3′ and 5′-TCGCGGCCGCTTATTTGCC GACTAC CTTGGTGAT-3′ (*Not*I restriction site underlined). The P*Ls*-*rrn* was joined to the 5′ end of the *aadA* gene by overlap extension and the P*Ls*-*rrn*/*aadA* fusion was cloned into the *Sac*I and *Not*I sites in the polylinker of pBS SK+ (Stratagene). The *HA* gene (mature coding region) was amplified using the PCR primer pair 5′-TCGCGGCCGCAGTTGTAGGGAGGGATTT-ATGCAAAAACTTCCCGGAAATGACAA-3′ and 5′-GGATCCTTAGTATCCTGACTTCAG-CTCAAC-3′ from cloned *HA* template DNA (Johnson *et al.*, 2000). These primers incorporate a *Not*I site (underlined) and a short leader sequence and ribosome-binding site at the 5′ end of the *HA* gene and a *Bam*HI site (underlined) at the 3′ end of the *HA* gene. The PCR fragment was cloned into the *Not*I/*Bam*HI sites in pBS SK+ to give pBS-HA. The lettuce *psbA* 3′UTR (3′*Ls*-*psbA*) was PCR amplified from lettuce chloroplast DNA (cv. Evola) using primers 5′-AACATTTAA-GGATCCGACTTTGGTCTTATTGTAATTGT-ATAG3′ and 5′-ATCTGCAGGGCGCGCCAT CCACTTGGCTACATCCGCC3′. These primers

incorporate a *Bam*HI site (underlined) at the 5′ end of the 3′*Ls-psbA* and *Pac*I and *Pst*I sites (underlined) at the 3′ end of the 3′*Ls-psbA*. The 3′*Ls-psbA* sequence was fused to the 3′ end of the *HA* gene by cloning into the *Not*I/*Pst*I sites in pBS-HA. The *HA*/3′*Ls-psbA* construction was excised from pBS SK+ and cloned downstream of the *aadA* gene in pBS SK+PL*rrn*/*aadA* using *Not*I and *Pst*I restriction sites. The entire LEC1 cassette (P*Ls-rrn*/*aadA*/*HA*/3′*Ls-psbA*) was excised from pBS SK+ and cloned into the *Pac*I/*Asc*I sites in pLCV2 to create pLCV2-LEC1.

Protoplast isolation, transformation and culture

Lettuce seeds cv. Flora were sterilized in 70% ethanol, 0.7% hypochlorite solution for 20 min, washed three times in sterile demineralized water, and sown on MS medium (0.7% microagar, Duchefa; Murashige and Skoog, 1962) supplemented with 2% sucrose, without hormones. Seeds were cultured at 15 °C for 2 days in the dark, after which they were transferred to 25 °C in the light (60 μmol m^{-2} s^{-1}, photoperiod 16 h light/8 h dark). When the first true leaves appeared, the shoot tips were transferred to MS medium with 3% sucrose without hormones and grown under the same conditions (25 °C, 16 h light/8 h dark). Protoplasts were isolated from 3-week-old shoot-culture leaves. The leaves were cut into pieces, approximately 25–50 mm^2 in size, and incubated in the dark for 1 h in PG solution (Glimelius, 1984). The PG solution was replaced with an enzyme solution consisting of 1% cellulase (Brunschwig, Switzerland) and 0.25% macerozyme (Brunschwig, Switzerland) in B5 medium (Gamborg *et al.*, 1968) and the leaf pieces were incubated for 16 h in the dark at 25 °C. The protoplast suspension was filtered through a 41 μm nylon mesh filter and washed with one third volume of CPW16S solution (Frearson *et al.*, 1973). Protoplasts were collected at the surface following centrifugation at $70 \times g$ for 8 min. Isolated protoplasts were washed in W5 solution (Menczel *et al.*, 1981) and pelleted following centrifugation at $70 \times g$ for 5 min. Protoplasts were diluted to a density of approximately $1–1.5 \times 10^6$ protoplasts per 0.4–0.6 ml of transformation buffer (Datta and Datta, 1999).

Ten microliters of transforming DNA (1 μg/μl in 10 mM Tris, 1 mM EDTA pH 8.0) and 0.4–0.6 ml PEG 6000 solution (Datta and Datta, 1999) was added to the protoplast suspension and incubated at room temperature for 10 min. The protoplasts were then diluted in wash solution (Datta and Datta, 1999), pelleted by centrifugation at $70 \times g$ for 8 min, then resuspended in ½B5 culture medium (Gamborg *et al.*, 1968) supplemented with 375 mg/l $CaCl_2 \cdot 2H_2O$, 18.35 mg/l NaFeEDTA, 270 mg/l sodium succinate, 103 g/l sucrose, 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), 0.3 mg/l 6-benzylaminopurine (BAP) and 0.1 g/l MES. The protoplasts were mixed with a 1:1 solution of ½B5 medium and 2% agarose to a culture density of $3–6 \times 10^4$ protoplasts per ml. The agarose/protoplast suspension was plated onto 3.5 cm Petri dishes, overlaid with 4 ml of liquid ½B5 culture medium and cultured at 25 °C in the dark without selection. Selection for transformed microcalli was initiated after 6 days by replacing the liquid culture medium with fresh medium containing spectinomycin dihydrochloride (500 mg/l) and incubating at 25 °C in the dark. One week after initiation of selection, the culture medium was replaced with fresh liquid medium containing spectinomycin dihydrochloride (500 mg/l) and the cultures were transferred to the light (16 h light/8 h dark) at 25 °C.

When the calli were approximately 0.5 mm in diameter they were transferred to callus-growth medium (Schenk and Hildebrandt, 1972) supplemented with 30 g/l sucrose, 5 g/l agarose, 0.1 mg/l 1-naphthalene acetic acid (NAA), 0.1 mg/l benzylaminopurine (BAP), and 500 mg/l spectinomycin dihydrochloride. When the calli had grown to a few mm in diameter they were transferred to regeneration medium (Schenk and Hildebrandt, 1972) supplemented with 15 g/l sucrose, 15 g/l maltose, 0.1 mg/l NAA, 0.1 mg/l BAP and 500 mg/l spectinomycin dihydrochloride. Culture conditions were as described above. Shoots regenerated after approximately 6 weeks and were transferred onto SH medium (Schenk and Hildebrandt, 1972) supplemented with 30 g/l sucrose, 8 g/l microagar and 500 mg/l spectinomycin dihydrochloride. Ploidy level of callus and regenerated plant tissue was determined by flow cytometric analyses (Partec, Munster).

PCR analyses of spectinomycin-resistant cell lines and shoots

The presence of the *aadA* gene was assessed in spectinomycin-resistant cell lines by PCR analysis using gene-specific primers (aadAF-5′-TATGACGGGCTGATACTGGGC-3′ and aadAR - 5′-AAGTCACCATTGTTGTGCACG-3′). The presence/absence of the *gfp* gene was assessed by PCR in pLCV2-GFP/aadA-transformed cell lines that showed no GFP expression using a *trnI* gene-specific primer (5′-TCGACAGTGAAGTAAGACCAAG-3′) and an *aadA*-gene specific primer (5′-CTACATTTCGCTCATCGGC-3′). The PCR products were sequenced commercially by AGOWA, GmbH, Germany. To confirm plastid integration of entire vector/expression cassette constructs in pLCV2-LEC1-transformed callus lines and shoots PCR was carried out using primers designed against genomic DNA sequences that flank the vector integration site on the lettuce plastid genome (For: 5′-ACTGGAAGGTGCGGCTGGAT-3′ and Rev: 5′-CTCGCCCTTAATT TTAAGGC-3′). In some cases, precise genome/vector junctions were confirmed by PCR using combinations of flanking primers and expression cassette-specific primers.

Immunoblot analysis

Total protein was extracted from frozen callus or leaf tissue in extraction buffer [60 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10 mM Na_2EDTA, 30 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride or 2 × Complete™ protease inhibitor (Roche Diagnostics, UK)]. The proteins were separated by SDS-12% PAGE, transferred to polyvinylidene difluoride (PVDF) membrane by electroblotting and the membrane was blocked overnight with 2% non-fat dry milk. For HA detection, the blots were incubated with sheep anti-HA Sichuan 1987 polyclonal antiserum (supplied by National Institute for Biological Standards and Control, UK) followed by donkey anti-sheep HRP-conjugated secondary antibody (Sigma). For GFP detection, the blot was incubated with rabbit antiserum to GFP (Abcam) and immunoreactive bands detected by enhanced chemiluminescence (Amersham). GFP protein standards were supplied by Clontech.

Microscopy analysis

A small piece of callus was chopped finely, teased out on a microscope slide and mounted in water. Plastids were examined by laser scanning confocal microscopy (TCS-NT, DM1RB light microscope stand, Leica). Excitation wavelengths were set at 488 nm and 568 nm, and images collected through FITC and TRITC filters, for chlorophyll and GFP fluorescence, respectively. In order to combine images for chlorophyll and GFP fluorescence, data were imported into Adobe Photoshop and merged.

Southern blot analysis

Total DNA was isolated from leaves of wild-type and transplastomic lettuce plants with a GenElute Plant Genomic DNA Kit (Sigma-Aldrich). Purified DNA (approx. 0.3 μg) was digested with *Sac*I overnight. Digested DNA fragments were separated by electrophoresis on a 0.7% agarose gel and transferred to a nylon membrane using standard blotting procedures (Southern, 1975). A *trnI* probe was generated by digesting pLCV2 with *Eco*RI and *Sac*I to release a 1.3 kb fragment that was gel purified using a Qiaquick Gel Extraction Kit (Qiagen). A 1.7 kb *HA* gene-specific probe was generated by PCR using pBS-HA as template and M13 forward and reverse primers. The PCR product was gel purified using a Qiaquick Gel Extraction Kit (Qiagen). Probe DNA was labeled with [α-^{32}P]dATP by random priming using a Prime-It II Random Primer Labeling Kit (Stratagene). Prehybridization, hybridization overnight at 65 °C to ^{32}P-labelled probes, and subsequent washing of the membrane was carried out according to the manufacturer's instructions. The membrane was probed initially with the *trnI* fragment, then stripped and re-probed with the *HA* gene fragment.

Northern blot analysis

Total RNA was extracted from leaves of wild-type and transplastomic lettuce plants using Tripure Isolation Reagent (Boehringer Mannheim). RNA (10 μg) was denatured by treatment with formaldehyde and separated by electrophoresis on a 1% agarose/formaldehyde gel. The gel was transferred to GeneScreen Plus nylon membrane overnight. The same HA probe described

above was used to probe the membrane for a HA transcript. The *aadA* probe was generated by PCR and the DNA fragment was gel isolated and labeled as described above. The membrane was prehybridized, hybridized overnight at 42 °C, and then washed according to the manufacturer's instructions. The membrane was probed initially with the HA gene fragment, stripped, and re-probed with the *aadA* fragment.

Testing seedlings for spectinomycin resistance

Seeds were surface sterilized and sown on MS medium (0.7% microagar, Duchefa; Murashige and Skoog, 1962) supplemented with 2% sucrose and 500 mg/l spectinomycin dihydrochloride. Seedlings were grown at 25 °C in the light (60 μmol m^{-2} s^{-1}, photoperiod 16 h light/8 h dark). After 2 weeks, non-transformed seedlings were yellow, whereas spectinomycin-resistant seedlings were green and showed vigorous growth.

Results

Approximately 8.2 kb of the lettuce chloroplast DNA inverted repeat region was sequenced (Figure 1A) and PCR primers based on these DNA sequence data were used to construct candidate lettuce plastid transformation vectors. pLCV2 contains a 2253 bp lettuce chloroplast DNA target sequence inserted into a pCR2.1 backbone vector (Figure 1B). pLCV2 targets insertions in the *trnI* and *trnA* intergenic region of the *rrn* operon and is based on the universal pSBL vector series (Daniell *et al.*, 1998; De Cosa *et al.*, 2001). The LCV2 insert sequence is 97% similar to the corresponding tobacco chloroplast DNA sequence over most of its length. However, the lettuce *trnI* intron has a 231 bp segment that is not present in the tobacco sequence and the *trnA* intron has a 101 bp segment that is also absent in the tobacco sequence.

A trial construct containing the aminoglycoside 3′adenylyltransferase (*aadA*) and green fluorescent protein *(gfp)* genes was used to develop a lettuce plastid transformation procedure and to demonstrate plastid-based expression of heterologous proteins in lettuce. The GFP/aadA expression cassette, containing the *gfp* and *aad*A genes under the control of identical tobacco *rrn* promoters (P*rrn*; Figure 1C), was introduced into pLCV2 at unique *Pac*I and *Asc*I cloning sites. The *aadA* gene confers resistance to spectinomycin and streptomycin when expressed in plastids and is used for the selection of stable transformants (Svab and Maliga, 1993). Lettuce plastid transformation was carried out by PEG-mediated uptake of pLCV2-GFP/aadA by leaf protoplasts. A protoplast-

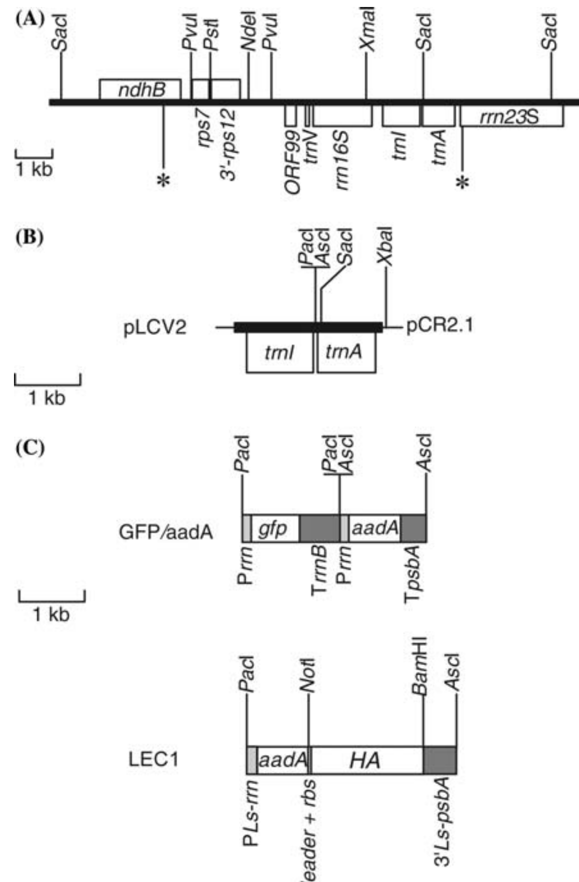

Figure 1. Vector and expression cassettes used for lettuce plastid transformation. (A) Physical and restriction map of the region of the lettuce chloroplast genome used for vector construction (region sequenced is delineated by *). (B) Map of pLCV2 showing engineered *Pac*I/*Asc*I sites in the intergenic spacer region between the *trnI* and *trnA* genes. The 1020 bp *trn*I gene has an intron at nucleotide position 39–985; the 874 bp *trnA* gene has an intron at nucleotide position 39–857. (C) Expression cassettes used for lettuce plastid transformation. The GFP/aadA expression cassette contains the *smgfp* gene under the control of the tobacco *rrn* promoter (P*rrn*) and the *aadA* gene under the control of a second tobacco *rrn* (P*rrn*) promoter. The LEC1 expression cassette contains the *aadA* gene and influenza virus A *HA* gene under the control of the lettuce *rrn* operon promoter (P*Ls-rrn*) and 3′ lettuce *psbA* 3′ sequence (3′*Ls-psbA*). An 18 bp leader sequence and ribosome-binding site (*rbs*) are present upstream of the *HA*-coding region.

mediated transformation system was preferred over biolistic transformation because stringent selection for transformed plastids was considered more feasible with single cells, or small clumps of cells, than with the larger tissues normally used for biolistic transformation. Protoplasts were initially cultured without selection in the dark before commencing selection on spectinomycin (500 mg/l) six days post-transformation. One week after initiation of selection, the protoplast cultures were transferred to the light. Green, spectinomycin-resistant callus material developed 4–5 weeks post-transformation (Figure 2A). Nine spectinomycin-resistant cell-lines were regenerated from a total of 5.6×10^6 treated viable protoplasts. The presence of the *aadA* gene in these cell lines was confirmed by PCR analyses (data not shown). Spectinomycin-resistant calli were propagated on culture medium containing spectinomycin. Plastid-based GFP accumulation was assessed in seven of the nine cell lines by immunoblot analysis of total soluble protein extracted from callus material and by confocal laser scanning microscopy. A 28 kDa immunoreactive protein was detected in protein extracts from two of the callus lines. The level of GFP accumulation in one callus line (GFP/aadA-2.2) was estimated at 1% total soluble protein (TSP) by comparison with known amounts of GFP protein (Figure 3A). Plastid-based GFP accumulation in the GFP/aadA-2.2 callus line was confirmed by confocal laser scanning microscopy which showed discreet plastid-localized GFP fluorescence (Figure 3B). Not all cells in the spectinomycin-resistant callus showed GFP fluorescence; however, in those that did, homogenous

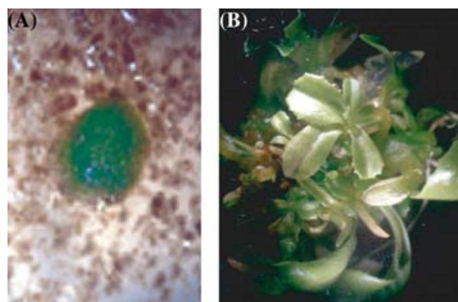

Figure 2. Regeneration of lettuce protoplasts on spectinomycin following PEG-mediated chloroplast transformation. (A) A green spectinomycin-resistant micro-callus on a background of bleached, non-spectinomycin-resistant protoplast colonies. (B) Lettuce shoots regenerating from spectinomycin-resistant callus.

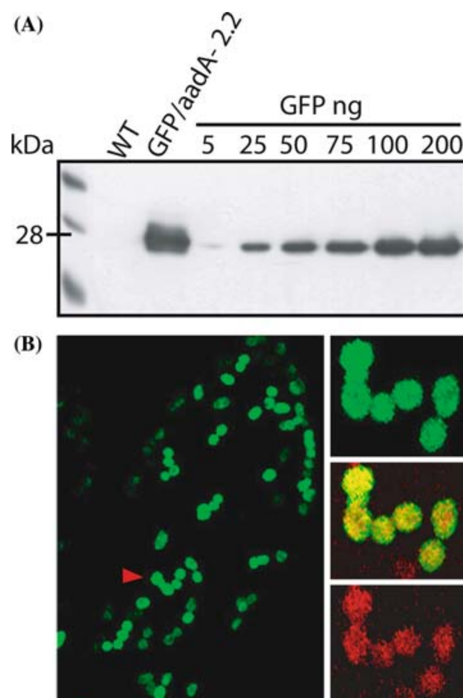

Figure 3. GFP accumulation in transplastomic lettuce callus cell lines. (A) Immunoblot of total soluble protein (10 μg) from wild-type (WT) and a transformed lettuce callus cell line (GFP/aadA-2.2) separated by SDS-12% PAGE. GFP standards (5, 25, 50, 75, 100 and 200 ng) were included in the gel. The membrane was incubated with rabbit antiserum to GFP and the immunoreactive bands were detected by chemiluminescence. (B) Laser scanning confocal microscope image of plastid-localized GFP in a single cell from transformed lettuce callus cell line GFP/aadA-2.2 (large image). A group of plastids (indicated by an arrow in large image) are shown under higher power on the right. Chloroplast localized GFP fluorescence (green, top panel); autofluorescence of chloroplasts (red, bottom panel), chloroplast localized GFP fluorescence superimposed on chloroplast autofluorescence (yellow, middle panel).

fluorescence was observed in all plastids. Both GFP-expressing cell lines were octoploid, as determined by flow cytometry analysis, and were not transferred to regeneration medium. Polyploid cell lines are the result of protoplast fusion events that can occur following the PEG treatment, no polyploid cell lines were generated from non-PEG treated protoplasts (data not shown). Two of the five callus lines that failed to show GFP accumulation were further assessed by PCR using *trnI* and *aad*A gene-specific primers. A 2 kb product was obtained in each case (data not shown) and the PCR products were gel purified and sequenced. The DNA sequence data confirmed a *gfp* excision

event in both callus lines mediated by recombination across the repeated P*rrn* region (data not shown).

One of the advantages of a plastid-based expression system in lettuce is the potential it offers both for the production and the delivery of human therapeutic proteins e.g. edible subunit vaccines. The influenza virus haemagglutinin (*HA*) gene was used as the first candidate antigen gene for expression in the lettuce plastid. HA is a major antigenic protein on the outer surface of influenza A virus and both HA subunit protein and *HA* DNA vaccines are known to elicit protective immune responses against viral challenge in mice (Saelens *et al.*, 1999; Johnson *et al.*, 2000; Ross *et al.*, 2000). The dicistronic LEC1 expression cassette (Figure 1C) contains the *aadA* gene and the *HA* gene under the control of the lettuce (*Ls*) *rrn* operon promoter (P*Ls-rrn*) and lettuce *psbA* 3′ sequence (3′*Ls-psbA*). A dicistronic construct was built to avoid having duplicated *rrn* promoter sequences in the expression cassette. Dicistronic constructs have been shown to generate significant levels of recombinant protein expression in tobacco plastids (Daniell *et al.*, 1998, De Cosa *et al.*, 2001, Lee *et al.*, 2003, Jeong *et al.*, 2004). The *HA* gene is preceeded by an 18 bp synthetic leader that includes a Shine-Dalgarno-like sequence (typically GGAGG) that is required for translation (Svab and Maliga, 1993; Hirose and Sugiura 2004). LEC1 was inserted into pLCV2 at the unique *Pac*I and *Asc*I cloning sites. PEG-mediated transformation experiments with pLCV2-LEC1 generated five independent spectinomycin-resistant cell lines that were all transplastomic and heteroplasmic for the LEC1 insertion as determined by PCR analyses using various combinations of PCR primers designed against vector, expression cassette, and flanking genomic DNA sequences (data not shown). Three independent plant lines (LEC1-1.2, 2.2, and 3.1) regenerated from the spectinomycin-resistant calli approximately 6 weeks after transfer to regeneration medium containing spectinomycin (500 mg/l; Figure 2B). Flow cytometry analyses showed that two of the transformed plant lines were diploid (LEC1-2.2 and 3.1) and the third (LEC1-1.2) was tetraploid. When these three lines were grown to flowering, the two diploid lines produced normal flowers that set seed (data not shown) whereas the tetraploid line produced abnormal flowers that failed to set seed even after selfing or reciprocal crossing with several lettuce cultivars.

Seed harvested from three self-pollinated LEC1-2.2 primary regenerants (A, B and C) produced 100% green, spectinomycin-resistant seedlings when sown on spectinomycin-containing medium (500 mg/l; Figure 4, and data not shown). T_1 plants from the LEC1-2.2A line were used to pollinate a male-sterile, wild-type lettuce line Ms 7 (Ryder, 1971). A batch of 1200 seeds obtained from this cross were cultured on spectinomycin-containing medium (500 mg/l) and produced 100% bleached, spectinomycin-susceptible, seedlings (Figure 4, and data not shown) indicating that the transformed plastids were not transmitted *via* pollen.

The ratio of transformed to non-transformed chloroplast DNA in T_1 plants from two of the

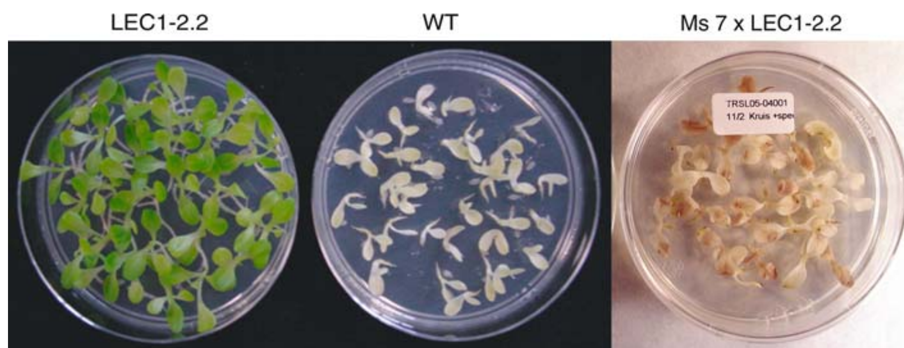

Figure 4. Spectinomycin-resistance in T_1 transplastomic lettuce transformed with pLCV2-LEC1. Seed harvested from the self-pollinated primary regenerant LEC1–2.2 showing 100% green, spectinomycin-resistant seedlings when cultured on spectinomycin-containing medium (500 mg/l). Wild-type lettuce seed showing 100% spectinomycin-susceptible seedlings when cultured on the same medium. Seeds obtained from a cross between male sterile, wild-type lettuce line Ms 7 and LEC1–2.2 also showing 100% spectinomycin-susceptible seedlings when cultured on the same medium.

three inbred lines (LEC1-2.2A and B) was assessed by Southern blot hybridization using a *trnI*-gene-specific probe (Figure 5A). As expected, the *trnI* probe hybridized to a single 9.9 kb DNA fragment in wild-type DNA cut with the restriction enzyme *Sac*I (Figure 5A). The *trnI* probe hybridized to a single 13.1 kb *Sac*I fragment in DNA isolated from the two T_1 transplastomic lines (Figure 5A), indicating that the entire LEC1 expression cassette was inserted into the plastid genome in these lines. The absence of the 9.9 kb wild-type band in DNA isolated from the transplastomic plants indicates that these plants were homoplasmic for the insertion (Figure 5A). An influenza *HA* gene-specific probe hybridized to the 13.1 kb *Sac*I fragment in the transformed plants and did not hybridize to wild-type DNA (Figure 5A).

Transcription of the *aadA* and *HA* genes in the same two LCV2-LEC1 transformed lines was assessed by Northern blot analysis (Figure 5B). Total RNA from the two transplastomic inbred lines and from wild-type lettuce plants was probed with *aadA* and *HA* gene-specific probes. Both probes hybridized to a single 3.2 kb transcript in the two transgenic lines, which is the expected size of the *aadA*/*HA* dicistronic transcript. Neither probe hybridized to wild-type RNA. HA protein accumulation was assessed in both young and mature leaf tissue from the two transgenic lines by immunoblotting, however, HA protein could not be detected in either case (data not shown). Other studies have also reported difficulty in achieving expression of a viral antigenic protein and a viral antigenic peptide in plant plastids (Birch-Machin *et al.*, 2004, Molina *et al.*, 2004). It is likely that the viral *HA* gene construct will require additional elements that could confer protein stability, such as candidate N-terminal or C-terminal fusions, to bring about protein accumulation in the lettuce plastid.

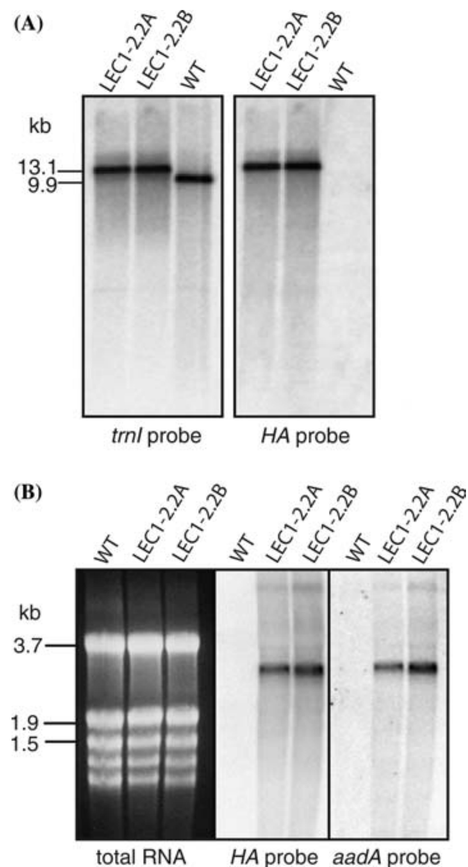

Figure 5. (A) Southern blot analysis of transplastomic lettuce plants. DNA from wild-type and T_1 plant lines (LEC1-2.2A and LEC1-2.2B) was cut with *Sac*I, separated by electrophoresis on a 1% agarose gel, and blotted onto nylon membrane. The blot was hybridized with a *trnI*-gene-specific probe then stripped and re-hybridized with a *HA* gene-specific probe. The *trnI* probe is expected to hybridize to a 9.9 kb DNA fragment in wild-type DNA cut with *Sac*I. (B) Northern blot analysis of transplastomic lettuce plants. Total RNA from wild-type (WT) and transplastomic T_1 lines LEC1-2.2A and LEC1-2.2B was separated by electrophoresis on a 1% agarose/formaldehyde gel and blotted onto nylon membrane. The blot was hybridized with a *HA* gene-specific probe then stripped and re-hybridized with an *aadA* gene-specific probe.

Discussion

Here we report PEG-mediated, stable transformation of lettuce plastids resulting in fertile, homoplasmic transgenic lines. Targeted integration of two expression cassettes was achieved using the pLCV2 vector that contains the lettuce *trnA* and *trnI* genes as target sites for homologous recombination into the inverted repeat region of the lettuce plastid genome. The pLCV2 vector is based on the universal chloroplast integration vector pSBL that has been used to transform the tobacco chloroplast genome (Daniell *et al.*, 1998, De Cosa *et al.*, 2001). pSBL has been proposed as a universal vector suitable for plastid transformation of other plant species because the *trnA* and *trnI* gene order is conserved in a wide range of plant species and because chloroplast DNA sequences are generally highly conserved at the

nucleotide sequence level (Daniell *et al*., 1998). Although gene order is conserved in lettuce, the DNA sequence of the lettuce *trnA* and *trnI* genes reveals two DNA segments of 231 and 101 bp that are absent from the corresponding tobacco sequence. Whether these sequence differences are sufficient to preclude lettuce chloroplast transformation with tobacco pSBL vectors has not been determined. However, at the very least, it might be expected that tobacco pSBL vectors might reduce the transformation frequency obtained.

On the other hand, this study shows that tobacco gene regulatory sequences are sufficent to achieve transgene expression in the lettuce plastid. Other researchers have demonstrated functionality of the tobacco chimeric *rrn* promoter (Svab and Maliga, 1993) in potato (Sidorov *et al*., 1999), tomato (Ruf *et al*., 2001), petunia (Zubko, *et al*., 2004), *Arabidopsis* (Sikdar *et al*., 1998, Sriraman *et al*., 1998), oilseed rape (Hou *et al*., 2003; Skarjinskaia *et al*., 2003), and soybean (Dufourmantel *et al*., 2004) plastids. Thus, these results indicate that tobacco gene regulatory sequences can be used for plastid-based expression of transgenes in a range of solanaceous and non-solanaceous plant species. Plastid-based GFP accumulation, to levels equivalent to 1% TSP, was observed in lettuce callus tissue transformed with a GFP/aadA construct. GFP accumulation in lettuce is less than the 5.5% TSP levels obtained in tobacco with a similar gene construct (Newell *et al*., 2003). The lower level of expression in lettuce is likely due to the fact that GFP was assessed in heteroplasmic callus tissue whereas in tobacco GFP was assessed in leaves of homoplasmic plant lines. Only two of the seven transplastomic cell lines assessed showed detectable GFP accumulation. PCR and DNA sequence analyses confirmed that lack of GFP in two of the non-expressing lines (the three others were not tested) is due to *gfp* excision events in these cell lines mediated by recombination across the repeated *rrn* promoter sequences upstream of the *gfp* and *aadA* genes (Figure 1C, Iamtham and Day, 2000). The *aadA* gene confers resistance to both spectinomycin and streptomycin (Svab *et al*., 1990) and selection of lettuce transformants was achieved using the non-lethal selective agent spectinomycin. The frequency of lettuce plastid transformation was estimated at 1–2 spectinomycin-resistant cell lines per 10^6 viable protoplasts (based on four independent transformation experiments that generated spectinomycin-resistant cell lines). This is significantly lower than the tobacco transformation frequency obtained using a comparable transformation approach that achieved 20–40 spectinomycin-resistant cell lines per 10^6 treated protoplasts (Koop *et al*., 1996). However, 13–55% of the tobacco spectinomycin-resistant cell lines were shown not to be true plastid transformants and spectinomycin resistance was conferred either by spontaneous mutation or nuclear transformation events (Koop *et al*., 1996). In contrast, 100% of the spectinomycin-resistant lettuce cell lines generated in this study were true plastid transformants (as determined by PCR) and no spectinomycin-resistant spontaneous mutants or nuclear transformants were obtained. The main limitation of the lettuce PEG-mediated transformation protocol is the high frequency of polyploid cell lines that were obtained and which were probably due to protoplast fusion events mediated by the PEG treatment. Consequently, only approximately 30% of the regenerated spectinomycin-resistant plants were diploid, and only the diploid plants were fertile and set seed.

One of the advantages of plastid transformation is the potential for high levels of accumulation of heterologous proteins. Several functional therapeutic proteins have been expressed in tobacco chloroplasts at levels that rival or exceed those of conventional animal or bacterial bioreactors (Fischer *et al*., 2004). A recent report described plastid-based expression of tetanus toxin fragment C from *Clostridium tetani* in tobacco to levels equivalent to 25% TSP (Tregoning *et al*., 2003). In this case, the plastid-expressed subunit vaccine was comparable to the conventional vaccine in terms of protection afforded against tetanus toxin challenge in a mouse model. The lettuce plastid offers several advantages as a production system for high value human therapeutic proteins compared to tobacco: (1) most of the plant is leaf tissue and this tissue contains the greatest number of plastids per cell; (2) unlike tobacco, lettuce has no toxic alkaloids that need to be removed, adding to protein purification and downstream processing costs; (3) lettuce is a relevant human foodstuff that can be consumed without cooking. Thus, lettuce plastid transformation offers the possibility of both a production and a delivery system for human health products such as subunit vaccines

that are capable of stimulating a therapeutic response *via* the gut. The influenza virus A haemagglutinin gene (*HA*) is the first potential subunit vaccine gene to be introduced into the lettuce plastid genome. The LEC1 dicistronic cassette contains the *aadA* gene and the *HA* gene under the control of the lettuce *rrn* operon promoter (P*Ls*-*rrn*). A similar dicistronic format was used to co-express *aadA* with various other transgenes in the tobacco plastid including *gfp* (Jeong *et al*., 2004), 5-enol-pyruvate shikimate-3-phosphate synthase (Daniell *et al*., 1998), the *Cry2*Aa2 operon (De Cosa *et al*., 2001), and a trehalose phosphate synthase gene (Lee *et al*., 2003). Although fertile, homoplasmic, plastid-transformed plant lines were obtained, only mRNA, and no HA protein, was detected in these lines (data not shown), even though HA protein was detected at a high level in *E. coli* containing this construct (data not shown). Recent studies have demonstrated instability of candidate human and viral proteins expressed in plant plastids (Leelavathi and Reddy 2003; Birch-Machin *et al*., 2004, Molina *et al*., 2004). At the moment there are still no hard-and-fast rules that can be applied to predict if a foreign protein will accumulate in plant plastids. Pre-screening of plastid transgene expression cassettes in *E. coli* is not a reliable predictor of plastid expression levels (Magee *et al*., 2004). Work in tobacco has shown that levels of plastid-based protein accumulation are very much dependent on optimizing expression cassette design, e.g. choice of promoter, choice of 5′ and 3′ untranslated regions, and choice of N-terminal fusions (Maliga, 2003). Molina *et al*. (2004) only achieved high levels of a viral antigenic peptide in tobacco plastids as a C-terminal translational fusion with cholera toxin B subunit or GFP. Now that a plastid transformation system has been developed for lettuce the future focus will be to generate a range of cassettes that utilize different types of translational control in order to maximise plastid-based protein production in this species.

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