A Guide to Choosing Vectors for Transformation of the Plastid Genome of Higher Plants

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Abstract

Plastid transformation, originally developed in tobacco (*Nicotiana tabacum*), has recently been extended to a number of crop species enabling *in vivo* probing of plastid function and biotechnological applications. In this article we report new plastid vectors that enable insertion of transgenes in the inverted repeat region of the plastome between the *trnV* and 3′*rps12* or *trnI* and *trnA* genes. Efficient recovery of transplastomic clones is ensured by selection for spectinomycin (*aadA*) or kanamycin (*neo*) resistance genes. Expression of marker genes can be verified using commercial antibodies that detect the accumulation of NPTII (neomycin phosphotransferase II), the *neo* gene product or the C-terminal c-myc tag of AAD (aminoglycoside-3′-adenylytransferase), encoded by the *aadA* gene. AAD, the spectinomycin inactivating enzyme is translationally fused with GFP in two vectors so that transplastomic clones can be selected by spectinomycin resistance and visually identified by fluorescence in UV light. The marker genes in the new vectors are flanked by target sites for Cre or Int, the P1 and phiC31 phage site-specific recombinases. When uniform transformation of all plastid genomes is obtained, the marker genes can be excised by Cre or Int expressed from a nuclear gene. Choice of expression signals for the gene-of-interest; complications caused by the presence of ptDNA sequences recognized by Cre; and loss of transgenes by homologous recombination via duplicated sequences are also discussed to facilitate a rational choice from among the existing vectors and to aid with new target-specific vector designs.
Plant cells contain DNA in three cellular compartments: the nucleus, plastids and mitochondria. The nucleus of Arabidopsis and rice, the dicot and monocot model species, encode ~ 29,454 (Alonso et al., 2003) and ~ 37,544 (IRGSP, 2005) genes, respectively, while their plastid (ptDNA) and mitochondrial genomes (mtDNA) carry only ~ 120 and ~57 genes, respectively (Hiratsuka et al., 1989; Unseld et al., 1997; Sato et al., 1999; Notsu et al., 2002). The plastid genome (plastome) of higher plants is highly polyploid. The number of organelles and genome copies per organelle depends on the species and the cell type. For example, a typical Arabidopsis leaf cell contains ~120 chloroplasts and a total of 1,000 to 1,700 ptDNA copies (Zoschke et al., 2007) while an average tobacco (Nicotiana tabacum) leaf cell carries ~100 chloroplasts and ~ 10,000 ptDNA copies (Shaver et al., 2006). Transformation of the nuclear genome is routine in higher plants and is reviewed in this Focus Issue of Plant Physiology. Plastid transformation is routine only in tobacco (Nicotiana tabacum) (Svab et al., 1990; Svab and Maliga, 1993), but has rapidly expanded to diverse crops including potato (Sidorov et al., 1999), tomato (Ruf et al., 2001), lettuce (Lelivelt et al., 2005; Kanamoto et al., 2006), soybean (Dufourmantel et al., 2004), cotton (Kumar et al., 2004), cauliflower (Nugent et al., 2006) and poplar (Okumura et al., 2006). Transformation of mtDNA remains a challenge for the future.

Delivery of transformation vectors to chloroplasts is by the biolistic process (Boynton et al., 1988) or by polyethylene glycol treatment (Golds et al., 1993). Transformation of ptDNA is based on targeted insertion of the transforming DNA by homologous recombination (Figure 1A), followed by enrichment of the transformed ptDNA copy by growing the cells on selective tissue culture medium. The gradual process of organelle (plastid) and genome (ptDNA) sorting ultimately yields genetically stable homoplastomic cells carrying only transformed ptDNA copies. Genetically stable plants are obtained by regenerating plants from the homoplastomic cells.

The objective of engineering the plastid genome is either to alter (or delete) the DNA sequence of native plastid genes or to incorporate new functions. Engineering of native plastid genes is accomplished by including a modified ptDNA sequence in the vector plastid-targeting region. Examples include testing a spinach psbF editing segment in tobacco chloroplasts (Bock et al., 1994) and replacing the tobacco rbcL gene with
cognate genes from sunflower or photosynthetic bacteria (Kanevski et al., 1999; Whitney and Andrews, 2001). When engineering of a native gene is the goal, the marker gene is always adjacent to the target gene to minimize the probability of recombination between the mutant sequence and the marker gene. The vectors for these manipulations are gene specific and are outside the scope of this article.

We report here on new vectors that are suitable for incorporation of novel functions in the plastid genome and are equipped with sequences for post-transformation removal of the marker genes (Figure 1B). Excision of marker genes by phage recombinases reported here is not the only approach to obtain marker-free transplastomic plants: the alternatives are excision by homologous recombination \textit{via} direct repeats (Iamtham and Day, 2000); transient cointegration facilitated by visual-assisted marker selection (Klaus et al., 2003; Klaus et al., 2004) and cotransformation-segregation (Ye et al., 2003); reviewed in (Lutz and Maliga, 2007a). For additional reviews on plastid transformation and its applications in basic science and biotechnology see (Maliga, 2004; Daniell et al., 2005; Herz et al., 2005; Bock, 2007).

This report is an update on Plastid Repeat Vectors (pPRV) described in 1994 (Zoubenko et al., 1994). It lists five vectors we still recommend from the earlier paper, four vectors described in the meantime and nine new vectors we report here for the first time. Novel features of the new vectors are sequences for post-transformation excision of the marker genes, a new insertion site in the \textit{trnI-trnA} intergenic region and vectors with alternative marker genes.

RESULTS AND DISCUSSION

Insertion Sites

The site of insertion in the plastid genome is determined by the choice of ptDNA segment flanking the marker gene and the gene of interest. Insertion of foreign DNA in intergenic regions of the plastid genome has been accomplished at more than 14 sites
Only three of the insertion sites have been developed into more sophisticated vectors in which a marker gene is adjacent to a polycloning site so that new chimeric genes can be directly assembled in the transformation vector in *E. coli*. Two of the plastid vectors target the 25-kb inverted repeat (IR) region between the *trnV*-3′-*rps12* and *trnl-trnA* genes and one targets the intergenic region between *trnfM-trnG* genes located in the large single copy (LSC) region of the 155-kb tobacco ptDNA (Figure 2). Any gene inserted into one of the repeats is rapidly copied over into the second repeat copy by gene conversion, thus a gene targeted to the IR is present in two copies per genome. (Both transformed and non-transformed copies may be templates for gene conversion, thus gene conversion may also eliminate a transgene.)

We selected the *trnV*-3′-*rps12* intergenic region in the IR for insertion of transgenes because there is no read-through transcription from the plastid ribosomal RNA (rrn) operon (Zoubenko et al., 1994) (Figure 2, pPRV111A). Thus, promoter activity at this site could be studied without interference by read-through transcription. Originally, we constructed two vectors with different expression signals for the *aadA* marker gene: in vectors pPRV111A/B *aadA* is expressed in a *psbA* cassette (PpsbA and TpsbA derive from the *psbA* gene promoter and terminator, respectively) and in vectors pPRV112A/B *aadA* is expressed in a Prrn/Trps16 cassette. Having alternative *aadA* expression signals enabled avoiding duplication of expression signals on the gene of interest, which may lead to deletion of sequences between the direct repeats by homologous recombination (Iamtham and Day, 2000; Kode et al., 2006). The pPRV vectors targeting insertions at the *trnV*-3′-*rps12* intergenic region are the most commonly used vectors. They yield high-levels of protein expression (Maliga, 2003), and are now endowed with signals for marker gene excision (see below)(Figure 3 and Table I).

Several laboratories have inserted transgenes between the *trnl* and *trnA* genes in the IR region of ptDNA. These two tRNAs are located between the small (*rrn16*) and large (*rrn23*) rRNA subunit genes and the operon is transcribed from promoters upstream of *rrn16* (Figure 2) (Vera and Sugiura, 1995; Suzuki et al., 2003). The polycistronic *rrn* operon mRNA is efficiently processed, releasing transgenic mRNA inserted between the two tRNAs. The first vector targeting insertions in the *trnl-trnA* intergenic region, pSBL-CTV2, was developed in the Daniell laboratory (Daniell et al., 1998) and was used to
express several proteins (Daniell et al., 2005). Transgenes in the Daniell laboratory are typically expressed by cloning genes and operons into an XbaI site downstream of aadA, which is expressed in a cassette consisting of the rrn operon promoter (Prrn) and the psbA gene 3’-UTR (TpsbA) (Figure 2). The aadA marker gene and the inserted transgenes are expressed from two mRNAs: the mRNA transcribed from ectopic Prm promoter driving the aadA marker gene and from the readthrough mRNA derived from the native rrn operon promoter. Stability of the transgenic mRNA is ensured by the 5’-UTR and 3’-UTR sequences of the Prrn-TpsbA cassette; protein accumulation from the transgene depends on the 5’-UTR inserted upstream of the reading frame encoding the gene(s) of interest. For information on protein expression with these vectors see ref. (Daniell et al., 2005).

Vectors targeting insertion of two complete genes in the trnI/trnA intergenic region were also reported from the Hanson laboratory (TSP)(Yu et al., 2007) and from our laboratory. Our 300-series PRV vectors give the freedom to either utilize readthrough transcription (pPRV323Clox, pPRV324Clox) or to insert a complete gene upstream (pPRV323Clox, pPRV324Clox) or downstream (pPRV323Blox) of the c-Myc-tagged aadA marker gene (Figure 4, Table I). Readthrough transcription was utilized, for example, for expression of the Cry9Aa2 protein in a pPRV323Clox (formerly pPRV312L) vector derivative (Chakrabarti et al., 2006). In our vectors the marker genes are flanked by loxP sites (floxed), thus they can be excised when transformation is complete. Transgene expression at this insertion site in the repeated region of the ptDNA benefits from duplication of the gene copy number and from increased mRNA levels due to readthrough transcription from the upstream rrn operon promoter. However, more important for protein accumulation is the choice of 5’-UTR that may affect protein yields in a 10,000-fold range (Maliga, 2003).

The trnfM-trnG intergenic region in the LSC region is utilized in the pRB94 and pRB95 plastid vectors developed in the Bock laboratory (Ruf et al., 2001) (Figure 2). This vector is used to study RNA editing (Bock, 1998) and metabolic engineering in chloroplasts (Bock, 2007). Recently a portable Intercistronic Expression Element was characterized using this vector that mediates intercistronic cleavage into stable monocistronic mRNAs. The short (50 bp) element derived from the psbT-psbH intergenic
region facilitates translation of monocistronic mRNAs at predictable levels (Zhou et al., 2007).

**Marker Genes**

There are two classes of plastid marker genes: primary selective markers that are suitable for direct selection of transplastomic clones and secondary selective markers that confer a phenotype when present in most ptDNA copies but are not suitable for recovery of transplastomic clones when present in only a few ptDNA copies (Maliga, 2004). Currently known primary markers are resistance to spectinomycin, streptomycin and kanamycin. Resistance to spectinomycin and streptomycin in plastids is conferred by the expression of the *aadA* gene (Goldschmidt-Clermont, 1991; Svab and Maliga, 1993); resistance to kanamycin is due to expression of the *neo (aph(3’)IIa)* (Carrer et al., 1993; Lutz et al., 2004) or *aphA-6* gene (Huang et al., 2002).

The original pPRV plastid vectors carry *aadA* genes, which express AAD (aminoglycoside-3’-adenylytransferase) at a relatively low level. The *aadA* genes in the new pPRV series are expressed from the PrrnLatpB+DS and PrrnLrbcL+DS Translation Control Regions (TCRs), which yield proteins in the 7% to 10% Total Soluble Protein (TSP) range (Kuroda and Maliga, 2001b). Recovery of transplastomic clones with the new markers is efficient and the AAD gene product can be quantified using the commercially available antibody to the C-terminal c-myc 9E10 (EQKLISEEDL) epitope tag (Kolodziej and Young, 1991). The Prrn promoter in the new vectors is in an inverted orientation relative to the native *rrn* promoter, an arrangement that prevents deletion of intervening sequences (Kittiwongwattana et al., 2007). The *aad-gfp* fusion gene is an *aadA* derivative that enables selection for spectinomycin resistance and is visually traceable by GFP fluorescence (Khan and Maliga, 1999). The new plastid vector pair pPRV131A and pPRV131B carry the *aadA-gfp* fusion gene as a selective marker (Figure 3, Table I).

Although kanamycin resistance could be used to recover transplastomic clones after bombardment with the plasmid pTNH32, the *neo* gene was not included in advanced
vectors because the transformation efficiency with this *neo* gene was low (Carrer et al., 1993). We report here a dramatic improvement in plastid transformation efficiency with a highly expressed (7% NPTII) *neo* gene derived from plasmid pHK30 (Kuroda and Maliga, 2001b). Bombardment of 25 leaves with the vector that carries the new *neo* gene (pAAK201) yielded 34 kanamycin resistant clones. Out of these, DNA gel blot analyses confirmed plastid transformation in 27 clones (supplemental Figure 1) whereas only 3 out of 99 kanamycin resistant clones were transplastomic after transformation with vector pTNH32. Interestingly, the kanamycin resistant clones appear later (after six to twelve weeks) than the spectinomycin resistant clones (three to twelve weeks). These data confirm the earlier report about the efficiency of kanamycin selection with the new *neo* gene in plastid transformation experiments (Lutz et al., 2004). The PrrnLatpB+DS promoter driving *neo* in plasmid pAAK201 is in tandem with the native Prrn promoter. We therefore shall release vectors pPRV145C and pPRV145D, in which the orientation of *neo* is inverted relative to the *rrn* operon (Figure 3).

**Expression of the Gene of Interest**

Levels of protein expression from plastid transgenes depend on mRNA abundance determined by promoter strength and mRNA turnover. The stability of mRNAs depends on protective stem-loop secondary structures in the 5’- and 3’-UTRs and their interactions with RNA binding proteins (Barkan and Goldschmidt-Clermont, 2000). More important for protein expression is the translatability of the mRNA determined by the 5’ translation control region that may affect protein yield in a 10,000-fold range (Maliga, 2003).

Thus far, expression signals, such as promoters and 5’- and 3’-UTRs are derived from the source organism, which results in duplicated ptDNA sequences. One option to minimize duplications is to build polycistronic expression units in which several genes may be expressed in the same promoter-terminator cassette. One example is pPRV110lox (formerly pPRV110L) (Figure 3, Table 1), a vector with a promoter-less *aadA* gene, in which transcription of the marker gene is from the operon inserted upstream. The utility
of the vector has been shown by introduction of herbicide resistance genes into the plastid genome, and subsequent removal of the marker genes by the Cre site-specific recombinase (Lutz et al., 2006a). Vectors suitable for polycistronic expression are pPRV323Clox and pPRV324Clox, in which suitably engineered operons may be expressed in the \textit{trnl-trnA} intergenic region. Again, these vectors are equipped for excision of the marker genes by the Cre-\textit{loxP} site-specific recombination system. The utility of the pPRV323Clox vector (Table I, Figure 4), formerly designated as pPRV312L vector, was shown by expression of the \textit{cry9Aa2} \textit{Bacillus thuringiensis} gene as part of the \textit{rrn} operon (Chakrabarti et al., 2006). A second option to minimize duplications is incorporation of expression signals from heterologous sources. An early example for successful use of a heterologous signal is the T7 phage gene 10 leader that promotes high-level translation in chloroplasts (Staub et al., 2000; Kuroda and Maliga, 2001a). A heterologous source of promoters may be prokaryotes or mitochondria, which have a transcription machinery similar to the plastid NEP. Interesting in this regard is the demonstration that the mitochondrial \textit{atpA} promoter is faithfully recognized in plastids (Bohne et al., 2007).

**Choices for Marker Excision**

Marker genes are essential for the selective amplification of the initially transformed ptDNA copies. When the transplastomic plants carry only transformed ptDNA, the marker gene is no longer needed. Most of the new vectors listed in Table I are equipped with sequences that are necessary for post-transformation removal of the marker genes utilizing phage site-specific recombinases. The transformed ptDNA in which recombinase target sites flank the marker gene is stable in the absence of the recombinase (Figure 1A). When removal of the marker gene is desired, the phage enzymes are expressed from a nuclear gene. Plastid targeting is achieved by fusing the recombinase at its N-terminus with the plastid-targeting region of a nuclear-encoded, plastid targeted gene, such as the rubisco small subunit transit peptide. The recombinase,
translated on cytoplasmic ribosomes, enters all plastids and simultaneously excises the marker genes (Figure 1B).

Thus far two recombinases have been tested for plastid marker gene excision, the Cre and the Int. The Cre enzyme derives from the P1 bacteriophage and excises target sequences flanked by directly oriented 34-bp loxP sites (Corneille et al., 2001; Hajdukiewicz et al., 2001; Kuroda and Maliga, 2003; Tungsuchat et al., 2006) (Lutz et al., 2006a). The Cre gene has been introduced into the plant nucleus by three methods: (i) stable transformation of the nucleus using an Agrobacterium binary vector (Corneille et al., 2001; Hajdukiewicz et al., 2001); (ii) by pollination (Corneille et al., 2001); (iii) or by transiently expressing Cre from T-DNA by Agroinfiltration (Lutz et al., 2006a). There are seven vectors in Table I that carry a floxed marker gene, each of which can be removed by any of the three approaches using a Cre vector listed in Table II.

The second site-specific recombinase that has been tested for marker gene excision is Int, the phiC31 phage site-specific recombinase (integrase). To facilitate excision of the aadA marker gene, it was flanked with directly oriented non-identical phage attP (215 bp) and bacterial attB (54 bp) attachment sites. Efficient excision of the marker gene was shown after transformation of the nucleus with an Int gene encoding a plastid-targeted Int enzyme (Kittiwongwattana et al., 2007). At this time two vectors, pPRV111Aatt and pPRV111Batt are available for release (Table I, Figure 3). The plastid-targeted nuclear Int is encoded in Agrobacterium Int vector pKO117 (Table II).

Out of the two site-specific recombinases, Int appears to be the better choice since the ptDNA contains pseudo lox sites recognized by the Cre but no sequences recognized by the Int (Corneille et al., 2003; Lutz et al., 2004; Kittiwongwattana et al., 2007). Thus Cre, but not Int, may induce deletions between target sites and ptDNA sequences. A second problem observed during recombinase-mediated marker excision was enhanced homologous recombination between repeated (non-target) ptDNA sequences (Corneille et al., 2001; Tungsuchat et al., 2006). Deletions via repeated sequences could be avoided when the repeated sequences were in an inverted orientation (Kittiwongwattana et al., 2007).
Loss of Transgenes via Repeated Sequences

We recently found that both the bar and aadA genes are lost in a small fraction of the seed progeny when the bar gene is expressed from a Prrn promoter after transformation with plasmid pMBC12, a pPRV111Batt vector derivative (Figure 5A). The deletion was noticed because loss of the bar gene restores the normal green color to leaf cells that are golden-yellow when expressing the bar gene in chloroplasts (Figure 5C)(Kittiwongwattana et al., 2007). Loss of the bar gene was detected by formation of green sectors and by faster growth in ~0.6% of the seed progeny (10 variegated seedlings found among 1,584 selfed seed progeny; Figure 5D). Sequencing of the PCR-amplified recombination junction revealed that the deletion occurred by homologous recombination between the Prrn promoter driving the bar gene and the native rrn operon promoter (data not shown). Deletion of sequences between the rrn operon repeats was also confirmed by DNA gel blot analyses in each of the 10 lines. A representative blot is shown in Figure 5B for one of the lines.

In plasmid pMBC12 the Prrn promoter and the rrn operon promoter are 2.7-kb apart sharing 84 bp sequence as a direct repeat (PP-BamHI promoter sequence deposited in Genbank under accession no. EF416278). In the pPRV112A/B vectors described in 1994 the Prrn promoter contains a 118 bp repeat 1.3 kb upstream of the native rrn operon promoter. Although never shown to be unstable experimentally, we assume that deletion of aadA and trnV also occurs in plants transformed with pPRV112 vectors via the direct Prrn repeats. That is because probability of deletion via direct repeats is dependent on the length and the distance of the repeats. To obtain reasonably frequent deletions 649 bp repeats placed 5.4 kbp apart were used because a 418 bp sequence spaced 1 kbp apart barely yielded any deletions (Iamtham and Day, 2000; Kode et al., 2006). We now only recommend vectors targeting the trnV-3′rps12 intergenic region, in which the Prrn promoter is in an inverted orientation relative to the native rRNA operon.

If essential plastid genes are deleted from the plastid genome, ptDNA copies lacking essential genes disappear in the absence of direct selection (Svab and Maliga, 1993). The trnV-3′rps12 insertion site is special because the trnV gene between the insertion site (nucleotide 102,312 in the tobacco plastid genome; GenBank accession
number z00044) and the rrn operon promoter is dispensable (Corneille et al., 2001; Hajdukiewicz et al., 2001; Tungsuchat et al., 2006). While it is worth taking note of the potential problem caused by tandemly repeated sequences, the adverse consequences can be readily avoided by constructing only inverted repeats or placing the duplicated sequence at a distance.

**Pros and Cons of Species-Specific Vectors**

Is it necessary to use vectors with species-specific targeting sequences and expression signals? The answer is no, although there are benefits in doing so. Targeting sequences are typically 1 kbp to 2 kbp long and flank the marker gene and gene of interest (Figure 1A). Recombination may take place via sequences adjacent to the marker gene (Sites #1 in Figure 1A) or via sequences distal to the marker gene (Sites #2 in Figure 1A). Sequencing of the ptDNA of multiple genetic lines indicates that there is significant intra-species sequence variation in the plastid genome in at least some taxonomic groups. For example, intra-species variation of the plastid genome in rice (Tang et al., 2004) and Arabidopsis (Sall et al., 2003) is well characterized. To obtain only one type of recombinant in such species requires transformation with strain-specific rather than species specific vectors. This is definitely not worth the effort, particularly since individual distinct ptDNA may be a useful identifier for patent protection.

When transformation is carried out with a vector having heterologous targeting sequences, there is sufficient sequence conservation in the coding region of all higher plant plastid genomes to ensure integration of the marker gene and the gene of interest in any region of the ptDNA. This does not mean that the new transplastomic plants contain any heterologous ptDNA sequence. If recombination is via sequences proximal to the marker gene (Sites #1 in Figure 1A), no sequences are incorporated from the vector targeting region. However, if recombination is via distal sequences (Sites #2 in Figure 1A), the vector targeting sequence may replace the native ptDNA sequence (in this case the *rrn16* and *trnV* genes). Caution is advised when the targeting region contains edited genes in which posttranscriptional C to U conversion restores a codon for a conserved
amino acid. There are about eighteen edited plastid genes carrying a total of ~ 30 edited C nucleotides (Tsudzuki et al., 2001; Kahlau et al., 2006). Highly edited genes make a bad targeting sequence for a heterologous transformation vector, as the new host may or may not have the capacity for editing the sites (Bock et al., 1994; Schmitz-Linnweber et al., 2005).

CONCLUSIONS

Plastid transformation vectors and marker excision systems are developed coordinately. Our intent has been to provide a simple vector system that enables transformation of the plastid genome in wild-type plants. While most of the vectors in Table I are new, the marker genes and the elements of the marker excision systems have been tested before. Both the Cre-lox and Int-att site-specific recombination systems are efficient; although Int appears to be the better choice.

High-level protein expression from plastid transgenes may come at a cost to the plant. Delayed development was shown in at least one transgenic line expressing the Cry9Aa2 B.t. protein in chloroplasts (Chakrabarti et al., 2006), but high-level protein accumulation is apparently compatible with normal growth in lines expressing other proteins. Delayed development is not a disadvantage if the goal is high-level expression of recombinant proteins but it may be a drawback in agronomic applications. The metabolic burden imposed by chloroplast expression is yet to be evaluated for most agronomic traits. It will be necessary to determine the protein level desired for each application and develop new tools that ensure protein accumulation at the desired level. Another new research direction will be exploration of the limits of increasing the size of ptDNA. Since insertion into the IR automatically doubles the size of the foreign DNA a task ahead will be development of new vectors for insertion of foreign DNA in the single copy regions.

MATERIALS AND METHODS
Vector Construction

The backbone of the new pPRV100 series vectors, targeting insertions in the \textit{trnV-3’rps12} region, is derived from plasmid pPRV1 (Zoubenko et al., 1994). The vectors were obtained by ligating the marker genes and multiple cloning sites into the ScaI site of vector pPRV1. The kanamycin resistance gene in vectors pPRV145C and pPRV145D derived from plasmid pHK30 and is expressed in a PrrnLatpB+DS promoter and TrbcL terminator cassette (Kuroda and Maliga, 2001b). The two (C, D) vectors differ with respect to the relative orientation of the multiple cloning sites. Plastid transformation vector pAAK201 is identical with vector pPRV145C or D, other than \textit{neo} was cloned in the inverted orientation relative to the ScaI site in plasmid pPRV1 and the \textit{neo} gene is flanked by \textit{loxP} sites (Supplemental Figure S1).

The pPRV123Clox vector was obtained by cloning the \textit{Swal} fragment present in plasmid pPRV323Clox (formerly pPRV312L)(Chakrabarti et al., 2006) into the ScaI site in plasmid pPRV1. Vectors pPRV123Blox and pPRV123Clox differ with respect to the position of multiple cloning site and the marker gene. (In vectors A and B the cloning sites are on the right; in vectors C and D the cloning sites are on the left of the marker gene.) In the vectors the \textit{aadA} coding region is translationally fused at its C-terminus with the 9E10 c-myc tag (EQKLISEDEEL) (Kolodziej and Young, 1991). The extension \textit{lox} indicates, that the \textit{aadA} marker gene is flanked by the 34-bp wild-type P1 phage \textit{loxP} sequences (Corneille et al., 2001). The pPRV124Clox vector is identical with pPRV123Clox, other that the \textit{aadA} gene is expressed from the \textit{rbcL} leader and downstream sequence (LrbcL+DS) (PrrnLrbcL+DS/TrbcL cassette) derived from plasmid pHK34 (Kuroda and Maliga, 2001b). Vector pPRV131A and pPRV131B are identical with vector pPRV111A and pPRV111B, other than the \textit{aadA} gene in the pPRV111 plasmids was replaced with the \textit{aadA-gfp} fusion gene from plasmid pMSK56 (Khan and Maliga, 1999).

Construction of the pPRV323Clox (formerly pPRV312L) has been described (Chakrabarti et al., 2006). Vector pPRV324Clox is identical with pPRV323Clox, other than \textit{aadA} is expressed from the PrrnLrbcL+DS promoter derived from plasmid pHK34
(Kuroda and Maliga, 2001b). Vector pPRV323Blox is identical with vector pPRV323Clox, other than the multiple cloning site is downstream of the marker gene.

The pMBC12 plastid transformation vector carries a bar gene that causes the aurea phenotype in a pPRV111Batt vector (Figure 5A). This construct is identical to the bar gene in plasmid pCK2, other than the bar gene in plasmid pCK2 was cloned into a pPRV111Aatt vector (Kittiwongwattana et al., 2007).

Plastid Transformation

Plastid transformation with plasmid pAAK201 was carried out as described (Lutz et al., 2006b; Lutz and Maliga, 2007b). Briefly, leaves of Nicotiana tabacum cv. Petit Havana grown in sterile culture were placed abaxial side up on filter paper and the transforming DNA was introduced by the biolistic process using 1.0 μm gold particles. Two days after bombardment the leaf sections were transferred to RMOP medium containing 50 mg/L kanamycin sulfate. Kanamycin inhibited growth and greening of leaf calli from the leaf sections. The kanamycin resistant clones were identified as green shoots and proliferating green calli six to 12 weeks after bombardment. Leaves from regenerating shoots transferred onto the same selective RMOP medium, regenerated and characterized by DNA gel blot analyses.

Plastid transformation and identification of transplastomic clones after bombardment with the pMBC12 vector was carried out as described for transformation with plasmid pCK2 (Kittiwongwattana et al., 2007).

DNA Gel Blot Analyses of ptDNA

DNA gel blot analysis was carried out as described (Svab and Maliga, 1993; Lutz et al., 2006b; Lutz and Maliga, 2007b). Briefly, total leaf cellular DNA was digested with the appropriate restriction endonucleases. The DNA fragments were separated by electrophoresis in 0.8% agarose gels and transferred to Hybond-N membranes (GE
Healthcare, Piscataway, NJ) using the PosiBlot Transfer apparatus (Stratagene, La Jolla, CA). Hybridization with the probes was carried out in Rapid Hybridization Buffer (GE Healthcare, Piscataway, NJ) overnight at 65°C. DNA probe was prepared by random-primed $^{32}$P-labeling. DNA isolated from Nt-pMBC12 leaves was digested with the EcoRI restriction endonuclease and probed with the Apal-StuI plastid targeting region probe (Figure 5B). DNA isolated from Nt-pAAK201 leaves was digested with the BamHI restriction endonuclease and probed with the 1.5-kb Apal-BsrEII fragment derived from the targeting region (Supplemental Figure S1).

**Identification of Seedlings with bar Gene Deletion**

~2,000 Nt-pMBC12 seed was spread on the surface of Pro-Mix general purpose growing medium Code 0432 (Premier Horticulture Inc., Quakertown, PA) in plastic flats (10 in x 20 in) and grown in the greenhouse with supplemental lighting (16 hours daylight). During the first five days of cultivation the germinating seedlings were kept moist by covering the flats with a plastic dome. Seedlings with green sectors could be identified three weeks after planting the seed (Figure 5D).

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**GenBank Accession Numbers**
GenBank accession numbers for the new plastid vectors are: pPRV131A, EU224427; pPRV131B, EU224428; pPRV123Blox, EU224424; pPRV123Clox, EU224425; pPRV124Clox, EU224426; pPRV323Blox, EU224429; pPRV324Clox EU224430; pPRV145C, EU224422; pPRV145D, EU224423.

Supplemental Data

Supplemental Figure S1. DNA gel blot analyses confirmed plastid transformation in plants selected by kanamycin resistance in pAAK201-transformed leaf cultures.

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FIGURE LEGENDS

**Figure 1.** Plastid transformation to obtain marker-free transplastomic plants. A, Insertion of the marker gene (mg) and the gene of interest (goi) at the trnV-3’rps12 insertion site of the plastid genome (ptDNA) by homologous recombination via the left and right plastid targeting sequences (LTR and RTR, respectively). Recombination sites are marked with dashed lines adjacent (#1) or distal (#2) to the marker gene. B, Excision of the marker gene from the ptDNA by the Int or Cre site-specific recombinase via the recombinase recognition sequence (RRS) to obtain marker-free transplastomic plant. Abbreviations: Int or Cre are site-specific recombinases; rrn16, trnV and 3’rps12 are plastid genes.

**Figure 2.** Plastid transformation vectors targeting insertions at three commonly used insertion sites. A, Plastid transformation vector pPRV111A targets insertion into the trnV-3’rps12 intergenic region (Zoubenko et al., 1994). A gene of interest (goi) can be cloned into the multiple cloning site upstream of the aadA marker gene. Red, wavy lines symbolize transcripts. Note that there is no read-through transcription from the rrn operon. Restriction sites marked with asterisks are not unique. B, Plastid transformation vector pSBL-CTV2 targets insertion into the trnI-trnA intergenic region (Daniell et al., 1998). Genes for expression are cloned into the XbaI site in the 3’-UTR (TpsbA) of the (aadA) marker gene. Transgenes are transcribed from the rrn operon promoter and from the promoter upstream of aadA. Vertical arrows above transcripts mark processed ends. C, Plastid transformation vector pRB94 targets insertion into the trnfM-trnG intergenic region. Genes for expression may be cloned into the multiple cloning site.

**Figure 3.** pPRV plastid vectors targeting insertion in the trnV-3’rps12 plastid region. Map positions are shown for: aadA, spectinomycin resistance gene; neo, kanamycin resistance gene; aadA-gfp, translational fusion of aadA and gene encoding the green fluorescent protein; rrn16, trnV and 3’rps12 are plastid genes; RBS, ribosome binding
site; triangles, \textit{loxP} sites; BB’ and PP’, \textit{attB} and \textit{attP} DNA sequences; black box, c-Myc tag. Restriction sites marked with asterisks are not unique.

**Figure 4.** Plastid pPRV vectors targeting insertion in the \textit{trnI-trnA} plastid region. Map positions are shown for: \textit{aadA}, the selectable spectinomycin resistance gene; \textit{rrn16}, \textit{trnI} and \textit{trnA} plastid genes. Triangles mark \textit{loxP} sites. Black box indicates c-Myc tag.

**Figure 5.** Deletion of \textit{bar} by homologous recombination via 84 bp direct repeat yields green sector in seedling. A, Plastid DNA map to show deletion via direct \textit{Prrn} promoter repeat. Shown are: \textit{aadA}, spectinomycin resistance gene; \textit{bar}, bialaphos resistance gene, delays growth and causes golden leaf color; \textit{PrrnPclpP} and \textit{PpsbA} are promoters; \textit{TrbcL} and \textit{TpsbA} are 3'-UTRs; BB’ and PP’ are the \textit{attB} and \textit{attP} sequences; \textit{rrn16}, \textit{trnV} and 3’\textit{rps12} are plastid genes. B, DNA gel blot analyses confirms deletion of \textit{bar} gene. Probing with \textit{ApaI-Stul} ptDNA fragment (Figure 5A) identifies 1.5-kb \textit{EcoRI} fragment resulting from deletion. Variegated Nt-pPMBC12 plant is heteroplasmatic and also contains the parental 4.1-kb Nt-pMBC12 fragment. C, Golden-yellow (aurea) Nt-pMBC12 parent. D, Larger variegated seedling among smaller aurea siblings.

**Supplemental Figure S1.** DNA gel blot analyses confirmed plastid transformation in plants selected by kanamycin resistance in pAAK201-transformed leaf cultures. A, Map of plasmid pAAK201, the cognate wild-type ptDNA (Nt-wt) and the transformed Nt-pAAK201 ptDNA. Shown are: \textit{rrn16}, \textit{trnV} and \textit{rps12/7}, plastid genes; \textit{neo}, kanamycin resistance gene; triangles, \textit{loxP} sites; \textit{ApaI}, \textit{BamHI}, \textit{EcoRI}, \textit{EcoRV}, \textit{HindIII}, \textit{KpnI}, \textit{NcoI}, \textit{SacI} and \textit{ScaI}, restriction sites. (B) DNA gel blot analysis confirmed plastid transformation in 27 out of 34 kanamycin resistant clones. Data are shown for 11 transplastomic clones (probe detects 4.5 kb transplastomic fragment). In two clones (3.3 kb wild-type fragment), kanamycin resistance is probably due to integration of \textit{neo} in the nucleus, as described earlier (Carrer et al., 1993). Total cellular DNA was digested with the \textit{BamHI} restriction enzyme and probed with the \textit{ApaI-EcoRV} plastid DNA fragment (heavy line, Supplemental Figure 1A).
| Plasmid     | Insertion Site | Plastid Marker Gene | Promoter | 5' - UTR | Antibiotic Resistance | 3' - UTR | Excision System | Accession Number | Reference                          |
|-------------|----------------|---------------------|----------|---------|-----------------------|---------|-----------------|-----------------|------------------|
| pPRV1       | trnV-rps12     | N/A N/A N/A N/A    | N/A      | N/A     | N/A                   | N/A     | N/A             | U12809          | (Zoubenko et al., 1994) |
| pPRV100A    | trnV-rps12     | N/A N/A N/A N/A    | N/A      | N/A     | N/A                   | N/A     | N/A             | U12810          | (Zoubenko et al., 1994) |
| pPRV100B    | trnV-rps12     | N/A N/A N/A N/A    | N/A      | N/A     | N/A                   | N/A     | N/A             | U12811          | (Zoubenko et al., 1994) |
| pPRV110lox  (pPRV110L) | trnV-rps12 | N/A rbcL aadA TrbcL Cre-loxP | N/A     | N/A     | N/A                   | N/A     | N/A             | U12810          | (Lutz et al., 2006a) |
| pPRV111A    | trnV-rps12     | PpsbA psbA aadA    | TpsbA    | N/A     | N/A                   | N/A     | N/A             | U12812          | (Zoubenko et al., 1994) |
| pPRV111B    | trnV-rps12     | PpsbA psbA aadA    | TpsbA    | N/A     | N/A                   | N/A     | N/A             | U12813          | (Zoubenko et al., 1994) |
| pPRV111Aatt | trnV-rps12     | PpsbA psbA aadA    | TpsbA    | Int-att | EF416277              |         |                 |                 | (Kittiwongwattana et al., 2007) |
| pPRV111Batt | trnV-rps12     | PpsbA psbA aadA    | TpsbA    | Int-att | EF416276              |         |                 |                 | (Kittiwongwattana et al., 2007) |
| pPRV131A    | trnV-rps12     | PpsbA psbA aadAgfp | TpsbA    | N/A     | EU224427              |         |                 |                 | This paper       |
| pPRV131B    | trnV-rps12     | PpsbA psbA aadAgfp | TpsbA    | N/A     | EU224428              |         |                 |                 | This paper       |
| pPRV123Blox (formerly pPRV123L) | trnV-rps12 | Prrn atpB+DS aadA-c-Myc TpsbA Cre-loxP | TpsbA    | N/A     | EU224424              |         |                 |                 | This paper       |
| pPRV123Clox (formerly pPRV123L) | trnV-rps12 | Prrn atpB+DS aadA-c-Myc TpsbA Cre-loxP | TpsbA    | N/A     | EU224425              |         |                 |                 | This paper       |
| pPRV124Clox | trnV-rps12     | Prrn rbcL+DS aadA-c-Myc TpsbA Cre-loxP | TpsbA    | N/A     | EU224426              |         |                 |                 | This paper       |
| pPRV323Blox (formerly pPRV312L) | trnI-trnA | Prrn atpB+DS aadA-c-Myc TpsbA Cre-loxP | TpsbA    | N/A     | EU224429              |         |                 |                 | This paper       |
| pPRV323Clox (formerly pPRV312L) | trnI-trnA | Prrn atpB+DS aadA-c-Myc TpsbA Cre-loxP | TpsbA    | N/A     | DQ489715              |         |                 |                 | (Chakrabarti et al., 2006) |
| pPRV324Clox | trnI-trnA     | Prrn rbcL+DS aadA-c-Myc TpsbA Cre-loxP | TpsbA    | N/A     | EU224430              |         |                 |                 | This paper       |
| pPRV145C    | trnV-rps12     | Prrn atpB+DS neo   | TrbcL    | N/A     | EU224422              |         |                 |                 | This paper       |
| pPRV145D    | trnV-rps12     | Prrn atpB+DS neo   | TrbcL    | N/A     | EU224423              |         |                 |                 | This paper       |
Table II. *Agrobacterium* binary vectors for Cre or Int expression in chloroplasts

TP+5aa and TP+22aa indicate that the recombinases are targeted to chloroplasts by fusion with the Rubisco small subunit transit peptide and 5 or 22 amino acids of the mature small subunit N-terminus.

| Plasmid | Agrobacterium vector | Plant Marker Gene | Antibiotics for plant selection | Promoter | TP + amino acids | Recombinase gene | 3’-UTR | Reference |
|---------|----------------------|-------------------|---------------------------------|----------|-----------------|-----------------|--------|----------|
| pKO27   | pPZP212              | *neo*             | Kanamycin 100 mg L⁻¹            | P2’      | TP+22aa         | Cre             | Tnos   | (Corneille et al., 2001) |
| pKO28   | pPZP212              | *neo*             | Kanamycin 100 mg L⁻¹            | P2’      | TP+5aa          | Cre             | Tnos   | (Corneille et al., 2001) |
| pKO30   | pPZP222              | *aacCI*           | Gentamycin 100 mg L⁻¹           | P2’      | TP+22aa         | Cre             | Tnos   | (Corneille et al., 2003) |
| pKO31   | pPZP222              | *aacCI*           | Gentamycin 100 mg L⁻¹           | P2’      | TP+5aa          | Cre             | Tnos   | (Corneille et al., 2003) |
| pKO117  | pPZP222              | *aacCI*           | Gentamycin 100 mg L⁻¹           | P2’      | TP+22aa         | *Int*           | Tnos   | (Lutz et al., 2004)     |
