Efficient targeting of foreign genes into the tobacco plastid genome

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ABSTRACT

The pPRV plasmids are vectors for targeted insertion of foreign genes into the tobacco plastid genome (ptDNA). The vectors are based on the pUC119 plasmid which replicates in E.coli but not in plastids. The spectinomycin resistance (aadA) gene and a multiple cloning site (MCS) are flanked by 1.8-kb and 1.2-kb ptDNA sequences. Biolistic delivery of vector DNA, followed by spectinomycin selection, yields plastid transformants at a reproducible frequency, ~1 transplastomic line per bombarded sample. The selected aadA gene and linked non-selectable genes cloned into the MCS are incorporated into the ptDNA by two homologous recombination events via the flanking ptDNA sequences. The transplastomes thus generated are stable, and are maternally transmitted to the seed progeny. The pPRV vector series targets insertions between the divergently transcribed trnV gene and the rps12/7 operon. The lack of readthrough transcription of appropriately oriented transgenes makes the vectors an ideal choice for the study of transgene promoter activity.

INTRODUCTION

Plastid transformation was first obtained in Chlamydomonas reinhardtii, a unicellular alga (1; reviewed in 2,3). More recently, stable transformation of the plastid genome of tobacco, a higher plant, has been accomplished. Transformation is a multistep process involving: (i) introduction of the transforming DNA on the surface of microscopic tungsten particles using the biolistic process (4–12) or polyethylene glycol treatment (13,14); (ii) integration of the transforming DNA into the plastid genome by homologous recombination (4–11); and (iii) selective elimination of wild-type plastid genome copies during the course of repeated cell divisions (4–11). Since tobacco cells contain up to 10,000 identical copies (15) of the circular 155,844 bp (16) plastid genome, the development of selectable marker genes has been critical for the recovery of a pure population of transplastomes. Selectable genes for tobacco plastid transformation confer resistance to spectinomycin based on mutations in the 16S rRNA gene (4,5) or by expression of a bacterial aadA gene (6). An additional marker is resistance to kanamycin based on the expression of the bacterial kan gene (8).

All characterized recombination events in plastids have been between homologous sequences. Targeted insertion of foreign genes into the plastid genome is obtained by flanking the heterologous sequence with homologous plastid DNA fragments (6–11). This homologous ptDNA is referred to as the targeting fragment since it directs the insertion of foreign DNA to a particular location in the plastid genome. Desirable characteristics of a plastid targeting fragment are: (i) sufficient flanking ptDNA sequence for efficient targeting of heterologous DNA; (ii) a carefully chosen insertion site so that the integrated heterologous DNA does not interfere with the expression of the adjacent plastid genes and (iii) the availability of convenient restriction sites for cloning.

We describe here an E.coli pUC119 plastid derivative, pPRV1, the backbone of a Plastid Repeat Vector family. The pPRV1 plasmid was developed into versatile plastid vectors by the addition of two important components: a selectable spectinomycin-resistance (aadA) gene for highly efficient recovery of plastid transformants (3) and a multiple cloning site (MCS) to facilitate the introduction of linked passenger genes. We have shown that the inserted foreign genes at the MCS are stable, and that there is no readthrough transcription from outside promoters when passenger genes are inserted in the appropriate orientation.

MATERIALS AND METHODS

Vector construction

Plasmid pPRV1 (GenBank acc. no. U12809) is based on a pUC119 plasmid (17) derivative, pZS192, in which the Scal site in the ampicillin resistance gene has been removed by oligonucleotide-directed mutagenesis (5'-AGTACT-3' changed to 5'-AGTATT-3'). The 3.0-kb EcoRI/BglII ptDNA fragment from pJS75 (5) was cloned into plasmid pZS192 digested with the EcoRI/BamHI restriction endonucleases. The EcoRI and BglII restriction sites in the tobacco ptDNA are at nucleotide positions 138,447 and 141,382, respectively (16). The spectinomycin-resistance mutation in the 16S rRNA gene (16SrDNA) was removed by replacing the EcoRI/ApaI fragment in the 3.0-kb...
**RESULTS**

**The pPRV plastid vector family**

In choosing a targeting region, we first sought a transcriptionally silent location for insertion of foreign genes. Since transcription is divergent from the rMv gene (22) and rps12/rps7 operon promoters (23, 24), the rMv—rps12/rps7 intergenic region was modified for targeting foreign genes. Therefore, the region was subcloned as a 3.0-kb EcoRI/BglII fragment in a pUC119 vector to yield plasmids pPRV1 and pPRV2. Plasmid pPRV1 has a unique Scal-site

**Construction of the uidA reporter genes**

The promoter-containing uidA transgene is contained in a 2.2-kb EcoRI/HindIII fragment in plasmid pLAA21, a pUC118 plasmid derivative. The gene was assembled from the Prrn promoter (SacI/NcoI fragment; ref. 6), the uidA coding region (the 1.87-kb NcoI/XbaI fragment from plasmid pJS25; ref. 7) and Trps16 (XbaI/HindIII fragment, above). The Prrn::uidA::Trps16 transgene was cloned in vector pPRV111A and pPRV111B to yield plasmids pLAA24A and pLAA24B (Fig. 5A).

The promoterless uidA transgene in plasmids pLAA25A and pLAA25B is identical with the uidA transgenes in plasmids pLAA24A and pLAA24B except that the SacI/NcoI Prrn promoter fragment was replaced with the EcoR1/NcoI linker 5'-GAATTCAGTTGATGGAGGATCCGATG-3' containing a consensus ribosome binding site (underlined).

**Production of transplastomic lines**

Tobacco (*N. tabacum*) plants were grown aseptically on agar-solidified medium containing MS salts (21) and sucrose (30 g/L). For plastid transformation, the DNA was introduced on the surface of microscopic tungsten particles (6) using the DuPont PDS1000He Biolistic gun. Typically, 25 bombardments were carried out with each of the plasmids. Spectinomycin resistant calli and shoots were selected on RMOP medium containing 500 μg/mL of spectinomycin dihydrochloride (6). Resistant shoots were regenerated on the same selective medium, and rooted on MS agar medium. Only 1 or 2 independently transformed lines was studied per plasmid construct.

**DNA and RNA gel blot analysis**

Total cellular DNA was digested with the appropriate restriction enzymes, electrophoresed on 0.7% agarose gels and transferred to nylon membrane (Amersham) using the PosiBiot Transfer apparatus (Stratagene) (7). Blots were probed using Rapid Hybridization Buffer (Amersham) with 32P labeled probes generated by random priming (Boehringer-Mannheim).

RNA was extracted from frozen leaf tissue with the TRIzol Reagent (BRL) following the manufacturer’s protocol. For Northern analysis total cellular RNA was separated on a 1% agarose-formaldehyde gel and transferred to nylon membrane as above. Labeling of DNA for hybridization, and probing was carried out as described for DNA gel blots (above). Membranes were stripped in boiling 0.1% SDS prior to reprobing.

**Testing of seedling phenotypes**

Seeding phenotypes were determined by plating surface-sterilized seeds on MS salt medium (6). On selective spectinomycin dihydrochloride medium (500 μg/mL) resistant progeny are green, whereas sensitive progeny are white (6).
Transformation of tobacco plastids with the pOVZ15 plasmid was carried out by the standard protocols. DNA gel blot analysis confirmed a uniform population of transformed plastid genome copies in the Nt-pOVZ15-2 and Nt-pOVZ15-3 independently transformed lines (Fig. 2).

Figure 1. The plastid targeting fragment in the pPRV plasmids. Abbreviations: 16SrDNA, trnV, rps7/12, are plastid genes (16). Restriction sites removed during construction are in brackets; those in the MCS are above the triangle. Note that all sites are unique except BamH1 and Sphl. Arrows mark the direction of transcription of each gene.

in the trnV-rps7/12 intergenic region, which was converted into a multiple cloning site (MCS) in plasmid pPRV100 (Fig. 1) by ligating a linker in both (A,B) orientations.

Plasmid pPRV1 was converted into a family of plastid transformation vectors by inserting spectinomycin resistance (aadA) genes in the MCS. The aadA coding region is expressed from the 5'- (P) and 3'- (T) regulatory regions of plastid genes, including those of the psbA gene (PspsbA – TpsbA) and the chimeric Prrn rRNA operon promoter and the 3' -region of the rps16 ribosomal protein gene (Trps16) in vectors pPRV111A/B and pPRV112A/B, respectively (Fig. 1). A and B refer to the relative orientation of the MCS. The aadA genes and the MCS are flanked by 1.8-kb and 1.2-kb ptDNA fragments to target the insertion of linked genes into the plastid genome by homologous recombination.

The utility of the pPRV vectors was confirmed by testing: (i) whether the plastid genomes carrying insertions in the trnV-rps7/12 intergenic region are stable and (ii) whether the MCS was inserted in a transcriptionally silent location. Experiments addressing these issues are described below.

Genetic stability of plants with aadA genes in the trnV-rps7/12 intergenic region

Genetic stability of aadA-carrying transplastomes was tested in plants transformed with plasmid pOVZ15, a progenitor of vector pPRV111 with aadA expressed from a psbA cassette. Transformation of tobacco plastids with the pOVZ15 plasmid was carried out by the standard protocols. DNA gel blot analysis confirmed a uniform population of transformed plastid genome copies in the Nt-pOVZ15-2 and Nt-pOVZ15-3 independently transformed lines (Fig. 2)

Figure 2. Integration of aadA into the tobacco plastid genome (ptDNA) after transformation with plasmid pOVZ15. Total cellular DNA was isolated from non-transformed tobacco leaves (wt), and from leaves of plants transformed with plasmid pOVZ15 (Nt-pOVZ15). The DNA was digested with the EcoRI and EcoRV restriction endonucleases, and the DNA gel blots were probed with (A) the 3.5-kb EcoRI/EcoRV ptDNA fragment (P1) that is part of the targeting fragment, and (B) the 0.8-kb aadA coding region (P2). (C) Map of the ptDNA region containing the pPRV targeting fragment, and of plasmid pOVZ15. Note that the P1 targeting sequences hybridize to a 2.4-kb fragment in the wild-type plants and a larger, 3.5-kb fragment in the transplastomic lines. The aadA probe, P2, hybridizes only to the larger transplastomic fragment. Note also, that the EcoRI site is blunted in the pOVZ15 plasmid, and the predicted size 3.5-kb fragment is obtained only if aadA has integrated in the ptDNA via flanking homologous sequences. Abbreviations: 16SrDNA, trnV, rps7/12 are plastid genes (16). Arrows indicate the direction of transcription.

Nt-pOVZ15-2 and Nt-pOVZ15-3 plants were transferred to the greenhouse where they were grown in soil, in the absence of spectinomycin selection. Seeds were collected after self-pollination, and after crossing with the wild-type parental plants. The seeds were germinated on a selective spectinomycin medium of spectinomycin selection. Seeds were collected after self-pollination, and after crossing with the wild-type parental plants. The seeds were germinated on a selective spectinomycin medium to determine whether maintenance of the plants under non-selective conditions resulted in the elimination of the transplastomic aadA gene, and therefore the loss of spectinomycin resistance.

Data on seed transmission of the transplastomic spectinomycin resistance trait are summarized in Table 1. Uniform resistance to spectinomycin in the selfed seed progeny, and in the cross with the transplastomic plants as the female parent, indicated that the transplastomic aadA marker is stable. Lack of pollen transmission of spectinomycin resistance in the reciprocal cross, using the wild-type non-transformed parent as female and the transplastomic as pollen parent, was expected since plastids are not transmitted via pollen in tobacco.

The pOVZ15 plasmid carries an aadA gene expressed in a psbA cassette. The psbA gene, the source of expression signals, is located relatively far away, ~18-kb, in the large single-copy region of the plastid genome. However, we were also interested...
in using transgenes bearing promoters of plastid genes located in the inverted repeat, in close proximity to the insertion site. Insertion of transgenes at these sites results in direct duplication of the promoter regions (Fig. 3). During evolution such short direct repeats were shown to mediate plastid genome rearrangements (25,26). We tested therefore if duplication of the promoter regions results in transplastome instability. Selectable aadA genes were cloned in expression cassettes with the promoters of the ribosomal RNA operon (Ptrn) and the trnV gene (PtrnV). The chimeric genes were cloned in the Scal site in a progenitor of pPRV1 to yield plasmids pZS208 (Ptrn::aadA::TpsbA) and pZS209 (PtrnV::aadA::TpsbA), and were introduced into the plastid genome. A uniform population of transplastomes was verified by Southern analysis in one transformed line per construct (data not shown).

To test if loss of aadA occurs by loop-out via the promoters, seeds were collected from pZS208- and pZS209-transformed plants. Seeds were then germinated on a selective spectinomycin medium to screen for spectinomycin-sensitive seedlings by their white color. No sensitive seedlings were found in a population of transplastomes was verified by Southern analysis in one transformed line per construct (data not shown).

Readthrough transcription of transgenes inserted in the trnV–rps7/12 intergenic region

The trnV–rps7/12 intergenic region contains ORF131 and ORF70B, two uncharacterized open reading frames which are unique to tobacco (16,27). The two overlapping ORFs are transcribed in a convergent orientation (Fig. 4). The Scal site in plasmid pJS75, a direct progenitor of pPRV1, was created by linker ligation of a HincII-site which altered the ORF131 reading frame but did not affect ORF70B (Fig. 4). This change in the ORF131 reading frame was without phenotypic consequences (5).

Transcriptional activity from the ORF promoters could result in readthrough transcription into transgenes located at the MCS. To test for readthrough activity, promoterless and promoter-containing uidA transgenes were introduced into the plastid genome in vectors pPRV111A and B (Fig. 5A). Transformation with the four plasmids was followed by selection for the linked spectinomycin resistance (aadA) gene. Integration of the uidA transgenes, and the lack of wild-type genome copies was confirmed by DNA gel blot analysis (not shown). Further studies were carried out only with one line.

When the promoterless uidA transgene was introduced into plastids using vector pPRV111A, no uidA message was detected with the uidA probe (Fig 5B, Nt-pLAA25A plants). However, when the uidA transgene was expressed from the engineered Prm promoter, the uidA message accumulated to high levels, as expected (Fig 5B, Nt-pLAA24A plants). Most of the mRNA is monocistrionic message derived form the Prm promoter. Note the minor, larger mRNA species which forms by termination downstream of the uidA gene at an uncharacterized site (Fig. 5A).

In plants transformed with the promoterless uidA construct in the B orientation two uidA-containing transcripts could be detected (Fig 5B, pLAA25B). The size of these transcripts is consistent

Table 1. The chimeric aadA gene is stable in the plastid genome of the seed progeny

<table>
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<th>Linea</th>
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<td>RF1</td>
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aNt and the plasmid name indicates with which plasmid the transplastomic line was obtained; the number added after the hyphen identifies an independently transformed line.

bF1, cross in which resistant is female; RF1, cross in which resistant is pollen parent.

Spectinomycin dihydrochloride at 500 µg/ml in the germination medium.

Figure 3. Transplastomes with promoters as short, direct repeat DNA elements. The 117 nucleotide Prm, and the 137 nucleotide PrmV direct repeats (heavy arrows below maps) in the plastid genome of plants transformed with plasmids pZS206 and pZS209, respectively. DNA sequence of the chimeric promoters is given below the maps. Nucleotides derived from the ptDNA are in upper case. The ribosome binding site (GGUGG) and translational initiation codon (AUG) are underlined.

Figure 4. Seal site is not limited to genes which are distant from the insertion sites, and that duplication of the promoters, via loop-out, cross in which resistant is female; RF1, cross in which resistant is pollen parent.
with their initiation at the promoter of the upstream endogenous ORF70B gene. The steady state level of these mRNAs was at least 20-fold less than that of the promoter-containing uidA gene. In plastids transformed with plasmid pLAA24B carrying the promoter-containing uidA genes two transcripts were detected on RNA gel blots with a uidA probe (Fig 5B). Both RNAs are transcribed from the uidA promoter and are terminated downstream of uidA and aadA genes, respectively, as diagrammed in Fig 5A.

DISCUSSION

We report here vectors that direct insertion of foreign genes in the inverted repeat region of the tobacco plastid genome. The selectable marker, the integrated aadA gene, was genetically stable even when its promoter region formed a short direct repeat with the endogenous rRNA operon or tmV gene promoters. Still, duplication of sequences is not desirable. Expression of aadA from alternative signals gives the option to choose a vector in which the aadA gene is expressed from signals other than the passenger gene.

The aadA gene was shown to be a highly efficient plastid marker yielding 1-2 independently transformed clones per bombarded leaf sample (6). No effort was made to determine the number of plastid transformants in the present experiment. However, the ease with which the plastid transformants are regularly obtained with the pPRV vectors in a sample of 25 bombarded leaves confirms the published efficiency data. Using longer aadA targeting fragments, 1.5+3.6-kb instead of 1.8+1.2-kb, yielded ~5 clones per bombardment in the tmV–tmM intergenic region (28). We believe therefore that the transformation efficiency may be further increased by increasing the size of the targeting fragment.

Figure 4. DNA sequence of the plastid targeting fragment surrounding ORF70B and ORF131. The numbers on the left refer to the relative position of the sequence in the ptDNA insert in pPRV1; numbers above the lines correspond to numbers in the plastid genome (16). The coding regions of the tmV gene, and of ORF70B and ORF131 are framed (11). Nucleotides derived from the ptDNA are in capital letters; those inserted during vector construction are in lower case. The Stul and ScaI cloning sites, the -10 and -35 conserved promoter elements for tmV (22), tmV (22) Seal cloning sites, the -10 and -35 conserved promoter elements for tmV (22), tmV (22)

Figure 5. Transcription pattern of uidA transgenes located in the tmV–rs7/12 intergenic region. (A) The map of transplastomes obtained by transformation with plasmids carrying the promoter-containing (pLAA24A,B) and promoterless (pLAA25A,B) uidA reporter genes. Black boxes indicate the Prrn (pLAA25A,B) and the leader of the promoterless uidA gene (pLAA25A,B); horizontally striped boxes depict Trps16. Wavy horizontal lines represent transcripts detected by the uidA probe. (B) RNA gel blot analysis of uidA mRNAs in the transplastomic lines. The amount of total cellular RNA (μg) loaded per lane is given in brackets. The membranes were probed with the uidA coding sequence (upper). Note the lack of transcripts in plants transformed with the pLAA25A plasmid, and monocistronic and dicistronic mRNAs in the other transplastomic lines, shown in Figure 5A. Position of monocistronic uidA, and dicistronic uidA/aadA mRNA is indicated. Asterisks mark transcripts initiated from an upstream plastid promoter in transplastomic line Ni-pLAA25B. The blot was stripped and probed with a 16S rRNA probe to control for amounts of RNA loaded (lower).

The 1.8+1.2-kb targeting fragments in the pPRV vectors were sufficient to direct the integration of a 3.3-kb block of heterologous sequence made up of the aadA (1.1 kb) and uidA
Given that the 3'-regions of plastid genes terminate transcription inefficiently (29,30), some degree of read-through transcription is expected at almost any location in the ptDNA. Indeed, the study of plastid transgene expression has been complicated by the formation of multiple, complex mRNAs (7,8). We report here that, when transgenes are oriented towards the \textit{rpsl2/7} operon in the pPRV vectors, there is no detectable read-through transcription from endogenous plastid gene promoters. The pPRV target site is therefore uniquely suited to study plastid promoter activity using reporter genes. In addition, applications that depend on tight transcriptional regulation of the introduced transgenes should now be feasible.

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