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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 reinhardttii*, 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 plasmid 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 *Sca*I 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 *Eco*RI/*Bgl*II ptDNA fragment from pJS75 (5) was cloned into plasmid pZS192 digested with the *Eco*RI/*Bam*HI restriction endonucleases. The *Eco*RI and *Bgl*II 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 *Eco*RI/*Apa*I fragment in the 3.0-kb

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*Eco*RI/*Bgl*II clone with the cognate wild-type ptDNA fragment (nucleotide positions 138,447 and 138,892, respectively, in ref. 16). The *Eco*RI-site at one end of the ptDNA fragment was eliminated by digestion with *Eco*RI and filling-in the single-stranded overhangs with the Klenow fragment. Subsequently, all remaining restriction sites of the pUC119 MCS were removed by digestion with *Xba*I and *Hin*dIII, filling-in the single-stranded overhangs with the Klenow Fragment and ligation. For convenience, the *Dra*I site in plasmid pJS75 (5) was converted to a unique *Stu*I restriction site by linker ligation (5'-CG-AGGCCTCG-3').

Plasmids pPRV100A (GenBank acc. no. U12810) and pPRV100B (GenBank acc. no. U12811) were obtained by ligating the multiple cloning site (MCS) as a synthetic double-stranded oligonucleotide into the *Sca*I site of pPRV1. The oligonucleotide was obtained by annealing the 5'-AAGCTTGCATGCCTGC-AGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCG-AATTC-3' sequence with its complementary strand, designed after the MCS in plasmid pUC18 (18). The linker was ligated in both (A,B) orientations. The *Eco*RI site is proximal to the *trnV* gene in derivatives with the A orientation.

Selectable markers in the pPRV family are *aadA* genes (6) expressed in different cassettes. The expression cassettes were set up to include the promoter (P) in an *Eco*RI/*Nco*I fragment and the 3'-regulatory region (T) in an *Xba*I/*Hin*dIII fragment. The *Nco*I site includes the translational initiation codon; the *Xba*I site is at the 5' end of the 3'-regulatory region. The P*psbA(S)*−T*psbA(S)* cassette is a truncated derivative of the *psbA(L)* cassette described in ref. 7. The 5'-regulatory region includes nucleotides 1735 through 1596; the 3-region includes nucleotides 533 through 345 of the tobacco ptDNA (16). The P*rrn*−T*rps16* cassette has the P*rrn* promoter described in ref. 6, and the 3'-regulatory region (T*rps16*) of the *rps16* ribosomal protein gene between nucleotides 5087 and 4939 in ref. 16. The chimeric *aad*A genes were obtained by ligating the coding region as an *BspHI*/*Xba*I fragment from plasmid pPM19 (19) in plasmids carrying the expression cassettes digested with *Nco*I and *Xba*I.

Plastid vectors pPRV111 and pPRV112 were obtained by ligating the P*psbA(S)*::*aadA*::T*psbA(S)* and P*rrn*::*aadA*::T*rps16* genes into the *Sca*I site, respectively, and a MCS adjacent to the *aadA* gene in both orientations (A,B), as described for plasmid pPRV100 above. The GenBank accession numbers for vectors pPRV111A,B and pPRV112A,B are U12812−U12815, respectively.

Plasmid pOVZ15 is a precursor of pPRV111 lacking the MCS but contains the *aadA* gene. Plasmids pZS208 and pZS209 also carry an *aadA* gene at the *Sca*I site but in a larger targeting fragment defined by *Sac*I and *Pst*I restriction sites at nucleotides 137,113 and 142,541, respectively. In plasmid pZS208, the *aadA* gene is expressed from a P*rrn* derivative in which the N-terminus of the *aadA* coding region is translationally fused with the 5 N-terminal amino acids of the ribulose-1,5-bisphosphate carboxylase large subunit (8) and T*psbA(L)* derived from plasmid pJS25 (7). In plasmid pZS209, *aadA* is expressed from a chimeric *trnV* promoter (P*trnV*) which is transcriptionally fused with a synthetic ribosome binding site to ensure translation of the mRNA, as described for the *Prrn* promoter (6) and T*psbA* from plasmid pJS25 (7). The *aadA* gene in both plasmids are ligated into the *Sca*I site. DNA sequence for the *aadA* promoters in plasmids pZS208 and pZS209 are included in the Results.

Plasmid construction was carried out by methods in ref. 20.

Construction of the *uidA* reporter genes

The promoter-containing *uidA* transgene is contained in a 2.2-kb *Eco*RI/*Hin*dIII fragment in plasmid pLAA21, a pUC118 plasmid derivative. The gene was assembled from the P*rrn* promoter (*Sac*I/*Nco*I fragment; ref. 6), the *uidA* coding region (the 1.87-kb *Nco*I/*Xba*I fragment from plasmid pJS25; ref. 7) and T*rps16* (*Xba*I/*Hin*dIII fragment, above). The P*rrn*::*uidA*:T*rps16* 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 *Sac*I/*Nco*I P*rrn* promoter fragment was replaced with the *Eco*RI/*Nco*I linker 5'-GAATTCAGTTGTAGGGAGGGATCCATGG-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 (30g/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 line 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 PosiBlot Transfer apparatus (Stratagene) (7). Blots were probed using Rapid Hybridization Buffer (Amersham) with ^{32}P 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

Seedling 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).

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 *trnV* gene (22) and *rps12/7* operon promoters (23,24), the *trnV*−*rps12/7* intergenic region was modified for targeting foreign genes. Therefore, the region was subcloned as a 3.0-kb *Eco*RI/*Bgl*II fragment in a pUC119 vector to yield plasmid pPRV1. Plasmid pPRV1 has a unique *Sca*I-site

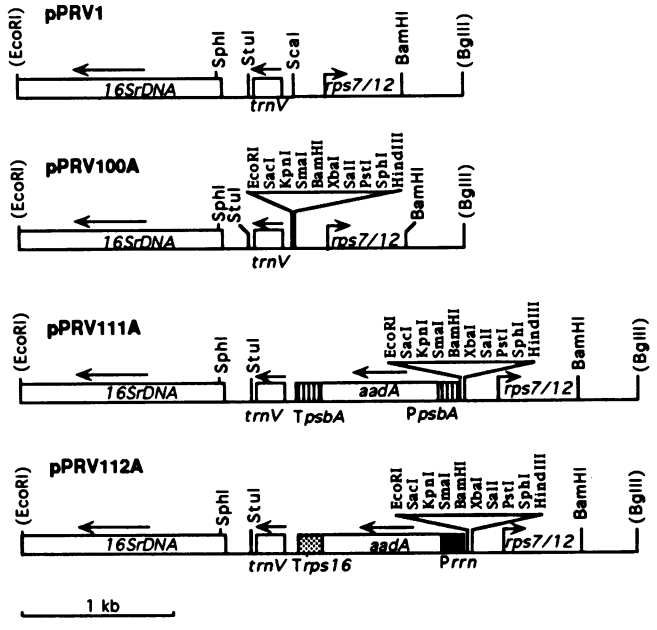

Figure 1. The plastid targeting fragment in the pPRV plasmids. Abbreviations: *16SrDNA*, *trnV*, *rps12/7*, are plastid genes (16). Restriction sites removed during construction are in brackets; those in the MCS are above the triangle. Note that all sites are unique except BamHI and *Sph*I. Arrows mark the direction of transcription of each gene.

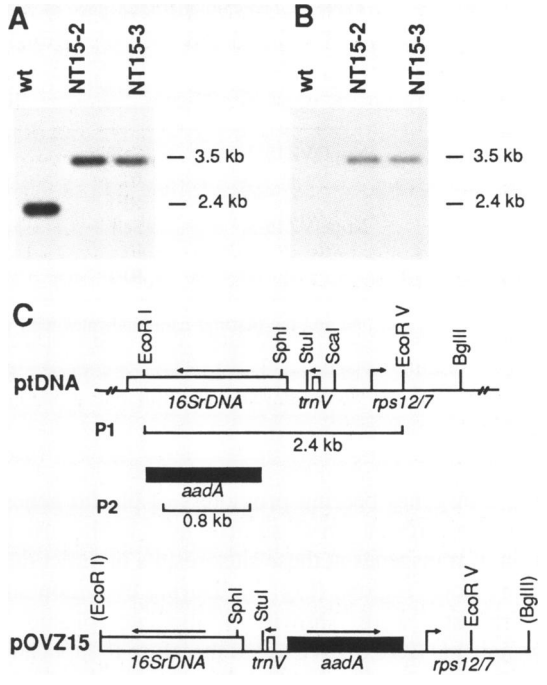

Figure 2. Integration of *aadA* into the tobacco plastid genome (ptDNA) after transformation with plasmid pOVZ15. Total cellular DNA was isolated from non-transformed tobacco leaves (wt), and from leaves of plants transformed with plasmid pOVZ15 (Nt-pOVZ15). The DNA was digested with the *Eco*RI and *Eco*RV restriction endonucleases, and the DNA gel blots were probed with (**A**) the 3.5-kb *Eco*RI/*Eco*RV 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 *Eco*RI 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*, *rps12/7* are plastid genes (16). Arrows indicate the direction of transcription.

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 *Sca*I-site. The *aadA* coding region is expressed from the 5′- (P) and 3′-(T) regulatory regions of plastid genes, including those of the *psbA* gene (P*psbA* – T*psbA*) and the chimeric P*rrn* rRNA operon promoter and the 3′-region of the *rps16* ribosomal protein gene (T*rps16*) 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)

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 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

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

Line[a]	Progeny[b]	No drug		Sp500[c]	
		Green	White	Green	White
Nt-pOVZ15-2	Self	960	0	720	0
	F1	704	0	784	0
	RF1	736	0	0	520
Nt-pOVZ15-3	Self	744	0	488	0
	F1	552	0	680	0
	RF1	760	0	0	800

[a]Nt and the plasmid name indicates with which plasmid the transplastomic line was obtained; the number added after the hyphen identifies an independently transformed line.
[b]F1, cross in which resistant is female; RF1, cross in which resistant is pollen parent.
[c]Spectinomycin dihydrochloride at 500 μg/ml in the germination medium.

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 (P*rrn*) and the *trnV* gene (P*trnV*). The chimeric genes were cloned in the *Sca*I site in a progenitor of pPRV1 to yield plasmids pZS208 (P*rrn*::*aadA*::T*psbA*) and pZS209 (P*trn*V::*aadA*::T*psbA*), 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 4,237 and 3,444 seedlings, respectively, indicating that both types of transplastomes are stable. We conclude therefore that study of transgenes at the *Sca*I site is not limited to genes which are distant from the insertion sites, and that duplication of the sequences does not result in easily detectable instability.

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 *Sca*I site in plasmid pJS75, a direct progenitor of pPRV1, was created by linker ligation of a *Hin*cII-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 transcription, 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

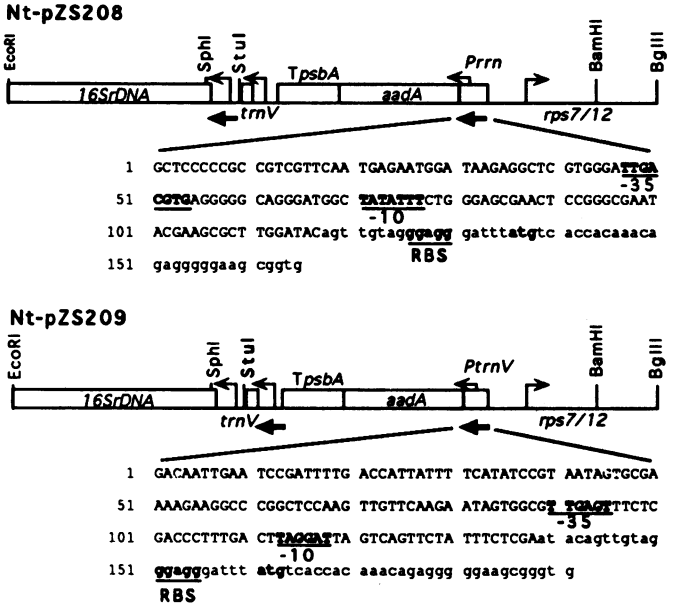

Figure 3. Transplastomes with promoters as short, direct repeat DNA elements. The 117 nucleotide P*rrn*, and the 137 nucleotide P*trnV* direct repeats (heavy arrows below maps) in the plastid genome of plants transformed with plasmids pZS208 and pZS209, respectively. DNA sequence of the chimeric promoters is given below the maps. Nucleotides derived from the ptDNA are in upper case. The −10 and −35 conserved promoter elements and DNA sequences encoding the ribosome binding site (GGUGG) and translational initiation codon (AUG) are underlined.

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 P*rrn* promoter, the *uidA* message accumulated to high levels, as expected (Fig. 5B, Nt-pLAA24A plants). Most of the mRNA is monocistronic message derived form the P*rrn* 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

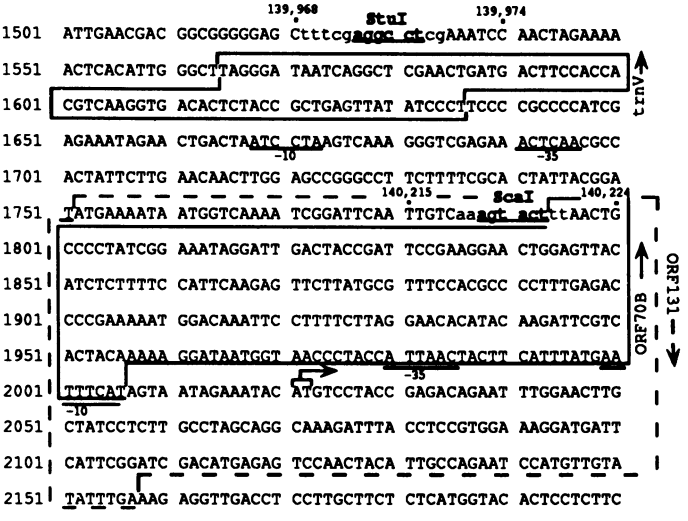

Figure 4. DNA sequence of the plastid targeting fragment surrounding ORF70B and ORF131. The numbers on the left refer to the relative position of the sequence in the ptDNA insert in pPRV1; numbers above the lines correspond to numbers in the plastid genome (16). The coding regions of the *trnV* gene, 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 *Stu*I and *Sca*I cloning sites, the −10 and −35 conserved promoter elements for *trnV* (22) and the *rps7/12* operon (23,24) are underlined.

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 those 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 selective marker, the integrated *aadA* gene, was genetically stable even when its promoter region formed a short direct repeat with the endogenous rRNA operon or *trnV* 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 trnG−trnfM intergenic region (28). We believe therefore that the transformation efficiency may be further increased by increasing the size of the targeting fragment.

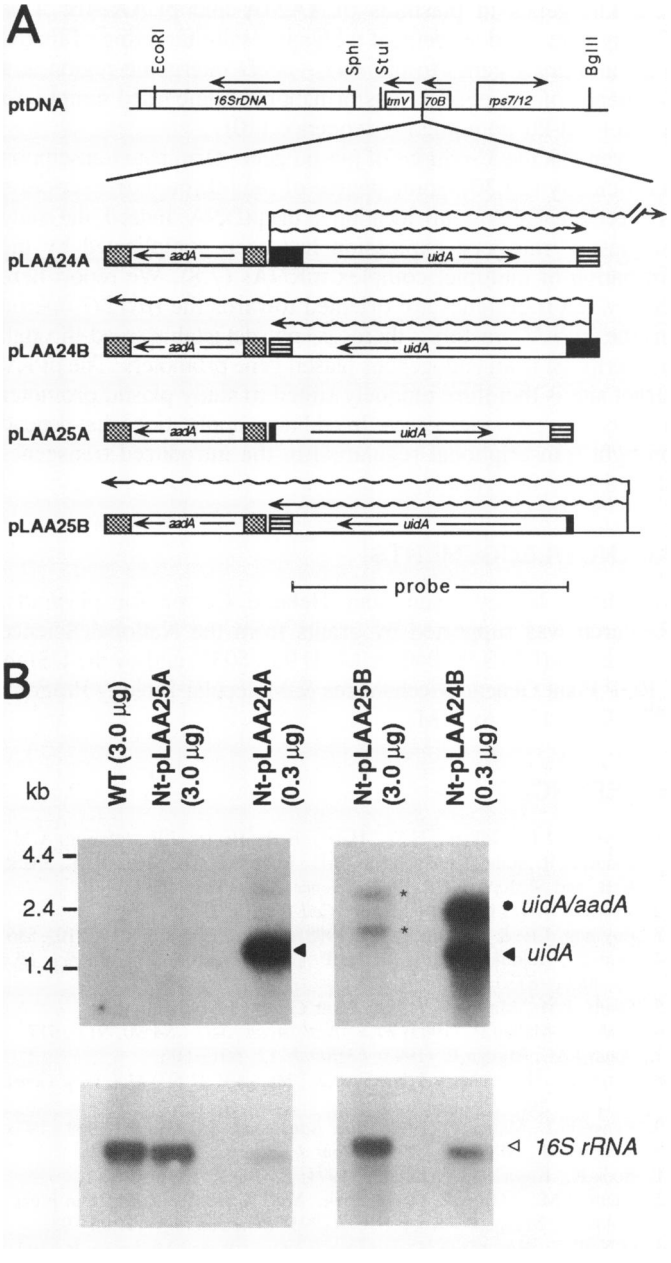

Figure 5. Transcription pattern of *uidA* transgenes located in the *trnV−rps7/12* intergenic region. (**A**) The map of transplastomes obtained by transformation with plasmids carrying the promoter-containing (pLAA24A,B) and promoterless (pLAA25A,B) *uidA* reporter genes. Black boxes indicate the P*rrn* (pLAA25A,B) and the leader of the promoterless *uidA* gene (pLAA25A,B); horizontally striped boxes depict T*rps16*. 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 Nt-pLAA25B. The blot was stripped and probed with a *16SrDNA* 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*

(2.2 kb) genes in plasmids pLAA24A and pLAA24B. This finding is in good agreement with successful targeting of linked *uidA* and *aadA* genes to the *rbcL−accD* intergenic region at a frequency of one plastid transformant per bombarded sample *via* a 1.6+1.3-kb homologous sequence (10).

Given that the 3′-regions of plastid genes terminate transciption 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 *rps12/7* 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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