SITE-SPECIFIC RECOMBINASES TO MANIPULATE THE PLASTID GENOME

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ABSTRACT OF THE DISSERTATION

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The plastid genome (ptDNA) of higher plants is highly polyploid and the 1,000 to 10,000 copies are compartmentalized in up to ~100 plastids per cell. I report here two different site-specific recombinases to manipulate the tobacco plastid genome, the P1 phage Cre recombinase and the phiC31 phage Integrase (Int). Thus far plastid transformation in higher plants was based on incorporation of foreign DNA in the plastid genome by the plastid's homologous recombination machinery. I describe here an alternative approach that relies on integration of foreign DNA by Int mediating recombination between bacterial *attB* and phage *attP* sequences. During the plastid transformation protocol marker genes are essential for selective amplification of rare transformed plastid genome copies to obtain genetically stable transplastomic plants. However, the marker gene becomes dispensable once homoplastomic plants are obtained. I describe plastid marker gene excision with both the Cre-*loxP* and the Int-*att* site-specific recombinases. The Cre-*loxP* approach uses a transiently expressed Cre, a novel protocol that enables rapid removal of marker genes from the ~10,000 plastid genome copies without transformation of the plant nucleus. The Int-*att* system uses a nuclear-encoded plastid-targeted Int to

efficiently excise plastid marker genes flanked by directly oriented *attB* and *attP* sites. Lastly, I describe use of the Int-*att* system to study plastid sorting with the conclusion that a newly arising minor plastid DNA type can get established in a developing tobacco shoot and transmitted to the seed progeny.

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Chapter 1

Introduction

Two site-specific recombinases, the P1 phage Cre and the phiC31 Integrase (Int) site-specific recombinases, have been adapted for manipulation of the plastid genome. Specific examples for the Cre and the Int will be discussed in the following chapters. As a follow-up to earlier work in the lab, I demonstrate that a transiently expressed Cre is effective for excision of plastid marker genes (Chapter 3) (Lutz et al., 2006a). The remaining Chapters (2, 4 and 5) describe uses for Int in the plastid genome. First I describe an alternative approach to plastid transformation that relies on integration of foreign DNA by Int mediating recombination between a plastid encoded bacterial *attB* and vector encoded phage *attP* sequence (Chapter 2) (Lutz et al., 2004). Then, I describe adaptation of Int for excision of plastid marker genes (Chapter 4) (Kittiwongwattana et al., 2007) and how Int can be used to study plastid sorting in a regenerating tobacco shoot (Chapter 5).

Plastid genome, evolution and gene content

Plastid bearing plant cells formed after an endosymbiotic event involving a cyanobacterium and a eukaryotic cell containing mitochondria. This event occurred billions of years ago and over time most of the genes encoded in the ancestral cyanobacterial genome were transferred to the plant nucleus. There are 120 plastid encoded genes and ~3000 nuclear genes whose proteins are targeted to chloroplasts. A typical tobacco leaf cell contains ~100 plastids, each plastid containing about 100 plastid

genome (ptDNA) copies (Shaver et al., 2006). Therefore there are ~10,000 ptDNA copies in a typical tobacco leaf cell, all of which are uniform.

Plastid transformation

The opportunity to express multiple genes in operons, exclusive uniparental-maternal inheritance, and readily obtainable high protein levels make engineering of the plastid genome attractive for applications in agriculture, metabolic engineering and molecular farming (Daniell et al., 2005; Bock, 2007). The ptDNA of higher plants is ~120-150-kb in size, highly polyploid and may be present in 1,000 to 10,000 copies per cell. Plastid transformation vectors are *E. coli* plasmids in which the gene of interest (*goi*) and a marker gene, encoding spectinomycin or kanamycin resistance, are flanked by plastid DNA sequences. Plastid transformation involves introduction of transforming DNA into chloroplasts on the surface of microscopic gold particles where the transgenes integrate into the ptDNA by two homologous recombination events *via* the flanking ptDNA (Figure 1-1). To obtain a genetically stable transplastomic plant all ptDNA copies must be changed. Such genetically stable, homoplastomic plants are obtained through a gradual process of ptDNA replication and sorting, and preferential maintenance of transgenic ptDNA copies on antibiotic-containing tissue culture medium. However, when plants with a uniformly transformed ptDNA population are obtained, the marker gene is no longer necessary to maintain the transplastomic state. For general reviews on plastid transformation see ref. (Bock, 2001; Maliga, 2004); for information on specialized vector designs see ref. (Herz et al., 2005; Maliga, 2005; Lutz et al., 2006b; Lutz and Maliga, 2007).

One reason to produce marker-free transplastomic plants is the potential metabolic burden imposed by high levels of marker gene expression. Marker genes are designed for high levels of expression so that the initial few integrated copies can protect the cells. As a consequence, when the homoplastomic state is reached, the marker gene product can make up 5% to 18% of the total cellular soluble protein (Khan and Maliga, 1999; Kuroda and Maliga, 2001b, 2001a). The second reason to remove the marker gene is the shortage of primary plastid selective markers, which at this point include only genes that confer resistance to spectinomycin and streptomycin (*aadA*) (Svab and Maliga, 1993) or kanamycin (*neo* or *kan*, (Carrer et al., 1993); and *aphA-6* (Huang et al., 2002)). If multiple engineering steps are required, the marker genes may have to be removed to enable repeated selection for the same marker. The third issue is opposition to having any unnecessary DNA in transgenic crops, especially antibiotic resistance genes (Lee and Natesan, 2006).

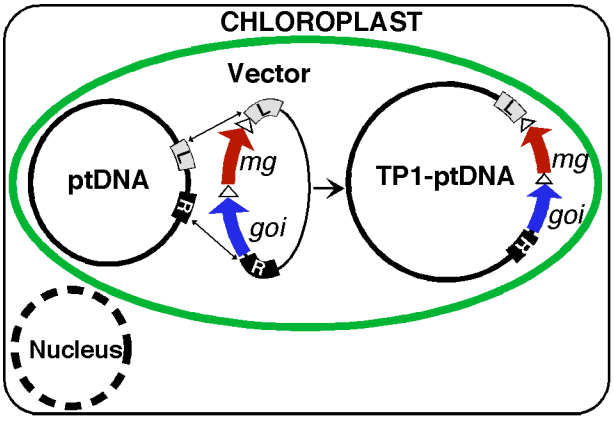

Figure 1-1. Homologous recombination yields transformed plastid genome in higher plants. Recombination between the left (L) and right (R) targeting sequence in the vector and cognate sequences in the plastid genome (ptDNA) lead to the incorporation of marker gene (*mg*) and the gene of interest (*goi*) in the ptDNA to yield the TP1 transplastome (TP1-ptDNA). Triangles flanking the marker gene symbolize recognition

sequences of site-specific recombinases to be used for marker gene excision. Based on ref. (Corneille et al., 2001; Hajdukiewicz et al., 2001).

Marker gene excision in plastids (based on. Curr. Opin. Biotechnol. 18:107-114, 2007)

I review here strategies developed for plastid marker gene excision including homology-based excision *via* directly repeated sequences, excision by phage site-specific recombinanses, transient cointegration of the marker gene and the cotransformation-segregation approach. At the end we shall discuss the relative merits of the different approaches and how marker excision technology will facilitate commercialization of transplastomic crops.

Homology-based marker gene excision *via* directly repeated sequences

The simplest approach to marker gene excision relies on the plastid's efficient native homologous recombination machinery, that excises any sequence between two directly oriented repeats. The feasibility of the approach was first shown in the chloroplast of the unicellular alga, *Chlamydomonas reinhardtii* (Fischer et al., 1996). Homology-based marker excision in tobacco chloroplasts was demonstrated after transformation with a construct carrying three transgenes: the *uidA* reporter gene encoding beta-glucuronidase; the *aadA* spectinomycin resistance marker gene; and *bar*, a herbicide resistance gene (Iamtham and Day, 2000). The repeats were the expression signals of the transgenes: two of the three genes shared the same (Prrn, the rRNA operon) promoter and all three genes had the same transcription terminator (TpsbA) (Figure 1-2). Initial heteroplastomic clones were identified by selection for spectinomycin and streptomycin resistance conferred by *aadA*. Herbicide resistant and sensitive derivatives were then obtained in the

absence of antibiotic selection. Ultimately two types of stable marker-free plants were obtained neither of which has repeated sequences: subclones carrying only the *uidA* gene (recombination R2 *via* T1 repeats) or the *bar* gene (recombination R1 *via* P1 repeat; Figure 1-2). Thus, homology-based marker excision relies on secondary recombination and segregation of ptDNA in an inherently genetically unstable, heteroplastomic plant.

A variant of homology-based marker excision technology enabled direct identification of marker free tobacco plants by herbicide resistance (Dufourmantel et al., 2007). The vector used for plastid transformation carried an *aadA* gene disrupting the herbicide resistance gene. The initial transplastomic clones were selected by spectinomycin resistance. Marker-free herbicide resistant derivatives were identified after excision of the *aadA* marker gene by homologous recombination within the overlapping region (403 nucleotides) of the N-terminal and C-terminal halves of the herbicide resistance gene. Excision of *aadA* led to reconstitution of a complete herbicide resistance gene and expression of the *Pseudomonas fluorescence* 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme that confers resistance to sulcotrione and isoxaflutole herbicides (Dufourmantel et al., 2007).

A second variant enables visual tracking of homology-based marker excision by formation of a pigment deficient sector due to loss of a plastid (*rbcL*) photosynthetic gene (Kode et al., 2006). Deletion of a relatively large segment, 6.1-kb DNA encoding the *uidA-aadA-rbcL* genes, could be obtained by recombination between two 649-bp direct repeats, the PatpB promoter driving expression of the *uidA* transgene and the PatpB promoter at is native location upstream of the *atpB* gene. The cells lacking *rbcL* could be visually identified by the pale green color; these cells also lack the *uidA* and *aadA* genes.

The marker-free knockout lines are useful as recipients in plastid transformation using the visually aided marker selection approach (see below).

Because the recombination frequency between direct repeats is dependent on the length of the repeat and the distance between the repeated sequences, the required length of the duplication is difficult to predict. If the excision frequency is too high, identification of the initial transplastomic clones by antibiotic selection may be a problem. If the frequency is too low, isolation of marker-free plants may not be feasible, or may take too many generations.

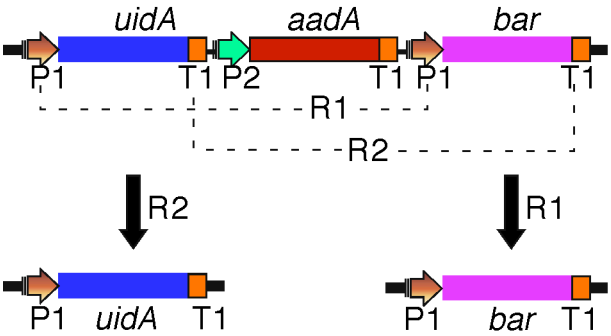

Figure 1-2. Homology-based marker gene excision *via* directly repeated sequences (Iamtham and Day, 2000). Shown are the *uidA* reporter gene encoding beta-glucuronidase; the *aadA* spectinomycin resistance marker gene; and *bar*, a herbicide resistance gene. The repeats were the promoters (P1) and transcription terminators (T1). Recombination *via* the P1 or T1 repeats yielded the two stable marker-free ptDNA carrying only the *uidA* (recombination R2) or the *bar* (recombination R1) gene. Note that no sequence is repeated in the stable product.

Marker gene excision by phage site-specific recombinanses

Generally applicable, efficient protocols for marker gene excision rely on nuclear encoded, plastid targeted phage site-specific recombinases, which utilize a two-step protocol. Step one is construction of transplastomic plants, which carry a marker gene flanked by two directly oriented recombinase target sites (Figure 1-1). The genome of the homoplastomic plants is stable in the absence of the site-specific recombinase. Step

two is removal of the marker gene by introduction of a gene encoding a plastid-targeted recombinase in the plant nucleus. 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 flanked by directly oriented target sites (Figure 1-3).

Thus far two recombinases were 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). The *Cre* gene has been introduced into the plant nucleus by three methods. (i) Stable transformation of the nucleus using an Agrobacterium binary vector that instantly yielded marker-free transplastomic plants. Although the plants regenerated from tissue culture were plastid marker free, now they carried a nuclear *Cre* transgene that had to be segregated away in the seed progeny (Corneille et al., 2001; Hajdukiewicz et al., 2001). (ii) In a second approach, the *cre* gene was introduced into the transplastomic plants by pollination (Corneille et al., 2001). Although introduction of *Cre* by pollination took longer, it appears that non-specific Cre-induced rearrangements between homologous ptDNA sequences were absent, or occurred significantly less often than in directly-transformed plants (Corneille et al., 2001) (see below). (iii) According to the third approach, Cre was expressed transiently from T-DNA introduced by Agroinfiltration, exploiting the observation that not every T-DNA delivery results in stable integration

(Lutz et al., 2006a). Useful for us is ~10% of the regenerated plants, which do not carry either a plastid (*aadA*) marker gene or a nuclear *Cre*.

The second site-specific recombinase tested for marker gene excision is Int, the phiC31 phage integrase, an enzyme that was used for insertion of the transforming DNA into the ptDNA (Lutz et al., 2004). 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 a plastid-targeted *int* (Kittiwongwattana et al., 2007).

When comparing Cre and Int for marker gene excision, Int appears to be the better choice. Testing Cre-mediated marker gene excision revealed two ptDNA sequences or pseudo *lox* sites (*lox-rps12* and *lox-psbA*) that share sequence similarity with *loxP* sites and are recognized by the Cre. As a consequence, when a *loxP* site is present, Cre mediates deletions between *loxP* and *lox-rps12* or *loxP* and *lox-psbA* sequences (Corneille et al., 2003). However, no ptDNA sequences appear to be present in the plastid genome that would be recognized by the Int (Lutz et al., 2004; Kittiwongwattana et al., 2007).

A second problem observed during Cre-mediated marker excision was deletion of ptDNA sequences by recombination *via* directly repeated non-*loxP* sequences that resulted in the loss of the *trnV* gene (Corneille et al., 2001; Hajdukiewicz et al., 2001). In one experiment this was observed in ~40% of the clones (Tungsuchat et al., 2006). The observation was explained by enhanced homologous recombination of the native plastid recombination machinery in the presence of Cre. Deletion of sequences *via* direct (and inverted) repeats adjacent to *loxP* sites has also been observed in *E. coli* expressing Cre

(Mlynarova et al., 2002). In contrast to *E. coli*, deletions in plastids occurred between direct repeats, but not between inverted repeats, at least in the presence of Int (Kittiwongwattana et al., 2007). Thus, deletions detected in the presence of Cre can possibly also be avoided by omitting directly repeated ptDNA sequences adjacent to *loxP* sites.

Homology-mediated marker excision is one of the approaches to obtain marker-free plants, as discussed above. The frequency of deletion is proportionate with the length of the repeat. Although the *loxP* sequences are relatively short, 34 bp in length, we expect that they still may cause infrequent loss of the marker gene in the absence of Cre. Since the *attB* and *attP* sequences are not homologous, plastid genomes carrying *att*-flanked marker genes are predicted to be more stable than those with marker genes flanked by identical *loxP* sequences. The absence of homology between the *attB* and *attP* sites and the absence of pseudo-*att* sites in ptDNA make Int a preferred alternative to Cre for plastid marker excision.

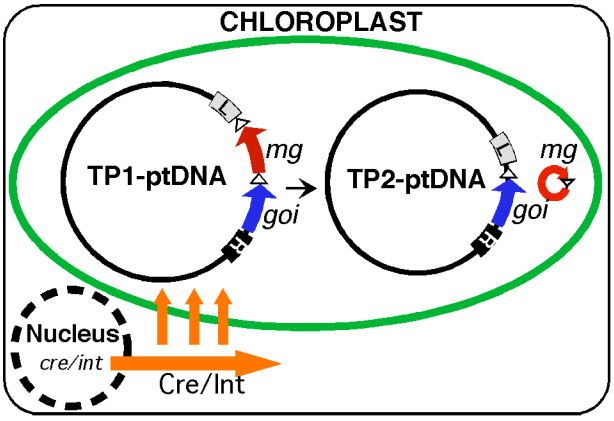

Figure 1-3. Site-specific recombinases (Cre/Int) expressed from nuclear genes (*cre/int*) excise marker genes (*mg*) from TP1-ptDNA after import into plastids. Excision of the marker gene by phage recombinases *via* the target sites (triangles) yields marker-free TP2-ptDNA carrying only the gene-of-interest (*goi*) and one recombinant copy of the recombinase recognition sequence (Corneille et al., 2001; Hajdukiewicz et al., 2001; Kittiwongwattana et al., 2007).

Transient cointegration of the marker gene to obtain marker free plants

During plastid transformation the marker gene and gene-of-interest are incorporated in the ptDNA together by two homologous recombination events *via* the targeting sequences (Figure 1-1). Placing the marker gene (*aphA-6*) outside the targeting region enables selection for a cointegrate structure that forms by recombination *via* only one of the targeting sequences (Figure 1-4). When selection for antibiotic resistance is stopped, the second recombination event may take place and the marker gene is lost. If the gene of interest is linked to a missing photosynthetic gene (*petA*, *rpoA*), and the recipient lacks this particular gene, marker free transplastomic plants can be directly recognized by reconstitution of the full gene set restoring green pigmentation (Klaus et al., 2004). This method is very convenient if repeated transformation is planned with variants of the same plastid gene, although this approach requires an initial investment, obtaining a knockout mutant for use in the visually aided complementation assay (Klaus et al., 2003).

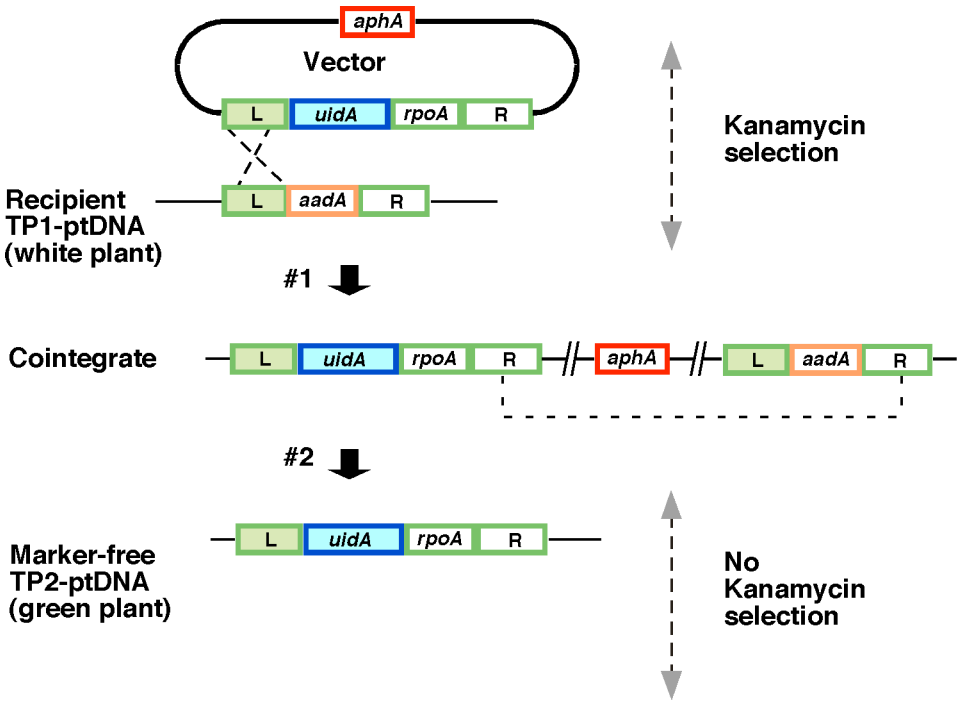

Figure 1-4. Transient cointegration of the marker gene to obtain marker-free plants (Klaus et al., 2004). The recipient plant is white because the plastid *rpoA* RNA polymerase subunit gene was replaced with a spectinomycin resistance (*aadA*) gene (TP1-ptDNA). Introduction of vector DNA by the biolistic process is followed by recombination (#1) *via* the left (L) targeting sequence that yields an unstable cointegrate structure on selective kanamycin medium. Transfer of cultures onto antibiotic-free medium allows second recombination (#2) to occur between the right (R) targeting repeats (marked by dashed line) that yields marker-free transplastomic plants carrying the *uidA* reporter gene. The marker free plants are readily recognized because incorporation of *rpoA* with the *uidA* gene restores green pigmentation.

Cotransformation-segregation to obtain marker-free plants

Cotransformation involves transformation with two plasmids targeting insertions at two different ptDNA locations: one plasmid carrying a selective marker and the second a non-selected gene. Selection for the marker yielded transplastomic clones, which also carried an insertion of the non-selected gene. The feasibility of the approach was shown in *Chlamydomonas reinhardtii*, a unicellular alga with a single chloroplast (Kindle et al., 1991; Newman et al., 1991; Roffey et al., 1991). Interestingly, when this approach was tested in tobacco, which has many more chloroplasts, twenty percent cotransformation

efficiency was obtained (Carrer and Maliga, 1995). Cotransformation enabled his-tagging of an unlinked *ndh* gene following spectinomycin selection (Rumeau et al., 2005).

The cotransformation-segregation approach to obtain marker-free transplastomic plants relies on the heteroplastomic ptDNA population obtained by cotransformation with two independently targeted genes. The approach was developed to obtain marker free plants that lack the antibiotic resistance gene and are resistant to the glyphosate or phosphinothricin herbicides (Ye et al., 2003) (Figure 1-5). Transplastomic clones cannot be directly selected by glyphosate or phosphinothricin resistance after transformation with the *CP4* or *bar* genes because the cells that carry only a few copies of the transgene die. However, when *CP4* and *bar* genes are introduced into the plastid genome by linkage to the spectinomycin resistance (*aadA*) gene and most ptDNA copies carry the genes, the cells in tissue culture and the plants under field conditions become resistant to high levels of the herbicides (Lutz et al., 2001; Ye et al., 2001). The transformation segregation scheme used two independent transformation vectors for a cotransformation approach and two different selective agents in a phased selection scheme. One transformation vector carried an antibiotic resistance (*aadA*) marker used for early selection, and the other transformation vector carried the herbicide (*CP4* or *bar*) resistance marker for use in the subsequent herbicide selection phase. About 50% of the clones selected by spectinomycin resistance also carried the non-selected herbicide resistance gene, with a high frequency (20%) of these giving rise to transformation segregants containing exclusively the initially nonselected herbicide resistance marker.

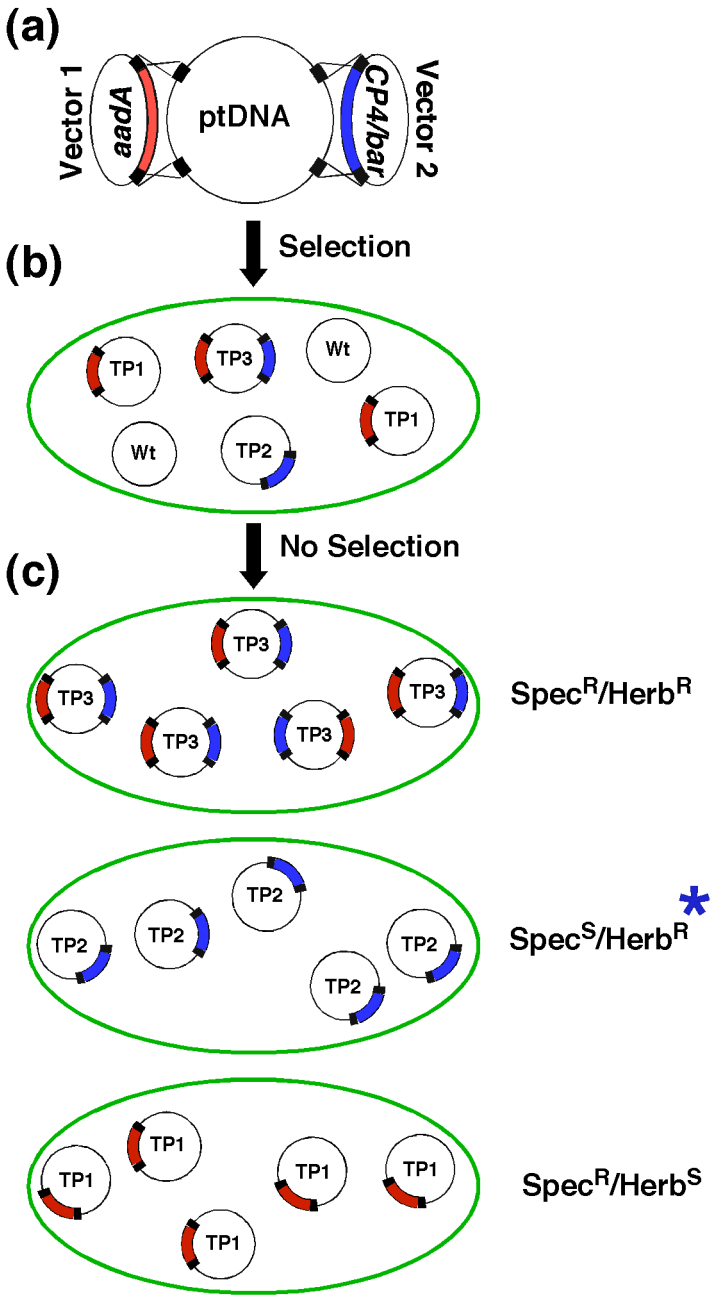

Figure 1-5. The cotransformation-segregation method to obtain marker-free transplastomic plants (Ye et al., 2003). (**a**) Transformation of the plastid genome (ptDNA) with the independently-targeted marker gene (*aadA* in vector 1) and the herbicide resistance gene (*CP4/bar* in vector 2). (**b**) Transplastomic clones are identified by selection for spectinomycin resistance (*aadA*). Heteroplastomic cell carries: wild-type ptDNA (wt), TP1-ptDNA obtained by transformation with Vector 1; TP2-ptDNA transformed with vector 2; TP3-ptDNA transformed with both vectors. (**c**) Replication and segregation of ptDNA on non-selective medium eventually yields chloroplasts with homoplastomic TP1-ptDNA, TP2-ptDNA and TP3-ptDNA. Desired herbicide resistant plants carry TP2-ptDNA and lack the antibiotic resistance marker (star).

Conclusions

All four protocols discussed here are suitable to obtain marker-free transplastomic plants. Ultimately the choice of approach will depend on the ease of use and the access to intellectual property rights. What homology-based excision and the cotransformation-segregation approaches share in common is that the final desired transplastomic line is identified in a genetically unstable, segregating ptDNA population. This makes it difficult to control production of the marker-free transplastomic clones. Transient cointegration to obtain marker-free plants is also difficult to control, unless selection is combined with "visual aided selection" by complementation of a knockout mutant, as originally described (Klaus et al., 2004). The need to obtain a knockout mutant first trades relatively high initial input for future convenience. By far the most convenient is recombinase-mediated marker excision that utilizes a two-stage approach. First transplastomic clones are obtained with marker genes that are flanked with site-specific recombinase recognition sequences. The lines marked for excision are genetically stable in the absence of the site-specific recombinase. However, marker gene excision can be initiated by introduction of a site-specific recombinase. The problems inherent to the system are potential native sequences (pseudo sites) recognized by the recombinase and enhanced homologous recombination between non-target repeats. Study of Cre and Int revealed that Int does not have the pseudo-target site problem. Thus, additional enzymes may be beneficially explored for application in plastids from the repertoire of site-specific recombinases tested for nuclear gene engineering (Keravala et al., 2006; Thomson and Ow, 2006).

While the technology to obtain marker-free transplastomic plants is available, no transplastomic crops are grown yet commercially. The reason is slow implementation of the tissue culture based plastid transformation technology outside the group of solanaceous species. Encouraging in this regard are recent additions to list in which genetically stable transplastomic plants have been obtained in a diverse group of species including: soybean (Dufourmantel et al., 2004), cotton (Kumar et al., 2004), lettuce (Lelivelt et al., 2005; Kanamoto et al., 2006), cauliflower (Nugent et al., 2006) and poplar (Okumura et al., 2006). The cereal crops rice, maize and wheat continue to be recalcitrant to plastid transformation. Given the information reviewed here transformation of the plastid genome with economically valuable genes can now be accomplished with vectors, which enable posttransformation excision of marker genes. Removal of the plastid marker gene will facilitate public acceptance of the new transplastomic crops which will be preferred over their nuclear counterparts due to elimination of transgene flow via pollen, an inherent characteristic of nucleus-transformed plants.

Site-specific recombinases for integration of transgenes

The Int system has been utilized not only for plastid marker gene excision, but also for efficient, unidirectional integration of foreign DNA in the nuclei of mammalian cells (Groth et al., 2000; Thyagarajan, 2001; Belteki et al., 2003; Allen and Weeks, 2005), yeast (Thomason et al., 2001) and higher plants (Ow, 2002). Although plastid transformation is feasible in tobacco (Svab et al., 1990; Svab and Maliga, 1993; Zoubenko et al., 1994) and other solanaceous species (Sidorov et al., 1999) (Ruf et al., 2001), plastid transformation in species such as *Arabidopsis* (Sikdar et al., 1998),

Brassica napus (Hou et al., 2003) and *Lesquerella fendleri* (Skarjinskaia et al., 2003) is not as practical. The reason for the lack of successful plastid transformation in these species is unknown, but may be due to difficulty with plant regeneration in tissue culture or due to the inefficiency of the plastids homologous recombination machinery, which is the general approach to plastid transformation in higher plants. The Int-based system may enable reproducible plastid transformation in species, in which homologous recombination rarely yields transplastomic clones.

Plastid sorting

Although the ~ 150-kb plastid genome is present in many copies, the ptDNA population is normally uniform. To determine how plastid populations can change, plastid sorting has been studied using plants with biparental plastid transmission (Hagemann, 2004). Crossing a pigment-deficient plant with a wild-type green plant allows for visual tracking of the plastid sorting (Chiu and Sears, 1993). Transmission of both plastid types yields a plant with two plastid types that need to sort out to create a stable homoplastomic plant. I use the Int site-specific recombinase to generate two unequal plastid populations that allows me to study plastid sorting in somatic cells. Plastid sorting could be visualized in the regenerated plants since the two ptDNA populations have distinct phenotypes in the developing leaves: ptDNA copies that retain a *bar* transgene cause an aurea phenotype on young leaves, whereas ptDNA copies that have the *bar* gene deleted yield plants with green leaf color.

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Chapter 2

A Novel Approach to Plastid Transformation Utilizes the phiC31 Phage Integrase

Introduction

Engineering the plastid genome is an attractive alternative to nuclear transformation because of readily obtainable high recombinant protein levels, natural containment due to lack of pollen transmission, the feasibility of expressing multiple genes from operons and the lack of position effects (Bock, 2001; Staub, 2002; Maliga, 2003, 2004). Plastid transformation has been most successful in the solanaceous species tobacco (Svab et al., 1990; Svab and Maliga, 1993; Zoubenko et al., 1994), potato (Sidorov et al., 1999) and tomato (Ruf et al., 2001). Plastid transformation in *Arabidopsis* (Sikdar et al., 1998), *Brassica napus* (Hou et al., 2003) and *Lesquerella fendleri* (Skarjinskaia et al., 2003) was feasible but inefficient. In rice, only heteroplastomic plants have been described (Khan and Maliga, 1999).

Stable integration of foreign DNA by the plastid's homologous recombination machinery is the general approach to plastid transformation in higher plants. Plastid transformation vectors are *E. coli* plasmid derivatives, which carry plastid DNA sequences (1-kb to 2-kb) to direct integration at specific sites in the plastid genome (Figure 2-1a). There are 1,000 to 10,000 plastid genome copies present in the 10 to 100 plastids per cell (Bendich, 1987; Pyke, 1999). The success of plastid transformation depends on amplification of transformed genome copies by selection for spectinomycin, streptomycin (Svab et al., 1990; Svab and Maliga, 1993; Zoubenko et al., 1994) or kanamycin resistance (Carrer et al., 1993; Huang et al., 2002) encoded in the marker genes.

We report here an alternative approach (Figure 2-1b) that is independent of the plastid's homologous recombination machinery and relies on the phiC31 *Streptomyces* phage site-specific integrase (Int) catalyzing recombination between the phage *attP* and bacterial *attB* attachment sites (Thorpe and Smith, 1998; Thorpe et al., 2000). The Int system has been utilized for efficient, unidirectional integration of foreign DNA in the nuclei of mammalian cells (Groth et al., 2000; Thyagarajan, 2001; Belteki et al., 2003), yeast (Thomason et al., 2001) and higher plants (Ow, 2002).

We tested the feasibility of plastid transformation utilizing Int-mediated recombination. A plastid vector carrying an *attP* sequence was inserted into recipient chloroplasts at a previously introduced *attB* site. Int function was provided either by expression from a nuclear gene which encoded a plastid-targeted Int or by expressing Int transiently from a non-integrating plasmid in plastids. Int-mediated plastid transformation will facilitate plastid transformation in species in which homologous recombination is the rate-limiting step.

Results

Construction of plants with the *attB* plastid genome

Minimal sequences required for *attB* and *attP* function *in vitro* were defined to be 34 bp and 39 bp, respectively (Groth et al., 2000). In mammalian cells, longer sequences were found to be more efficient (Groth et al., 2000). Therefore, we utilized 55-bp *attB* and 217-bp *attP* regions.

To create the *attB* recipient plastid genome, a suitable plastid transformation vector was designed. Plasmid pSAC114 contains plastid targeting sequences, an *attB*

sequence and a linked spectinomycin resistance (*aadA*) gene flanked by *loxP* sites (floxed) (Figure 2-2a). Vector pSAC114 was introduced into tobacco leaf chloroplasts by the biolistic process to obtain the R1 recipient plastid genome. Plastid transformants containing the *attB* site were obtained by selection for spectinomycin resistance. The R1 recipient plastid genome structure was verified by DNA gel blot analysis (Figure 2-2b). Four pSAC114 transplastomic lines were purified to the homoplastomic state: Nt-pSAC114-2A, Nt-pSAC114-3B, Nt-pSAC114-5A and Nt-pSAC114-63A. The four lines are considered identical.

To obtain marker-free *lox/attB* plants (R2 recipient), a plastid-targeted *Cre* gene (pKO30) (Corneille et al., 2003) was introduced into the nucleus of the Nt-pSAC114-63A plant by *Agrobacterium* transformation. When all copies of the floxed *aadA* marker gene were removed (Figure 2-2a,c), the integrated nuclear *Cre* gene was segregated out in the seed progeny yielding the Nt-LA63 recipient line.

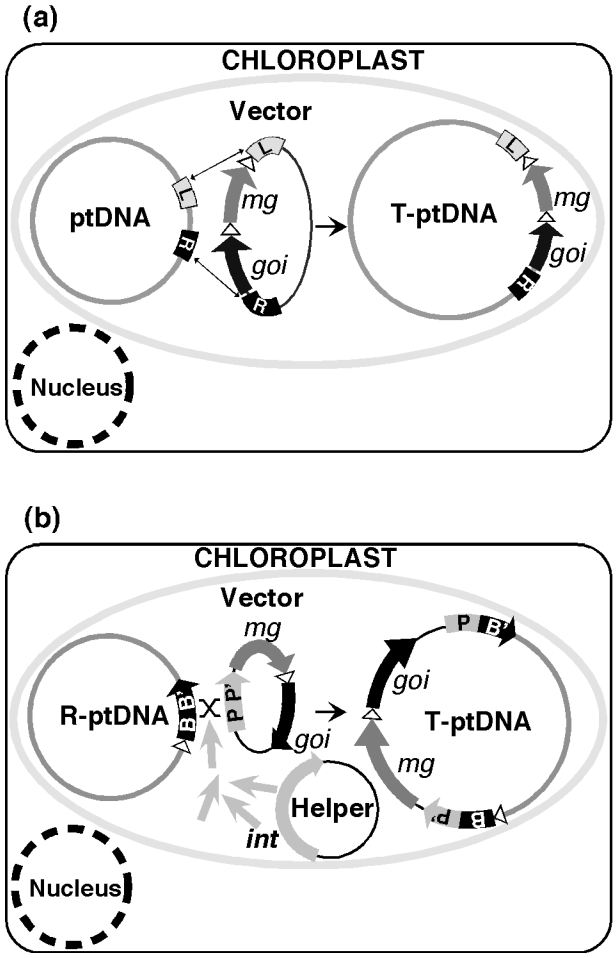

Figure 2-1. Approaches to plastid transformation. **(a)** Plastid transformation based on the plastid's recombination machinery and homologous targeting. Note: wild-type plastid genome (ptDNA), left (L) and right (R) targeted regions; transformation vector with L and R targeting regions, marker gene (*mg*) flanked by *loxP* sites (floxed; open triangles) and gene of interest (*goi*); and the engineered plastid genome (T-ptDNA).
(b) Plastid transformation mediated by Int. Shown are: recipient plastid genome (R-ptDNA) with *attB* (BB') and *loxP* sites; plastid vector with *attP* site (PP'), marker gene (*mg*), *loxP* site and gene of interest (*goi*); the Int plasmid and engineered plastid genome (T-ptDNA) with *attL* (BP') and *attR* (PB') recombination junctions.

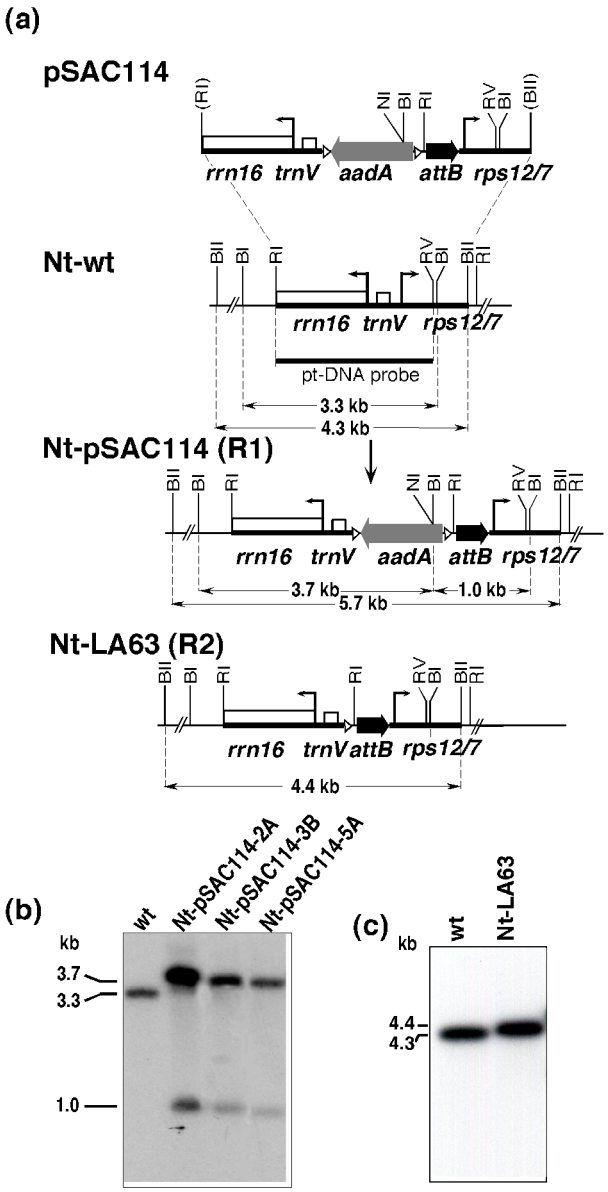

Figure 2-2. Verification of the *attB* plastid genome structure. **(a)** Maps of the pSAC114 vector, the wild type (Nt-wt), the Nt-pSAC114 (R1) and Nt-LA63 (R2) plastid genomes. Positions of the plastid genes *rrn16*, *trnV*, *rps12/7*, the *aadA* gene, the *attB* sequence and the *Bam*HI (BI), *Bgl*II (BII), *Eco*RI (RI), *Eco*RV (RV) and *Nco*I (NI) restriction sites are marked. **(b)** DNA gel blot analysis confirms ptDNA transformation. Total cellular DNA was digested with the *Bam*HI restriction endonuclease and probed with the wild-type *Eco*RI-*Eco*RV plastid DNA fragment (ptDNA probe, heavy line, Figure 2-2a).
(c) DNA gel blot analysis to confirm *aadA* excision. Total cellular DNA was digested with the *Bgl*II restriction endonuclease and probed as Figure 2-2b.

Plastid *attP* vectors

The pKO103 *attP* plastid vector was obtained by incorporating an *attP* site in an *E. coli* (pBSIIKS+) plasmid and replacing the ampicillin resistance gene with a *neo* gene that confers kanamycin resistance to both *E. coli* and plastids. Since the vector cannot replicate in plastids, kanamycin resistant plants will only be obtained if the vector integrates into the plastid genome. An alternative *attP* vector, pKO193, was obtained by replacing the ampicillin resistance gene with a spectinomycin resistance (*aadA*) gene as a selective marker; a *loxP* site for excision of the marker gene; and a *bar* gene that confers herbicide resistance.

Stable nuclear Int for plastid transformation

Since construction of the Nt-LA63 recipient line took time, we decided to test Int-mediated transformation using R1 recipients (Figure 2-3). The R1 Int recipients were obtained by *Agrobacterium* transformation of the R1 recipient (Nt-pSAC114) with vector pKO117, which encodes a plastid-targeted *Int* and a gentamycin resistance (*aacC1*) gene (Figure 2-4). The Int lines with the R1 plastid genome are designated N1-N18 (Table 2-1).

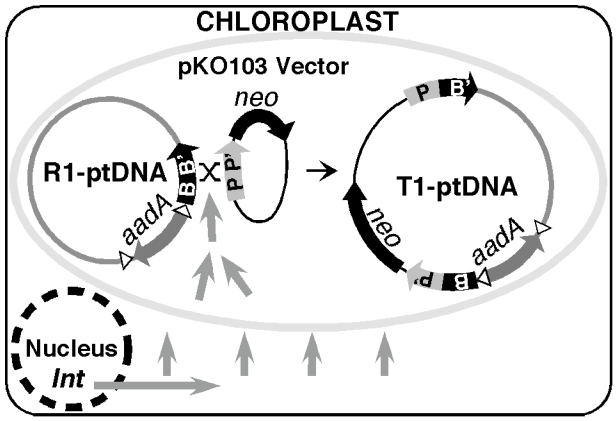

Figure 2-3. Plastid transformation mediated by Int expressed from a stably integrated nuclear gene. Shown are: recipient plastid genome (R1-ptDNA) with *attB* site (BB') and floxed *aadA* marker gene; plastid vector with *attP* site (PP') and a *neo* marker gene; and engineered plastid genome (T1-ptDNA) with *attL* (BP') and *attR* (PB') recombination junctions.

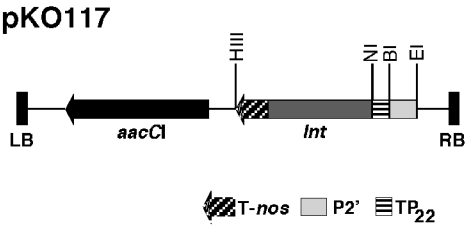

Figure 2-4. Schematic map of the pKO117 *Agrobacterium* binary vector T-DNA region with the plastid-targeted *Int* gene. Abbreviations: LB and RB, left and right border sequences; *aacC1*, gentamycin resistance gene; P2' and Tnos *Agrobacterium* P2' promoter and *nos* terminator; TP_{22}, Rubisco small subunit transit peptide, with 22 amino acids of the mature small subunit N-terminus.

To test the feasibility of Int-mediated plastid transformation, the pKO103 plastid vector (Figure 2-5b) was bombarded into the leaves of N1-N18 Int recipient lines. Two days after bombardment the leaves (~7 cm long) were cut into ~1 cm^2 pieces (20 to 30 pieces per leaf) and selected for kanamycin resistance (50 mg/L). The number of kanamycin resistant clones per bombarded leaf was between 1 and 19. In 45 bombarded leaf cultures 237 independent kanamycin resistant lines were obtained. Of these, 113 were evaluated for integration of the pKO103 vector at the *attB* site (Table 1). The individual transformed lines were identified by the Int line and a serial number, e.g. N1-49. Letters after the serial number distinguish subclones derived from the same event.

Int-mediated insertion of the *attP* vector at the *attB* site was confirmed in 81 of the 113 clones (~72%; Table 2-1). Integration was verified by PCR amplification of border fragments obtained with primer pairs 01-02 and 03-04, one which annealed to the

pKO103 vector part and one which annealed to the recipient plastid DNA (Figure 2-5c). Sequencing of PCR-amplified junction fragments revealed *attL* and *attR* junction sequences, the hallmarks of Int-mediated recombination (Figure 2-5e). DNA gel blot analysis in the 16 clones tested confirmed integration at the *attB* site as the *neo* probe recognized the predicted 6.1-kb fragment in the transplastomic clones (Figure 2-5d). DNA gel blots of 16 kanamycin resistant clones, in which no insertion at the *attB* site was found, were also probed with *neo*. No high-copy signal was detected on these blots therefore, no integration of *attP* vectors at alternative plastid target sites occurred (data not shown).

In one sample (N1-49A) a smaller than expected fragment (4.7 kb) was seen (Figure 2-5d). This smaller fragment is the result of a post-integration deletion event between 18-bp direct repeats in the pKO103 multiple cloning site (MCS) and restriction sites adjacent to the *attR* site (verified by sequencing PCR-amplified DNA).

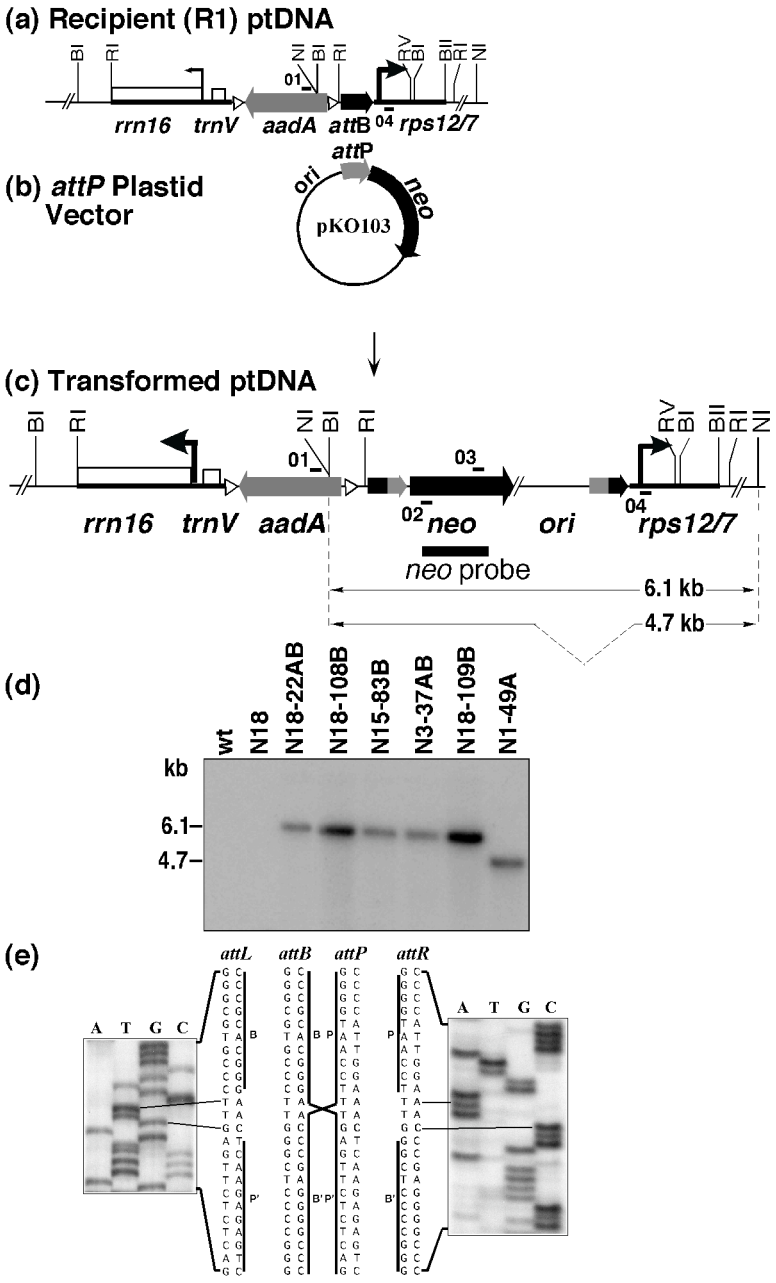

Figure 2-5. Int-mediated insertion of the pKO103 *attP* vector at the plastid *attB* site. **(a)** Map of R1 plastid genome with an *attB* sequence (heavy black arrow) and floxed *aadA*. Position of plastid genes *rrn16*, *trnV*, *rps12/7*, the *Bam*HI (BI), *Bgl*II (BII), *Eco*RI (RI), *Eco*RV (RV) and *Nco*I (NI) restriction sites and 01 and 04 primers are marked. **(b)** Map of the pKO103 *attP* plastid vector. Note *attP* site, *neo* marker gene and *Col*E1 replication origin (*ori*). **(c)** Map of T1-ptDNA. Fragments detected by the *neo* probe and the positions of O1-O4 primers are shown. **(d)** Probing of DNA gel blots with *neo* confirms integration of the pKO103 vector at the *attB* site. wt and N18 are DNA samples from wild-type and N18 recipient (R1) plants. Total cellular DNA was digested with the *Nco*I restriction endonuclease and probed with *neo* (heavy line, Figure 2-5c). **(e)** The *attL* and

attR junction sequences confirm Int-mediated insertion of the pKO103 vector at the *attB* site.

Transformation mediated by Int transiently expressed in plastids

When a nuclear gene provides Int function, as described in the previous section, *Int* has to be removed when transformation is accomplished. To avoid this tedious process, we tested the feasibility of providing Int function transiently, from a non-integrating plasmid. This was accomplished by incorporating an *int* gene with plastid expression signals in an *E. coli* (pBSIIKS+) plasmid, which could be introduced together with the *attP* plastid vector (Figure 2-6). Two *int* helper plasmids, pKO107 and pKO111, were tested. Translation of Int from the pKO111 mRNA was dependent on the creation of an AUG translation initiation codon from an ACG codon by mRNA editing, whereas no editing was required for the translation of the pKO107 mRNA.

The transient Int system was tested by introducing two plasmids, the pKO193 *attP* plastid vector (Figure 2-7c) and an *int* helper plasmid (pKO107 or pKO111)(1:2 ratio), into the chloroplasts and selecting for spectinomycin resistance. Bombardment of five leaves of the Nt-LA63 recipient line with the mix containing the pKO107 helper plasmid yielded five spectinomycin resistant lines. Of these, three were confirmed as products of Int-mediated insertion (Figure 2-7e). Bombardment of five Nt-LA63 leaves with the mix containing the pKO111 plasmid yielded seven spectinomycin resistant lines of which one was the product of Int-mediated insertion (Figure 2-7e). The plastid *int* gene encoded in the helper plasmids did not incorporate in the plastid or nuclear genomes, as confirmed by PCR analysis (data not shown).

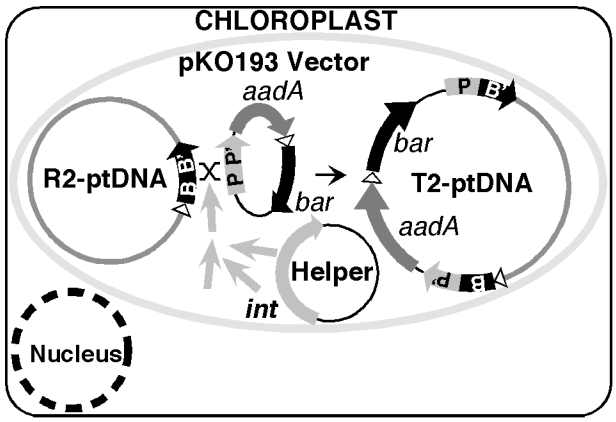

Figure 2-6. Plastid transformation mediated by Int transiently expressed in plastids. Note: recipient plastid genome (R2-ptDNA) with *attB* (BB') and *loxP* sites; plastid vector pKO193 with *attP* site (PP'), *aadA*, *loxP* site and *bar* gene; the Int plasmid and the engineered plastid genome (T2-ptDNA) with *attL* (BP') and *attR* (PB') recombination junctions.

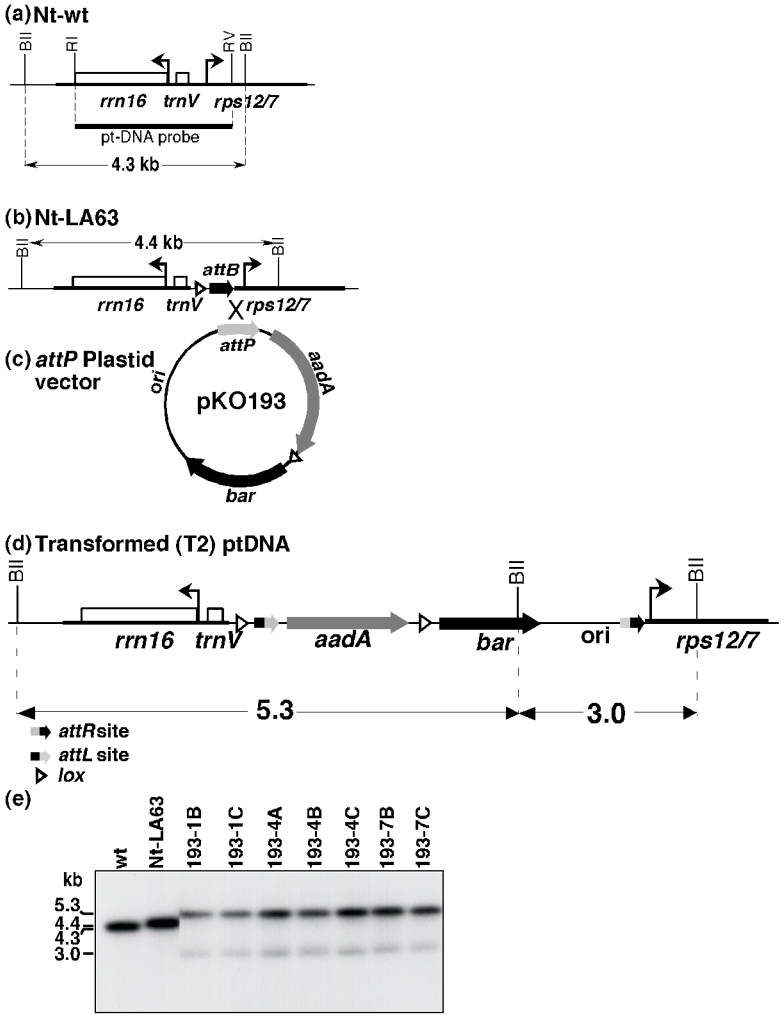

Figure 2-7. Transiently expressed Int mediates plastid transformation with pKO193. **(a)** Map of the wild-type (Nt-wt) plastid genome. Positions of plastid genes *rrn16*, *trnV*, *rps12/7*, and the *Bgl*II (BII), *Eco*RI (RI), and *Eco*RV (RV) restriction sites are marked. **(b)** Map of Nt-LA63 recipient plastid genome with a *loxP* site (open triangle) and an *attB* sequence (heavy black arrow). Positions of plastid genes *rrn16*, *trnV*, *rps12/7*, the *attB* sequence and the *Bgl*II (BII), restriction site are marked. **(c)** Map of the pKO193 *attP* plastid vector. Marked are the *attP* site, the *aadA* marker gene, the *loxP* site, the *bar* gene and the *Col*E1 replication origin (*ori*). **(d)** Map of the plastid genome transformed with the pKO193 vector. The 5.3 kb and 3.0 kb fragments detected by the *rrn16-rps12/7* targeting region probe. **(e)** DNA gel blot analysis confirms integration at the *attB* site. Wt and Nt-LA63 are DNA samples from wild-type plants and R2 recipient lines. Total cellular DNA was digested with the *Bgl*II restriction endonuclease and probed with the *Eco*RI-*Eco*RV *rrn16/rps12/7* targeting region (heavy line, Figure 2-7a). Position of hybridizing fragments is shown in Figure 2-7d.

Discussion

We report here efficient, unidirectional integration of foreign DNA into the genome of a eukaryotic organelle, the tobacco plastid by the phiC31 phage site-specific integrase. Implementation of the system is dependent on the availability of a recipient line carrying an *attB* (or *attP*) site introduced into the plastid genome by homologous recombination. We have shown here that Int mediates plastid transformation when it is provided either from a nuclear-encoded plastid targeted gene or from a non-replicating helper plasmid.

Approaches to providing Int function

Incorporation of Int into the nucleus, although effective, requires that the gene be removed once recombination is complete. Transient expression of the recombinase allows for sufficient amounts of Int protein to be expressed without the need to remove the *int* gene once the reaction is complete. Plastid transformation with the transiently expressed Int was relatively efficient yielding 4 independently transformed transplastomic lines after bombardment of 10 leaves, five leaves each with the pKO107 (3 lines) and pKO111 (1 line) helper. In a preliminary experiment, we obtained 19 transplastomic clones in 8 bombarded leaf samples confirming that transient expression of Int reproducibly yields transplastomic clones. The preliminary experiment was carried out with the R1 recipient, the pKO103 *attP* transformation vector and the pKO111 Int helper plasmid. Thus, in the transient system, kanamycin (pKO103) and spectinomycin (pKO193) resistance markers were both suitable to recover transplastomic clones and both the edited (pKO111) and unedited (pKO107) *int* genes provided Int function.

We report here that we did not find integrated copies of the helper plasmids in the plastid or nuclear genomes. However, it is possible that the helper plasmid was transiently maintained during the initial stage of transformation resulting in maintenance of Int activity for a relatively long period of time. Possible mechanisms for transient maintenance of the helper plasmid are cointegrate formation by recombination *via* ptDNA sequences driving Int expression, or independent replication of the helper plasmid which has been shown to occur in *Chlamydomonas reinhardtii* chloroplasts (Boynton et al., 1988). These transiently maintained helper plasmids, if present at all, were lost by the time we tested *int* by PCR in homoplastomic plants.

The *attB* recipient lines

Proof of concept for Int mediated plastid transformation was obtained with the R1 recipient plastid genome carrying a floxed *aadA* plastid marker gene. Removal of the floxed *aadA* by Cre yielded the marker free R2 recipient genome, which can now be transformed with an *attP* vector carrying either *aadA* or *neo* as the selective marker. The R2 recipient genome and the *attP* transformation vector each carry one suitably positioned *loxP* site so that the integrated marker gene can be excised *via* the directly oriented *loxP* sites (Figure 2-7d) when uniform transformation of all plastid genomes is obtained, as reported earlier (Corneille et al., 2001; Hajdukiewicz et al., 2001).

No functional *attB* sites were found in the plastid genome

We have previously identified functional *lox* sites in the plastid genome, which yielded recombination products with *loxP* sites (Corneille et al., 2003). Therefore, we tested if Int

-mediated integration can occur into plastid sequences that function as *attB* sites. Candidates for integration at plastid *attB* sequences were kanamycin-resistant clones, in which no *attP* vector insertion was found at the *attB* site. Probing of total cellular DNA of these plants with *neo* did not yield a high-copy signal that would suggest integration of the vector in the plastid genome at alternative sites (data not shown). Thus, the kanamycin resistant clones were probably obtained by fortuitous integration of *neo* in the nucleus (Carrer et al., 1993).

Alternative *att* vector designs

Int-mediated transformation efficiency in plastids may be further improved by incorporating *attP* instead of *attB* in the plastid genome as a target site, because it was shown that the *attP* sequence could be a more efficient target (Thyagarajan, 2001; Belteki et al., 2003).

An alternative approach to integrating an entire plastid transformation vector would be the cassette exchange system where only sequences flanked by *attP* (or *attB*) sites are integrated into the plastid genome *via* two *attB* (or *attP*) sites suitably positioned in the plastid genome (Thomason et al., 2001; Ow, 2002; Belteki et al., 2003). By this design only sequences between the *attP* (or *attB*) sites would be integrated in the plastid genome. However, even if the entire vector is incorporated in the plastid genome, remaining vector sequences may be removed along with the marker gene by Cre when transformation is accomplished *via* strategically positioned *loxP* sites. For this, the relative position of *ori* and *attP* sequences should be swapped in the *attP* vector shown in Figure 2-7c.

Application of the Int/*attB*/*attP* system for plastid transformation

We have shown here that the Int system is a useful alternative to homologous targeting for plastid transformation in tobacco. Plastid transformation efficiency in some of the stable nuclear Int lines was unusually high, up to 17 transplastomic clones per bombarded sample (Table 2-1). Homologous recombination-based plastid transformation efficiency numbers range from 0.5 to 5.0 per bombarded sample (Maliga, 2003, 2004). Since only a relatively small number of experiments was performed, it would be premature to draw the conclusion that Int-based plastid transformation is more efficient than homologous-recombination based transformation in tobacco. However, we expect that the Int-based system may enable reproducible plastid transformation in species, in which homologous recombination rarely yields transplastomic clones. Combination of the Int /*attB*/*attP* system for insertion of the transforming DNA and the Cre-*loxP* site-specific recombination system for removal of the marker gene (Corneille et al., 2001; Hajdukiewicz et al., 2001; Kuroda and Maliga, 2003) will facilitate applications of plastid transformation in basic science and biotechnology (Bock, 2001; Maliga, 2004).

Experimental procedures

Plastid vector for construction of *attB* plants

Plastid vector pSAC114 is a pPRV1 vector derivative (Zoubenko et al., 1994) in which an *aadA* gene, flanked by two directly oriented *lox* sites, and an *attB* sequence are targeted for insertion at the *trnV-rps12/7* intergenic region. Directly oriented *loxP* sites were available in plasmid pSAC61 (pBSIIKS+ backbone; Stratagene, La Jolla, CA) in a

*Kpn*I-*Hin*dIII fragment (5'-ggtaccATAACTTCGTATAATGTATGCTATACGAAGTTATagatctATAACTTCGTATAATGTATGCTATACGAAGTTATaagctt-3'). Plasmid pSAC61 was obtained by cloning a *Kpn*I-*Sal*I adapter (5'-ggtaccATAACTTCGTATAATGTATGCTATACGAAGTTATagatctgtcgac-3') in a pBSIIKS+ vector (Stratagene, La Jolla, CA) to obtain plasmid pSAC60, then ligating a *Bgl*II-*Hin*dIII adapter (5'-agatctATAACTTCGTATAATGTATGCTATACGAAGTTATaagctt-3') in plasmid pSAC60. Nucleotides added to aid construction are shown in lower case. The *aadA* gene was cloned into the *Bgl*II site located between the *loxP* sequences in pSAC61. The *aadA* gene derives from plasmid pZS176, which contains an identical *aadA* gene in plasmid pZS197 (Svab and Maliga, 1993) other than it has an *Eco*RI site in the 5'-UTR. The *attB* sequence was cloned upstream of *aadA* as a *Pst*I-*Spe*I linker 5'-CTGCAGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACTAGT-3'.

Agrobacterium vector with nuclear *Int* gene

Tobacco *attB* plants expressing a nuclear *Int* were obtained by transformation with *Agrobacterium* binary vector pKO117. Plasmid pKO117 is a pPZP222 vector derivative with gentamycin resistance (*aacC1*) as selective marker (Hajdukiewicz et al., 1994) and a plastid-targeted *Int* in the multiple cloning site. Plasmid pKO117 was obtained by replacing the *Cre* coding region in plasmid pKO27 (Corneille et al., 2001) with an *Int* coding region. The *Int* gene was then cloned into a pPZP222 vector. The *Streptomyces*

ssp. phiC31 phage *Int* coding segment derives from plasmid pSET152 (Bierman et al., 1992)(GenBank Accession No. AJ414670) obtained from Mark Buttner. To facilitate *Int* expression, an *Nco*I site was added as an *Nco*I-*Sun*I linker at the N-terminus to include the ATG translation initiation codon (5'-ccATGgctagcGACACGTACG-3') and an *Xba*I site was created downstream of the stop codon by PCR amplification (5'-TAGtctaga-3'). *Int* is expressed in a cassette consisting of the *Agrobacterium* P2' promoter and Tnos terminator, and is translationally fused with the pea Rubisco small subunit transit peptide (Timko et al., 1985) and twenty-two amino acids of the mature small subunit.

Helper plasmids for transient Int expression in plastids

Plasmid pKO107 is a pUC118 vector derivative obtained by replacing the *aadA* coding segment in plasmid pZS176 (above) with the *Int* coding segment (*Nco*I-*Xba*I fragment). Plasmid pKO111 was obtained by replacing the *neo* coding segment with an *Int* coding segment (*Nhe*I-*Xba*I fragments) in plasmid pSC4 (Chaudhuri and Maliga, 1996).

Plastid *attP* vectors

Plastid *attP* vector pKO103 is a pBSII KS+ (Stratagene, La Jolla, CA) derivative that contains an *attP* sequence, a *neo* marker gene, a *loxP* site (not shown in Figure 2-5) and a multiple cloning site (MCS). The *attP* sequence was PCR amplified from plasmid pSET152 (primers: 5'-caactcGAGCAATCGCCCTGGGTG-3' and 5'-caaaagcttCCCGGTCACAACCCCTTG-3') and cloned as a *Xho*I-*Hin*dIII fragment in the multiple cloning site of the pBSIIKS+ plasmid. The kanamycin resistance (*neo*) gene from plasmid pHK10 (blunt *Sac*I/*Hind*III fragment; *Xba*I site removed)(Kuroda and

Maliga, 2001) was ligated in the *Hin*dIII site. The *attP-neo* fragment (*Xho*I-*Eco*RV blunt) was cloned into the *Kpn*I site of plasmid pSAC60. The ampicillin resistance gene and the f1 replication origin were removed by religating the *Ahd*I and *Sac*I digested plasmid.

Plastid *attP* vector pKO193 is pKO103 derivative, in which the *neo* gene was replaced with an *aadA* gene from plasmid pZS176 (see above) and the *bar* gene from plasmid pKO18 (*Eco*RI-*Hin*dIII fragment) (Lutz et al., 2001) was cloned into the multiple cloning site.

Plastid transformation

Transforming DNA was introduced into tobacco leaves on the surface of tungsten particles by the biolistic process (Svab and Maliga, 1993). Transplastomic clones transformed with plasmids pSAC114 or pKO193 were selected on RMOP medium containing 500mg/L spectinomycin HCl (Svab and Maliga, 1993). Clones transformed with plasmid pKO103 were selected on RMOP medium containing 50mg/L kanamycin sulfate (Carrer et al., 1993). Plastid transformation was confirmed by DNA gel blot analysis of total cellular DNA (Svab and Maliga, 1993). PCR amplification of junction sequences of pKO103-transformed clones was performed with primers O1 5'-CCGCCAGCGTTCATCCTGAGC-3' and O2 5'-TGACAGCCGGAACACGGCGGC-3' for the left (*attL*) junction or primers O3 5'-TGAAGAGCTTGGCGGCGAAT-3' and O4 5'-GAGATGTAACTCCAGTTCC-3' for the right (*attR*) junction.

Agrobacterium-mediated transformation

The *Int* gene was introduced into the nucleus of Nt-pSAC114 transformed plants by cocultivation of Nt-pSAC114 leaves with *Agrobacterium* containing the pKO117 binary vector (Hajdukiewicz et al., 1994). Nuclear gene transformants were selected by gentamycin resistance on RMOP shoot regeneration medium containing 100mg/L gentamycin. Transformation was confirmed by rooting shoots on selective plant maintenance (Murashige and Skoog salts, 3% sucrose, pH 5.6)(Murashige and Skoog, 1962) medium containing 100mg/L gentamycin.

Sequencing of *attB/attP* junctions

Junction sequences of pKO103-transformed clones were PCR amplified using primers O1 5'-CCGCCAGCGTTCATCCTGAGC-3' and O2 5'-TGACAGCCGGAACACGGCGGC-3' for the left junction or primers O3 5'-TGAAGAGCTTGGCGGCGAAT-3' and O4 5'-GAGATGTAACTCCAGTTCC-3' for the right junction. PCR products were sequenced using oligonucleotides O1-O4 as primers with the T7 Sequenase PCR Product Sequencing Kit (USB, Cleveland, OH).

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Table 2-1 Integration of the pKO103 *attP* vector at the plastid *attB* site

INT Line	**No.of Leaves**	**Kan^r**	**Kan^r /leaf**	**Insertion at *attB****	**Effici-ency****
N1	3	10	3.3	10/10	3.3
N2	3	14	4.7	6/10	2.8
N3	2	24	12.0	7/7	12.0
N4	1	5	5.0		
N5	1	2	2.0		
N6	4	13	3.3	8/10	2.6
N7	1	9	9.0		
N8	4	17	4.3	0/16	0.0
N9	1	11	11.0	5/10	5.5
N10	1	1	1.0		
N11	2	14	7.0	10/10	7.0
N12	2	13	6.5	10/10	6.5
N13	4	4	1.0		
N14	4	3	0.8		
N15	3	22	7.3	10/10	7.3
N16	4	25	6.3	6/10	3.8
N17	3	12	4.0		
N18	2	38	19.0	9/10	17.1

* Number of transplastomic clones/Number of clones tested.
** Efficiency = (Kan^r/leaf) x Insertion at *attB*. Values were calculated to adjust for differences in the fraction of transplastomic clones in INT lines.

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Chapter 3

Plastid Marker Gene Excision by Transiently Expressed Cre Recombinase

Introduction

Because of readily obtainable high protein levels and natural containment, the plastid genome (ptDNA) is an attractive target for biotechnological applications. The ~ 150-kb ptDNA is highly polyploid and may be present in 1,000 to 10,000 copies per cell. Plastid transformation involves targeted insertion of the transforming DNA into the plastid genome by two homologous recombination events (Figure 3-1a) and amplification of the rare, transformed copies by selection for antibiotic resistance encoded in the vector (Bock, 2001; Maliga, 2004). Once transformation of ptDNA is accomplished, the marker gene is no longer desirable because of the metabolic burden imposed by high levels of marker gene product, the need to use the same marker gene for multi-step engineering and to meet consumer expectations (Maliga, 2004).

There are multiple protocols for the excision of plastid marker genes. Most protocols are tedious because transformation and marker gene elimination occur simultaneously (Iamtham and Day, 2000) (Ye et al., 2003), or the protocols may depend on the availability of specialized plant lines (Klaus et al., 2004). Generally applicable, efficient protocols for marker gene excision rely on a nuclear encoded, plastid targeted Cre recombinase derived from the P1 bacteriophage, which excises marker genes flanked by directly oriented 34-bp *loxP* sites (Figure 3-1b) (Corneille et al., 2001; Hajdukiewicz et al., 2001). When removal of the plastid marker gene has been accomplished, the nuclear gene encoding Cre has to be removed by segregation in the seed progeny. Removal of the nuclear *Cre* is a time consuming process and is not practical in

vegetatively propagated crops, in which seed propagation is incompatible with variety preservation. We report here an alternative protocol relying on excision of the multi-copy plastid marker genes by a transiently expressed Cre recombinase in *Nicotiana tabacum* L. (tobacco).

Results

Transplastomic tester plants

Vector pPRV110L is a member of the Plastid Repeat Vector (pPRV) family (Zoubenko et al., 1994) targeting insertions between the *trnV* and 3'-*rps12* intergenic region (Shinozaki et al., 1986) and carrying a selectable spectinomycin resistance (*aadA*) gene without a promoter but with a ribosome binding site (RBS) and the 3'-UTR of the plastid *rbcL* gene (TrbcL) for stabilization of mRNAs. Vector pPRV110L is a *loxP* vector because *loxP* sites flank *aadA* so that the marker gene can be excised when the homoplastomic state is achieved (Figure 3-1b, Figure 3-2a). Plastid transformation vectors pMHB10 and pMHB11 are dicistronic and encode the *bar* and *aadA* genes (Figure 3-2a), the expression of which confers herbicide (Iamtham and Day, 2000; Lutz et al., 2001; Ye et al., 2003) and spectinomycin resistance (Goldschmidt-Clermont, 1991; Svab and Maliga, 1993), respectively. The pMHB10 and pMHB11 plasmids differ with respect to the leader sequence of the *bar* gene. The *bar* gene (*b-bar2*) N-terminus in plasmid pMHB10 is translationally fused with the PrrnLatpB+DS, the *rrn* operon promoter fused with the plastid *atpB* leader and 14 of the N-terminal *atpB* codons (Kuroda and Maliga, 2001). In vector pMHB11, the *bar* coding segment is translationally fused with the *psbL* gene N-terminus (E*bar* gene). Translation of the tobacco plastid *psbL*

gene is dependent on conversion of an ACG codon to an AUG translation initiation codon by mRNA editing (Kudla et al., 1992). The 73 nt segment (-63 to +10, plasmid pSC4) containing the *psbL* initiation codon, used in this study, was sufficient to direct efficient editing of the initiation codon of a chimeric transcript enabling expression of the encoded protein (Chaudhuri and Maliga, 1996). Therefore, we expected that the chimeric pMHB11 E*bar* mRNA would also be edited and herbicide resistance be expressed.

Plasmids pMHB10 and pMHB11 were introduced into the plastid genome and uniform transformation of plastid genomes was confirmed by DNA gel blot analysis (Figure 3-2b). Three pMHB10 (Nt-pMHB10-13, Nt-pMHB10-14 and Nt-pMHB10-23) and two pMHB11 (Nt-pMHB11-12, Nt-pMHB11-18) independently transformed lines were obtained. The transplastomes in these plants will be termed TP1-ptDNA (Figure 3-2a).

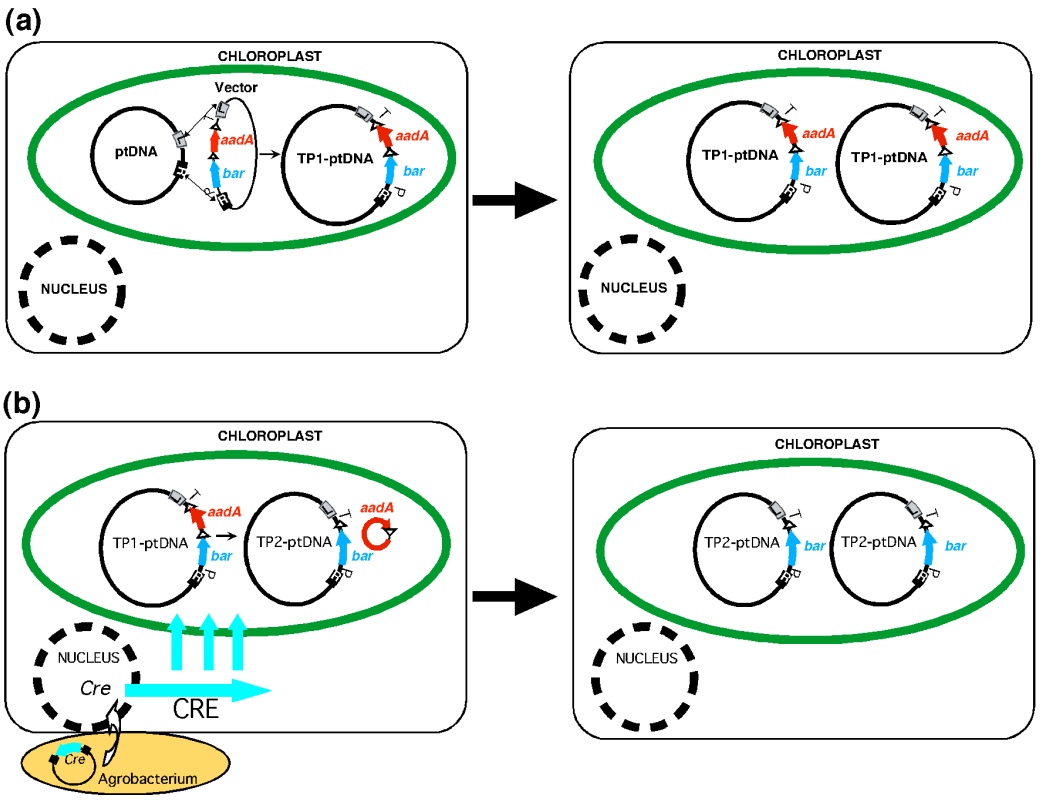

Figure 3-1. Plastid transformation to obtain marker-free transplastomic plants.
(a) Insertion of transforming DNA into the plastid genome (ptDNA) by homologous recombination to yield transplastome (TP1-ptDNA).
(b) Marker excision by transiently expressed Cre. *Cre* introduced into the plant nucleus on an Agrobacterium T-DNA is expressed in the cytoplasm, enters chloroplasts and excises floxed *aadA* from TP1-ptDNA to yield TP2-ptDNA.
Abbreviations: *bar* gene, blue; *aadA*, red; L and R, left and right targeting sequences; P, promoter; T, terminator; triangles, *lox* sites; *Cre*, gene for Cre recombinase.

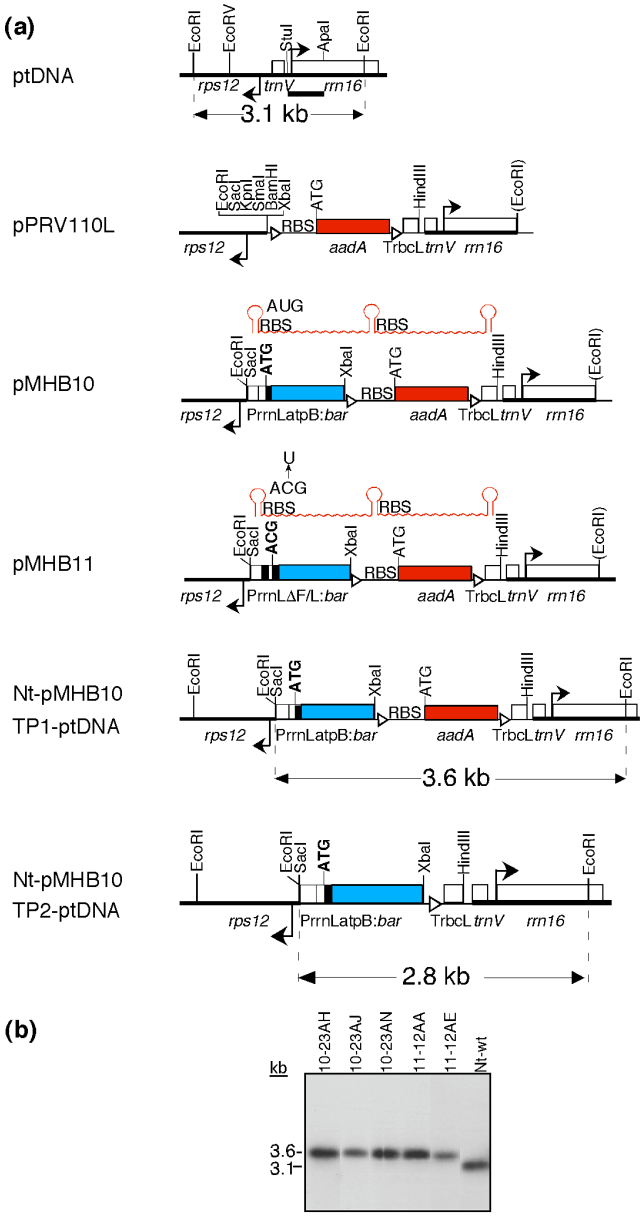

Figure 3-2. Introduction of *bar* genes into the plastid genome.

(a) Maps of ptDNA, plastid vectors pPRV110L, pMHB10 and pMHB11, and the TP1-ptDNA and TP2-ptDNA (after *aadA* excision) of Nt-pMHB10 plants. Transcripts from vectors pMHB10 and pMHB11 with 5' and 3' stem-loop structures are shown as wavy lines. Abbreviations: *rrn16*, *trnV* and *rps12* are plastid genes; RBS, ribosome binding site; PL, promoter-leader cassette; T, transcription terminator. Map distances and restriction enzyme recognition sites are marked.
(b) DNA gel blot analysis to confirm TP1-ptDNA structure. Total leaf cellular DNA was digested with *Eco*RI and probed with the *Stu*I/*Apa*I plastid DNA fragment (P1 probe, heavy line, Figure 3-2a). The position and size of hybridizing fragments is marked in Figure 3-2a. For plant designation see Experimental procedures.

Editing-dependent *bar* is expressed in chloroplasts but not in *E. coli*

The newly obtained transplastomic Nt-pMHB10 and Nt-pMHB11 plants were tested for herbicide resistance. The TP1-ptDNA leaf segments were resistant to 4 mg l^{-1} phosphinothricin in cell culture (Figure 3-3a). The regenerated plants were resistant to 2% Liberty herbicide, a commercial formulation of phosphinothricin, when sprayed in the greenhouse (Figure 3-3b). In accordance with the herbicide resistance phenotype, PAT activity was detected *in vitro* by acetylation of phosphinothricin in the leaves of both Nt-pMHB10 and Nt-pMHB11 plants (Figure 3-4). Expression of E*bar* in Nt-pMHB11 plants was enabled by posttranscriptional conversion of an ACG to an AUG translation initiation codon (Figure 3-5). Editing of the *bar* segment was efficient, because most signal (>85%) is found in the T lane (edited nucleotide) instead of C lane (non-edited nucleotide) (Figure 3-5).

We also tested PAT activity in *E. coli* carrying the plastid transformation vectors. PAT activity could be readily detected in *E. coli* cells carrying the pMHB10 construct, which does not require mRNA editing for expression, but not in cells carrying the

pMHB11 construct (Figure 3-4) indicating that the E*bar* gene is not edited, and therefore is not expressed in *E. coli*.

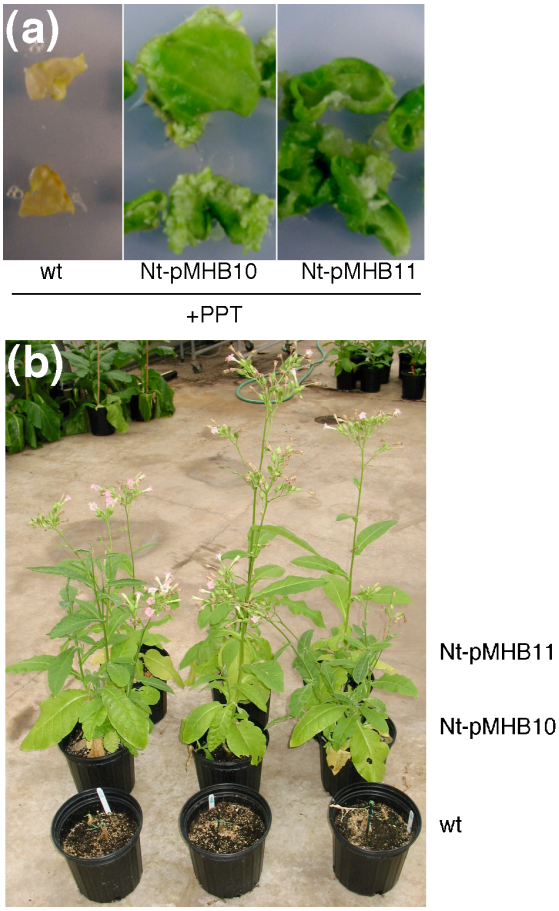

Figure 3-3. Expression of E*bar* confers herbicide resistance. (a) Leaf assay of wt, Nt-pMHB10 and Nt-pMHB11 plants grown in the presence 4 mg l^{-1} of PPT.
(b) Wild-type (wt), Nt-pMHB10 and Nt-pMHB11 plants one month after spraying with Liberty (2%; BayerCropScience, Research Triangle Park, NC).

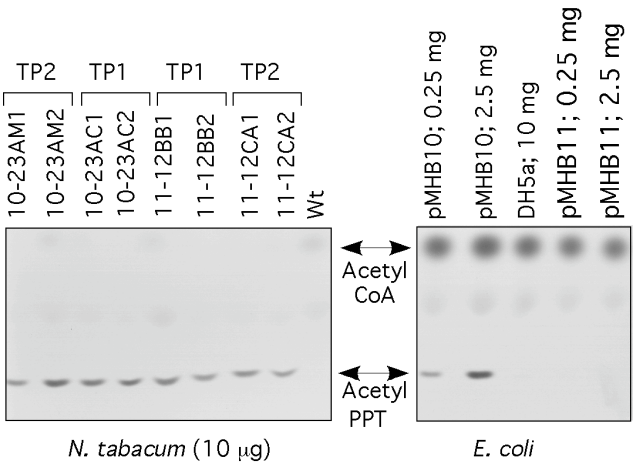

Figure 3-4. PAT assay confirms that E*bar* is expressed in *N. tabacum* plastids but not in *E. coli* cells. The *N. tabacum* assays were performed with 10 μg protein extracts from wild type (wt) and transplastomic TP1-ptDNA and their TP2-ptDNA derivatives with the marker gene excised. Assays are also shown for expression of *bar* from the transformation vectors in *E. coli.* The amount of extract in the *E. coli* assay is next to the plasmid name. PAT activity was determined by conversion of phosphinotricin (PPT) into acetyl-PPT using radiolabeled ^{14}C-Acetyl-CoA. Unincorporated ^{14}C-Acetyl CoA and labeled Acetyl-PPT are indicated with arrows. The faint, unmarked spot between them is an acetyl CoA degradation product.

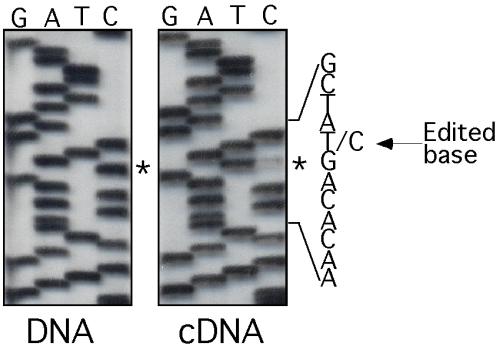

Figure 3-5. Editing of the *psbL* site in the chimeric E*bar* mRNA. The cDNA was PCR amplified and directly sequenced. Asterisk marks the position of the edited nucleotide. Note edited T in cDNA sequence at position of C in DNA.

Marker excision in agroinfiltrated leaves

The main question we address here is whether or not transient expression yields sufficient Cre levels to excise a significant fraction of the marker genes from the ~10,000 TP1-

ptDNA copies to enable recovery of marker-free (TP2-ptDNA) plants, as shown in Figure 3-1b. Agroinfiltration is a protocol that is suitable for high-level nuclear gene expression from a transiently introduced T-DNA region. Agroinfiltration involves vacuum treatment of leaf segments in an Agrobacterium suspension, and subsequent release of the vacuum to facilitate entry of bacterium cells into the inter-cellular space (Kapila et al., 1997). Protein expressed from the T-DNA accumulates two to four days after agroinfiltration (Johansen and Carrington, 2001; Voinnet et al., 2003). To test the efficiency of marker gene excision in TP1-ptDNA, Nt-pMHB10 and Nt-pMHB11 leaf segments were infiltrated with Agrobacterium strain EHA101 (Hood et al., 1986) containing the pKO31 binary vector encoding a plastid-targeted Cre (Corneille et al., 2003). We tested marker gene excision in TP1-ptDNA leaf segments up to four days after agroinfiltration. Marker gene excision was observed as early as three days after agroinfiltration. By day four, a significant fraction (10% to 20%) of plastid genome copies lacked the marker gene (Figure 3-6a).

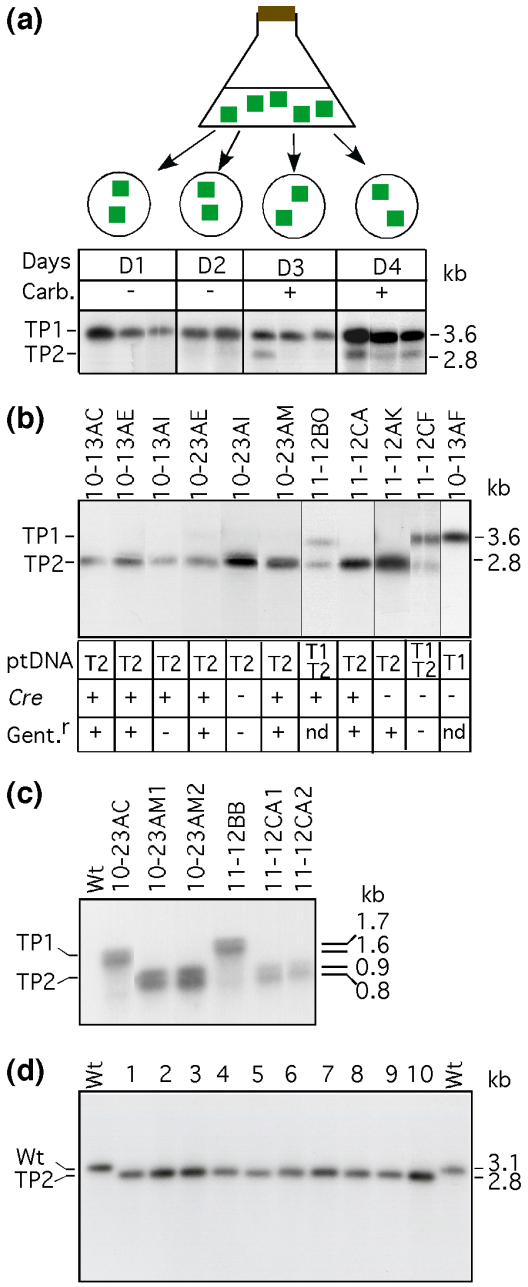

Figure 3-6. Excision of floxed *aadA* by transiently expressed Cre. (a) Short-term testing of excision. Vacuum infiltration of leaf disks (squares) in Agrobacterium solution was followed by culture of leaf disks (1-4 days; D1-D4) on RMOP shoot regeneration medium. Probing of *Eco*RI-digested total cellular DNA isolated from leaf disks with *Stu*I/*Apa*I ptDNA fragment confirmed *aadA* excision: TP1-ptDNA yields a 3.6-kb, TP2-ptDNA a 2.8-kb diagnostic fragment (Figure 3-2a). Agrobacterium was killed on day 3 by transferring the leaf sections onto RMOP medium containing carbenicillin (500 mg l^{-1}). (b) TP1-ptDNA and/or TP2-ptDNA in regenerated shoots was identified by Southern blot analysis (see Figure 3-6a). Presence of *Cre* determined by PCR and the T-DNA plant

marker gentamycin resistance (Gent^r) determined by leaf tissue culture assay is listed below. Abbreviations: +, present; –, absent; nd, not determined. (c) RNA gel blot analysis with the *bar* probe confirms reduction in size of mRNAs due to marker gene excision. Note double band, probably caused by two independent transcription termination/processing downstream of *loxP* site and *rbcL* 3'-UTR: TP1-ptDNA, 1.6 kb and 1.7 kb; TP2-ptDNA, 0.8 kb and 0.9 kb. (d) Southern analysis indicates that TP1-ptDNA copies are absent in the Nt-pMHB10-23AI seed progeny. Probing (Figure 3-6a) identifies 3.1-kb wild-type and 2.8-kb TP2-ptDNA fragments.

Marker-free plants from agroinfiltrated leaves

Encouraged by marker gene excision from TP1-ptDNA in the short-term experiment, we attempted to obtain marker-free transplastomic plants from Agrobacterium-treated leaves of Nt-pMHB10-13A, Nt-pMHB10-23A, Nt-pMHB11-12A, Nt-pMHB11-12B and Nt-pMHB11-12C plants. Plant regeneration was carried out on a medium containing PPT to select for the *bar* gene as a second round of purification and carbenicillin (500 mg l^{-1}) to kill the Agrobacterium cells. Plants regenerated on the PPT medium were named by a second letter and randomly screened for *aadA* excision. Southern blots identified marker-excised (TP2-ptDNA) plastid genomes in 19 of the 61 regenerated plants (11 of 27 Nt-pMHB10; 8 of 34 Nt-pMHB11). Examples for marker excision by DNA and RNA gel blot analysis are shown in Figure 3-6b and 3-6c, respectively. The nuclear *Cre* gene was absent in 7 clones (Nt-pMHB10-13AB, Nt-pMHB10-23AB, Nt-pMHB10-23AI, Nt-pMHB11-12AK, Nt-pMHB11-12BV, Nt-pMHB11-12BW, Nt-pMHB11-12CF), as determined by PCR analysis. One of these (Nt-pMHB11-12AK) carries the unselected gentamycin resistance gene encoded in pKO31 (data not shown).

Overall, excision of *aadA* by a non-integrated nuclear *Cre* was observed in ~10% (6 out of 61) of the regenerated plants. Examples for this category in Figure 3-6b are the Nt-pMHB10-23AI and Nt-pMHB11-12CF lines. Although a small fraction of ptDNA

copies still may contain *aadA* in blots shown in Figure 3-6b, these copies are typically lost during plant development. As an example for the absence of TP1-ptDNA, the Nt-pMHB10-23AI seed progeny is shown in Figure 3-6d.

Discussion

Polycistronic *loxP* expression vector

Plastid vector pPRV110L is the prototype of a polycistronic plastid vector with an excisable marker gene. The vector is polycistronic, because it was designed for expression of multiple genes organized in an operon. The operon with its own promoter is cloned upstream of *aadA*, which becomes the last reading frame of the enlarged operon (Figure 3-2a). Such polycistronic expression units are advantageous because they minimize the number of promoters and terminators, which are necessary for expression of multiple transgenes. Constructs described in this paper (pMHB10, pMHB11) are dicistronic and encode genes for herbicide and spectinomycin resistances. *LoxP* sites flank the *aadA* marker gene in vector pPRV110L so that it can be excised when the homoplastomic state is achieved.

Expression of herbicide resistance is dependent on mRNA editing

Others (Iamtham and Day, 2000; Ye et al., 2003) and we (Lutz et al., 2001) have reported that expression of PAT (phosphinothricin acetyl transferase) confers resistance to glufosinate herbicides when expressed in chloroplasts. The *bar* gene in vector pMHB10 has the same promoter as plasmid pKO18 (PrrnLatpB+DS), a monocistronic gene we studied earlier (Lutz et al., 2001). In vector pMHB11 the PAT coding segment is

translationally fused with the *psbL* gene N-terminus. The -63 to +10 *psbL* fragment was sufficient to ensure mRNA editing of the chimeric *bar* mRNA (Figure 3-5) so that expression of the *Ebar* gene confers herbicide resistance in Nt-pMHB11 plants. Similar editing-dependent genes were described by fusing *psbL* editing segments with *aadA* and *neo* coding regions, which conferred spectinomycin and kanamycin resistances when the chimeric mRNA was edited in chloroplasts (Chaudhuri et al., 1995). Translation of edited mRNAs in a chimeric context thus far has been reported only for the *aadA* and *neo* genes. PAT expression and the associated herbicide resistance phenotype demonstrate the general applicability of editing-dependent genes.

The editing-dependent *Ebar* gene (pMHB11) was not expressed in *E. coli* (Figure 3-4b), because prokaryotes lack the capacity for mRNA editing (Smith et al., 1997). Dependence of E*bar* expression on editing creates an effective expression barrier when the gene is transferred to prokaryotes, a phenomenon shown to occur under laboratory conditions at a very low frequency (Kay et al., 2003; Tepfer et al., 2003). Since no mRNA sequence is edited in the plant nucleus (Smith et al., 1997), *Ebar* mRNA will not confer herbicide resistance if the gene accidentally escapes to the plant nucleus (Huang et al., 2003; Stegemann et al., 2003). However, this *Ebar* will be expressed in the plastids of tobacco, and other higher plant species which share the capacity to edit the initiation codon of *psbL* mRNA (Kudla et al., 1992) (Chateigner-Boutin and Hanson, 2002; Hegeman et al., 2005).

Excision of marker genes by transiently expressed Cre

Since there are 1,000 to 10,000 ptDNA copies in a tobacco cell (Bendich, 1987), the challenge we have been facing was whether or not transient Cre expression is sufficient for excision of plastid marker genes from most genome copies. We report here that transient Cre expression is sufficient to obtain marker-free tobacco plants in the absence of *Cre* integration in the plant nucleus. The frequency of plastid marker-free plants in this study, 10%, is comparable to the ~12% excision frequency (13 out of 57) of a floxed plastid *codA* gene in an independent experiment (data not shown).

Although excision of marker genes from the plant nuclear genome is routine (Hare and Chua, 2002; Ow, 2002; Gilbertson, 2003), the system we describe here is the first for excision of organellar DNA by a transiently expressed Cre. So far only constitutively expressed Cre was used for the excision of plastid marker genes (Corneille et al., 2001; Hajdukiewicz et al., 2001; Corneille et al., 2003; Lutz et al., 2004) and to probe plastid gene function (Kuroda and Maliga, 2003). Data in this paper show that a short burst of Cre expression is sufficient to eliminate target sequences from all genome copies in the highly polyploid plastid genetic system. Plastid marker gene excision by a transiently expressed Cre is advantageous because it saves time, as there is no need to remove the Cre integrated in the nucleus. Transient Cre expression limits the time during which Cre may come in contact with ptDNA, thereby reducing the probability of undesired ptDNA rearrangements, including deletions involving fortuitous ptDNA sequences that function as *loxP*-sites (Hajdukiewicz et al., 2001; Corneille et al., 2003) and repeat-mediated recombination events increased in the presence of Cre (Corneille et al., 2001; Mlynarova et al., 2002). Transient expression of Cre for marker gene removal

now enables production of marker free transplastomic plants in vegetatively propagated species such as potato, in which variety preservation is incompatible with seed propagation.

Experimental procedures

Vector construction

Plastid transformation vectors pPRV110L (GenBank Accession No. DO211347), pMHB10 (GenBank Accession No. DO211346) and pMHB11 (GenBank Accession No. DO211345) have been constructed by standard molecular biology protocols and their maps are shown in Figure 3-2a. Vector pPRV110L targets insertions between the *trnV* and 3'-*rps12* intergenic region (Shinozaki et al., 1986) (insertion site No. 1, first digit of 110L) carrying a selectable spectinomycin resistance (*aadA*) gene (Chinault et al., 1986; Svab and Maliga, 1993) (marker gene No. 1, second digit) without a promoter but with a ribosome binding site (RBS) and the 3'-UTR of *rbcL* gene (TrbcL) (Shinozaki and Sugiura, 1982) (expression signals No. 0, third digit). Vector pPRV110L is a *loxP* (L) vector because *loxP* sites (Hoess et al., 1982; Van Duyne, 2001) flank *aadA* so that the marker gene can be excised when the homoplastomic state is achieved (Figure 3-1b, Figure 3-2a). Plastid transformation vectors pMHB10 and pMHB11 are dicistronic and encode the *bar* (Thompson et al., 1987) and *aadA* (Chinault et al., 1986) genes. Plasmid pMHB10 carries the *b-bar2* gene in which the *bar* N-terminus is translationally fused with the PrrnLatpB+DS (Kuroda and Maliga, 2001). Vector pMHB11 carries the E*bar* coding segment in which *bar* is translationally fused with the *psbL* gene N-terminus

including 63 nucleotides upstream and 10 nucleotides downstream of the *psbL* translation initiation codon as in plasmid pSC4 (Chaudhuri and Maliga, 1996).

Plastid transformation

Plastid transformation and identification of transplatomic clones was carried out as previously described (Svab and Maliga, 1993). Briefly, sterile leaves were bombarded with DNA coated tungsten particles using the biolistic gene gun (PDS-1000, BioRad, Hercules, CA). Plastid transformants were identified on RMOP medium containing 500 mg l^{-1} spectinomycin dihidrochloride by green color and shoot formation and uniform transformation of ptDNA genome copies was verified by DNA gel blot analysis. Plants were designated by: the plasmid name, a serial number and letters to indicate the number of regeneration cycles on a selective medium to segregate away non-transformed ptDNA. An example for line designation is Nt-pMHB10-23AH, which involved two cycles of plant regeneration on a selective medium.

Agroinfiltration

The vacuum infiltration was performed as described by Kapila et al (Kapila et al., 1997). Agrobacterium transformed with pKO31 (Corneille et al., 2003), carrying the plastid-targeted *cre* gene, was inoculated into 100 ml YEB medium (5 g l^{-1} Beef extract, 1 g l^{-1} Yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sucrose, 2 mM MgSO4) supplemented with 100 mg l^{-1} spectinomycin and grown overnight at 27°C. 1 ml from the above culture was inoculated into fresh YEB medium containing 10 mM MES 2-(N-morpholino)ethanesulfonic acid, pH adjusted to 5.6, 20 mM acetosyringone, and 100 mg

l^{-1} spectinomycin, and grown overnight at 27°C. The culture was centrifuged at 6000 rpm for 15 minutes, resuspended in MMA medium (MS salts, 10 mM MES, 20g l^{-1} sucrose, pH 5.6 and 200 mm acetosyringone) to a final OD_{660}=2.4 and incubated at room temperature for 1 hour. Nt-pMHB10 and Nt-pMHB11 leaves were cut into small pieces ($1cm^2$) and submerged in the Agrobacterium culture. The flask containing the culture was placed under continuous vacuum of 2 Torr for 20 minutes while shaking gently. After vacuum infiltration the leaf samples were incubated on RMOP medium for two days, then transferred to RMOP medium containing carbenicillin (500mg l^{-1}). DNA was isolated daily from individual leaf samples for four days after the vacuum infiltration. Southern blots were performed on total leaf cellular DNA samples from individual leaf pieces digested with the *Apa*I and *Eco*RI restriction enzymes and probed with the plastid targeting region (1.9 kb *Apa*I-*Stu*I ptDNA fragment containing the *rrn16* gene; Figure 3-2a)(Svab and Maliga, 1993). The remaining leaf pieces were further incubated on RMOP shoot regeneration medium containing carbenicillin (500mg l^{-1}) to kill Agrobacterium cells. After four weeks individual shoots were removed from the cultures and rooted on RM medium containing carbenicillin (500mg l^{-1}). Total cellular leaf DNA was probed for marker gene (*aadA*) excision as described above.

Testing mRNA editing

Isolation of total cellular RNA and testing of RNA editing was performed by direct sequencing of reverse-transcribed, PCR amplified (RT-PCR) cDNA as described by Bock (Bock, 1998). For RNA isolation, leaf tissue was ground in a mortar with a pestle and extracted with TRIzol (Invitrogen, Carlsbad, CA). Total cellular RNA was treated

with Proteinase K (1x; RNA grade, Invitrogen, Carlsbad, CA) and with DNase I (2x; Roche, Indianapolis, IN) as described by Kudla et al. (Kudla et al., 1992). The absence of DNA contamination was confirmed by the lack of a PCR product in the control tube, which lacked reverse transcriptase. Complementary DNA was prepared with AMV reverse transcriptase (GE Healthcare, Piscataway, NJ) using random hexanucleotide primers. The cDNA samples were PCR amplified using 1 μg each of the primers 5'-GCGAATACGAAGCGCTTGG-3' and 5'-GGCGACCTCGCCGTCCAC-3' and one unit of the Taq polymerase (Applied Biosystems, Foster City, CA). Amplification was performed as follows: 3 min at 92 ^{o}C, followed by 28 cycles of 1 min at 92 ^{o}C, 1 min at 55 ^{o}C and 1.5 min at 72 ^{o}C; 1 cycle of 1 min at 92 ^{o}C, 1 min at 55 ^{o}C and 11 min at 72 ^{o}C and 1 min at 30 ^{o}C. PCR amplification products were directly sequenced with the T7 sequenase PCR product sequencing kit (United States Biochemical Co., Cleveland, OH) using the above primers.

PAT assay

The PAT assay was performed as described by Spencer *et. al*. (Spencer et al., 1990). 100 mg of leaf tissue was homogenized in 1 volume of extraction buffer (10 mM Na_2HPO_4, 10 mM NaCl). The supernatant was collected after spinning in a microfuge for 10 minutes. *E.coli* was grown to stationary phase levels (OD_{550} > 1.3). 400 μl of lysate was collected and pelleted. The pellet was resuspended in 300 μl of BugBuster protein extraction reagent (Novagen, Madison, WI), incubated for 15 minutes on a shaker at room temperature and microfuged for 20 minutes. The Bio-Rad Protein Assay reagent kit (Hercules, CA) was used to determine protein concentrations with bovine serum albumin

as a reference; PAT activity in leaf extracts was determined using 10 μg of protein. Protein extracts from bacteria were diluted 10 fold (2.5 or 0.25 μg protein per assay). 1 mg ml^{-1} PPT and ^{14}C-labeled Acetyl CoA were added to the protein samples and incubated at 37°C for 30 minutes; the entire reaction was spotted onto a TLC plate. Ascending chromatography was performed in a 3:2 mixture of 1-propanol and NH_4OH, and radioactivity was detected by exposure to Kodak XAR6 film.

RNA gel blot analysis

Total cellular RNA was prepared from the leaves of plants grown in sterile culture (Stiekema et al., 1988). The RNA (5 μg per lane) was electrophoresed on 1.2% agarose/formaldehyde gels, and then transferred to Hybond N membranes (GE Healthcare, Piscataway, NJ) using the PosiBlot Transfer apparatus (Stratagene, La Jolla, CA). Hybridization with the *bar* probe was carried out in Rapid Hybridization Buffer (GE Healthcare, Piscataway, NJ) overnight at 65 ˚C. Double-stranded DNA probe was prepared by random-primed ^{32}P-labeling. The template for probing *bar* was a gel-purified *Nhe*I-*Bgl*II fragment excised from plasmid pMHB10.

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Chapter 4

Plastid Marker Gene Excision by the phic31 Phage Site-Specific Recombinase

Introduction

Plastids are plant cellular organelles with a ~120-150-kb genome present in 1,000 to 10,000 copies per cell (Bendich, 1987). The plastid genome (ptDNA) has become an attractive alternative to nuclear gene transformation due to the obtainable high recombinant protein levels, the lack of pollen transmission of the transgene and the opportunity to express multiple genes in operons. Plastid transformation has been extensively used to probe plastid gene function, plastid transcription and mRNA editing. Exploratory research has shown that plastids are also suitable for biotechnological applications, including expression of genes that confer insect and herbicide resistance, and for the expression of recombinant proteins. Although plastid transformation has been achieved in many species, it is routine only in tobacco. For references see reviews in (Maliga, 2004, Daniell*, et al*., 2005, Bock, 2007).

Plastid transformation vectors are *E. coli* plasmids that contain ptDNA flanking the marker gene and the gene of interest. The transforming DNA is introduced into plastids by the biolistic process, where it integrates into the ptDNA by homologous recombination via the flanking targeting sequences. Since ptDNA is present in many copies, selectable marker genes are critically important to achieve uniform transformation of all genome copies during an enrichment process that involves gradual sorting out of non-transformed plastids on a selective medium (Maliga, 2004). However, when all genome copies are transformed and a homoplastomic plant is obtained, the marker gene is no longer necessary. There are multiple reasons for post-transformation removal of

plastid marker genes. First, a highly expressed marker gene may impose a metabolic burden on the plant. An example for a highly expressed marker gene is FLARE, which accumulates to 18% of the total soluble cellular protein (Khan and Maliga, 1999). Secondly, there are only two efficient selective markers available for plastid transformation: resistance to the antibiotics spectinomycin (Goldschmidt-Clermont, 1991, Svab and Maliga, 1993) and kanamycin (Carrer*, et al.*, 1993, Huang*, et al.*, 2002). Thus, multiple engineering steps depend on removal of the marker gene before a new cycle of engineering may be initiated. Thirdly, the marker gene would not be acceptable in any commercial crop.

There are three approaches to obtain marker free transplastomic plants. The first system relies on deletion of marker genes by homologous recombination via direct repeats flanking the marker gene (Iamtham and Day, 2000, Kode*, et al.*, 2006). The second approach utilizes transient cointegration vectors combined with visually aided complementation of knockout mutants (Klaus*, et al.*, 2004, Herz*, et al.*, 2005). The third approach relies on marker excision by the Cre-*loxP* site-specific recombination system. According to this approach, *loxP* sites in the transformation vector flank the marker gene. The marker gene is stable in the absence of Cre. When excision of the marker gene is required, a plastid-targeted Cre gene is expressed in the nucleus and the Cre protein is imported into chloroplasts where it excises the plastid marker gene (Corneille*, et al.*, 2001, Hajdukiewicz*, et al.*, 2001, Lutz*, et al.*, 2006a). Problems encountered with Cre-mediated marker excision were recombination of *loxP* sequences with fortuitous pseudo *lox* sites in the plastid genome (Corneille*, et al.*, 2003), and enhanced homologous

recombination adjacent to *loxP* sites that resulted in the loss of ptDNA segments between directly repeated sequences (Corneille*, et al*., 2001, Tungsuchat*, et al*., 2006).

Complex engineering requires the use of multiple site-specific recombinases that promote excision and integration of target sequences (Ow, 2002). Thus far in higher plant plastids ptDNA manipulation was carried out with two phage recombinases. The Cre/*loxP* system derived from the P1 phage was shown to be useful for the excision of marker genes (see above). Int, the phiC31 phage integrase (Thorpe and Smith, 1998, Thorpe*, et al*., 2000), has been employed for the insertion of transforming DNA (Lutz*, et al*., 2004). Here we report a different use of Int, efficient excision of marker genes. Since the *attB* and *attP* sequences are not homologous, plastid genomes carrying *att*-flanked marker genes are predicted to be more stable than those with marker genes flanked by identical *loxP* sequences.

Results

Construction of transplastomic tobacco to test Int-mediated marker gene excision

We designed the experiment as a two-stage process. First we constructed transplastomic tester tobacco in which the marker gene is flanked with *attB* and *attP* sites. When the homoplastomic tester was obtained, we introduced Int into the chloroplasts to test excision of the marker gene. As part of the project, we constructed pPRV111A*att* and pPRV111B*att* plastid transformation vectors (Fig. 4-1) where the spectinomycin resistance (*aadA*) genes are flanked by directly oriented non-identical phage *attP* (215 bp) and bacterial *attB* (54 bp) attachment sites targeting insertions in the *3'-rps12-trnV* intergenic region. To facilitate cloning, a multiple cloning site was inserted adjacent to

the *aadA* gene cassette. The pCK2 plastid transformation vector derives from a progenitor of vector ppRV111A*att* in which a *bar* herbicide resistance gene is cloned divergent to the *aadA* gene (Fig. 4-2).

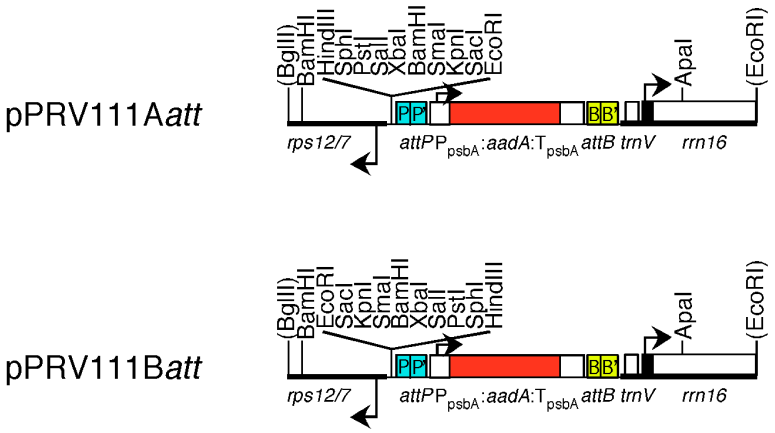

Figure 4-1. Plastid vectors pPRV111A*att* and pPRV111B*att*. Plastid targeting regions (heavy lines) target insertions in the *rps12/7* and *trnV* intergenic region; *rrn16* is a plastid gene (Shinozaki, *et al*., 1986). The spectinomycin resistance marker gene (*aadA*) expressed in the PpsbA and TpsbA cassette, the *attB* (BB') and *attP* (PP') sequences and multiple cloning sites are marked. Restriction sites in brackets have been removed during construction. Site of transcription initiation is marked with horizontal arrow.

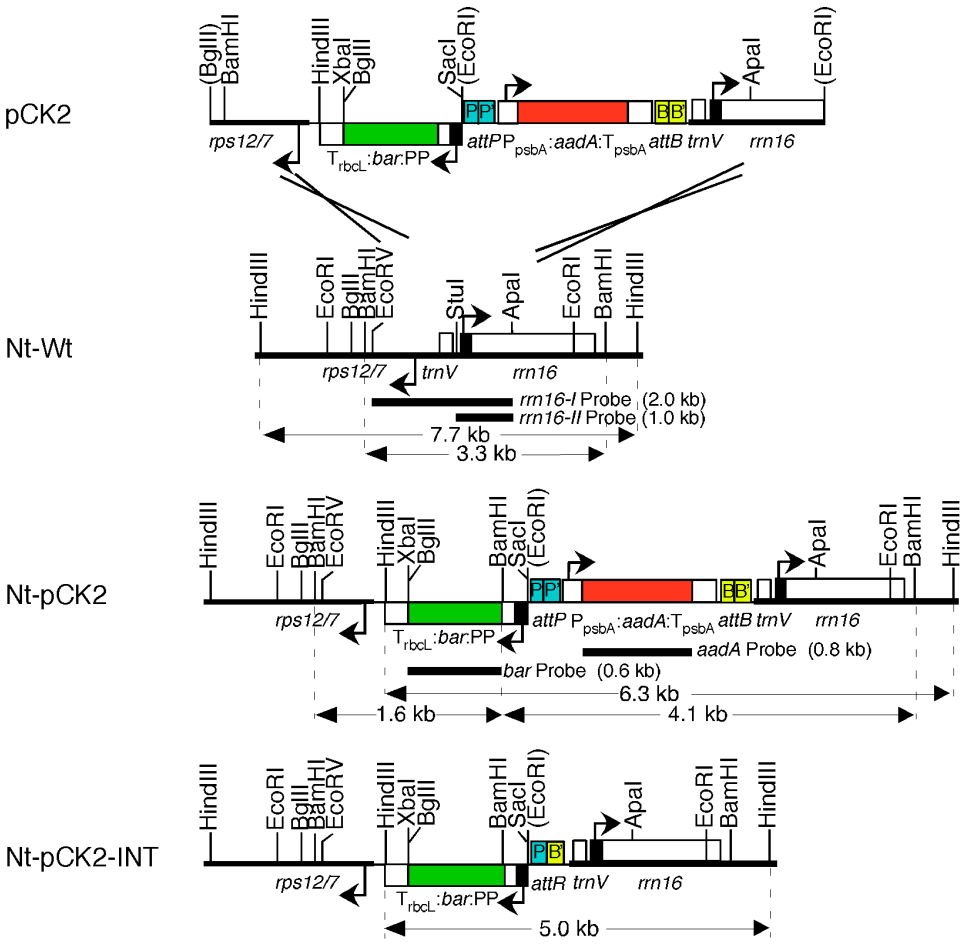

Figure 4-2. Plastid DNA maps to show Int-mediated marker excision. Plastid targeting region of vector pCK2, the cognate wild-type ptDNA (Nt-Wt), the region of ptDNA transformed with vector pCK2 (Nt-pCK2) and its derivative with *aadA* excised (Nt-pCK2-Int) are shown. Position and size of DNA probes and fragment sizes are also shown. Note *bar* herbicide resistance gene adjacent to *aadA* flanked with *attB* (BB') and *attP* (PP') sites. For further details see caption to Fig. 4-1.

The pCK2 plasmid DNA was introduced into tobacco plastids by the biolistic process and transformed transplastomic plants were obtained by selection for spectinomycin resistance then by selection for both spectinomycin and phosphinothricin resistance. Uniform transformation of the plastid genomes was confirmed by DNA gel blot analysis and probing with the *rrn16*-I probe. This probe in *Bam*HI digested total leaf DNA detected only the transplastomic 4.1 kb and 1.6 kb fragments in lines Nt-pCK2-

4BB, Nt-pCK2-4BC, Nt-pCK2-20AA, Nt-pCK2-30CA and Nt-pCK2-30CC instead of the 3.3 kb wild type fragment, indicating that these transformed plants are homoplastomic (Fig. 4-3A). Subsequent experiments were carried out with one transplastomic line, Nt-pCK2-30CA, which will be referred to as the Nt-pCK2 line. The presence of the *bar* and *aadA* genes was also confirmed in the Nt-pCK2 line in *Hin*dIII digested leaf DNA that yielded a 6.3 kb fragment with the targeting sequence (*rrn16*-II), *bar* and *aadA* probes (lane CK2-30CA in the left of Fig. 4-3B). The homoplastomic Nt-pCK2 plants were used as testers for Int-mediated plastid marker excision.

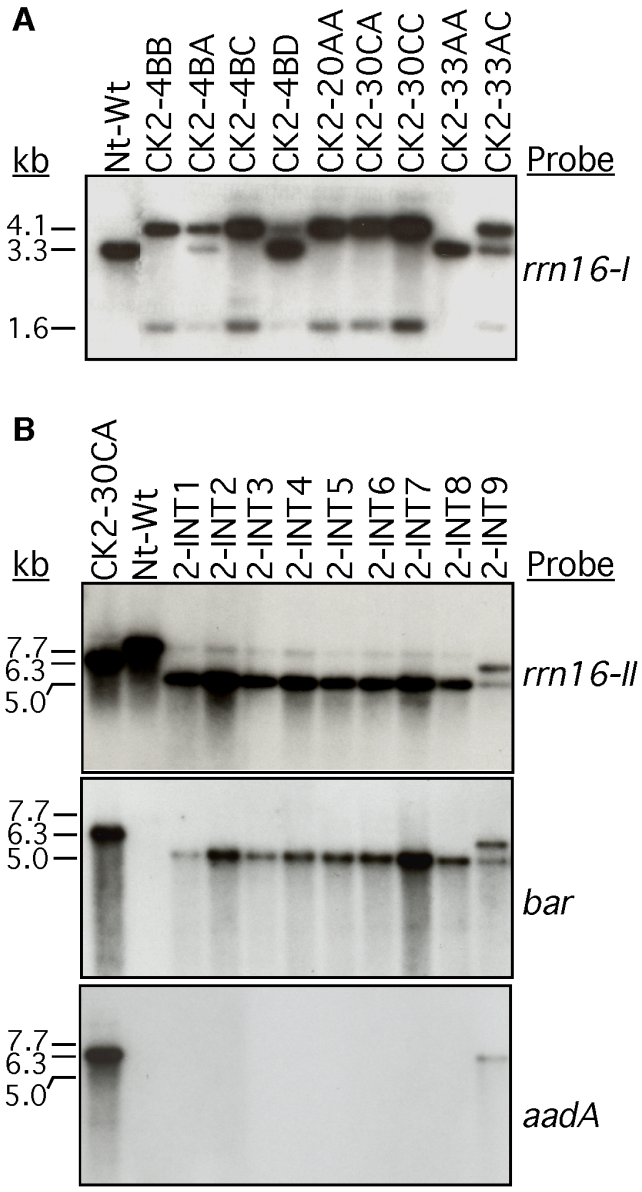

Figure 4-3. DNA gel blot analyses to detect Int-mediated *aadA* excision in Nt-pCK2 plants. **(A)** Identification of homoplastomic Nt-pCK2 lines. The *Bam*HI-digested total cellular DNA was probed with a 2.0-kb *Eco*RV-*Apa*I targeting region (*rrn16-I*). **(B)** Verification of Int-mediated excision of *aadA* gene. *Hind*III-digested total cellular DNA was probed with 1.0-kb *Apa*I-*Stu*I targeting region (*rrn16-II*), 960-bp *aad*A coding region, and 600-bp *bar* coding region.

Int mediated *aadA* gene excision

To test Int-mediated marker gene excision, Nt-pCK2 leaf pieces were cocultivated with Agrobacterium harboring pKO117, a binary nuclear transformation vector. Plasmid

pKO117 encodes a plastid-targeted Int. Plastid targeting of Int is ensured by a translational fusion with the rubisco small subunit transit peptide (Lutz*, et al*., 2004). Transgenic lines were selected for gentamycin resistance, the plant marker that is linked to the *int* gene in the binary vector. Altogether 32 independently transformed gentamycin resistant lines were obtained. The shoots were excised from the cocultivation plates, transferred to rooting medium, and immediately tested for *aadA* excision. Complete excision was obtained in 27 lines, indicated by hybridization to a 5 kb fragment with the *rrn16*-II and *bar* probes and the absence of a signal when probing with *aadA* (Fig. 4-3B). In three clones *aadA* excision was incomplete. An example in Fig. 4-3B is the Nt-pCK2-Int9 line, in which the unexcised 6.3 kb fragment is detected by all three probes, including *aadA*. Partial excision in the three lines is probably due to low level Int expression and testing excision relatively early after nuclear gene transformation. In the Int lines a weak ~7.7 kb fragment is also visible above the 5.0 kb transgenic fragment with the *rrn16*-II targeting region probe. We believe that this signal derives from ptDNA integrated in the nucleus (Ayliffe and Timmis, 1992), since this fragment does not hybridize with the *bar* or *aadA* probes and no wild-type ptDNA is detected with the sensitive phenotypic assay (see below).

Phenotypes of the marker free plants

Wild type, Nt-pCK2 and *aadA*-free Nt-pCK2-Int plants have similar phenotypes in culture. However, when they are transferred to greenhouse, the rapidly growing young leaves of Nt-pCK2 and *aadA*-free Nt-pCK2-Int plants have a distinct yellowish-golden (aurea) phenotype (Fig. 4-4A). Sectors with wild-type plastids, or with plastids lacking

bar at this growth stage are readily recognized by their normal green color (data not shown). Leaves of older flowering Nt-pCK2 and Nt-pCK2-Int plants are normal green that is indistinguishable from the wild type (data not shown). A variant of the PP-BamHI promoter that drives *bar* expression in the pCK2 construct has already been shown to cause a chlorotic phenotype due to interference with maturation of the intron-containing *clpP1* mRNA (Kuroda and Maliga, 2002). The promoter used in this study is slightly different from the one earlier described: it contains a shorter *clpP* sequence and drives expression of *bar* and not the *neo* (kanamycin resistance) gene.

Leaves of greenhouse grown wild type, Nt-pCK2 and Nt-pCK2-Int plants were tested for spectinomycin, phosphinothricin and gentamycin resistance. As expected, Nt-pCK2 leaves are resistant to spectinomycin and phosphinothricin due to expression of *aadA* and *bar* (Fig. 4-4B). Nt-pCK2-Int plants are resistant to phosphinothricin conferred by *bar* expression and to gentamycin due to expression of the plant marker in the Agrobacterium pKO117 binary vector, but sensitive to spectinomycin due to excision of *aadA* by Int (Fig. 4-4B).

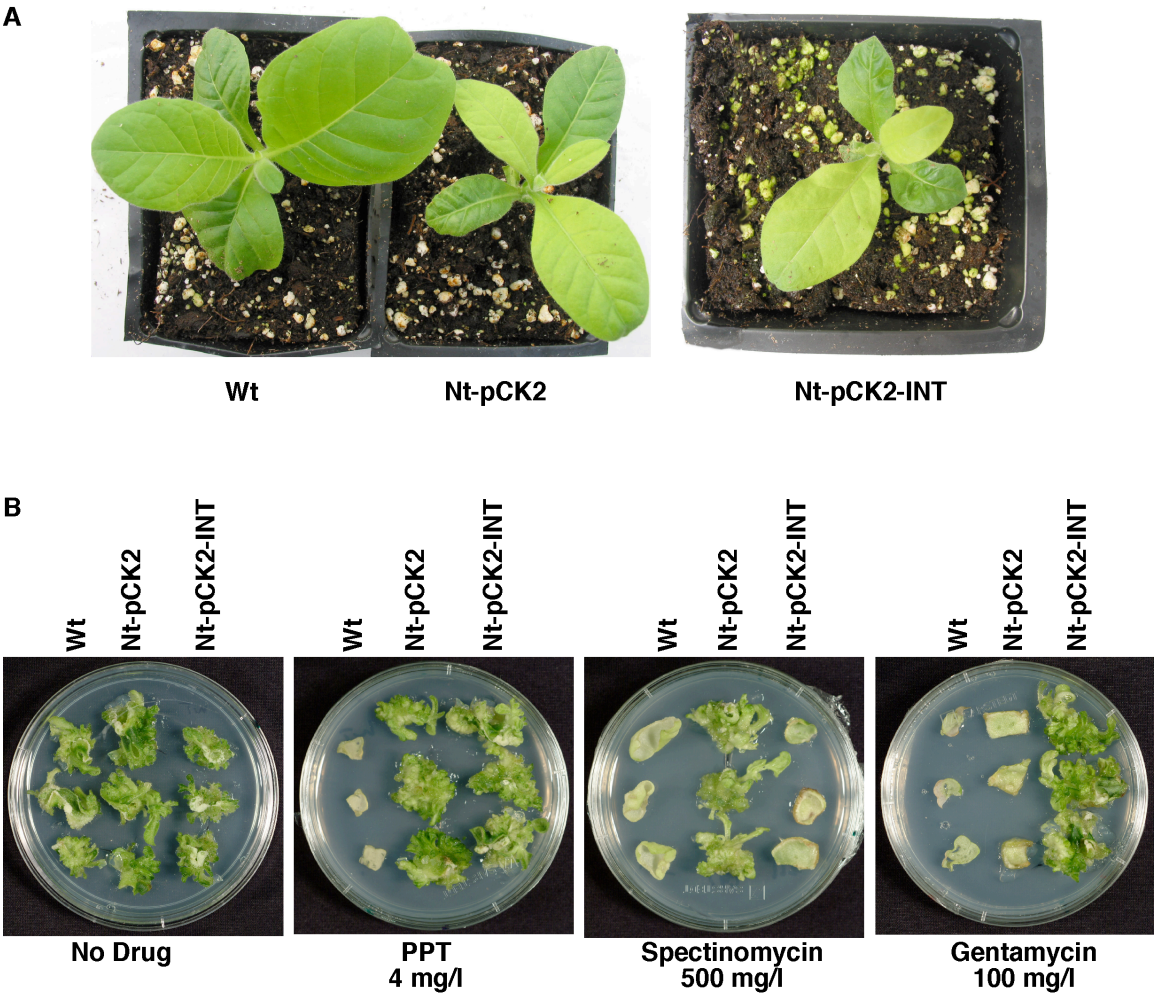

Figure 4-4. Phenotypes of wild type, Nt-pCK2, and *aadA*-free Nt-pCK2-Int plants. **(A)** Young dark green wild type and aurea Nt-pCK2 and Nt-pCK2-Int22 plants in the greenhouse. **(B)** Testing of leaves for antibiotic resistance confirms excision of *aadA* gene in Nt-pCK2-Int22 plant. Note, that the Nt-pCK2-Int22 plant is resistant to gentamycin, the plant marker linked to *int*.

Discussion

We report here efficient excision of the *aadA* marker gene by the Streptomyces phage phiC31 integrase. Efficient excision is indicated by absence of the *aadA* target sequence in 27 out of the 32 lines transformed with the Int construct. The phiC31 integrase is the second site-specific recombinase after Cre that has been shown to excise plastids marker genes. There are several different prokaryotic site-specific recombinases used for

engineering the nucleus of plants and animals (Ow, 2002, Branda and Dymecki, 2004, Groth and Calos, 2004, Keravala*, et al.*, 2006, Thomson and Ow, 2006), many of which may be adoptable for marker gene excision in plastids.

Testing Cre-mediated marker gene excision revealed fortuitous ptDNA sequences or pseudo *lox* sites in the plastid genome that are recognized by the Cre. As the consequence, when a *loxP* site is present, Cre mediates deletions between *loxP* and *lox-rps12* or *loxP* and *lox-psbA* sequences (Corneille*, et al.*, 2003). In this study no deletions were found on DNA blots that would be indicative of recombination between *attP* or *attB* sequences and fortuitous ptDNA sequences that function as pseudo-*attP* or pseudo-*attB* sites. When Int was used for plastid transformation with a vector carrying an *attP* site, the vector always inserted into the bacterial *attB* sequence that was incorporated in the plastid genome (Lutz*, et al.*, 2004). Thus no fortuitous sequences appear to be present in the plastid genome that would be recognized by the Int.

A second problem observed during Cre-mediated marker excision was deletion of ptDNA sequences by recombination via directly repeated non-*loxP* sequences that resulted in the loss of the *trnV* gene (Corneille*, et al.*, 2001, Hajdukiewicz*, et al.*, 2001). The observation was explained by enhanced homologous recombination of the native plastid recombination machinery in the presence of Cre. Deletion of sequences via direct (and inverted) repeats adjacent to *loxP* sites has also been observed in Cre-expressing *E. coli* (Mlynarova*, et al.*, 2002). Deletions in plastids were dependent on the presence of direct repeats, such as the rRNA operon promoter (Prrn), adjacent to the *loxP* sites. By altering the vector design we could avoid deletions via repeated ptDNA sequences in the presence of Int. Although the pCK2 plasmid also carries duplicated Prrn sequences (as

part of the PP promoter), these are now in an inverted orientation and apparently are not prone to deletion by the homologous recombination machinery in the presence of Int. However, when the Prrn promoters are in a direct orientation adjacent to *attB* and *attP* sites, Int also enhances homologous recombination via the Prrn repeats (data not shown). Thus, deletions detected in ~40% of the clones in the presence of Cre (Tungsuchat*, et al.*, 2006) could also be avoided by omitting directly repeated ptDNA sequences adjacent to *loxP* sites.

Deletion of plastid marker genes by homologous recombination via relatively long, 200-bp to 400-bp direct repeats is one of the approaches to obtain marker-free plants (Iamtham and Day, 2000, Kode*, et al.*, 2006). The frequency of deletion is proportionate with the length of the repeat. Although the *loxP* sequences are relatively short, 34 bp in length, we expect that they still may cause infrequent loss of the marker gene in the absence of Cre. Since the *attB* and *attP* sequences are not homologous, plastid genomes carrying *att*-flanked marker genes are predicted to be more stable than those with marker genes flanked by identical *loxP* sequences. The absence of homology between the *attB* and *attP* sites and the absence of pseudo-*att* sites in ptDNA make Int a preferred alternative to Cre for plastid marker excision.

Experimental procedures

Construction of the pCK2 plastid vector

The pPRV111A*att* (GenBank Accession no. EF416277) and pPRV111B*att* (GenBank Accession no. EF416276) excision vectors are pPRV100A and pPRV100B vector derivatives (Zoubenko*, et al.*, 1994) in which the spectinomycin resistance (*aadA*) marker

gene is flanked by 215 bp *attP* and 54 bp *attB* sites. Plasmid pCK2 is based on a progenitor of pPRV111A*att* that carries an herbicide resistance (*bar*) gene (Lutz*, et al.*, 2001) expressed in a cassette with the PP-*Bam*HI promoter (a variant of the rRNA (*rrn*) operon PEP promoter and *clpP*-53 NEP promoter fusion described earlier (Kuroda and Maliga, 2002)) and TrbcL, the plastid *rbcL* gene 3' untranslated region. The DNA sequence of the PP-*Bam*HI promoter is deposited in GenBank (accession no. EF416278).

Plastid transformation and testing of resistance phenotype

Plastid transformation was carried out by the biolistic protocol, as previously described (Svab and Maliga, 1993, Lutz*, et al.*, 2006b). Briefly, 1μm gold particles were coated with pCK2 vector DNA for bombardment of sterile *Nicotiana tabacum* cv Petit Havana leaves with the PDS-1000 biolistic gun (Bio-Rad, Hercules, CA, USA). Transplastomic clones were selected by spectinomycin resistance manifested as formation of green shoots on RMOP medium containing 500 mg/l spectinomycin dihydrochloride. Leaf peices from the shoots were transferred for a second cycle of shoot regeneration on a selective RMOP plant regeneration medium containing 500 mg/l spectinomycin dihydrochloride and 4 mg/l phosphinotricin. The shoots were rooted on RM plant maintenance medium in sterile culture. Transformation of the plastid genome was confirmed by DNA gel blot analysis of total leaf DNA. The transplastomic plants are designated by the plasmid name, a serial number to identify independent transformation events, and letters identifying shoots derived from the same event. The number of letters indicates the number of regeneration cycles on selective medium.

To test the resistance phenotypes, leaves of greenhouse-grown plants were sterilized by rinsing in 70% ethanol, soaking in 10-fold diluted Clorox (0.5% sodium hypochloride) for 5 minutes and rinsing in sterile water three times. Small (5 mm x 5 mm) leaf pieces were cultured on RMOP medium containing no drugs, 500 mg/l spectinomycin dihydrochloride, 100 mg/l gentamycin or 4 mg/l phosphinothricin. Sensitive leaf pieces bleached whereas formation of green callus indicated resistance.

Transformation of the plant nucleus with the *int* gene

An integrase gene (*int*) engineered for expression in the plant nucleus in plasmid pKO117 is available in a binary plasmid in the EHA101 Agrobacterium strain (Lutz*, et al.*, 2004). To provide Int activity for marker gene excision, leaf pieces of the Nt-pCK2-30CA transplastomic plant were co-cultivated with Agrobacterium carrying binary plasmid pKO117 for two days, then transferred onto RMOP medium containing 100 mg/l gentamycin and 500 mg/l carbenicillin. On this medium shoots formed only if transformed with the *aacC1* gentamycin resistance gene carried in the pKO117 plasmid as the plant marker linked to the *int* gene. Carbenicillin was provided to kill the Agrobacterium. For a more detailed protocol see (Hajdukiewicz*, et al.*, 1994). Plants regenerated on the selective medium are designated as Nt-pCK2-Int and a serial number.

DNA gel blot analysis of ptDNA

DNA gel blot analysis was carried out as described (Svab and Maliga, 1993). 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. A double-stranded DNA probe was prepared by random-primed ^{32}P-labeling. The templates for probing were prepared from the targeting region (*rrn16-I* probe, 2.0 kb *Apa*I-*Eco*RV fragment; *rrn16-II* probe, 1.0 kb *Apa*I-*Stu*I fragment), *aadA* (*Spe*I-*Spe*I fragment) and *bar* (*Nco*I-*Bgl*II fragment).

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Chapter 5

Plastid Genomes in a Regenerating Tobacco Shoot Derive From A Small Number of Copies Selected Through A Stochastic Process

Introduction

The plastid genome (ptDNA) of higher plants is represented as circular DNA, which ranges from 120-150 kb in size. The plastid genome is highly polyploid. Each tobacco cell contains from 10 to 100 plastids and each plastid contains about 100 copies of the ptDNA (Shaver et al., 2006). Therefore there may be as many as 10,000 identical copies of ptDNA present in a leaf cell. Engineering of the plastid genome is an attractive alternative to nuclear transformation because of the readily obtainable high protein levels, the feasibility of expressing multiple genes in operons and natural containment due to the lack of pollen transmission. The engineered plants are genetically stable only if all the ptDNA copies are uniformly altered. Since the inception of plastid transformation there is unsubstantiated fear that a small reservoir of wild-type ptDNA may be maintained in the transplastomic plants making the plants genetically unstable (Maliga and Nixon, 1998; Ruf et al., 2000; Maliga, 2004). Furthermore, the rules of plastid sorting by which new mutations or a few ptDNA copies transmitted by pollen can be incorporated in the germline are not yet understood (Ruf et al., 2007; Svab and Maliga, 2007). Therefore, we address the question whether or not newly arising, minor ptDNA populations can become established in a developing shoot and transmitted to the seed progeny, or is formation of heteroplastomic plants always the rule.

Study of plastid genome sorting requires that new, alternative, genetically distinct populations are generated at a defined time during development. In our system, a linked

antibiotic resistance gene and a site-specific recombinase are introduced into a leaf cell that is regenerated into a shoot on a selective medium. The site-specific recombinase creates unequal ratios of alternative ptDNA recombination products that are the starting point for our study on ptDNA segregation. The precision tool we employed here to obtain alternative ptDNA forms is Int, the phiC31 phage site-specific recombinase. When a gene encoding a suitably engineered Int is introduced into the nucleus, Int is efficiently imported into plastids (Lutz et al., 2004). Based on experiments with Cre-mediated marker excision, we anticipated two reactions upon Int expression: excision of a marker gene (*aadA*) between two directly oriented target sites (*attB* and *attP*) and enhanced homologous recombination between directly repeated (non-target) sequences (Corneille et al., 2001; Hajdukiewicz et al., 2001; Tungsuchat et al., 2006) (Figure 5-1). The direct repeat in our construct is the rRNA operon promoter; deletion of sequences between the repeats leads to the loss of a spectinomycin resistance (*aadA*) and herbicide resistance (*bar*) genes. The parental transplastomic plants have a yellowish-golden (aurea) phenotype due to *bar* expression (Kittiwongwattana et al., 2007); deletion of the region containing *bar* and *aadA* genes yields green leaf sectors and green plants. Thus, ptDNA sorting can be readily tracked by phenotype, a fortuitous discovery made during the course of the experiments enabling extension of a study on plastid marker gene excision to a study on ptDNA sorting.

We report here that Int generates two unequal ptDNA populations: a major one maintaining the aurea phenotype and a minor population restoring normal green color. We also report formation of homoplastomic green plants with the minor ptDNA type suggesting that the plastid genome population in a regenerating shoot apical meristem

derives from a small number of copies selected through a stochastic event. Furthermore the minor ptDNA type can be transmitted to the seed progeny yielding genetically stable, homoplastomic plants.

Results

Int-mediated ptDNA Recombination to Track Plastid Sorting

The phiC31 *Streptomyces* phage site-specific recombinase mediates deletion of sequences flanked by directly oriented bacterial *attB* and phage *attP* attachment sites (Thorpe et al., 2000; Thyagarajan, 2001). Plastid transformation vector pMBC12 was designed to test Int-mediated excision of a spectinomycin resistance (*aadA*) marker gene flanked by a 54-bp *attB* and a 215-bp *attP* site. The plasmid also carries a *bar* gene encoding phosphinothricin acetyltransferase, an enzyme that confers resistance to phosphinothricin herbicides when expressed in plastids (Iamtham and Day, 2000; Lutz et al., 2001). Vector pMBC12 targets insertions in the *trnV* and *3'-rps12/7* intergenic region (Figure 5-1, Figure 5-2). The *bar* gene is expressed from the PrrnPclpP double promoter obtained by fusing the plastids native rRNA (*rrn*) operon PEP promoter with the *clpP*-53 NEP promoter. Plasmid pMBC12 was introduced into tobacco plastids by the biolistic process where the *aadA* and *bar* genes integrated into the plastid genome by two homologous recombination events (Figure 5-2). In line Nt-pMBC12-8AC the structure of the transplastomic TP1-ptDNA was confirmed by DNA gel blot analysis (see below).

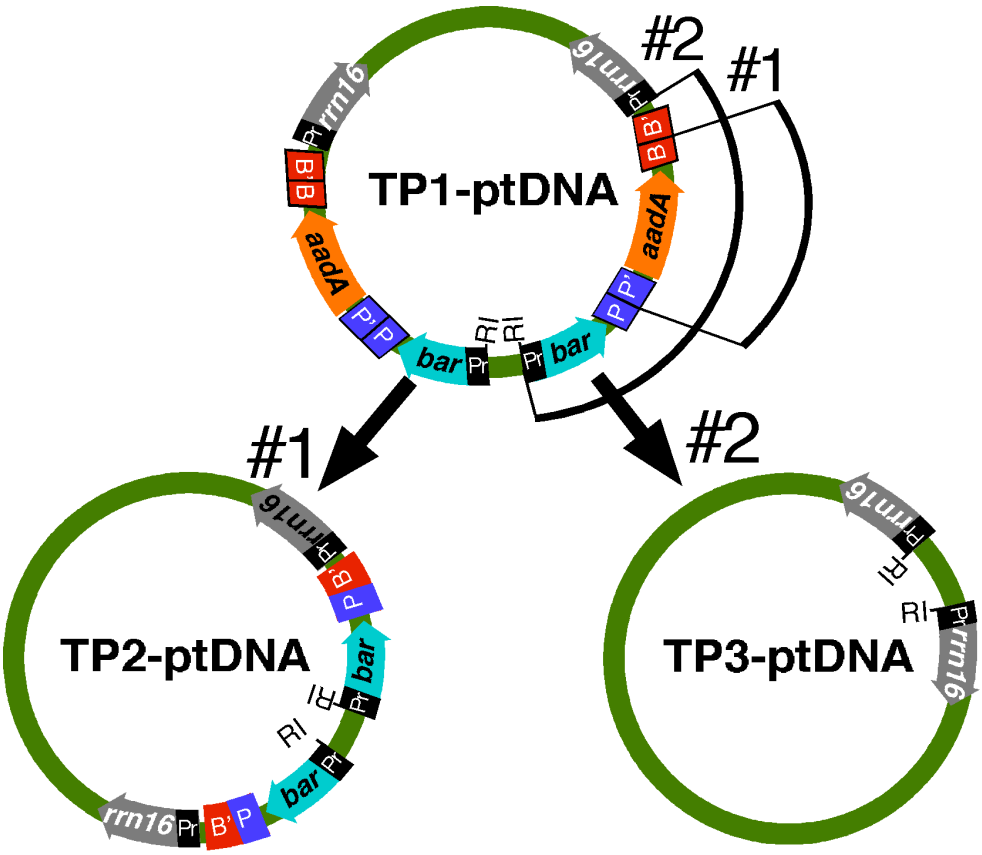


Figure 5-1. Simplified map of the Nt-pMBC12 plastid genome (TP1-ptDNA), TP2-ptDNA formed by Int-mediated excision (Recombination #1) and TP3-ptDNA formed by recombination (Recombination #2) *via* directly oriented rRNA operon (Pr) promoter sequences. Engineered Pr upstream of *bar* gene is distinguished with *Eco*RI (RI) site. Abbreviations: *attP* site, PP'; *attB* site, BB'; *attR*, PB'; *bar*, bialaphos resistance gene. The transgenes are incorporated in the repeat region of the plastid genome (Shinozaki et al., 1986).

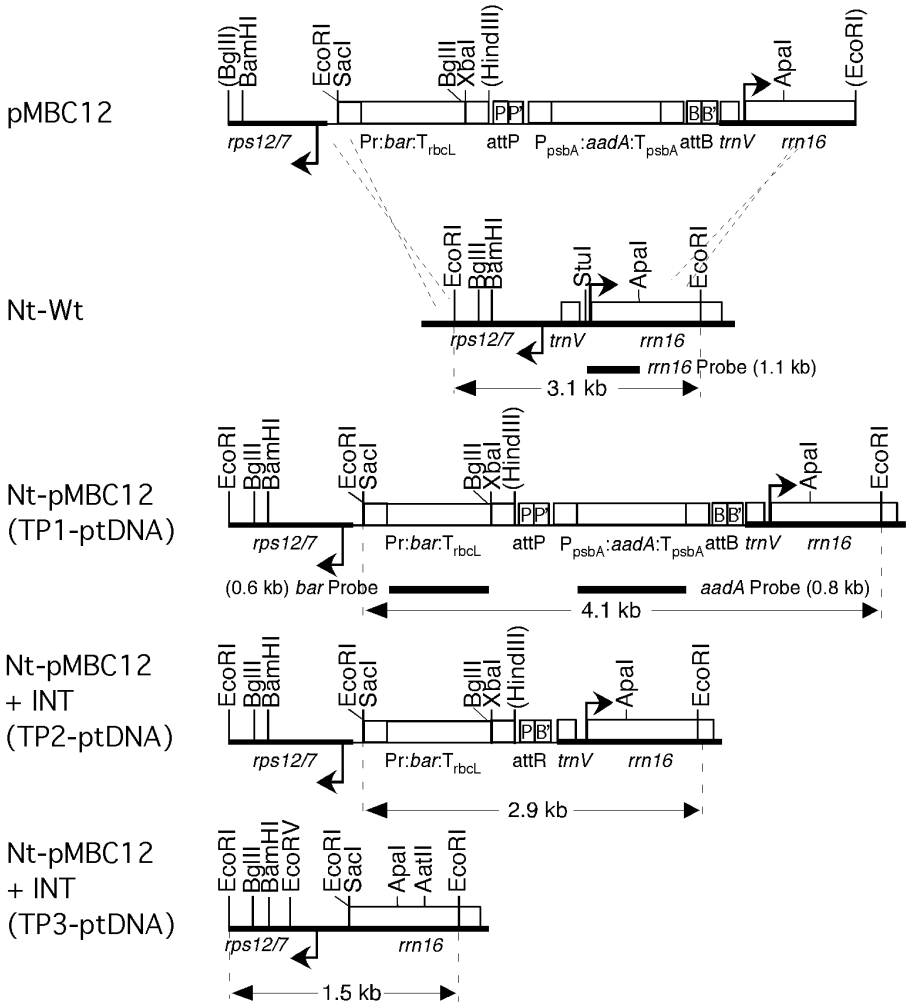


Figure 5-2. Maps of plastid transformation vector pMBC12, the cognate region of wild-type ptDNA (Nt-Wt), Nt-pMBC12 TP1-ptDNA, TP2-ptDNA (after *aadA* excision) and TP3-ptDNA formed by deletion of *aadA*, *bar* and *trnV via* repeated Prrn sequences. Abbreviations: *rrn16*, *trnV* and *3'-rps12*/*rps7* are plastid genes; Pr, PrrnPclpP promoter; TrbcL, 3'UTR of plastid *rbcL* gene, transcription terminator; PpsbA, promoter region of plastid *psbA* gene; TpsbA, 3'UTR of plastid *psbA* gene, transcription terminator; *attB* site, BB'; *attP* site, PP'; *attR* site, PB'. Size and map position of DNA fragments are shown.

Transient Assay to Probe Ratio of ptDNA Recombination Products

Int was expected to generate two alternative derivatives of the TP1-ptDNA: The products of Int-mediated recombination via the *attB*/*attP* target sites (TP2-ptDNA, Figure 5-1) and

deletion derivatives formed by recombination via directly oriented 84 bp Prrn promoter sequences (TP3-ptDNA, Figure 5-1). In the past we could detect excision of target sequences from the ptDNA in a transient assay using Cre (Lutz et al., 2006). To determine the relative ratio of the two Int-mediated recombination products, a transient assay involving agroinfiltration was carried out with Nt-pMBC12-8AC leaf disks. Agroinfiltration is a protocol that is suitable for high-level transient (2 to 4 days) nuclear gene expression of INT from a transiently introduced T-DNA (Kapila et al., 1997; Johansen and Carrington, 2001; Voinnet et al., 2003). Formation of ptDNA recombination products in agroinfiltrated leaf disks was tracked by DNA gel blot analysis over a five-day period. Probing *Eco*RI digested total DNA isolated from individual leaf disks with the 1.1-kb targeting sequence probe indicated *aadA* excision as early as 2 days after agroinfiltration (Figure 5-3). In the blots only the 2.9-kb fragment generated by Int-mediated excision via *attB* and *attP* sequences was present indicating that deletion *via* the directly repeated Prrn sequences, that would yield a 1.5-kb fragment, is relatively infrequent.

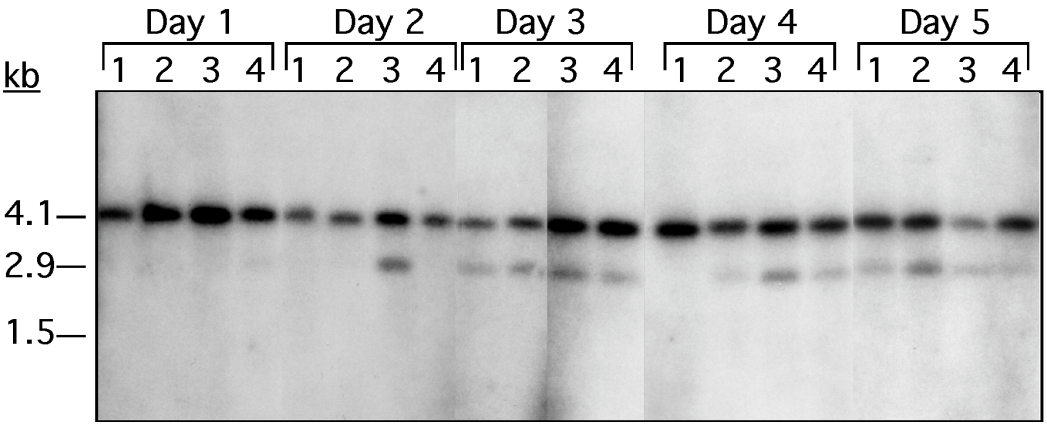


Figure 5-3. DNA gel blot analysis after transient Int expression shows deletion *via* direct repeats is a rare event. Total leaf cellular DNA was digested with the *Eco*RI restriction

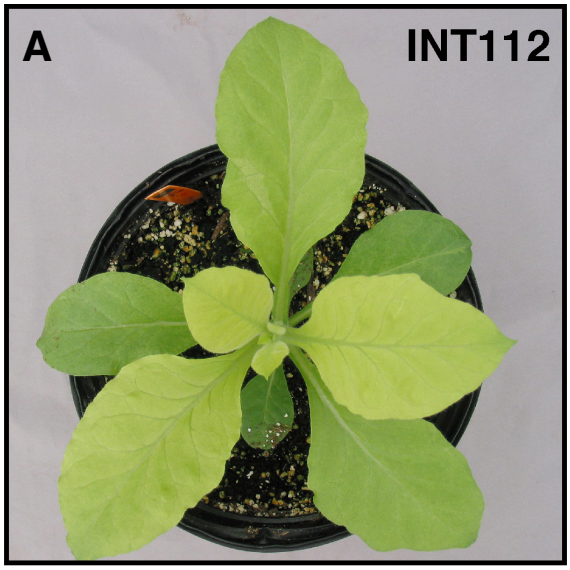
endonuclease and probed with the *rrn16* plastid region (*Apa*I-*Stu*I fragment, Figure 5-2). Size and map positions of DNA fragments are shown in Figure 5-2.

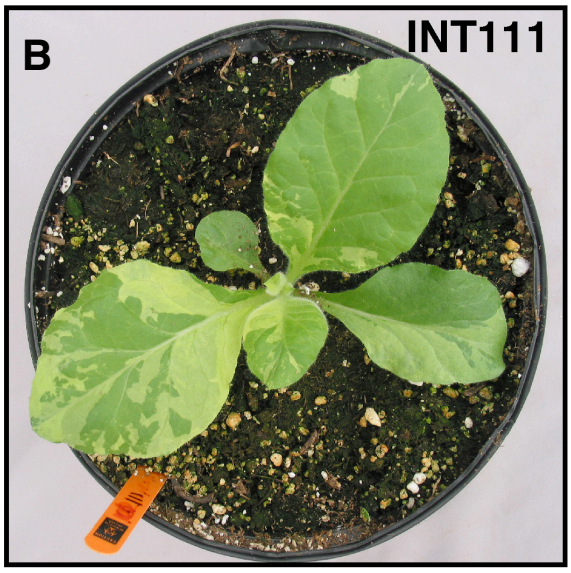
Plastid Sorting in Regenerating Shoots

Plastid sorting was studied in regenerating shoots of the tester strain Nt-pMBC12-8AC after Int was expressed from a stably integrated nuclear gene. The starting point for both plastid sorting and shoot regeneration was introduction of T-DNA into a leaf cell by cocultivation with Agrobacterium carrying Int-plasmid pKO117 and placing the leaf on a shoot regeneration medium. Shoot regeneration from cells, which did not acquire Int was blocked by exposure of cells to gentamycin; the selective agent to which the plant marker in the T-DNA conferred resistance. Plastid sorting in the regenerating shoots was tracked by leaf color. The tester strain with the TP1-ptDNA had the aurea phenotype due to expression of the *bar* gene. Deletion of the *aadA* marker gene by the Int via the *attB*/*attP* sequences did not affect leaf color and the plants retained the aurea phenotype. However, loss of both transgenes by homologous recombination via the Prrn direct repeat restored wild-type green color. Differentiation of the plastid types was feasible only in the greenhouse on rapidly expanding, young leaves (Figure 5-4). On older leaves the color difference gradually diminished and ultimately disappeared.

When transferred to the greenhouse, the 53 regenerated plants could be assigned to three phenotypic classes (Table 5-1). Leaves of the 24 plants, which lacked *aadA,* had a uniform aurea phenotype; the three plants, which lacked both transgenes, were uniformly green and the 25 lines with mixed TP2-ptDNA and TP3-ptDNA were variegated with normal green and aurea sectors (Figure 5-4). Correlation of ptDNA type and pigment phenotype was confirmed by probing total cellular DNA (Figure 5-5). In the

24 aurea plants DNA gel blot analysis confirmed efficient excision of the *aadA* marker gene via *attB* and *attP* sequences, as shown schematically in Figure 5-1, yielding TP2-ptDNA. Excision of *aadA* is indicated by a smaller 2.9-kb fragment instead of the parental (TP1-ptDNA) 4.1-kb fragment when using the targeting sequence (*rrn16*) or *bar* probes, and the absence of a signal when using the *aadA* probe. Examples in Figure 5-5 are lines INT22, INT24, INT26, INT27; for complete listing of lines see Table 5-2. DNA gel blots for three of the lines, INT13 and INT15 and INT119, indicated absence of both *aadA* and *bar* transgenes, as shown schematically for the TP3-ptDNA in Figure 5-1. Deletion of both transgenes is indicated by (i) a 1.5-kb EcoRI fragment when probing with the 1.1-kb *rrn16* targeting region fragment and (ii) the absence of the signal when probing with *bar* or *aadA* probes (Figure 5-5). Study of DNA samples of 25 of the clones indicated presence of mixed TP2-ptDNA and TP3-ptDNA in leaves. Examples for this in Figure 5-5 are lanes containing INT7, INT9 and INT23 DNA. In the remaining one gentamycin resistant plant no ptDNA rearrangement could be detected indicating that Int is not expressed in this plant. The reasons for the lack of Int activity in INT3 line have not been investigated.


A
INT112


B
INT111

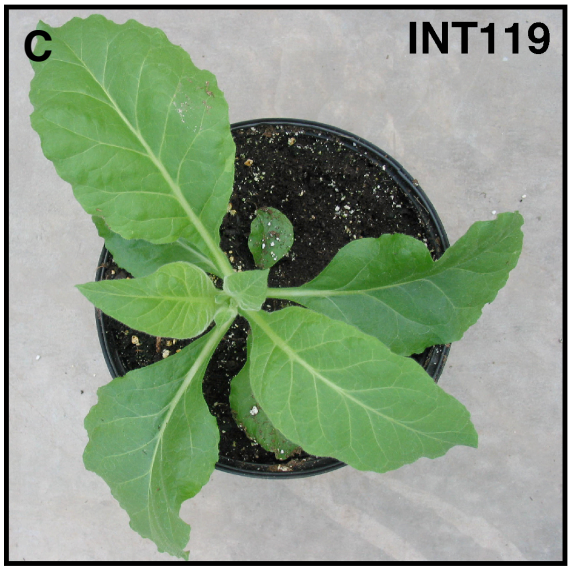

C
INT119

Figure 5-4. Tracking chloroplast segregation in Nt-pMBC12 plants transformed with INT by leaf color. **(A)** Nt-pMBC12-INT112 plant with TP2-ptDNA that confers the aurea phenotype. **(B)** Nt-pMBC12-INT111 plant with light-green and dark-green sectors carrying TP2-ptDNA and TP3-ptDNA. **(C)** Nt-pMBC12-INT119 plant with TP3-ptDNA that confers normal green color.

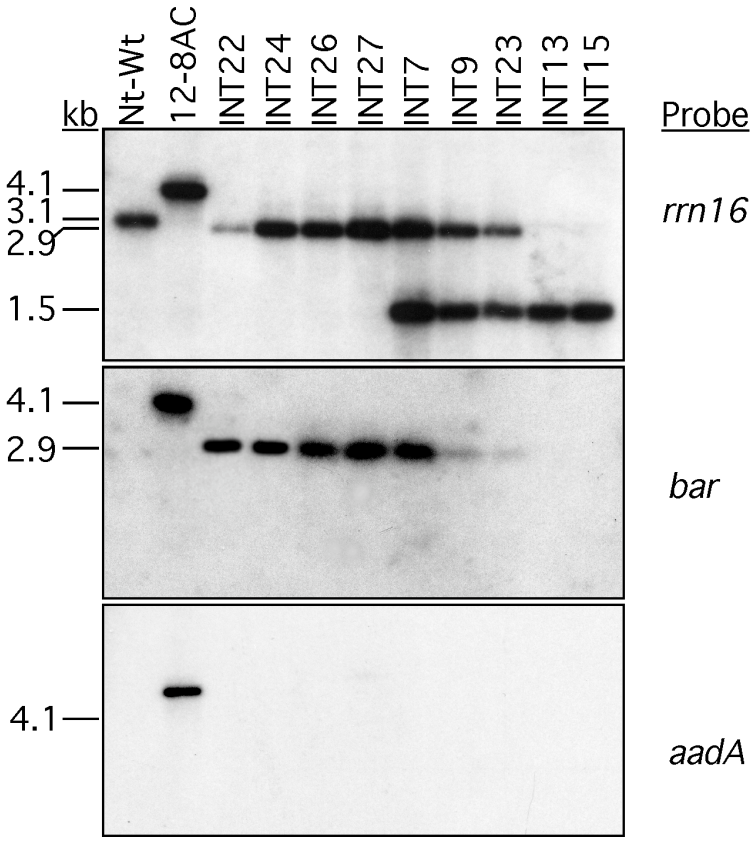


Figure 5-5. DNA gel blot analysis confirms *aadA* excision. Total leaf cellular DNA was digested with the *Eco*RI restriction endonuclease and probed with the *rrn16* plastid region (*Apa*I-*Stu*I fragment), *aadA* gene (*Nco*I-*Xba*I fragment of plasmid pZS315) or *bar* gene (*Nco*I-*Bgl*II fragment of plasmid pMBC12); probes are shown in Figure 5-2.

Plastid Type in the Seed Progeny of Int-Activated Plants

The regenerated aurea and green plants looked phenotypically uniform. The question was: are rare copies of the minor ptDNA type still present in the plant. Since the cytoplasm of seed progeny derives from a single cell, testing the seed progeny is a reliable way to test for ptDNA homogeneity. We germinated seed from one capsule each

of the Int-activated plants and grew plants to obtain preliminary information about the uniformity of ptDNA. We than classified the plants as aurea, green or variegated (Figure 5-6, Table 5-3). The progeny of the two green plants (INT13, INT15) was uniformly green indicating that only TP3-ptDNA was present in the germline of the parental plants. In seven of the 12-aurea plants lacking *aadA* no green sectors were identifiable in the leaves of the seed progeny indicating uniformity of the TP2-ptDNA. However, in five aurea clones a total of six variegated seedlings were observed. DNA gel blot analyses confirmed that the green sectors are products of homologous recombination via the Prrn promoter repeat (Figure 5-7A). Out of the six variegated plants four yielded non-segregating progeny; three aurea and one green. The progeny of two remaining variegated plants (INT7, INT9) yielded aurea progeny with a few variegated seedlings. However, two variegated seedlings were found in 204 seed progeny of the non-transformed tester line. Deletion of both transgenes in these sectors was confirmed by DNA gel blot analyses (Figure 5-7B). We are certain that the green plants are the products of homologous recombination via the directly repeated Prrn sequence (and not residual non-transformed ptDNA) because (i) they carry an *Eco*RI site absent in wild-type ptDNA (Figure 5-2, Figure 5-7B) and (ii) because loss of the *trnV* gene has been confirmed by direct sequencing of PCR-amplified DNA (data not shown). The ~1% frequency found in the tester line is similar to the 0.25% to 0.5% frequency found in the progeny of Int-activated lines. Thus, we believe that the green sectors in the variegated progeny of aurea and variegated plants formed by the secondary loss of the *bar* gene by homologous recombination rather than segregation of residual TP3-ptDNA copies.

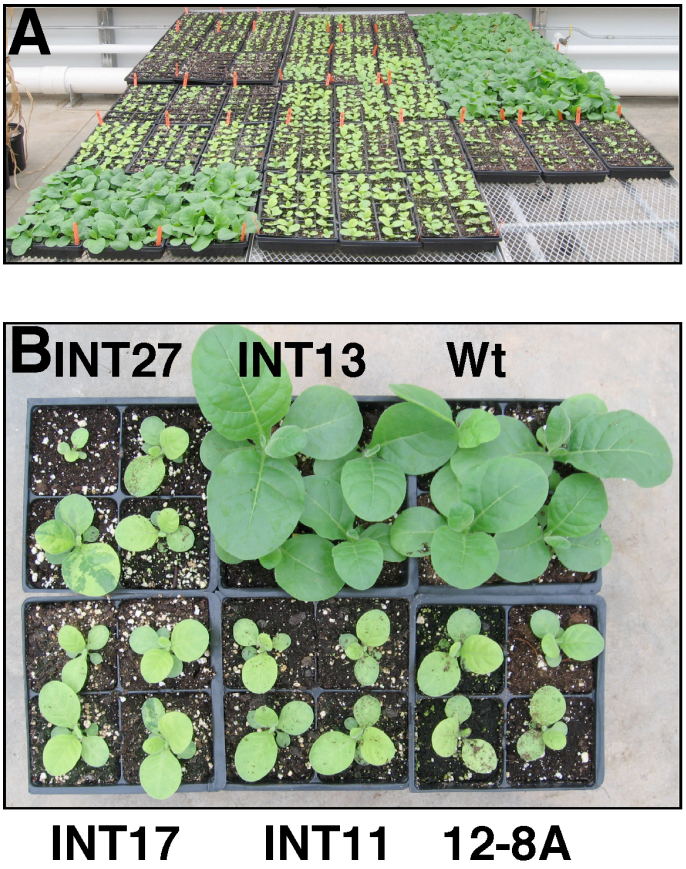


Figure 5-6. Seedlings from selfed crosses are mostly homoplastomic. **(A)** Overview of seedlings from wild-type and multiple Int lines. **(B)** Set of 4 seedlings from the following lines: INT27, INT13, wild-type (Wt), INT17, INT11, 12-8A.

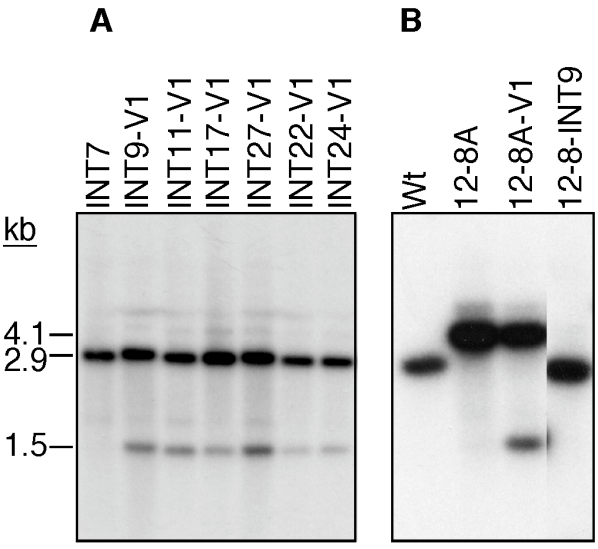


Figure 5-7. DNA gel blot analyses of ptDNA in the seed progeny. **(A)** Green sectors in rare variegated progeny of aurea plants lack *aadA* and *bar*, but have the *Eco*RI site

unique to transplastomic ptDNA(TP3-ptDNA). Note 1.5-kb diagnostic *Eco*RI fragment after probing with the 1.1-kb *rrn16* probe (Figure 5-2). **(B)** Deletion of *aadA* and *bar* in Nt-pMBC12-8A-V1, a variegated progeny of non-transformed Nt-pMBC12-8A tester plant (TP3-ptDNA) Note heteroplasmy for diagnostic 1.5-kb TP3-ptDNA and 4.1-kb TP1-ptDNA fragment in *Eco*RI-digested DNA. Autoradiographs of blots of *Eco*RI digested wild-type tobacco DNA (Nt-wt; 3.1-kb fragment) and an aurea TP2-ptDNA from line Nt-pMBC12-8-INT9 (2.9-kb fragment) are shown for reference.

Discussion

We report here that a newly arising minor ptDNA population can get established in a developing tobacco shoot and transmitted to the seed progeny implying that the originally minor ptDNA is present in all developmental layers of the shoot apex. Green plants were a minority, represented by three individuals, among the regenerated plants. The rest of the plants were either homoplastomic for the major aurea (TP2-ptDNA) type, or variegated in about equal numbers (24 to 25, respectively). The variegated plants had large patches of homoplastomic sectors locked in different developmental layers of the shoot apex, in line with the expectation that heteroplastomic state is unstable and somatic segregation yields homoplastomic cells. Homoplastomic state of germline cells is reflected in the uniformity of their seed progeny, most capsules yielding a pure population of either plastid types.

Reduction in organelle number and ptDNA copy number during shoot regeneration creates the bottleneck that allows contribution of only of a small number of ptDNA copies to the shoot apex. The tobacco leaf mesophyll cells we transformed have about 100 chloroplasts. In the experiment described here leaf mesophyll cells in tissue culture become meristematic, form a shoot apex and regenerate a shoot. Mersitematic cells have only 9-16 (mean of 12) proplastids (Thomas and Rose, 1983; Shaver et al.,

2006). Although the initial ratio of TP2-ptDNA to TP3-ptDNA is not known. the TP3-ptDNA must have been significantly less abundant since it was below detection limit on over-exposed gels in the transient excision assay. Still, we obtained 3 plants (~5% of all shoots) which carried exclusively TP3-ptDNA. Thus, ptDNA copies are selected for inclusion in the shoot apex through a stochastic event and may be either type; the probability of their inclusion in the germline is proportionate with their relative abundance. Selection of the minor type for inclusion in the shoot apex thus may lead to rapid genetic changes of the plastid genetic makeup. We interpret these rapid changes as the result of genome rearrangement, replication and sorting and not the amplification of minor subgenomic forms assumed to exist in mitochondria (Small et al., 1987; Arrieta-Montiel et al., 2001; Kmiec et al., 2006).

The utility of Int, the phiC31 phage site-specific recombinase was shown for targeted insertion of transforming DNA into the plastid genome at inserted *attB* target sites (Lutz et al., 2004). We have shown here that Int mediates efficient excision of target sequences flanked by directly oriented bacterial *attB* and phage *attP* sequences. Int, as Cre, also facilitate homologous recombination via direct (Prrn) repeats adjacent to its target sites (this study). This property proved fortuitous, enabling study of segregation of alternative recombination products with a distinct phenotype. However, no deletion *via* the duplicated Prrn sequences was detected using the same marker system, when the Prrn promoters were divergent (Kittiwongwattana et al., 2007).

Mutations conferring pigment deficient phenotypes are powerful genetic tools that allowed tracking plastid inheritance in genetics crosses with relative ease; for review see (Hagemann, 2004). Isolation of new plastome-encoded pigment mutations is labor

intensive and the outcome is unpredictable. Targeted manipulation of the plastid genome enabled engineering of photosynthetic target genes with predictable pigment-deficient phenotypes. Most of the known pigment mutants are gene knockouts that are recessive, loss of function mutants; for reviews see (Bock, 2001; Maliga, 2004). Deletion of the *rbcL* gene, for example, was used to track repeat-mediated deletions in the plastid genome (Kode et al., 2006). The *bar* gene used in this study is unique because it is a dominant marker; it causes pigment deficiency by interference with maturation of the plastid *clpP1* mRNA (Kuroda and Maliga, 2002). The interference is caused by expression of the plastid rRNA operon promoter-*clpP1* leader construct. Since no photosynthetic gene is mutated, the plants grow in the greenhouse without grafting, albeit at a slower rate. Rapid sorting out of alternative ptDNA recombination products reported here was already suspected in previous studies, but could not be confirmed, since DNA probing was not giving the same level of resolution that could be achieved with the visual pigment marker in this study.

The power of this new marker is shown by the discovery of the loss of the *bar* transgene when Prrn, the 84-bp rRNA operons promoter is in direct orientation with the native rRNA operon promoter, affecting only 0.25% to 0.5% of the seed progeny. The sequence arrangement that yielded the deletion is commonly used in transplastomic experiments, and until now an 84-bp direct repeat spaced at ~1.3 kb was considered stable. For reference, to obtain repeat-mediated deletion of a 5.4-kb fragment a 649-bp duplication was used because a 418-bp duplication at 1 kb apart yielded barely detectable deletions (Kode et al., 2006). Furthermore, the structure inserted in the plastid genome is special, since only one plastid gene, *trnV*, is found between the two duplicated sequences

and *trnV* can be deleted from the plastid genome without an apparent consequence (Corneille et al., 2003; Tungsuchat et al., 2006). Placing the Prrn promoters at a greater distance and including essential plastids genes between them would solve the problem since deletions, if they would occur, would eliminate essential genes and thus those ptDNA copies would be eliminated (Drescher et al., 2000; Kuroda and Maliga, 2003; Kode et al., 2005). A simple, alternative solution is placing the same 84-bp Prrn sequence in an inverted orientation which do not mediate loss of the aurea-inducing *bar* gene (Kittiwongwattana et al., 2007)(unpublished).

Experimental procedures

Vector Construction

Plasmid pMBC12 is a pPRV100B derivative (Zoubenko et al., 1994) and targets insertions into the *trnV-rps12/7* intergenic region of the tobacco plastid genome. The PrrnPclpP promoter which is used to express the *bar* genes, was PCR amplified using oligonucleotides O1 5'-TATGAGCTCGCTCCCCCGCC-3' and O2 5'-TATGGATCCCTCCCTACAACTATACTATATTTCACTTTGAGGTGG-3' and plasmid pHK13 (Kuroda and Maliga, 2002) as a template. The PCR product was cloned into plasmid pBSII KS+ (Stratagene) using *Sac*I and *Bam*HI restriction endonucleases to create plasmid pMHB118.

Plastid Transformation

Transforming DNA was introduced into tobacco leaves on the surface of tungsten particles by the biolistic process (Svab and Maliga, 1993). Transplastomic clones

transformed with plasmids pMBC12 or pCK2 were selected on RMOP medium containing 500mg/L spectinomycin HCl (Svab and Maliga, 1993). Plastid transformation was confirmed by DNA gel blot analysis of total cellular DNA (Svab and Maliga, 1993).

Agrobacterium-mediated Transformation

The *Int* gene was introduced into the nucleus of Nt-pSAC114 transformed plants by cocultivation of Nt-pSAC114 leaves with *Agrobacterium* containing the pKO117 binary vector (Hajdukiewicz et al., 1994; Lutz and Maliga, 2007). In plasmid pKO117 *Int* is translationally fused with the Rubisco small subunit transit peptide to ensure its efficient targeting to chloroplasts. Nuclear gene transformants were selected by gentamycin resistance on RMOP shoot regeneration medium containing 100mg/L gentamycin and 500 mg/L carbenicillin to kill Agrobacterium. Transformation was confirmed by rooting shoots on selective plant maintenance (Murashige and Skoog salts, 3% sucrose, pH 5.6) (Murashige and Skoog, 1962) medium containing 100mg/L gentamycin. The independently transformed lines are identified by the extension INT and a serial number, for example Nt-pMBC12-8AC-INT22.

Agroinfiltration

The vacuum infiltration was performed as described by Kapila et al (Kapila et al., 1997; Lutz and Maliga, 2007). Agrobacterium transformed with pKO31 (Corneille et al., 2003), carrying the plastid-targeted *cre* gene, was inoculated into 100 mL YEB medium (5 g/L Beef extract, 1 g/L Yeast extract, 5 g/L peptone, 5 g/L sucrose, 2 mM MgSO4)

supplemented with 100 mg/L spectinomycin and grown overnight at 27°C. 1 mL from the above culture was inoculated into fresh YEB medium containing 10 mM MES 2-(N-morpholino)ethanesulfonic acid, pH adjusted to 5.6, 20 mM acetosyringone, and 100 mg/L spectinomycin, and grown overnight at 27°C. The culture was centrifuged at 6,000 rpm for 15 minutes, resuspended in MMA medium (MS salts, 10 mM MES, 20g/L sucrose, pH 5.6 and 200 mM acetosyringone) to a final OD_{660}=2.4 and incubated at room temperature for 1 hour. Nt-pMBC12 leaves were cut into small pieces ($1cm^2$) and submerged in the Agrobacterium culture. The flask containing the culture was placed under continuous vacuum of 2 Torr for 20 minutes while shaking gently. After vacuum infiltration the leaf samples were incubated on RMOP medium for two days, then transferred to RMOP medium containing carbenicillin (500mg/L). DNA was isolated daily from individual leaf samples for ten days after the vacuum infiltration. Southern blots were performed on total leaf cellular DNA samples from individual leaf pieces digested with the *Eco*RI restriction enzyme and probed with the plastid targeting region (1.9 kb *Apa*I-*Stu*I ptDNA fragment containing the *rrn16* gene; Figure 5-2A) (Svab and Maliga, 1993).

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Table 5-1. Phenotypic classification of Int-activated tester plants

	Yellow Δ*aadA*	Green Δ*aadA* Δ*bar*	Variegated Δ*aadA*/Δ*aadA* Δ*bar*
Expt. #1	16	2	7
Expt. #2	8	1	18
Total	**24**	**3**	**25**

Table 5-2. Plastid types in Int-activated tester plants

INT Line	**Phenotype**	**Genotype**
1	Aurea	ΔaadA
2	Variegated	ΔaadA/ΔaadAΔbar
3	Aurea	No deletion
5	Variegated	ΔaadA/ΔaadAΔbar
6	Aurea	ΔaadA
7	Variegated	ΔaadA/ΔaadAΔbar
9	Variegated	ΔaadA/ΔaadAΔbar
10	Variegated	ΔaadA/ΔaadAΔbar
11	Aurea	ΔaadA
12	Aurea	ΔaadA
13	Green	ΔaadAΔbar
14	Aurea	ΔaadA
15	Green	ΔaadAΔbar
16	Aurea	ΔaadA
17	Aurea	ΔaadA
18	Aurea	ΔaadA
20	Aurea	ΔaadA
21	Aurea	ΔaadA
22	Aurea	ΔaadA
23	Variegated	ΔaadA/ΔaadAΔbar
24	Aurea	ΔaadA
25	Aurea	ΔaadA
26	Aurea	ΔaadA
27	Aurea	ΔaadA
28	Aurea	ΔaadA
30	Variegated	ΔaadA/ΔaadAΔbar
100	Aurea	ΔaadA
101	Variegated	ΔaadA/ΔaadAΔbar

102	Aurea	ΔaadA
103	Variegated	ΔaadA/ΔaadAΔbar
104	Variegated	ΔaadA/ΔaadAΔbar
105	Variegated	ΔaadA/ΔaadAΔbar
106	Aurea	ΔaadA
107	Aurea	ΔaadA
108	Aurea	ΔaadA
109	Variegated	ΔaadA/ΔaadAΔbar
110	Variegated	ΔaadA/ΔaadAΔbar
111	Variegated	ΔaadA/ΔaadAΔbar
112	Variegated	ΔaadA/ΔaadAΔbar
113	Variegated	ΔaadA/ΔaadAΔbar
114	Variegated	ΔaadA/ΔaadAΔbar
115	Variegated	ΔaadA/ΔaadAΔbar
116	Variegated	ΔaadA/ΔaadAΔbar
117	Aurea	ΔaadA
118	Variegated	ΔaadA/ΔaadAΔbar
119	Green	ΔaadAΔbar
120	Variegated	ΔaadA/ΔaadAΔbar
121	Variegated	ΔaadA/ΔaadAΔbar
122	Aurea	ΔaadA
123	Variegated	ΔaadA/ΔaadAΔbar
124	Aurea	ΔaadA
125	Variegated	ΔaadA/ΔaadAΔbar
126	Variegated	ΔaadA/ΔaadAΔbar

Table 5-3. Plastid types in the seed progeny of Int-activated tester plants

Line	**Parental phenotype**	**Total No.**	**Aurea**	**Variegated**	**Green**
1	Aurea	249	249	0	0
2	Variegated	214	214	0	0
3	Aurea*	89	89	0	0
5	Variegated	374	374	0	0
7	Variegated	276	275	1	0
9	Variegated	228	226	2	0
10	Variegated	315	315	0	0
11	Aurea	211	210	1	0
13	Green	194	0	0	194
14	Aurea	393	393	0	0
15	Green	204	0	0	204
17	Aurea	298	296	2	0
18	Aurea	46	46	0	0
20	Aurea	146	146	0	0
21	Aurea	206	206	0	0
22	Aurea	412	411	1	0
24	Aurea	387	386	1	0
25	Aurea	146	146	0	0
27	Aurea	186	185	1	0
28	Aurea	236	236	0	0
30	Variegated	218	0	0	218
Tester 12-8A	Aurea*	206	204	2	0

*Parental TP1-ptDNA

Author contributions to manuscript on which Chapter 5 is based: Kerry Lutz and Pal Maliga designed research. KL performed experiments, Mark Clark helped with construction of vector pMBC12, Massimo Bosacchi made the PrrnPclpP promoter; KL and PM analysed data and wrote the manuscript.

Appendix

Introduction

The following Appendices include work that does not conform to the subject matter of my thesis and also describes protocols for plastid transformation. In the previous four chapters I described how the Cre and the Int site-specific recombinases have been adapted for manipulation of the plastid genome. In the following chapters I will give an example of plastid transformation yielding high-level protein (Cry9Aa2) expression (Chapter A1), describe protocols for construction of marker-free transplastomic tobacco using the Cre-l*ox* site-specific recombination system (Chapter A2) and plastid transformation to study RNA editing (Chapter A3). Lastly I will describe a novel system to provide an abundant source of totipotent embryogenic cells that could potentially be a source tissue for plastid transformation in *Arabidopsis thaliana* (Chapter A4).

Transformation of the plastid genome is an attractive alternative to nuclear gene transformation because of the readily obtainable high protein levels, the opportunity for the expression of multiple genes in operons, lack of gene silencing and natural containment due to the lack of plastid transmission *via* pollen (Bock, 2001; Maliga, 2004). The ability to routinely express proteins at high levels will allow for expression of recombinant proteins on a commercial scale. Plastid transformation has been used for the production of proteins ranging from 5-25% of the total soluble protein (Maliga, 2003). In Chapter A1 I describe expression of a gene isolated from *Bacillus thuringiensis* that confers resistance to the potato tuber moth (*cry9Aa2* gene) at high levels, ~ 10% of the total soluble cellular protein and ~ 20% in the membrane fraction. These levels were more than sufficient to confer resistance to the potato tuber moth, but we found that the

high levels significantly delayed plant development. For the plants to be useful, the level of expression will have to be reduced.

The following two chapters (Chapter A2 and A3) are detailed protocols on plastid transformation of tobacco. Chapter A2 describes plastid transformation and the subsequent removal of the marker gene by the Cre site-specific recombinase as described in Chapter 2 and in (Corneille et al., 2001; Hajdukiewicz et al., 2001; Lutz et al., 2006). Chapter A3 describes how plastid transformation can be used to study RNA editing. Since plastid transformation is a tissue-culture based approach, these protocols directly apply only to *Nicotiana tabacum* cultivar (cv.) Petit Havana (Svab and Maliga, 1993); *Nicotiana sylvestris* (unpublished) and Petunia hybrida (Zubko et al., 2004) and plants that can be regenerated by the same protocol, *N. tabacum* cv. Samsun and cv. Xanthi and *Nicotiana plumbaginifolia.* However, modification of tissue culture conditions may allow for plastid transformation in other cultivars of *N. tabacum*, for example in *N. tabacum* cv. Wisconsin 38, and other plants species.

Unfortunately, plastid transformation is not routine in one of the most important model plants, *Arabidopsis thaliana*. Although Arabidopsis plastid transformation has been obtained, it is very inefficient, and the transplastomic plants were sterile (Sikdar et al., 1998). Chapter A4 describes a novel system to provide an abundant source of totipotent embryogenic cells for plastid transformation that will regenerate into a fertile plant. Although we were unable to show plastid transformation using this system, its utility was shown by recovery of plants carrying a plastid-encoded spectinomycin-resistant mutation in the 16S rRNA that were fertile. Efficient transformation of the plastid genome in Arabidopsis will enable us to study the function of plastid-encoded

genes and regulatory interactions between the plastid and nuclear genes. Since the biology of Arabidopsis is similar to the related Brassica crop species this technology may find applications in oilseed rape and vegetable brassicas.

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Appendix 1

Expression of the *cry9Aa2 B.t.* Gene in Tobacco Chloroplasts Confers Resistance to Potato Tuber Moth

Introduction

Potato tuber moth (PTM; *Phthorimaea operculella,* Lepidoptera, Gelechiidae) is one of the most destructive insect pests of potato with a pandemic distribution. In the field, the moths lay their eggs on the potato foliage and the larvae mine the foliage and the stems. Larvae attack the tubers through infected stems or may enter the tubers directly. Development of cultivars resistant to potato tuber moth through conventional breeding has not been successful because of lack of reliable resistance sources in potato germplasm. However, considerable degree of protection has been achieved by using insecticidal crystal proteins of the soil bacterium *Bacillus thuringiensis* (*B.t.*). Insecticidal crystal proteins are susceptible to UV damage necessitating frequent sprays on the standing crop. To overcome this, transgenic potato lines with tuber moth resistance have been developed by engineering *cry1* class *B.t.* genes. Transgenic potato lines with variable level of PTM resistance have been obtained by expressing the native *cry1Aa* (Chan et al., 1996), *cry1Ab* (Jansens et al., 1995) and *cry1Ac* (Ebora et al., 1994) genes. Nuclear transformation with native *cry* genes in plants, however, results in very low levels of *B.t.* protein expression due to instability of prokaryotic transcripts in plant systems (Murray et al., 1991). Relatively high level of *B.t.* protein expression with better PTM control could be achieved by using codon modified and truncated *cry1Ac9* (Beuning et al., 2001; Davidson et al., 2002), *cry 1Ia1* (Mohammed et al., 2000; Douches

et al., 2002), and a hybrid Bt toxin (SN19) gene consisting of domain I and III of *cry1Ba* and domain II of *cry1Ia* (Naimov et al., 2003). Gleave *et al*. cloned and sequenced a *B.t.* gene, later named *cry9Aa2*, from *Bacillus thuringiensis* var. *galleriae* (strain DSIR517) that showed strong insecticidal activity to *P. operculella* (LC_{50} 80 ng/ml) (Gleave et al., 1992). The amino acid sequence of this new *B.t*. protein was significantly different from those belonging to Cry1 class and, therefore, it was placed under the new class of Cry9 (Crickmore et al., 1998). In their later work, Gleave *et al.* transformed tobacco with the native and the modified versions of the *cry9Aa2* gene and found significant improvement in *B.t*, expression as well as PTM resistance in those expressing the truncated and codon modified versions (Gleave et al., 1998). It is apparent from the published work that sequence modification of *cry* nuclear genes is an essential requirement for achieving satisfactory levels of toxin expression and PTM control in transgenic plants.

We report here an alternative approach, expression of the Cry9Aa2 crystal protein gene in plastids to obtain higher levels of protein expression. Expression of transgenes in plastids involves placing the coding segment under control of prokaryotic-type plastid expression signals and incorporating the transgene in the plastid genome by two homologous recombination events via plastid targeting sequences. There are 1,000 to 10,000 identical copies of the circular, double-stranded plastid genome ~150-kb in size. Uniform alteration of all genome copies is obtained by selective amplification of transformed copies and gradual loss of non-transformed copies during plant regeneration on a selective tissue culture medium (Bock and Khan, 2004; Maliga, 2004). Expression of *B.t.* insecticidal protein genes in the plastid genome was found useful to obtain high protein levels of *cry1Ac* (McBride et al., 1995), *cry2Aa2* (Kota et al., 1999; De Cosa et

al., 2001) and *cry1Ia5* (Reddy et al., 2002) from native bacterial genes without a reported impact on plant fitness or imposing a yield penalty. We report here that expression of the *cry9Aa2* gene also results in high expression levels, ~ 10% of the total soluble cellular protein and ~ 20% in the membrane fraction, and conferred resistance in feeding trials to potato tuber moth. However, unlike in the earlier cases, we found that high Cry9Aa2 expression levels significantly delayed plant development, calling for optimized plastid expression signals in practical applications.

Results

Construction of transplastomic tobacco plants with *cry9Aa2* genes

The engineered *cry9Aa2* gene was introduced into the plastid genome in the pPRV312L plastid vector, which targets insertions in the *trnI-trnA* intergenic region located between the *rrn16* and *rrn23* genes in the plastid rRNA operon. The pPRV312L vector carries a selectable spectinomcyin resistance (*aadA*) gene flanked by *lox*-sites for marker gene excision, and a multiple cloning site for passenger genes (Figure A1-1A). For expression in plastids, 48 nucleotides of the *cry9Aa2* leader and a DNA segment encoding 734 N-terminal amino acids (82.1 kDa) was linked to the 3'-untranslated region of the plastid *rbcL* gene (TrbcL) and cloned upstream of the selective marker (*aadA*) (Figure A1-1A). The promoter-less *cry9Aa2* construct in this vector relies on readthrough transcription from the plastid rRNA operon promoter. Plastid transformation vector pSKC84 encodes the Cry9Aa2 peptide. In vector pSKC85 the Cry9Aa2 C-terminus was fused with a c-myc tag to enable detection by the commercial c-myc antibody. Transplastomic plants were obtained by bombardment of 20 leaves with plasmids pSKC84 and pSKC85, which

yielded 26 and 21 spectinomycin resistant clones; of these plastid transformation was confirmed in 13 and 12 clones, respectively. Uniform transformation of plastid genomes was verified by DNA gel blot analysis (Figure A1-1B). Plants derived from an independent transformation event are designated by a serial number; letters distinguish plants regenerated from the same clonal event; multiple letters indicate successive cycles of plant regeneration.

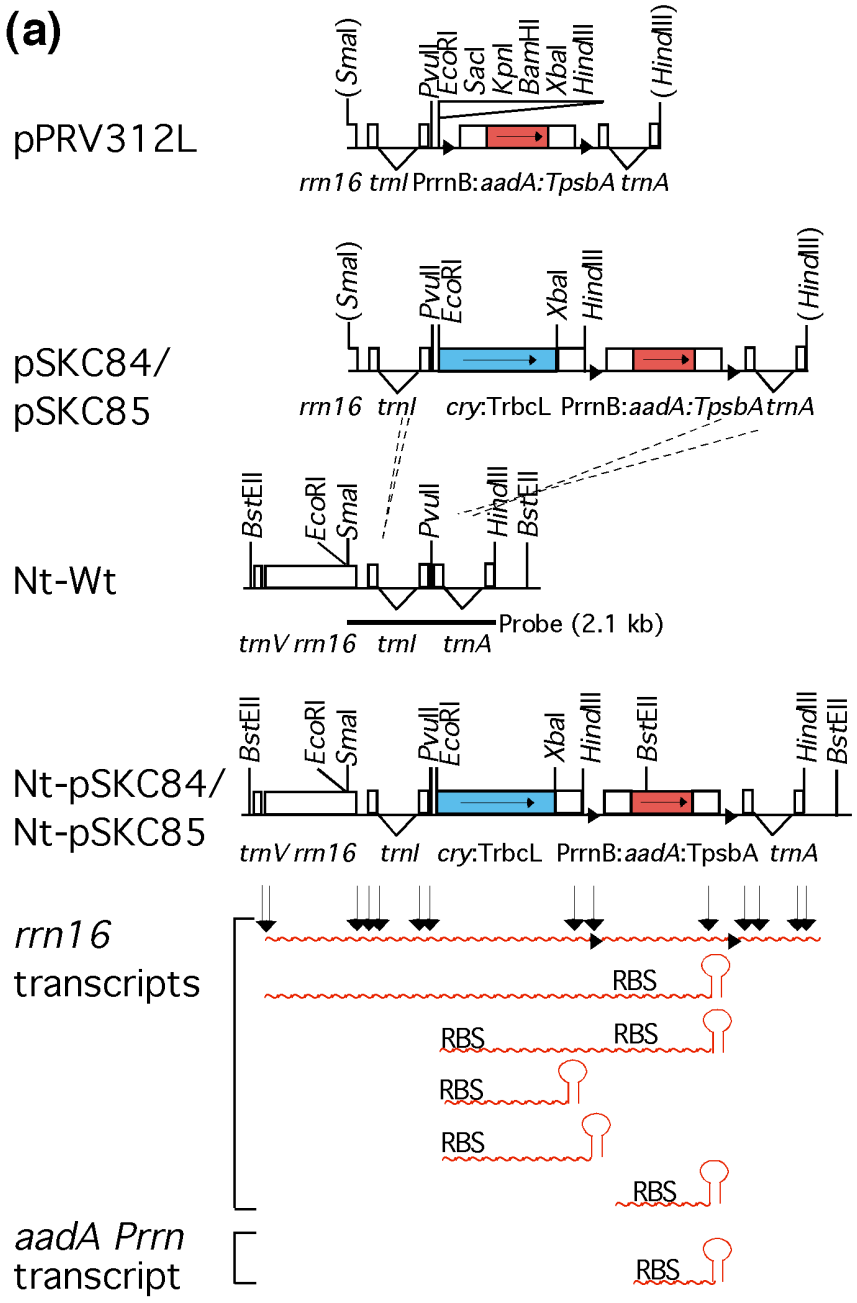
(a)
pPRV312L
(SmaI)
PvuII
EcoRI
SacI
KpnI
BamHI
XbaI
HindIII
(HindIII)
rrn16 trnI PrrnB:aadA:TpsbA trnA
pSKC84/
pSKC85
(SmaI)
PvuII
EcoRI
XbaI
HindIII
(HindIII)
rrn16 trnI cry:TrbcL PrrnB:aadA:TpsbA trnA
Nt-Wt
BstEII
EcoRI
SmaI
PvuII
HindIII
BstEII
Probe (2.1 kb)
trnV rrn16 trnI trnA
Nt-pSKC84/
Nt-pSKC85
BstEII
EcoRI
SmaI
PvuII
EcoRI
XbaI
HindIII
BstEII
HindIII
BstEII
trnV rrn16 trnI cry:TrbcL PrrnB:aadA:TpsbA trnA
rrn16
transcripts
RBS
RBS
RBS
RBS
RBS
RBS
RBS
aadA Prrn
transcript
(b)
Nt-Wt
84-19A
84-19B
84-19C
85-5BA
85-5BB
85-5BC
85-6BA
kb
5.9
4.6
2.2

Figure A1-1. Introduction of the *cry9Aa2* gene into the tobacco plastid genome. **(A)** Map of the plastid-targeting region in plastid vector pPRV312L and *cry9Aa2* vectors pSKC84 (Cry9Aa2 not tagged) and pSKC85 (Cry9Aa2 c-myc tagged) and of the cognate regions in the wild-type (wt) and transplastomic plants. Map positions are shown for: the *cry9Aa2* gene; *aadA*, the selectable spectinomycin resistance gene; *rrn16*, *trnI* and *trnA* plastid genes. Wavy lines symbolize transcripts; vertical arrows above the line mark processed ends; triangles indicate *loxP* sites; RBS, marks position of ribosome binding site. Map position of the 2.1-kb *Eco*RI-*Hin*dIII probe is also shown. **(B)** DNA gel blot confirms *cry9Aa2* integration in the tobacco plastid genome. Data are shown for transplastomic lines transformed with plasmids pSKC84 and pSKC85 and the wild-type parental line. *Bst*EII digested total cellular DNA was probed with the 2.1-kb *Eco*RI-*Hin*dIII ptDNA fragment to detect the wild type (4.6 kb) and transgenic (5.9 kb and 2.2 kb) *Bst*EII fragments.

Expression of *cry9Aa2* genes in chloroplasts

RNA gel blot analysis was carried out to test *cry9Aa2* mRNA accumulation. The *cry9Aa2* transgene is transcribed from the *rrn* operon promoter (Prrn). Probing of the RNA blot with the *cry9Aa2* coding segment revealed mRNAs 2.4- and 2.5-kb in size (Figure A1-2). We have observed accumulation of two mRNA species differing with 0.1-kb in size when TrbcL was combined with a *loxP* site, which forms a 13-nt stem-loop structure (Tungsuchat et al., 2006). Therefore, we assume that the 5'-end of the transcript was generated by maturation of *trnI* and the 3'-end by processing within the TrbcL or at the *loxP*-site. In RNA samples from young leaves additional, partially processed mRNA species are visible, which are processing intermediates of the *rrn* operon transcript (Figure A1-1A). Degradation of *cry9Aa2* mRNA is apparent in each of the samples.

The selective marker *aadA* is transcribed from two promoters: its own promoter (PrrnLatpB+DS) and the native *rrn* operon promoter upstream of *rrn16*. Probing with *aadA* revealed two ~1.1-kb monocistronic messages, which did not separate on the blot shown in Figure A1-2. These transcripts derive from the *aadA* gene promoter and from processing of the *rrn* operon readthrough transcript.

We tested protein accumulation by separating leaf protein extracts in SDS-PAGE. Staining with Comassie Blue revealed a novel band, ~82 kDa in size (Figure A1-3A). The novel protein was present in both soluble and membrane protein fractions of transgenic plants. Antibody to the c-myc tag in Nt-pSKC85 plants confirmed the identity of the novel band as a Cry9Aa2 insecticidal protein. The protein in the Comassie Blue stained soluble extracts was quantified with Alpha Innotech Alphaimager IS-2200 using the 1D-Multi Lane densitometry. The Nt-pSKC84 and Nt-pSKC85 leaves contained comparable amounts of Bt protein, ~ 10% of total soluble protein (Figure A1-3A). Immunoblot analysis showed that the Cry9Aa2 protein concentration in the membrane fraction was higher, ~ 20% (Figure A1-3B).

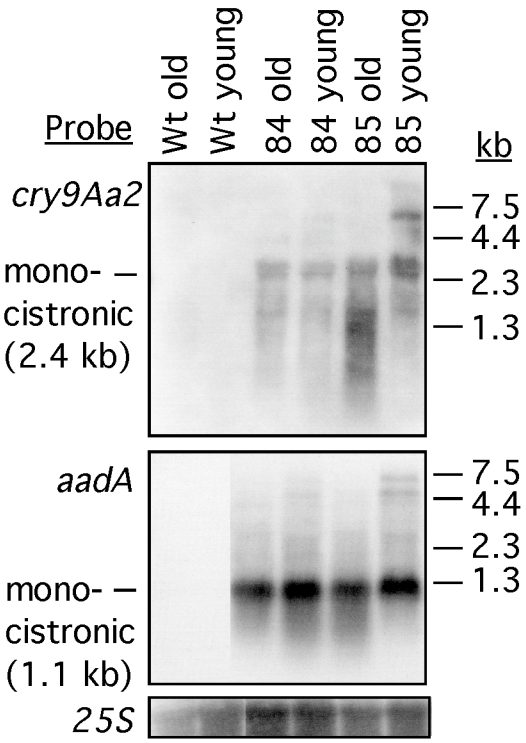

Figure A1-2. Testing mRNA accumulation by probing total cellular RNA with **(A)** *cry9Aa2 (*1.3-kb *SwaI* fragment), **(B)** *aadA* (0.8-kb *Nco*I-*Xba*I fragment) and **(C)** cytoplasmic 25S rRNA (loading control) probes.

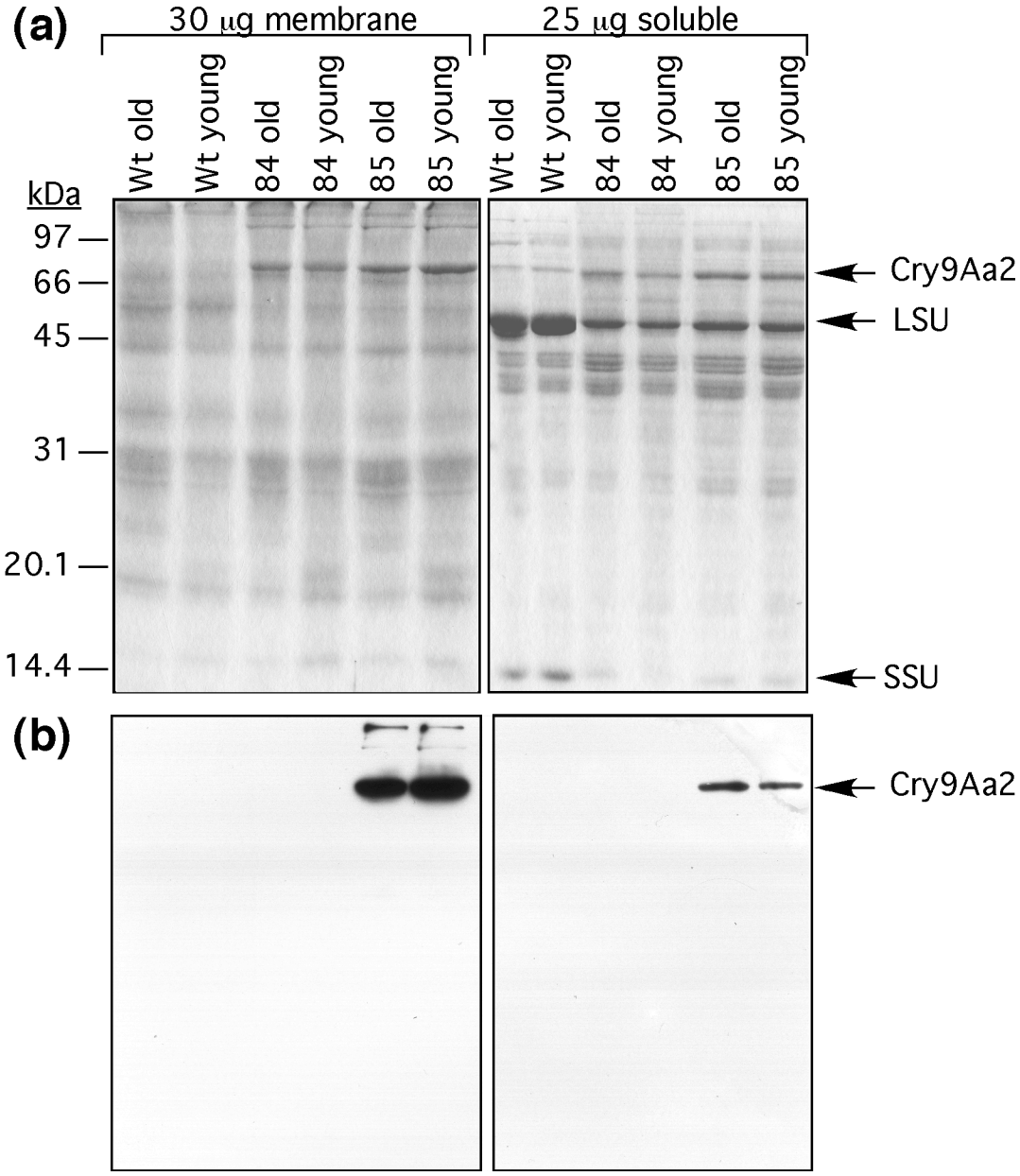

Figure A1-3. Cry9Aa2 protein accumulation in tobacco leaves. **(A)** Soluble (25 μg per lane) and membrane protein (30 μg per lane) fractions were separated by SDS-PAGE and stained with Coomassie blue R250. The position of Cry9Aa2 and the large (LSU) and small (SSU) rubisco subunits are marked by arrows. **(B)** The c-myc antibody recognizes the tagged Cry9Aa2 protein in Nt-pSKC85 extracts.

Potato tuber moth bioassay on tobacco leaves

Transgenic plants have been transferred to the greenhouse where they flowered and produced seed. When grown from seed, development of the Nt-pSKC84 and Nt-pSKC85 transgenic plants was significantly delayed and the young leaves had a pale green color (Figure A1-4A). However, the older leaves were normal green and plants eventually reached maturity, at which stage they were indistinguishable from wild-type plants. Out of the 12 and 13 independently transformed clones (see above) plants from four lines were studied in greater detail in the greenhouse: Nt-pSKC84-19CA, Nt-pSKC84-16DA and Nt-pSKC85-5BA and Nt-pSKC85-6BB. Seed progeny of each of the lines behaved similarly. Since several, independently transformed lines have the same phenotype, we believe, that the delay in plant development is due to high levels of Cry9aA2 protein accumulations. Furthermore, based on experience with tobacco plants regenerated from hundreds of independently derived transplastomic lines (Bock and Khan, 2004; Maliga, 2004) we are very certain that the delay in plant development is not caused by the transformation method.

Detached leaves of Nt-pSKC84 and Nt-pSKC85 tobacco plants expressing the *cry9Aa2* gene have been tested for insecticidal activity against neonate potato tuber moth larvae (Figure A1-4B). Transplastomic lines gave complete control of leaf mining by potato tuber moth larvae: 100% larval mortality was observed within 48-72 hrs, whereas no mortality was seen on the control plants for at least 120 hours.

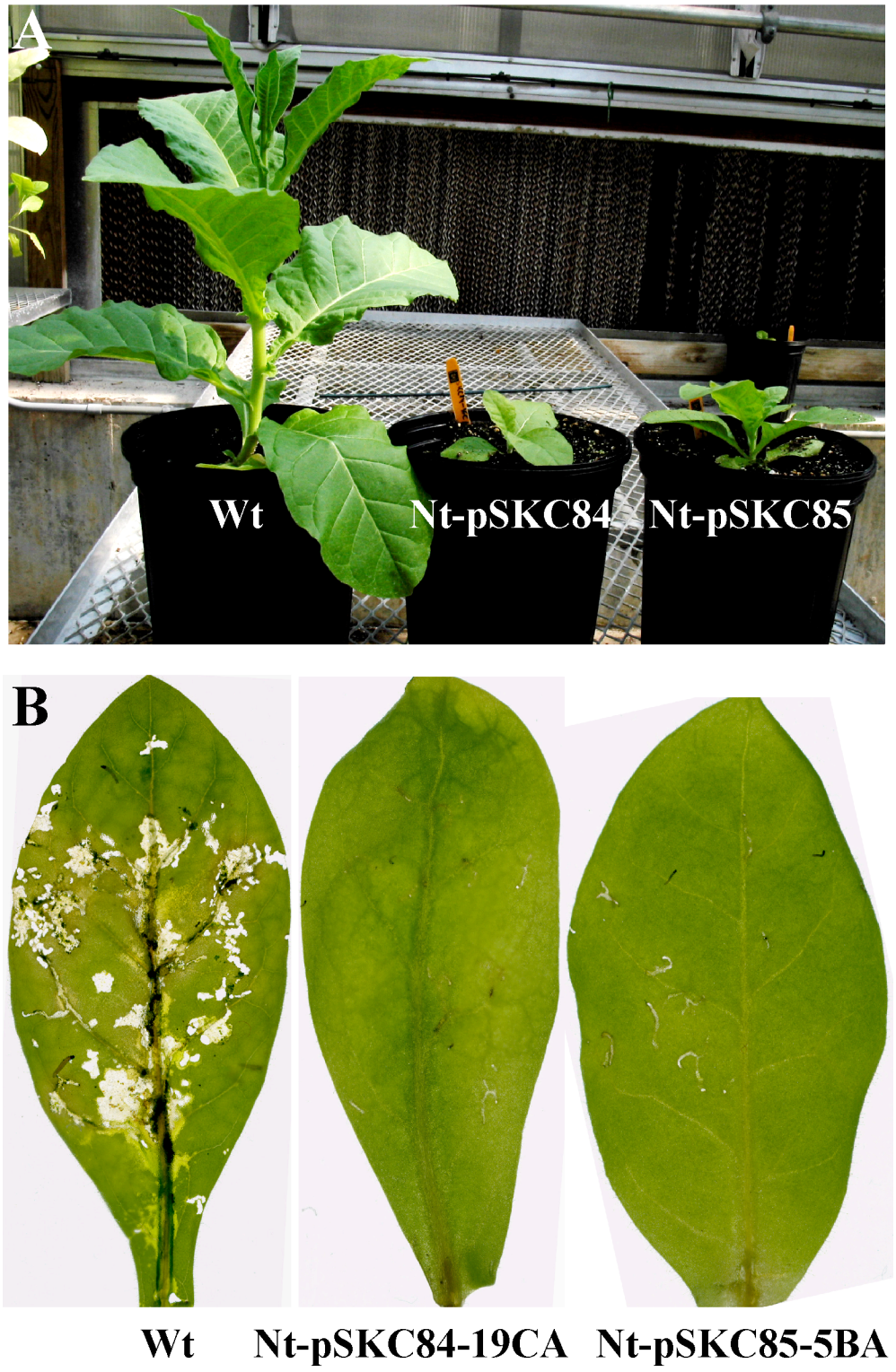

Figure A1-4. The transplastomic Nt-pSKC84 and Nt-pSKC85 plants. **(A)** Expression of Cry9Aa2 gene delays development of transplastomic plants. Shown are one-month old

seedlings. **(B)** Potato tuber moth bioassay on detached transplastomic tobacco leaves expressing the Cry9Aa2 crystal protein. All larvae died on the leaves of transplastomic tobacco lines Nt-pSKC84-19CA and Nt-pSKC85-5BA.

Discussion

The pPRV312L plastid vector

The new vector pPRV312L has a multiple cloning site and *loxP*-flanked (L) *aadA* marker gene for excision by the Cre recombinase (Corneille et al., 2001; Hajdukiewicz et al., 2001; Lutz et al., 2006). Vector pPRV312L shares the feature of an excisable marker gene with vector pPRV110L that targets insertions in the *trnV-rps12* intergenic region (Lutz et al., 2006). Vector pPRV312L in this study targets insertions between the *trnI*/*trnA* genes in the plastid *rrn* operon. The *trnI*/*trnA* intergenic region has been used for insertion of heterologous genes; reviewed in (Daniell et al., 2005). Genes of interest inserted at this site thus far always had their own promoter. Significant levels of protein expression have been obtained only from the rRNA operon (*rrn*) promoter and the promoter of the *psbA* gene (Maliga, 2003; Daniell et al., 2005). Expression of multiple genes from polycistronic mRNAs is a more desirable approach, reducing the requirement for promoters. We have shown already that transcriptional fusion with the *rbcL* mRNA yields significant levels of protein expression (Staub and Maliga, 1995). Expression of the gene of interest in our vector pPRV312L relies on readthrough transcription from the strong *rrn* operon promoter. High-level accumulation of Cry9aA2 indicates that transcription from an upstream promoter and translation from a processed 5'-UTR is sufficient for protein expression. In plastids, posttranscriptional gene regulation is important and high-level protein accumulation is dependent on the choice of 5'-UTR (Kuroda and Maliga, 2001a, 2001b); for review see (Maliga, 2003). Thus, the 49

nucleotide *B.t. cry9aA2* 5'-UTR is a leader that promotes high-level protein expression in chloroplasts. The *B.t. cry2Aa2* leader also promotes high-level protein expression (De Cosa et al., 2001). The 49-nucleotide *cry9aA2* leader sequence reported here is unrelated to the *cry2Aa2* leader and lacks the upstream open reading frames, which are present in the cry2Aa2 operon construct.

We used here a 49 nucleotide segment of the native *cry9aA2* 5'-UTR, which apparently promotes high levels of translation. However, mRNA degradation is also apparent on the RNA gel blot. This may be caused by *cry9aA2* sequences, which target mRNAs for degradation.

Although the native *rrn* operon transcript is large (includes *rrn16, trnI, trnA, rrn23, rrn4.5, rrn5* genes)(Strittmatter and Kossel, 1984), the large precursor is efficiently processed (Kishine et al., 2004), creating the 5'-UTR of *cry9aA2* mRNA. Normally, transcription termination and/or processing of mRNAs within the TrbcL segment used here is inefficient, yielding significant amounts of dicistronic transcripts due to TrbcL readthrough (Serino and Maliga, 1997; Kuroda and Maliga, 2001a; Tregoning et al., 2003). Interestingly, in this case dicistronic ~3.5-kb *cry9aA2-aadA* mRNAs were absent in mature leaves, suggesting efficient transcription termination and/or mRNA processing due to having *loxP* downstream of TrbcL (Tungsuchat et al., 2006).

Insect biocontrol with plastid-expressed Cry9aA2

Because of its strong insecticidal activity against larvae (LC50=80 ng per ml diet for 5 days), Cry9Aa2 Bt insecticidal protein is highly desirable for the biocontrol of potato

tuber moth (Gleave et al., 1998). Lethality from a codon-modified nuclear gene yielded at least 75% larval mortality in nine days. Larval mortality caused by expression of Cry9Aa2 in plastids in our study was significantly higher, 100% within two-to-three days.

High-level expression (10% of soluble, 20% of membrane protein) of Cry9Aa2 protein came at a price of significantly delayed plant development. In earlier publications, plastid-expressed Bt insecticidal proteins also accumulated to relatively high levels in leaves: 5% *cry1Ac* (McBride et al., 1995), 3% (Kota et al., 1999) and 45.3% (De Cosa et al., 2001) *cry2Aa2* and 3% *cry1Ia5* (Reddy et al., 2002). Interestingly, no adverse affect of *B.t.* protein expression was reported on plant development in any of these studies. 100% larval mortality was observed even if the *B.t.* proteins were expressed at relatively modest (3%-5%) levels (McBride et al., 1995; Kota et al., 1999; Reddy et al., 2002). Thus, expression of Cry9Aa2 at significantly lower levels should be sufficient to achieve efficient insecticidal control. To create a useful *cry9Aa2* construct one is facing the unusual task of down-regulating translation efficiency.

Materials and methods

Construction of transformation vectors

Plastid transformation vector pPRV312L targets insertions in the *trn*I/*trn*A intergenic region in the plastid ribosomal RNA operon (GenBank Accesion Number DQ489715). Vector pPRV312L is a pUC118 plasmid derivative in which the *Pvu*II fragment was replaced with the *Sma*I-*Hin*dIII tobacco ptDNA fragment (nucleotides 104,093-106,202; GenBank Accession No. Z00044) (Shinozaki et al., 1986). The polycloning site and

marker gene were introduced as a *Pvu*II-*Sac*I (blunted with T4 DNA polymerase) fragment. The *aadA* marker gene is expressed in a cassette consisting of the PrrnLatpB+DS promoter (Kuroda and Maliga, 2001a) and TpsbA, the 3'-UTR of *psbA* gene (Shinozaki et al., 1986). The *aadA* gene is flanked by the P1 phage *loxP* sites to facilitate its excision by the Cre site-specific recombinase (Corneille et al., 2001; Lutz et al., 2006).

Plasmid pNZA10 carrying the *cry9Aa2* gene (GenBank Accession number X58534) was obtained from the Bacillus Genetic Stock Center, The Ohio State University, Columbus, Ohio. First the *Hin*cII/*Bam*H1 fragment (2.2 Kbp) encoding 49 nucleotides of the leader sequence and the N-terminal half of the *cry9Aa2* gene was excised from plasmid pNZA10 and sub-cloned into a pBluescriptKS (Stratagene, La Jolla, CA) plasmid. The C-terminally truncated *cry9Aa2* gene was converted into an *Eco*RI-*Xba*I fragment for expression in pPRV312L. The *Xho*I site upstream of the *Hin*cII was converted into an *Eco*RI site by blunting and linker ligation and an in-frame stop codon was introduced in a *Bam*HI-*Xba*I linker (5'-GGATCCAtaattctaga-3'). The modified *cry9Aa2* gene was excised as an *Eco*RI-*Xba*I fragment and cloned in a plasmid pPRV312L derivative, which carried the 3'UTR of the *rbcL* gene (TrbcL; *Xba*I-*Hin*dIII fragment) to yield transformation vector pSKC84. Plastid vector pSKC85 is similar to pSKC84, except that it has a C-terminal c-myc tag (amino acids 410-419; EQKLISEEDL) (Kolodziej and Young, 1991) introduced in a *Bam*HI-*Xba*I fragment (5'-GGATCCgaacaaaaactcatttctgaagaagacttgtgattctaga-3') with the stop codon.

Plastid transformation

DNA for plastid transformation was prepared using the QIAGEN Plasmid Maxi Kit (QIAGEN Inc., Valencia, CA). Transforming DNA was introduced into leaf chloroplasts on the surface of tungsten particles (1 μm) using the Du Pont PDS1000He Biolistic gun (Svab and Maliga, 1993). Transplastomic plants were selected on RMOP medium containing 500 mg/L spectinomycin dihydrochloride. The transgenic plants were grown on MS (Murashige-Skoog) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.6% (w/v) agar in sterile culture condition. A uniform population of transformed plastid genome copies was confirmed by DNA gel blot analysis after digestion with the *Bst*EII restriction enzyme. Double-stranded DNA probes were prepared by random-primed ^{32}P-labeling using the Ready-To-Go DNA Labeling Beads (Amershem Pharmacia Biotech, Piscataway, NJ). The probe was the *trnI-trnA* plastid targeting region, encoded in an *Eco*RI-*Hin*dIII ptDNA fragment.

RNA gel blot analysis

For RNA gel blot analysis (Silhavy and Maliga, 1998) 5 μg total cellular RNA was loaded per lane. Probes were prepared by random-primed ^{32}P-labeling (see above). The *cry9Aa2* and *aadA* probes were prepared using *Swa*I and *Nco*I-*Xba*I coding region fragments, respectively.

SDS-PAGE and immunoblotting

Leaves for protein extraction were taken from greenhouse plants. To obtain total soluble leaf protein, about 200 mg leaf was homogenized in 0.1 ml buffer containing 50 mM

Hepes/KOH (pH 7.5), 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 10 mM DTT and 2 mM PMSF. Insoluble material from the soluble fraction was removed by centrifugation. The insoluble material was solubilized by adding 0.1 ml solubilization buffer containing 50 mM Hepes/KOH (pH 7.5), 2% lithium dodecyl sulfate and heating for 10 minutes at 95 °C. The insoluble material was then removed by centrifugation. Soluble protein concentrations were determined by the Bradford Protein Assay Reagent kit (Bio-Rad, Hercules, CA); membrane proteins were quantified with the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). The protein in the Comassie Blue stained soluble extracts was quantified with Alpha Innotech (San Leandro, CA) Alphaimager IS-2200 using the 1D-Multi Lane densitometry. Immunoblot analysis of Cry9Aa2 accumulation was carried out as described (Carrer et al., 1993) using commercial c-Myc antibody purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Insect Bioassay

First, a homogenous, laboratory population of the potato tuber moth was established (Raman and Palacios, 1982). For the detached leaf bioassay fully expanded, young leaves were excised with a sharp blade and placed singly in sterile tissue culture plate on a moist filter paper disk. Five neonate larvae were released on each leaf. Following their incubation at 26 ºC for 5 days, the leaf damage and feeding area was recorded. Five leaves were used for each transplastomic line and the experiment was repeated twice.

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Appendix 2

Construction of Marker-Free Transplastomic Tobacco Using the Cre-l*oxP* Site-specific Recombination System

Introduction

Plastids in higher plant cells are semi-autonomous cellular organelles which carry multiple identical copies of a ~150-kb circular genome that may be present in 1,000 to 10,000 copies per cell. The plastid genome is an attractive alternative to nuclear gene transformation because of the readily obtainable high protein levels, the opportunity for the expression of multiple genes in operons, lack of gene silencing and natural containment due to the lack of plastid transmission *via* pollen (Bock, 2001; Maliga, 2004). We describe here a protocol for plastid transformation using biolistic DNA delivery into leaves (Fig. A2-1) and construction of marker free transplastomic plants in tobacco (*Nicotiana tabacum*), the model species for plastid biology.

Since marker genes are required to obtain transplastomic plants (Box A2-1), marker-free transplastomic plants can be obtained only if plastid transformation is carried out with specialized vectors enabling marker gene excision (Fig. A2-2) (Box A2-2). Therefore, we shall discuss transformation with the specialized vectors (Fig. A2-1) as well as marker gene excision. The three protocols described here for marker gene excision rely on expressing a plastid-targeted Cre in the nucleus (Box A2-3, Fig. A2-3). The Transformation Protocol involves introduction of *Cre* into the nucleus by Agrobacterium transformation(Corneille et al., 2001); according to the Pollination Protocol, *Cre* is introduced by fertilization with pollen of a transgenic plant(Corneille et

al., 2001); whereas the Transient Protocol for marker excision depends on transient expression of *Cre* delivered on a non-integrated T-DNA by Agroinfiltration(Lutz et al., 2006). When the Transformation and Pollination Protocols are used for marker gene excision, the nuclear *Cre* gene still has to be segregated away in the seed progeny. Therefore, the Transient Protocol is preferred in heterozygous, vegetatively propagated crops such as potato or poplar tree, because with this protocol it is not necessary to go through a seed progeny to obtain plastid and nuclear marker-free plants.

The limitation of the protocol is that it is directly applicable only to *Nicotiana tabacum* cultivar (cv.) Petit Havana(Svab and Maliga, 1993); *Nicotiana sylvestris* (unpublished) and Petunia hybrida(Zubko et al., 2004). Since plants can be regenerated by the same protocol, plastids could also be transformed in *N. tabacum* cv. Samsun and cv. Xanthi and *Nicotiana plumbaginifolia*. However, modification of tissue culture conditions would be required to transform plastids in other cultivars of *N. tabacum*, for example in *N. tabacum* cv. Wisconsin 38, and other plants species.

Protocols for tobacco plastid transformation have been available since 1990(Svab et al., 1990; Svab and Maliga, 1993) (Fig. A2-1). Plastid transformation has been used in basic science and biotechnology to study plastid gene transcriptional regulation, plastid promoter elements, mRNA elements that control translation efficiency, mRNA stability, RNA editing, and for the deletion and replacement of plastid genes. For further information on plastid transformation and its uses see recent reviews by(Bock, 2001; Maliga, 2004; Herz et al., 2005; Maliga, 2005). Protocols for Cre-mediated plastid marker excision are relatively recent(Corneille et al., 2001; Hajdukiewicz et al., 2001;

Lutz et al., 2006) as are the first practical vectors to obtain marker-free transplastomic plants(Chakrabarti et al., 2006; Lutz et al., 2006; Tungsuchat et al., 2006) (this paper).

Recombinase mediated marker excision is not the only option to obtain marker free transplastomic plants. Marker free transplastomic plants have also been obtained by selection for a transiently cointegrated gene(Klaus et al., 2004; Herz et al., 2005) and by deletion *via* directly oriented repeat sequences(Iamtham and Day, 2000; Kode et al., 2006). These alternative approaches may be useful for specialized applications but, in general, offer less control over the timing and the speed of excision.

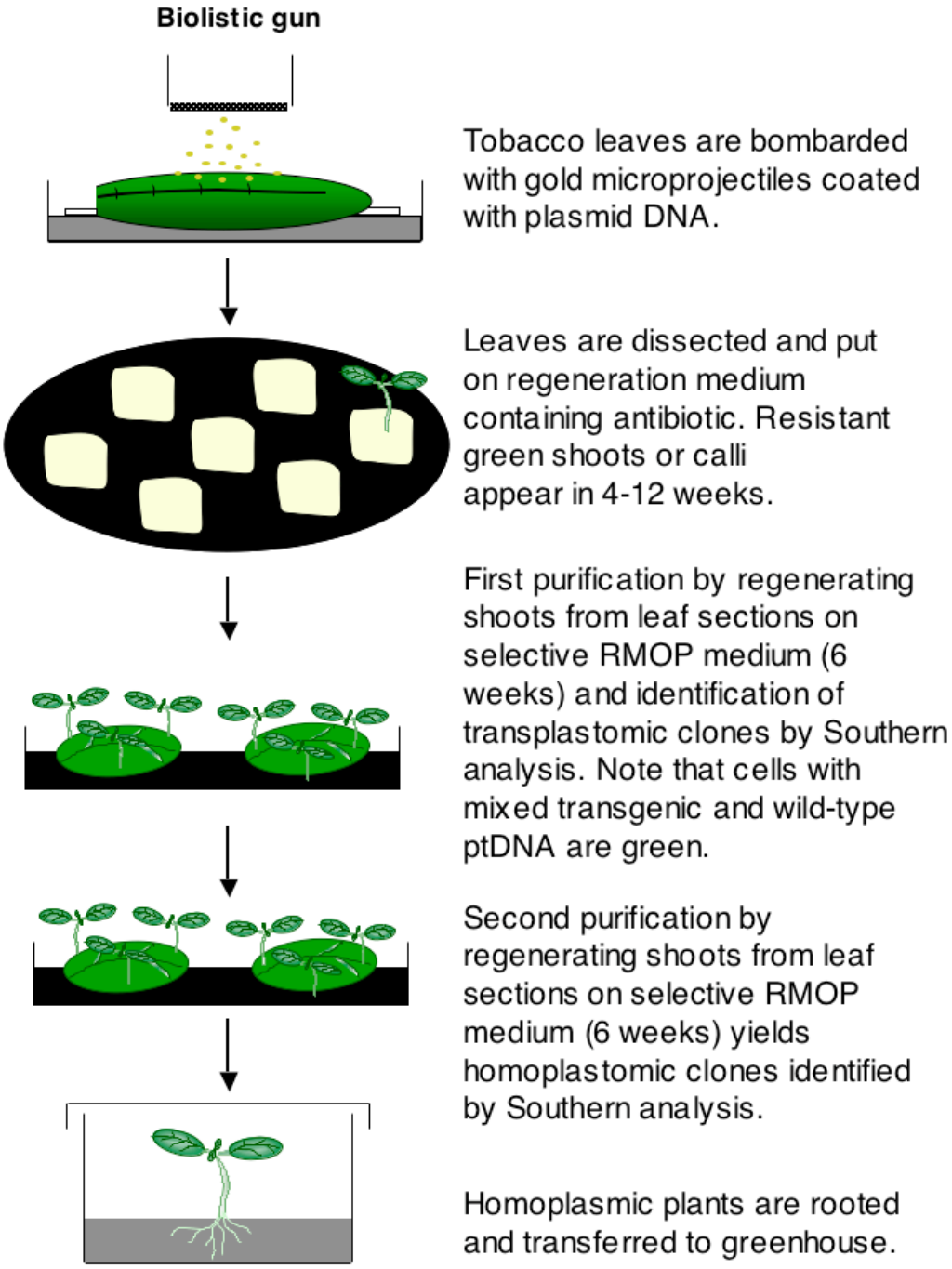

Figure A2-1. Transformation and regeneration of transplastomic plants. Based on Fig. 2 in ref.(Svab and Maliga, 1993).

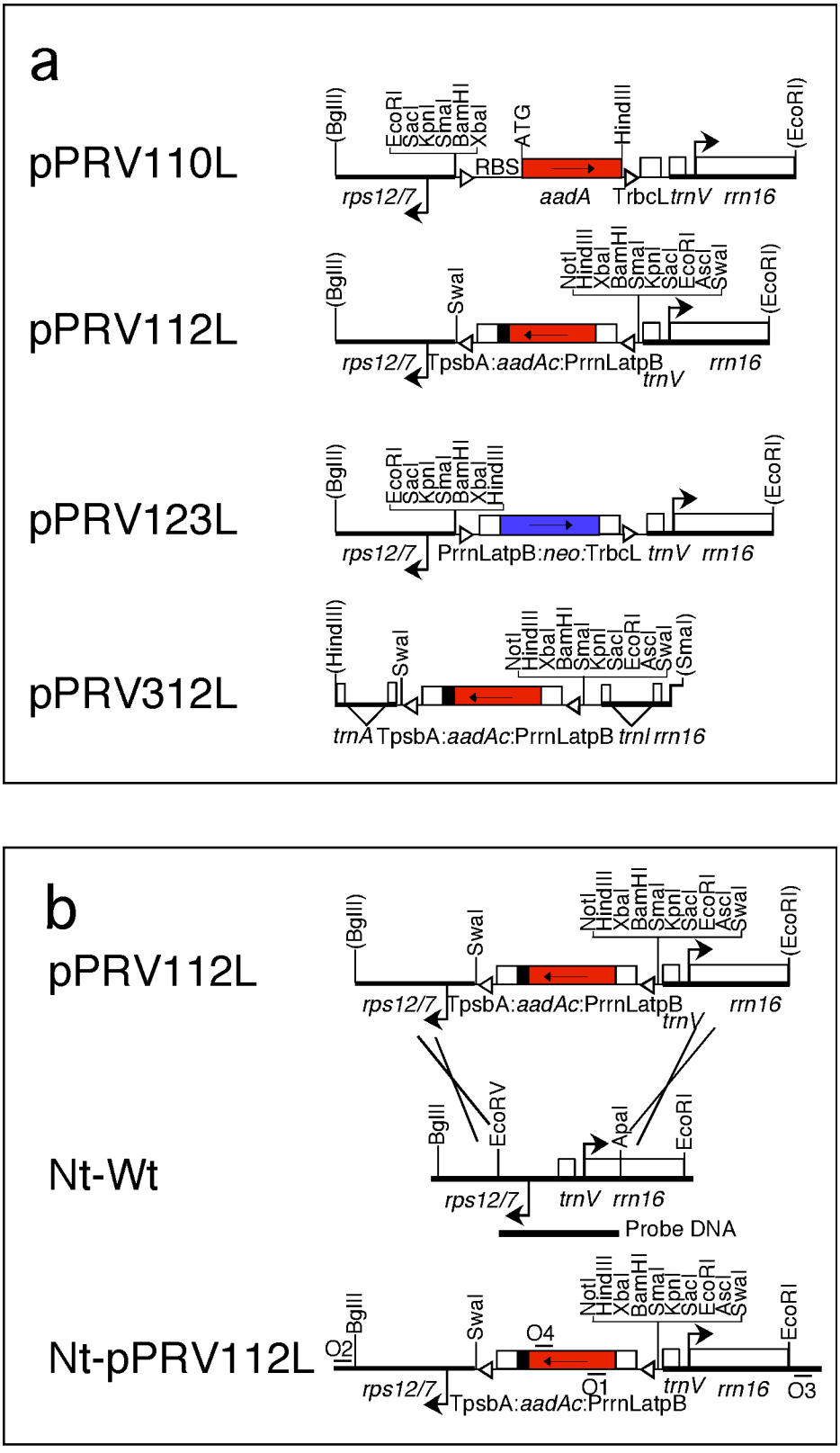

Figure A2-2. Plastid *loxP* vectors for marker gene excision. (**a**) Map of plastid *loxP* vectors listed in Table A2-1. Shown are: the spectinomycin resistance (*aadA*) and

kanamycin resistance (*neo*) genes; the plastid *rrn16*, *trnV* and *3'rps12* genes; triangles symbolizing *loxP* sites; promoters (P) and terminators (T) as listed in Table A2-1. Horizontal arrow marks direction of transcription. (**b**) Integration of *aadA* marker gene into the wild type plastid genome after transformation with the pPRV112L vector DNA. Crossover sites of homologous recombination are marked. Position of O1-O4 oliglonucleotides used to confirm integration is marked on transplastomic ptDNA.

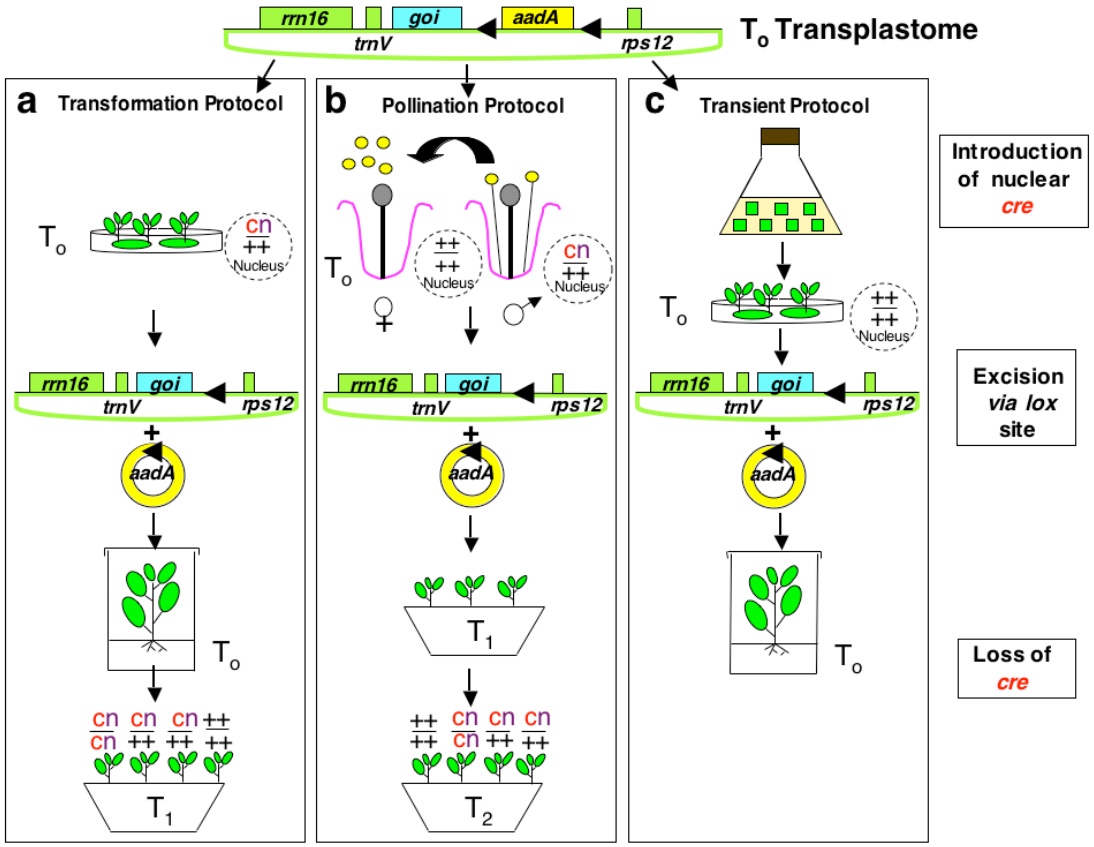

Figure A2-3. Approaches for plastid marker gene excision. (**a**) The Transformation Protocol (**b**) the Pollination Protocol and (**c**) the Transient Protocol for marker excision. On top is the map of ptDNA containing the floxed *aadA* and the gene of interest, and the plastid *rrn16*, *trnV* and *3'rps12* genes. Triangles symbolize *loxP* excision sites through which circular *aadA* excision products form. Modified from Fig. 5 in ref.(Maliga, 2002).

MATERIALS

Reagents

Nicotiana tabacum, c.v. Petit Havana

Agrobacterium tumefaciens, EHA101, EHA105

Acetosyringone

Agar, plant tissue culture tested (Sigma, A7921)

AmpliTaq DNA Polymerase (US Biochemical)

dNTP mixture (2.5 mM each dATP, dCTP, dGTP, dTTP)

Ethanol 70% (v/v)

Helium, 99.999% pure, moisture free

Glycerol 50% (v/v)

Plasmid DNA

Calcium chloride; 2.5 mM

Carbenicillin

Kanamycin sulfate (Sigma)

Rapid-hyb Buffer (GE Healthcare)

Gentamycin sulfate (Sigma)

Spermidine free base 0.1M (Sigma S4139)

Ethanol 100% (v/v)

Spectinomycin dihydrochloride pentahydrate (Sigma)

Streptomycin sulfate (Sigma)

Southern hybridization solutions (see Reagents setup)

REAGENTS SETUP

Loading dye, 5x (40 mM EDTA, pH 8.0; 1% SDS, 15% Ficoll, Bromophenol Blue, 0.12% Xylene Cyanole FF)

CTAB DNA extraction Buffer (2% C-tetradecyl-trimethyl-ammonium bromide (CTAB, Sigma T4762), 1.4M NaCl, 20 mM EDTA pH 8, 100 mM Tris-HCl, pH 8, 100 mM β-merkaptoethanol)

Minimal A medium (Per liter add: 1 g $(NH_4)_2SO_4$, 4.5 g KH_2PO_4, 10.5 g K_2HPO_4 and 0.5 g sodium citrate·$2H_2O$, autoclave and when cool add 1 ml 1M $MgSO_4 \cdot 7H_2O$ and 10 ml 40% sucrose)

RM plant maintenance medium (MS medium, ref.(Murashige and Skoog, 1962))(Per liter add: 100 ml 10X macronutrients, 10 ml 100X micronutrients, 5 ml 1% Fe-EDTA, 30 g sucrose, pH 5.6-5.8 with 1M KOH; 7 g agar)

RMOP shoot regeneration medium(Cséplö and Maliga, 1984) (Per liter add: 100 ml 10X macronutrients, 10 ml 100X micronutrients, 5 ml 1% Fe-EDTA, 1 ml thiamine (1mg/ml), 0.1 ml alpha-naphthaleneacetic acid (NAA, 1mg/ml in 0.1 M NaOH), 1 ml 6-benzylaminopurine (BAP, 1mg/ml in 0.1M HCl), 0.1 g myo-inositol, 30 g sucrose, pH 5.8 with 1M KOH), 7 g agar)

RM Medium 10x Macronutrient solution (Per liter add: 19 g KNO_3, 3.7 g $MgSO_4 \cdot 7H_2O$, 4.4 g $CaCl_2 \cdot 2H_2O$, 1.7 g KH_2PO_4, 16.5 g $(NH_4)NO_3$)

RM Medium 100x Micronutrient solution (Per liter add: 169 mg $MnSO_4 \cdot 2H_2O$, 62 mg H_3BO_3, 86 mg $ZnSO_4 \cdot 7H_2O$, 8.3 mg KI, 2.5 ml $Na_2MoO_4 \cdot 2H_2O$ (1 mg/ml), 2.5 ml $CuSO_4 \cdot 5H_2O$ (1 mg/ml), 0.25 ml $CoCl_2 \cdot 6H_2O$ (1 mg/ml))

YEB Agrobacterium culture medium (Per liter add: 5 g Beef extract, 1 g Yeast extract, 5 g peptone, 5 g sucrose, 2 mM $MgSO_4$) supplemented with 100 mg/l spectinomycin

MMA (Per liter add: 100 ml macronutrients, 10 ml micronutirents, 10 mM MES, 20g sucrose, pH 5.6 then add 200 uM acetosyringone)

Southern hybridization: For 20X SSPE solution, per liter add: 175.3 g NaCl, 27.6 g $NaH_2PO4{\cdot}H_2O$, 40 ml 0.5 M EDTA, pH to 7.4 with 10M NaOH. For the depurination solution use 0.25M HCl. For the denaturation solution use 0.5 M NaOH and 0.8 M NaCl. For the neutralization solution use 0.5 M Tris-HCl, pH7.0 and 0.5 M NaCl. For the wash solution use 0.1X SSPE and 0.2% SDS.

EQUIPMENT SETUP

Culture room, temperature set to 27 °C, illuminated with cool white fluorescent bulbs for 16 hours at 2000 lux

EQUIPMENT

DNA ladder, 1 kb ladder (Invitrogen)

HandiWrap, cut into 2.5 cm strips

Hybridization mesh (GE Healthcare)

Microcentrifuge

Microspin G-50 column (GE Healthcare)

^{32}P-dCTP

dCTP Ready to go DNA labeling beads (GE Healthcare)

Particle gun gold microcarrier, 0.6 micron (Bio-Rad)

Particle gun macrocarriers (Bio-Rad)

Particle gun PDS-1000/He (Bio-Rad)

Particle gun rupture disks, 1,100 psi

Particle gun stopping screens (Bio-Rad)

Posiblot Pressure blotter (Stratagene)

UV Stratalinker 1800 (Stratagene)

PROCEDURE

Coating of gold with plasmid DNA (Modified from Bio-Rad Bulletin 9075)

1l Add 1 ml 70% ethanol to 30 mg gold microcarrier in a 1.5 ml Eppendorf tube.

2l Place tube in a Vortex microtube holder and vortex vigorously for 5 minutes. Store tube at room temperature for 15 minutes to let particles settle.

3l Compact gold by spinning in microcentrifuge at 3000 rpm (600*g*) for 1 minute.

4l Remove ethanol with pipette and add 1 ml ice-cold sterile distilled water.

5l Vortex tube to suspended particles and store tube at room temperature for 10 minutes to let particles settle.

6l Compact gold by spinning in microcentrifuge at 3000 rpm (600*g*) for 1 minute.

7l Remove water with pipette and add 1 ml ice-cold sterile distilled water.

8| Repeat washing gold with water by repeating Steps 5, 6 and 7.

9| Vortex to ensure all particles are resuspended and store at room temperature for 10 minutes to let particles settle.

10| Microcentrifuge at 5000 rpm (1700*g*) for 15 seconds and remove the water completely.

11| Add 500 μl 50% glycerol and vortex for 1 minute to ensure all particles are resuspended. Gold concentration will be 60 mg/ml. ■ **PAUSE POINT:** Clean gold can be stored for 2 weeks at room temperature.

12| Place ten 1.5 ml Eppendorf tubes in a rack. Place Eppendorf tube with gold in Vortex microtube holder and shake at setting 3. While tube containing gold is shaking, aliquot 50 μl of gold into each of the ten tubes.

13| Place tubes with gold aliquots in Vortex microtube holder and shake at setting 3. While tubes are shaking, add 5 µl DNA (1 μg/μl), 50 μl 2.5M $CaCl_2$ and 20 μl 0.1M spermidine. ▲ **CRITICAL STEP:** Add in the order described and make sure contents are thoroughly mixed before adding next item.

14| Shake on Vortex at setting 3 for 5 minutes.

15| Sediment gold by spinning in microcentrifuge at 3000 rpm (600*g*) for 1 minute.

16| Discard the supernatant and add 140 μl 70% ethanol to each tube with coated gold.

17| Lightly tap tube until the pellet just starts to come into solution to make sure pellet is not tightly packed.

18| Sediment gold by spinning in a microcentrifuge at 3000 rpm (600*g*) for 1 minute.

19| Discard the ethanol and add 140 μl ice-cold 100% ethanol to each tube with coated gold.

20| Lightly tap tube until the pellet just starts to come into solution. If pellet is tightly packed, break up pellet by pipetting.

21| Sediment gold by spinning in a microcentrifuge at 5000 rpm (1700*g*) for 15 seconds.

22| Resuspend coated gold pellet in 50 μl 100% ethanol by gently tapping tube. Pellet should easily come into solution. Shake tubes at setting 3 while waiting to use them for bombardment. If tubes are sitting for a long period of time before bombardment, replace ethanol in tube with fresh 100% ethanol.

Leaf transformation

23| To prepare a tobacco leaf for biolistic transformation, place a sterile Whatman No. 4 filter paper on thin Petri plate of solid RMOP medium (20 ml) and place one leaf or a leaf section abaxial side up on the filter paper. If the leaf is small more than one can be used to cover the central area. Leaves may be harvested from plants grown aseptically in Magenta boxes or from greenhouse-grown plants. (Box A2-4). *For one DNA construct typically 20-30 plates are bombarded. We expect to obtain one transplastomic clone per plate (range 5-0.5).* ■ **PAUSE POINT: Stack of thin plates containing cut leaves is bagged in plastic and can be left in the culture room overnight**.

24| Sterilize gun main chamber, rupture disk retaining cap, microcarrier launch assembly and target shelf by wiping off with a cloth containing 70% ethanol. The gun is stored and used in a sterile laminar flow hood.

25| Sterilize rupture disks (1100 psi), flying disks, flying disk holders and stopping screens by soaking in 100% ethanol (5 minutes) then air dry them in tissue culture hood in open container.

26| Turn on helium tank and set delivery pressure in regulator (distal to tank) for 1300 psi (200 to 300 psi above rupture disk value).

27| Turn on vacuum pump and gene gun. Set the vacuum rate on the gene gun to 7 and the vent rate to 2.

28| Prepare DNA-coated gold particles as described above. Pipette 10 μl of DNA-coated gold onto one flying disk (placed in holder) and let air dry for 5 minutes. 5 samples may be made up at one time.

29| Place rupture disk into retaining cap and screw in tightly.

30| Put stopping screen and flying disk (face down) in microcarrier launch assembly and place in chamber just below rupture disk. For description see Biorad Bulletin 9075.

31| Place leaf on thin RMOP plate into chamber 9 cm (4^{th} shelf from top) below the microcarrier launch assembly and close the door.

32| Open vacuum valve. When vacuum reaches 28 in. Hg press fire button and hold down until the pop from the gas breaking the rupture disk is heard. **▲ CRITICAL STEP:** If gun is fired at lower pressure, DNA coated particles will lack momentum to penetrate cells and no transplastomic lines will be obtained. Test efficiency of coating and DNA delivery using transient expression of nuclear *uidA* gene, that encodes β-glucuronidase, an enzyme the activity of which can be readily detected by histochemical staining, as recommended by manufacturer.

➨ TROUBLESHOOTING

33| Immediately release the vacuum and remove the leaf sample.

34| Repeat steps 28-33 until all leaf samples are bombarded. When finished, turn off helium tank, and release pressure by holding down the fire button while vacuum is on.

35| Place plastic bag over plates containing bombarded leaf samples and place in culture room. Incubation in the culture room allows time for expression of marker gene before commencing selection.

36| After 2 days, cut bombarded leaves into small (1 cm square) pieces and place abaxial side up in deep RMOP-Spectinomycin (500 mg/l) plates. ▲ **CRITICAL STEP:** Place only 7 pieces per plate as the leaf pieces will grow and expand. Typically leaf pieces from 1 leaf bombardment fit on 3-5 thick plates. If leaf sections are too large, there is insufficient nutrient in medium to support growth for up to 12 weeks, the time frame within which transplastomic clones appear. Diagnostic sign of overcrowding is absence of spontaneous spectinomycin resistant mutants (see Step 39) and transplastomic clones. Overcrowding may be also caused by less than the desired 50 ml culture medium in a deep plate.

➨ **TROUBLESHOOTING**

37| Individually wrap each plate with plastic wrap and place in culture room. ■ **PAUSE POINT:** incubate plates for 4-12 weeks.

38| Green shoots appear on the bleached leaf sections between 4-12 weeks after bombardment. Shoot formation indicates spectinomycin resistance that may be due to

expression of the spectinomycin resistance (*aadA*) gene or mutation in the plastid small ribosomal RNA (*rrn16*) gene. Each shoot that forms at a distinct location derives from an independent event, and is identified by a serial number.

➨ **TROUBLESHOOTING**

Plant regeneration and classification by antibiotic resistance

39| To distinguish transgenic clones from mutants, from each shoot that appears, place leaf sections abaxial side up in deep plates on selective spectinomycin (500 mg/l; RMOP) and streptomycin-spectinomycin (500 mg/l each; RMOP) media. The *aadA* gene confers resistance to both, spectinomycin and streptomycin, whereas the spontaneous spectinomycin resistant mutants are resistant only to spectinomycin. Thus, putative transplastomic clones are resistant to both antibiotics and form green regenerating calli on both media in three to six weeks, whereas plastid mutants will be sensitive and bleach out on streptomycin-containing medium. The *aadA* gene rarely inserts and expresses in the nuclear genome; therefore transplastomic clones are positively identified by showing incorporation of *aadA* in the plastid genome by PCR or DNA gel blot analysis.

40| Take ten shoots each from three or four putative transplastomic clones and root them on RM deep plates. The shoots will root and form multiple leaves in ~3 weeks. Shoots regenerated from the same initial shoot are considered subclones and are distinguished with letters. Normally, we characterize further only three to four plants. We pick ~ 10 shoots for rooting because not all shoots develop into a plant.

41| When plants are identified as transplastomic, they are transferred to soil in the greenhouse after gently breaking up the agar, and washing off the agar-solidified RM medium.

42| Cover plantlets with plastic foil to prevent desiccation. Grow plants in shade for about a week, then expose to full sunlight.

43| Identify transplastomic clones by showing incorporation of *aadA* in the plastid genome by DNA gel blot analysis (A) or PCR (B).

A) Southern analyses

i Cut part of 2 leaves (20-100 mg) from plants growing on RM plates for DNA isolation by the CTAB protocol modified from (Murray and Thompson, 1980).

ii Grind leaf tissue in a 1.5 ml microfuge tube using a 1.5 ml pestle. **▲ CRITICAL STEP:** If grinding is insufficient, no DNA will be obtained, but overgrinding of DNA will cause DNA breakage.

iii Add 0.4 ml of CTAB buffer and place the eppendorf tube in a Dri-Bath Incubator at 60°C for 30 minutes.

iv Remove eppendorf tube from 60°C, homogenize again with pestle, and add another 0.4 ml CTAB buffer to the tube.

v Vortex the tube vigorously to mix.

vi Extract the DNA by adding 0.5 ml chloroform ($HCCl_3$)/isoamyl alcohol (24:1), vortexing vigorously for 30 seconds and spinning in a Microcentrifuge for 1 minute at 14000 rpm (13000*g*).

vii Transfer supernatant to a clean 1.5 ml microfuge tube by suction with pipette.

viii Repeat DNA extraction (Steps vi, vii). **▲ CRITICAL STEP:** If white precipitate is present in interphase repeat extraction again.

ix Precipitate the DNA by transfering supernatant (~ 0.5 ml) to a clean 1.5 ml microfuge tube and adding 2/3 volume (0.4 ml) of isopropanol.

x Pellet the DNA by spinning precipitated DNA in a Microcentrifuge at 14000 rpm (13000*g*) for 30 minutes at room temperature.

xi Remove the supernatant and wash the pelleted DNA with 70% ethanol by adding 0.5 ml 70% ethanol and spinning in a Microcentrifuge for 5 minutes at 14000 rpm (13000*g*).

xii Repeat DNA wash with 70% ethanol (Step xi) one time.

xiii Remove all of the 70% ethanol and place open tube on benchtop for 5 minutes to dry the DNA pellet.

xiv Once the DNA pellet is dry, suspend pellet in 50 μl of double-distilled sterile water.

xv Digest 5 µl total cellular DNA (~1 microgram) with restriction endonuclease(s) in a 20 microliter reaction for 5 hours. Suitable enzymes to distinguish wild type (size in brackets) and transgenic fragments by Southern probing are: *Eco*RI (3.1 kb), *Bam*HI (3.3 kb) *Apa*I-*Eco*RV (2.0 kb) in the *trnV/3'rps12* region; and *Bst*EII (4.6 kb) and *Hinc*II (4.9 kb) in the *trnI/trnA* region.

xvi Add loading dye to digested DNA sample and load it along with a DNA ladder onto a 0.8% agarose gel. Run gel until dye runs almost to the end.

xvii Stain the gel with ethidium bromide and take a photo with a cm ruler along the DNA ladder.

xviii Depurinate the DNA by placing the gel in 250 ml of Southern blot depurination solution for 15 minutes on a shaker.

xix Remove the depurination solution; rinse the gel briefly with water and place gel in Southern blot denaturation solution. Place gel on a shaker for 30 minutes.

xx Remove the denaturation solution; rinse the gel briefly with water and place gel in Southern blot neutralization solution. Place gel on a shaker for 30 minutes. ▲ **CRITICAL STEP:** The gel will be very slippery; handle carefully.

xxi Transfer the DNA from the gel onto a nitrocellulose membrane using a PosiBlot pressure blotter (Stratagene) for 1 hour.

xxii Rinse the nitrocellulose membrane briefly in 2X SSPE and crosslink DNA using 1200 μjoules in a UV Stratalinker 1800 (Stratagene). ■ **PAUSE POINT:** Membrane can be stored at 4°C at least a month.

xxiii Place the membrane in 2XSSPE briefly to wet it.

xxiv Place wet membrane between 2 sheets of hybridization mesh (GE Healthcare) and put it into a 50 ml Falcon tube. Add 7.5 ml Rapid Hybridization Buffer (GE Healthcare) to the Falcon tube. Place Falcon tube in glass hybridization tube and place in a hybridization oven at 65°C for 0.5-1 hour.

xxv To label a DNA fragment add 20 μl of denatured DNA (30 ng, 3 minutes at 95°C) to a dCTP Ready to go DNA labeling Bead (GE Healthcare) resuspended in 27 μl

water. Add 2.5 μl ^{32}P-dCTP (50 µCi) and incubate at 37°C for 45 minutes. Remove unincorporated isotope by spinning in a Microspin G-50 column (GE Healthcare). ▲ **CAUTION** Observe radiation safety guide.

xxvi To determine the percentage of transformed ptDNA copies probe with the plastid-targeting region. A typical ptDNA probe is the ApaI/EcoRV fragment (Fig. A2-1b). Add 10 μl denatured labeled probe DNA (3 minutes at 95°C) to the tube containing the membrane. Place the membrane back into the hybridization oven and leave overnight.

xxvii Remove the membrane from the Falcon tube and rinse filter briefly with 150 ml 2X SSPE.

xxviii Wash membrane by placing it in 250 ml of Southern hybridization wash solution and placing in a shaker at 55°C for 30 minutes. Repeat 1 time.

xxix Rinse the membrane briefly with 2X SSPE at room temperature.

xxx While membrane is still moist wrap it in Saran Wrap and expose to X-ray film. Identify transplastomic plants by hybridization pattern.

➨ **TROUBLESHOOTING**

xxxi If it is necessary to probe with a different DNA fragment the membrane can be stripped of the DNA probe by rinsing the blot 2-3 times in near boiling (90-95°C) 0.1-0.5% SDS for 5 minutes. Check removal of radioactivity by exposing film.

B) PCR analyses

i To check for plastid transformation by PCR, add 0.5 µl DNA (from step 43A xiv.), 4 µl 10 mM dNTP's (2.5 mM each dNTP), 5 µl AmpliTaq 10X PCR Buffer,

1 µl of each primer (100 pmol/µl), 0.5 µl AmpliTaq DNA Polymerase, 38 µl water to a 0.2 ml eppendorf tube. Oligo pairs are either O1 (5'-ACTACCTCTGATAGTTGAGTCG-3') and O2 (5'-AGAGGTTAATCGTACTCTGG-3') or O3 (5'-GCTCCTATAGTGTGACG-3') and O4 (5'-CTTGGCCTCGCGCGCAGAT-3'). One oligonucleotide (O2 and O3) is located in the plastid genome, outside of the targeting region. The other oligonucleotide (O1 and O4 is located in the *aadA* gene (Fig. A2-2). Use of PCR to detect integration was described in (Khan and Maliga, 1999).

ii Place 0.2 ml eppendorf tube in PCR machine and run program of 3 min at 94ºC, followed by 30 cycles of 30 seconds at 94ºC, 1 minute at 55ºC and 1 min at 72ºC; and 1 cycle of 11 minutes at 72ºC.

iii Check PCR reaction by adding loading dye to PCR reaction and running 5 µl of PCR reaction on a 0.8% agarose gel alongside a DNA ladder. Stain gel with Ethidium bromide and visualize under UV light.

Testing antibiotic resistance in seed progeny

44l Verify genetic homogeneity (homoplastomic state) and maternal inheritance by germinating seed on selective medium. Sterilize seed by placing seed in a 1.5 ml eppendorf tube and storing it overnight in a desiccator with a flask containing 100 ml bleach and 3 ml of concentrated (37.8%) HCl. Flasks should be placed in desiccator immediately after bleach and HCl are mixed. Sprinkle seed onto RM plates containing spectinomycin (500 mg/l) and incubate the plates in the culture room. Since plastids are

maternally inherited, selfed seed progeny should be uniformly resistant (green). If seed derives from cresses between wild type and transplastomic plants, seedlings should be uniformly resistant (green) when they derive from a cross with the transplastomic plant as maternal parent and uniformly sensitive (bleached) when the maternal parent is the wild type plant.

Plastid marker excision

45| Plastid marker excision can be achieved via : A) transformation, B) pollination or C) transient protocol; For overview and time required see Table A2-3.

A) Plastid marker excision by the Transformation Protocol

i For cocultivation, grow a patch of Agrobacterium from single-cell purified stock for two days at 27°C on solid minimal A plate containing spectinomycin (100 mg/l) to select for the binary plasmid and kanamycin (50 mg/l) or chloramphenicol (50 mg/l) dependent on the virulence strain used (Box A2-5).

ii Cut leaf from plastid transformed plant into 1 cm x 1 cm leaf disks and wound leaf disks by gently squeezing with sterile forceps. Prevent desiccation of leaf sections by floating them on distilled sterile water.

iii Prepare *Agrobacterium* suspension by resuspending *Agrobacterium* cells in Minimal A medium at the concentration of 1-2 x 10^9 bacteria per ml. Transfer leaf disks into Agrobacterium suspension for 1 minute then blot leaf discs on filter paper to remove as much Agrobacterium as possible.

iv Place leaf discs abaxial side up on a Whatman No. 4 filter paper on solid RMOP medium. Seal plates with Handi Wrap strip and place in the culture room. ▲ **CRITICAL STEP:** If sections are placed on medium directly, Agrobacterium may overgrow leaf sections.

v After 2 days transfer leaf discs to RMOP plates containing antibiotics that select for the plant nuclear marker encoded in the T-DNA (100 mg/l kanamycin sulphate, pKO27 and pKO28; or 100 mg/l gentamycin sulphate, pKO30 and pKO31) and control Agrobacterium growth (carbenicillin, 500 mg/l).

vi Shoots will form 2 to 3 weeks after cocultivation at the cut and wounded leaf surfaces.

vii Excise shoots and insert them into selective RM medium containing the drug used for plant selection (kanamycin or gentamycin) and carbenicillin to control Agrobacterium growth. Transgenic shoots will produce new green leaves and form roots in 7 to 15 days. Wild-type shoots bleach and will not form roots. Root formation on the shoots is a reliable assay of antibiotic resistance.

viii Isolate leaf DNA (Steps 43Ai-xiv) and perform a Southern blot (Steps 43Axv-xxx) to identify plants lacking the plastid marker gene due to Cre-mediated excision. Cre-mediated excision via non-*loxP* sequences may also be observed (Box A2-3).

ix Put plastid marker free plants in soil after gently breaking up the agar and washing off the agar-solidified RM medium. Cover plantlets with plastic foil for about a week. Grow plants in shade initially, as described in Step 42.

x Let plants self fertilize and collect seed.

➨ **TROUBLESHOOTING**

xi The objective is to identify plants in which the nuclear *Cre* gene has been segregated away by the absence of the linked plant nuclear marker in the selfed seed progeny. Sterilize seed as described in Step 44. Sprinkle seed onto RM plates containing kanamycin (200 mg/l) or gentamycin (200 mg/l) and incubate the plates in the culture room. Bleaching will identify seedlings lacking the plant nuclear marker gene linked to *Cre*. Transfer bleached seedlings to non-selective RM medium where they will green up and can be transferred to the greenhouse. These plants are both plastid and nuclear marker free and only contain the gene of interest.

B) Plastid marker excision by the Pollination Protocol

i Emasculate transplastomic flower by cutting it open and removing the anthers. If pollen is visible, a younger flower should be chosen. Take open flower from nuclear *Cre* transformed plants and touch stigma of the emasculated flower with the anthers of *Cre* flower. Tag pollinated flower and collect seed when capsules turn brown.

ii Sterilize seed (Step 44) and sow it on RM medium containing the drug that was used to select for the introduction of nuclear *Cre* (kanamycin or gentamycin; see Step 45Axi) to identify progeny carrying the *Cre*.

iii Isolate DNA (Steps 43Ai-xiv) from seedlings that carry the *Cre* gene and test total leaf DNA for plastid marker gene excision by Southern blot analysis (Steps 43Axv-xxx).

iv Transfer seedlings lacking the plastid marker gene to the greenhouse.

v Let plants self fertilize and collect seed.

➨ **TROUBLESHOOTING**

vi Screen seed progeny for absence of nuclear marker linked to *Cre* gene as described in Step 45Axi.

C) Plastid marker excision by the Transient Protocol

Agroinfiltration performed as described by Kapila et al (Kapila et al., 1997).

i Inoculate 100 ml YEB medium with Agrobacterium carrying a plastid-targeted *Cre* gene (Step 45Ai) and grow overnight at 27°C. ▲ **CRITICAL STEP:** Agrobacterium binary plasmids can be lost or rearranged; check plasmid in miniprep before experiment.

ii Inoculate 1 ml from culture of Step 45Ci into fresh YEB medium supplemented with 10 mM MES 2-(N-morpholino)ethanesulfonic acid, adjust the pH of the YEB medium to 5.6, then add 20 μM acetosyringone and 100 mg/l spectinomycin. Grow Agrobacterium culture overnight at 27°C.

iii To pellet the Agrobacterium cells centrifuge the culture for 15 minutes at 6000 rpm (4000*g*), room temperature. Suspend the cells in MMA medium to obtain a final OD_{660}=2.4. Incubate at room temperature for 1 hour.

iv Cut leaves from transplastomic plants into small pieces (1cm^2) and submerge in the Agrobacterium culture of Step 45Ciii.

v Place the flask containing the Agrobacterium culture and the leaf disks under continuous vacuum of 2 Torr for 20 minutes while shaking gently.

vi Remove the leaf pieces from the Agrobacterium culture, dab them on filter paper to remove as much agrobacterium as possible and place them on a Whatman No. 4 filter paper on solid RMOP medium. Wrap the plates with HandiWrap and place in the culture room for two days.

vii Transfer the agroinfiltrated leaf disks to RMOP medium containing carbenicillin (500mg/l). Wrap the plates with HandiWrap and place in the culture room. Shoots should appear within 3 to 4 weeks.

viii For rooting, cut shoots of Step 45Cvii and insert them into RM medium containing carbenicillin.

ix Isolate leaf DNA (Steps 43Ai-xiv) from plants of Step 45Cviii and test for plastid marker gene excision by Southern blot analysis (Steps 43Axv-xxx). ~30% of the regenerated plants are expected to be free of the plastid selectable marker. In ~10% of the plants plastid marker excision is expected to be from a transiently expressed Cre; ~20% from an integrated Cre. Plants that are heteroplastomic should be purified one more time by regeneration on RMOP medium repeating Steps 45Cvii—ix.

x To determine if Cre expression is from a transiently expressed or integrated *Cre*, PCR amplify the *Cre* gene with primers: 5'-TAGCTCCAATTTACTGACCGT-3' and 5'-CTAATCGCCATCCTCGAGCA-3'. The 50 µl PCR reaction contains: 1µl DNA, 1 µl of each primer (100 pmol/ul), 4 µl 10mM dNTP mixture, 5 µl 10X AmpliTaq PCR Buffer (Applied Biosystems) and 0.5 µl AmpliTaq (5U/µl; Applied Biosystems). Amplification was performed as follows: 3 min at 94ºC, followed by 45 cycles of 30 seconds at 94ºC, 45 seconds at 56ºC and 1 min at

72ºC; and 1 cycle of 11 minutes at 72ºC. Also a tissue culture assay for antibiotic resistance (kanamycin or gentamycin) can be performed on leaf disks from the rooted shoots (Step 45Av).

xi To obtain seed from the plastid and nuclear marker free plants, plant the rooted shoots in soil after gently breaking up the agar, and washing off the agar-solidified RM medium. Cover plantlets with plastic foil for about a week. Grow plants in shade initially.

➨ **TROUBLESHOOTING**

ANTICIPATED RESULTS

The anticipated result of plastid transformation with *loxP* vectors and excision of marker genes is to obtain marker-free transplastomic tobacco plants carrying the gene of interest(Hajdukiewicz et al., 2001; Lutz et al., 2006). Excision of a blocking sequence, such as a marker gene disrupting a gene of interest, results in expression of the gene of interest (Hajdukiewicz et al., 2001). Plastid gene activation may also be obtained by linking up the coding region of a gene of interest with the promoter, 5'-UTR and translation initiation codon of the selective marker by CRE-mediated excision of the marker gene's coding region (Tungsuchat et al., 2006). In addition, CRE-mediated excision can be used for deletion of an essential plastid gene to gain insights into its function (Kuroda and Maliga, 2003).

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BOX A2-1. THE CELL BIOLOGY OF PLASTID TRANSFORMATION

Since plastids carry many identical copies of their genome, marker genes are required to obtain genetically stable transplastomic plants. DNA is introduced into plastids on the surface of microscopic tungsten or gold particles by the biolistic process (Klein et al., 1987). An alternative protocol for DNA introduction is polyethylene glycol (PEG) treatment of protoplasts (Golds et al., 1993; O'Neill et al., 1993); reviewed in (Kofer et al., 1998). Plastid transformation involves insertion of the marker gene into the plastid genome by two homologous recombination events *via* the flanking plastid DNA that targets the insertions. Culturing the bombarded leaf on a selective spectinomycin- or kanamycin-containing shoot regeneration medium enables enrichment of the few initially transformed ptDNA copies. Spectinomycin or kanamycin inhibit greening and shoot regeneration. Transplastomic clones are recognized by greening of cultured cells and shoot regeneration. The cells in the regenerated shoots are chimeric, but leaf sectors with uniformly transformed plastid genome copies are present as the result of replication and sorting of transformed ptDNA (Maliga, 2004). Uniformly transformed plants are obtained by purification involving shoot regeneration from a transplastomic sector on a selective medium.

BOX A2-2. CHOICE OF PLASTID VECTORS

Plastid transformation vectors are *E. coli* pUC118 or pUC119 vector derivatives (*Sca*I site removed) in which plastid *trnV/3'rps12* or *trnI/trnA* targeting sequences flank a selectable spectinomycin (*aadA*) or kanamycin (*neo*) resistance gene and a polycloning site (Table A2-1)(Fig. A2-2). In the vectors, directly oriented 34-bp *loxP* sites flank the marker gene so that it can be excised with the P1 phage CRE site-specific recombinase. All vectors (except pPRV110L) have high levels of marker gene expression that yield visually detectable bands after SDS-PAGE on Coomassie Brilliant Blue stained gels. There is commercial antibody to detect neomycin phosphotransferase II, the *neo* gene product while AAD, the *aadA* gene product can only be detected by the C-terminal *c-myc* tag in plasmids pPRV112L and pPRV312L. The choice of promoters for expression of the marker genes and the gene-of-interest should be carefully considered to avoid sequence duplications because CRE mediates deletions *via* directly oriented repeat sequences. One such duplication is use of the Prrn promoter for transgene expression at the *trnV/3'rps12* insertion site, which is located near the native Prrn promoter (Tungsuchat et al., 2006). Note that vector pPRV110L has no promoter, thus the marker gene is expressed by read through transcription from the upstream gene of interest. In vector pPRV312L the polycloning site is positioned to take advantage of the gene of interest being transcribed as part of the rRNA operon from the upstream *rrn* operon promoter (Chakrabarti et al., 2006).

BOX A2-3. CHOICE OF APPROACH FOR PLASTID MARKER GENE EXCISION

Each of the three protocols for marker gene excision has advantages and disadvantages. The Transformation Protocol (Fig. A2-3a) involves introduction of *Cre* into the plant nucleus by Agrobacterium transformation to accomplish excision of the plastid marker genes. The nuclear *Cre* gene is then segregated away in the T_1seed progeny. The advantage of this approach is speed: because once the genetically stable homoplastomic state is achieved, the marker gene can be instantly excised without going thorough a seed generation. The disadvantage of the approach is that this protocol yields the largest fraction of undesirable Cre-mediated deletions via non-*loxP* sequences (Corneille et al., 2001; Hajdukiewicz et al., 2001; Corneille et al., 2003; Tungsuchat et al., 2006). Since deletions *via* direct repeats affect only about 30%-40% of the clones, marker-free transplastomic clones can be readily recovered by this approach. Using promoters other than Prrn close to the *trnV/3 'rps12* insertion site, or choosing an alternate insertion site away form the *trnV/3' rps12* intergenic region can minimize this problem. According to the Pollination Protocol (Fig. A2-3b), the nuclear *Cre* gene is introduced by pollination. With this approach we did not see CRE-mediated non-*loxP* excision events. This approach, however, takes the longest time, because plastid and nuclear marker-free plants are recovered only in the T_2 seed generation (Corneille et al., 2001). The Transient Protocol for marker excision (Fig. A2-3c) depends on transient expression of *Cre* delivered on a non-integrated T-DNA introduced by Agroinfiltration. This is the fastest approach, because plastid and nuclear marker-free transplastomic plants are obtained already in the T_0 generation, but it is the most labor intensive.

BOX A2-4. CHOICE OF LEAVES FOR BOMBARDMENT

Leaves may be harvested from plants grown aseptically in Magenta boxes containing RM medium solidified with agar. Typically, one leaf is used per plate. Alternatively, leaves may be harvested from greenhouse-grown plants that are surface sterilized by first rinsing the leaf with water and then with 70% Ethanol and placing in diluted commercial bleach (0.5% Sodium hypochlorite) for 3 minutes. Rinse the leaf thoroughly 3X with sterile distilled water. Cut out a section from the leaf to fit the plate. The leaf surface should touch the medium therefore, if necessary, nick it at the midrib to allow it to lie flat. Do not use leaves from plants that have entered the flowering stage.

BOX A2-5. CHOICE OF AGROBACTERIUM VIRULENCE STRAINS TO MATCH MARKERS IN BINARY VECTOR

Agrobacterium binary plasmids pKO27(Corneille et al., 2001), pKO28(Corneille et al., 2001), pKO30(Corneille et al., 2003) and pKO31(Corneille et al., 2003) carry a *Cre* gene engineered for expression in the plant nucleus and encode a Cre enzyme targeted to plastids by fusion with a Rubisco small subunit transit peptide. The virulence plasmid in strain EHA101 carries a kanamycin resistance gene (Hood et al., 1986), whereas virulence strain EHA105 carries a chloramphenicol resistance gene (Hood et al., 1993). Binary plasmids pKO27 and pKO28 carry a kanamycin resistance plant marker gene; binary plasmids pKO30 and pKO31 carry a gentamycin resistance plant marker gene. All four CRE binary plasmids, pKO27, pKO28, pKO30 and pKO31 can be combined with the EHA105 Agrobacterium virulence strain. Agrobacterium virulence strain EHA101 is compatible only with binary plasmids pKO30 and pKO31.

Table A2-1. Tobacco plastid *loxP* vectors*

Plasmid	**Insertion site**	**Marker Gene**	**Promoter/ Leader**	**Terminator**	**Ref.**
pPRV110L	*trnV/3'rps12*	*aadA*	N/A	TrbcL	Ref.(Lutz et al., 2006)
pPRV112L	*trnV/3'rps12*	*aadA-c-myc*	PrrnLatpB+DB	TpsbA	Unpbl.
pPRV123L	*trnV/3'rps12*	*neo*	PrrnLatpB+DB	TrbcL	Upubl.
pPRV312L	*trnI/trnA*	*aadA-c-myc*	PrrnLatpB+DB	TpsbA	Ref.(Chakrabarti et al., 2006)

*Plasmids are available pending on execution of a Materials Transfer Agreement with Rutgers University.

Table A2-2. Troubleshooting

Problem	Possible reason	Solution
Steps 36 and 38 No spectinomycin resistant shoots are obtained in 12 weeks.	Crowding affect: too many leaf pieces, not enough culture medium in plate.	Transform new leaves and place only 7 leaf pieces per plate. Make sure deep Petri dish has ~50 ml of medium per plate.
Steps 32 and 38 Spectinomycin resistant shoots form due to mutations in the 16S rRNA gene but no transplastomic clones are obtained.	Insufficient vacuum when firing gun. Problem with DNA coating and delivery.	Transform new leaves and make sure vacuum reads at least 28 mm Hg before firing the gun. Coat gold particles with plasmid encoding nuclear *uidA* gene and test coating and delivery by histochemical staining for GUS in bombarded leaves
Step 43xxix Wild type and transgenic ptDNA is detected in spectinomycin-streptomycin resistant plants.	Incomplete sorting out of wild type ptDNA copies.	Purify new subclones by regenerating shoots from chimeric leaves on selective spectinomycin RMOP- medium.
Step 43xxix Weak, persistent wild type ptDNA fragment detected after 2-3 rounds of purification.	ptDNA fragments are present in nucleus and/or in mitochondria.	Ignore. For explanation see ref. (Maliga and Nixon, 1998; Ruf et al., 2000; Swiatek et al., 2003)
Step 43xxix Only wild type ptDNA is detected in spectinomycin-streptomycin resistant plants	Regenerated shoot is chimeric; DNA was isolated from wild type sector. Integration of transgene in the nucleus.	Make new subclones by regenerating shoots on selective spectinomycin RMOP- medium from resistant callus formed on spectinomycin-streptomycin medium. Select another clone.
Steps 45Ax; 45Bv and 45Cxi Plants fail to set seed.	Tissue culture induced sterility due to lack of pollen, or shorter stamens.	Pollination with wild type pollen yields seed and in most cases restores fertility in progeny.

Table A2-3. Time required to obtain seed of marker-free transplastomic plants

Transformation	**Pollination**	**Transient**
Transplastomic plants 2 x purification 5 months	Transplastomic plants 2 x purification 5 months	Transplastomic plants 2 x purification 5 months
Stable Agrobacterium transformation with Cre 2 months	Seed from cross with Cre line in greenhouse 3 months	Transient Agrobacterium transformation with Cre 2 months
Seed in greenhouse 3 months	Identification of plastid marker free seedling 1 month	Seed in greenhouse 3 months
Identification of nuclear marker free seedling 1 month	Seed in greenhouse 3 months	
Seed in greenhouse 3 months	Identification of nuclear marker free seedling 1 month	
	Seed in greenhouse 3 months	
Total: 14 months	**Total: 16 months**	**Total: 10 months**

Lutz, K.A., Svab, Z. and Maliga P. (2006) Construction of marker-free transplastomic tobacco using the CRE-*loxP* site-specific recombination system. Nat. Prot. 10.1038/nprot.2006.118.

Appendix 3

Transformation of the Plastid Genome to Study RNA Editing

Introduction

In land plants RNA editing has been reported in the plastids and mitochondria, two DNA-containing cytoplasmic organelles with their own prokaryotic-type transcription and translation machinery (Maier et al., 1996). In lower plants RNA editing was shown in some (Kugita et al., 2003; Miyata and Sugita, 2004; Sugita et al., 2006), but not all taxonomic groups (Freyer et al., 1997) indicating that editing is an evolved trait. In bryophytes both C to U as well as U to C editing has been reported; RNA editing in the plastids of higher plants only involves C to U conversion. RNA editing in plastids of higher plants was discovered in 1991 (Hoch et al., 1991), and since then has been shown in all higher plant species tested so far (Bock, 2000; Tsudzuki et al., 2001). Editing in most cases occurs in the coding region and restores a conserved amino acid at the mRNA level. Examples for creation of translation initiation or stop codons are also known. Among the higher plant species tobacco has the highest number (37) of identified editing sites (Hirose et al., 1999; Sasaki et al., 2003; Kahlau et al., 2006). In bryophytes the number of editing sites can be as high as 942 (Kugita et al., 2003). Comparison of editing sites indicates that some sites are highly conserved across large taxonomic groups such as monocots and dicots, while others vary even among closely related species (Sasaki et al., 2003; Calsa Junior et al., 2004; Kahlau et al., 2006). A comprehensive listing of editing sites is available for a number of species, including the allotetrapoid *Nicotiana tabacum* (Hirose et al., 1999; Sasaki et al., 2003; Kahlau et al., 2006) and its progenitor species *N.*

sylvestris and *N. tomentosiformis* (Sasaki et al., 2003), and the related solanacous plant tomato (Kahlau et al., 2006). A catalogue of editing sites is also available for the cereal crops maize (Maier et al., 1995), rice (Corneille et al., 2000) and sugarcane (Calsa Junior et al., 2004), and the model species *Arabidopsis thaliana* (Lutz and Maliga, 2001; Tillich et al., 2005) and pea (Inada et al., 2004).

First we shall give an overview of RNA editing in plastids. We shall cover how genetic approaches, such as plastid transformation, complementation by protoplast fusion and the study of mutants advanced our understanding of the editing process. The Introduction will be followed by two protocols. In the first protocol we shall discuss construction of transplastomic plants for studies on RNA editing. In the second protocol we shall describe methods to detect RNA editing *in vivo*. A separate chapter in this volume is dedicated to the description of *in vitro* systems to study plastid RNA editing. The protocols we discuss have been developed in tobacco (*Nicotiana tabacum*), the only species in which plastid transformation is routinely obtained (Maliga, 2004; Bock, 2007).

Identification of Unique cis*-Sequences for RNA editing*

Plastid transformation enabled *in vivo* testing of chimeric mRNAs containing sequences surrounding the edited C nucleotide. Testing a series of deletion derivatives defined the sequences required for editing. The most complete information is available for the *psbL* editing site. *psbL* is a plastid photosynthetic gene, in which the translation initiation codon is created by conversion of an ACG codon to an AUG codon at the mRNA level. *In vivo* dissection using plastid transformation revealed that information

required for editing is contained within a 22-nucleotide fragment including 16 nucleotides upstream and 5 nucleotides downstream (-16 to +5) of the edited C (Chaudhuri and Maliga, 1996). Importance of the upstream and downstream sequences for *psbL* editing has been confirmed *in vitro* (Hirose and Sugiura, 2001). Study of *ndhB* sites IV and V indicates that essential elements are located between the -12 to -2 position (Bock et al., 1996). Scanning mutagenesis of the same sites revealed that the upstream sequences are important for editing and that spacing of the upstream recognition sequence relative to the edited C is important (Bock et al., 1997; Hermann and Bock, 1999). The 3rd site for which detailed *in vivo* transgenic data are available is the tobacco *rpoB* site II (NtrpoB C473). Sequences minimally required for editing were located in the -20 to +6 region surrounding the edited C (Reed et al., 2001b), although longer sequences were edited more efficiently (Hayes et al., 2006).

In the early studies, no consensus sequence was recognized by alignment of sequences adjacent to the edited C nucleotide. Thus, it was assumed that each of the editing sites is individually recognized. Later on specificity clusters have been identified, which share short (2-3 nucleotide) group-specific sequence elements (Hayes et al., 2006).

Editing trans-*Factors*

Protein factors involved in editing have been identified in many systems. In higher plant plastids, most information for the involvement of protein factors is indirect: the existence of species-specific, organelle-specific and site-specific factors was inferred from genetic experiments. The first evidence for a species-specific editing *trans*-factor

was obtained when a spinach (*psbE*) editing sequence was incorporated in tobacco plastids where it was not edited (Bock et al., 1994), unless the spinach nuclear *trans*-factor was provided by cell fusion (Bock and Koop, 1997). Similarly, maize *rpoB* site IV, which is absent in the tobacco *rpoB* gene, was not editied in transplastomic tobacco. However, the maize *rpoB* site I sequence, a site that is edited in tobacco, was edited when incorporated in the tobacco ptDNA (Reed and Hanson, 1997). Also, the tobacco *atpA* editing site, that is absent in *Atropa belladonna*, was not recognized in tobacco plastids when introduced by cell fusion into the *Atropa belladonna* nuclear background and, as a consequence, the plants were pigment deficient. This was the first time that the molecular mechanism of nucleus-cytoplasm incompatibility could be explained by the lack of RNA editing (Schmitz-Linneweber et al., 2005). Based on these findings it is assumed that each species has the capacity to edit the sites that it carries, but lacks the capacity for editing the sites that it does not have. Exceptions to this rule have been found in *Nicotiana sylvestris,* which has the capacity to edit *ndhA* site 1 even though it does not have the editing site encoded in the DNA. This was explained by conservation of the editing capacity present in related species (Tillich et al., 2006).

Evidence for organelle-specific factors was obtained when an edited Petunia mitochondrial *coxII* sequence was expressed in tobacco chloroplasts, where none of the seven sites were edited (Sutton et al., 1995). Since editing of the cognate sequence has not been studied in tobacco, the lack of editing could also be due to species-specific differences in the editing capacity of the two closely related species.

Existence of site-specific factors was inferred from competition between transgenic and native mRNAs. In early studies with over-expressed *psbL* editing

segments, competition was found only with the native *psbL* segment (Chaudhuri et al., 1995; Chaudhuri and Maliga, 1996). Based on this, individual recognition of each of the plastid editing sites was proposed. More extensive studies with the *rpoB* and *ndhF* editing sites led to the conclusion that at least some of the editing sites can be grouped in specificity clusters (Reed et al., 2001a; Hayes et al., 2006). The best studied is the NtrpoB C473, NtpsbL C2 and Ntrps14 C80 cluster in which group-specific response is attributed to short (2-3 nucleotide) group-specific sequence elements (Hayes et al., 2006). *In vivo* competition data have been corroborated *in vitro* for the *psbL* (Hirose and Sugiura, 2001), *rpoB* and *ndhF* genes (Reed et al., 2001a; Hayes et al., 2006).

Although the existence of general and site-specific factors is documented *in vitro*, none of these proteins have been cloned to date. The only exception is a pentatricopeptide repeat protein encoded in the *crr4* gene involved in editing *ndhD* site 1 of *Arabidopsis thaliana*, which was identified in a mutant screen (Kotera et al., 2005).

Protocols for Construction of Transplastomic Plants

Transformation of the plastid genome (ptDNA) provided the first experimental tool to study RNA editing. The ptDNA of higher plants is ~120-150-kb in size, is highly polyploid and may be present in 1,000 to 10,000 copies per cell (Bendich, 1987; Wakasugi et al., 2001; Shaver et al., 2006). Plastid transformation vectors are *E. coli* plasmids in which the transgenes are flanked by plastid DNA sequences. Plastid transformation involves introduction of transforming DNA into tobacco leaf chloroplasts on the surface of microscopic gold particles (0.6 μm) by the biolistic process. The

transgenes integrate into the ptDNA by two homologous recombination events *via* flanking ptDNA. To obtain a genetically stable transplastomic plant all ptDNA copies must be changed. Such genetically stable, homoplastomic plants are obtained through a gradual process of ptDNA replication and sorting, and preferential maintenance of transgenic ptDNA copies on a selective tissue culture medium (Fig. A3-1).

To ensure preferential maintenance of plastids carrying transgenic ptDNA a selective marker is incorporated adjacent to the gene of interest so that the two form a heterologous block (Fig. A3-2). The selective agents in the culture medium are spectinomycin, streptomycin or kanamycin, inhibitors of protein synthesis on the plastid's prokaryotic type 70S ribosomes. (The cells survive because protein synthesis on eukaryotic 80S cytoplasmic ribosomes is not sensitive to these drugs and the cells are supplied with a reduced carbon source, sucrose, in the culture medium.) The *aadA* gene confers resistance to spectinomycin and streptomycin (Svab and Maliga, 1993); the *neo* or *kan* (Carrer et al., 1993) or *aphA-6* (Huang et al., 2002) genes confer resistance to kanamycin. Cells of the bombarded leaves form scanty white callus on the selective medium due to inhibition of chlorophyll biosynthesis. Transplastomic clones are recognized by formation of green cells and shoots (Fig. A3-1B). The cells in the regenerated shoots are chimeric with transgenic and non-transformed sectors, but leaf cells within the transgenic sectors carry only uniformly transformed ptDNA copies as the result of replication and sorting of transformed ptDNA. Homoplastomic, uniformly transformed plants are obtained during a second cycle of plant regeneration from the resistant sectors (Fig. A3-1D). The shoots are rooted and the plants are transferred to the greenhouse (Fig. A3-1E). For reviews on plastid transformation see ref. (Maliga, 2004;

Herz et al., 2005; Maliga, 2005; Bock, 2007); for protocols on tobacco plastid transformation see ref. (Bock, 1998; Lutz et al., 2006a).

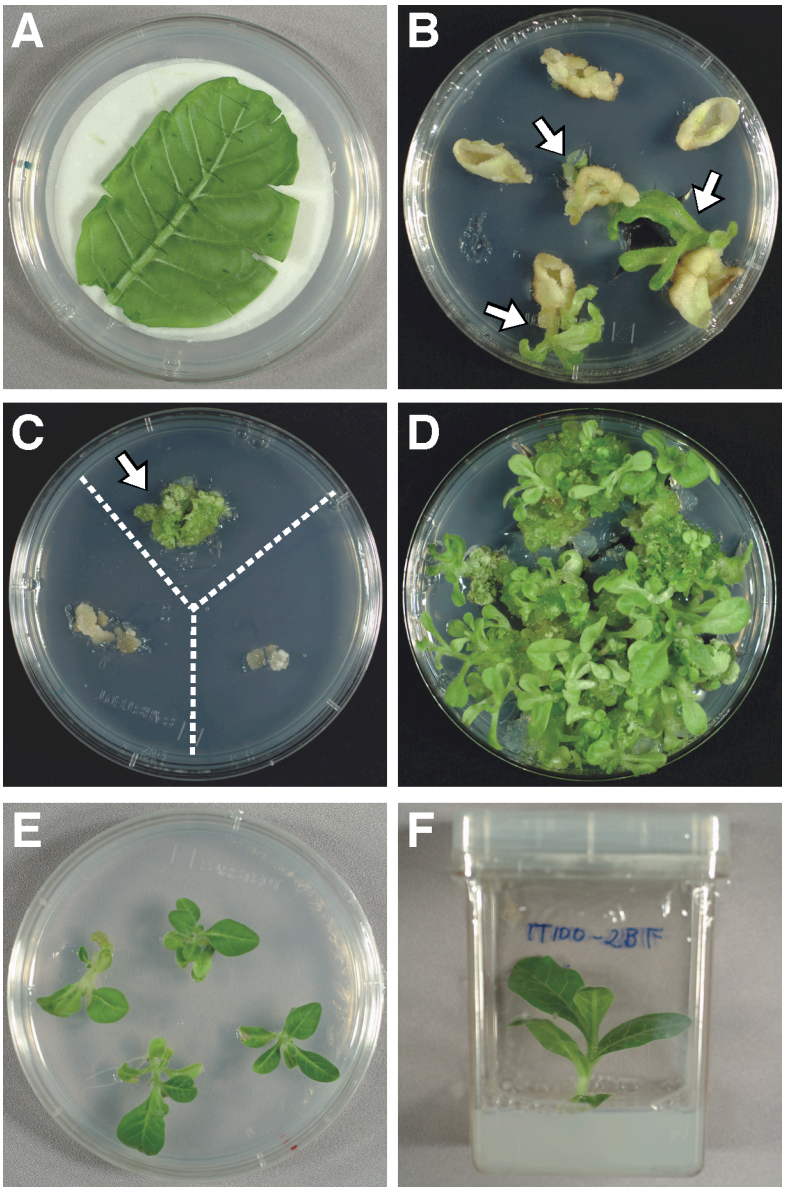

Figure A3-1. Transformation of tobacco leaves to obtain transplastomic plants. (A) Leaves prepared for bombardment with DNA coated gold particles. (B) Resistant shoots and/or calli appear in 4-12 weeks on leaf cut into 1 cm^2 pieces on selective RMOP regeneration medium. (C) Classification of spectinomycin resistant lines as putative transplastomic clones by resistance to streptomycin on RMOP medium containing streptomycin and spectinomycin (500 mg/l each). Plate is shown after 6 weeks. (D) Shoot regeneration from leaf sections on RMOP spectinomycin medium (500 mg/l) to obtain homoplastomic shoots. Plate is shown after 6 weeks. (E) Homoplastomic shoots rooted in plate or (F) in Magenta box.

Plastid Transformation Vector Design

In plastid transformation vectors the marker gene and the gene of interest, the heterologous block to be introduced, are flanked by ptDNA "targeting sequences" to ensure integration at the target site (Fig. A3-2). Three approaches were used to test mRNA editing: minigenes, translational fusion with a reporter gene, and incorporation of an editing segment in the 3'-untranslated region (3'-UTR). Conceptually the simplest design was construction of minigenes that involved insertion of an editing fragment in a plastid expression cassette linked to a marker gene. The progenitor plasmid was vector pLAA24A, a transformation vector in which a *uidA* reporter gene is expressed in a Prrn-Trps16 promoter-terminator cassette (Zoubenko et al., 1994). The editing constructs were obtained by PCR amplification of the editing segments with NcoI (5') and XbaI (3') sites at the ends that were used to replace the *uidA* reporter gene to create plasmid pMR210 (Fig. A3-2A). This approach was used to test editing, for example, of *rpoB* sites I, II, and IV (Reed and Hanson, 1997; Reed et al., 2001a; Reed et al., 2001b; Hegeman et al., 2005b; Hayes et al., 2006) and *ndhF* site 2 (Reed et al., 2001a). Editing segments as short as 27 nucleotides could be studied in minigenes because fusion with the 5'-untranslated region (5'-UTR) of the promoter and the 3'-UTR of the terminator increased the transcript size to 0.2 kb and the transcript was stable (Reed et al., 2001b).

The second approach, translational fusion with a reporter gene (Fig. A3-2B), was used to study the *psbL* and *ndhD* editing events that create a AUG translation initiation codon by editing of an ACG codon at the mRNA level (Chaudhuri et al., 1995;

Chaudhuri and Maliga, 1996). Editing of the two sites was studied in chimeric *aadA* or *kan* genes that confer spectinomycin or kanamycin resistance, respectively. Expression of the antibiotic resistance phenotype in plastids could be used to verify RNA editing because the reporter gene segment lacked an initiation codon, therefore translation of the chimeric mRNA was dependent on RNA editing. Vector pSC2 carries an editing dependent *kan* gene fused with a *psbL* editing fragment and allowed testing a series of editing fragments by replacing the *psbL* (*psbF/psbL*) editing segment (NcoI-NheI fragment) with new test segments (Chaudhuri and Maliga, 1996). Replacement of the *kan* coding segment with alternate genes, for example a *bar* gene (Lutz et al., 2006b), facilitated testing of editing fragments in a different sequence context.

The third approach to test editing was incorporation of editing segments in the 3'UTR of the *aadA* marker gene (Fig. A3-2C) where editing status of the segment does not affect expression of the marker gene. In plasmid pRB51 the editing segment can be conveniently cloned in as an XbaI-BamHI fragment (Bock et al., 1996). Derivatives of pRB51 have been used to study editing of *ndhB* sites (Bock et al., 1996; Bock et al., 1997; Hermann and Bock, 1999).

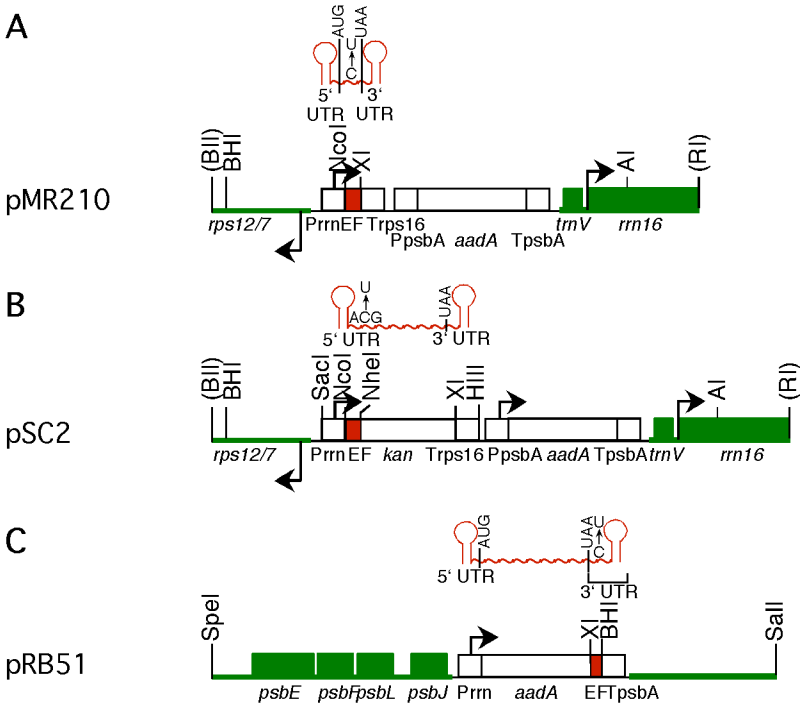

Figure A3-2. Plastid vectors to test *in vivo* RNA editing. (A) The pMR210 minigene vector with the maize *rpoB* Site I editing segment (EF, NcoI-XbaI fragment) (Reed and Hanson, 1997). (B) The pSC2 *kan* fusion vector with the *psbL* editing segment (NcoI-NheI fragment) (Chaudhuri and Maliga, 1996). (C) The pRB51 3'-UTR vector to accept editing segments as XbaI-BamHI fragments (Bock et al., 1996). The left and right plastid targeting sequences are in bold. The 5'-UTR and 3'-UTR of mRNA above the edited genes is depicted as a stem-loop structure; the position of edited nucleotide is also marked. Shown are: the spectinomycin resistance (*aadA*) and kanamycin resistance (*kan*) genes; the plastid *rrn16*, *trnV, psbE, psbF, psbL, psbJ* and *3'-rps12* genes; EF, editing fragment; Prrn promoter of the plastid ribosomal RNA operon, Trps16, 3'-UTR of plastid *rps16* gene, PpsbA, promoter of the plastid *psbA* gene; TpsbA, 3'-UTR of plastid *psbA* gene. Arrows mark transcription initiation sites.

Materials

RM plant maintenance medium (MS medium, ref.(Murashige and Skoog, 1962))(Per liter add: 100 ml 10X macronutrients, 10 ml 100X micronutrients, 5 ml 1% Fe-EDTA, 30 g sucrose, pH 5.6-5.8 with 1M KOH; 7 g agar)

RMOP shoot regeneration medium (Per liter add: 100 ml 10X macronutrients, 10 ml 100X micronutrients, 5 ml 1% Fe-EDTA, 1 ml thiamine (1mg/ml), 0.1 ml alpha-

naphthaleneacetic acid (NAA 1mg/ml in 0.1MNaOH), 1 ml 6-benzylaminopurine (BAP 1mg/ml in 0.1M HCl), 0.1 g myo-inositol, 30 g sucrose, pH 5.8 with 1M KOH, 7 g agar)

RM Medium 10x Macronutrient solution (Per liter add: 19 g KNO_3, 3.7 g $MgSO_4 \cdot 7H_2O$, 4.4 g $CaCl_2 \cdot 2H_2O$, 1.7 g KH_2PO_4, 16.5 g $(NH_4)NO_3$)

RM Medium 100x Micronutrient solution (Per liter add: 169 mg $MnSO_4 \cdot H_2O$, 62 mg H_3BO_3, 86 mg $ZnSO_4 \cdot 7H_2O$, 8.3 mg KI, 2.5 ml $Na_2MoO_4 \cdot 2H_2O$ (1 mg/ml), 2.5 ml $CuSO_4 \cdot 5H_2O$ (1 mg/ml), 0. 25 ml $CoCl_2 \cdot 6H_2O$ (1 mg/ml))

Nicotiana tabacum, c.v. Petit Havana

Agar, plant tissue culture tested (Sigma, A7921)

Spermidine free base 0.1M (Sigma S4139)

Cleaning of Gold Particles (This protocol was modified from Bio-Rad Bulletin 9075.)

1. Weigh out 30 mg 0.6 μm gold microcarrier (Bio-Rad Catalog No. 165-2262) in a 1.5 ml Eppendorf tube and add 1 ml ice-cold 70% ethanol.

2. Place tube in a Vortex microtube holder and vortex vigorously for 5 minutes. Let particles settle at room temperature (20-25°C) for 15 minutes.

3. Spin in microcentrifuge at 3000 rpm (600*g*) for 1 minute to compact gold.

4. Remove ethanol with pipette and add 1 ml ice-cold sterile distilled water.

5. Vortex tube to suspended particles. Allow particles to settle at room temperature for 10 minutes.

6. Sediment gold by spinning in microcentrifuge at 3000 rpm (600*g*) for 1 minute.

7. Remove water with pipette and add 1 ml ice-cold sterile distilled water.

8. Repeat washing gold with water by repeating Steps 5, 6 and 7.

9. Resuspended particles by vortexing and store tube at room temperature for 10 minutes to let particles settle.

10. Microcentrifuge at 5000 rpm (1700*g*) for 15 seconds then remove water completely.

11. Add 500 µl 50% glycerol and vortex for 1 minute to resuspended particles. Gold concentration will be 60 mg/ml. Clean gold can be stored for 2 weeks at room temperature.

Coating Gold Particles with Plasmid DNA

With one DNA construct 20 to 30 leaf samples are bombarded. Each tube (30 mg) of gold is sufficient for 50 bombardments (two DNA constructs). You may use freshly

prepared gold particles, or stored gold. If using stored gold, vortex tube for 5 minutes before coating with DNA. This protocol was modified from Bio-Rad Bulletin 9075.

1. Place Eppendorf tube containing gold in a Vortex microtube holder and shake at setting 3. While tube is shaking, remove 50 µl aliquots of gold and pipette into ten 1.5 ml Eppendorf tubes in a rack.

2. Place tubes with gold aliquots in Vortex microtube holder and shake at setting 3. While tubes are shaking, add 5 µl DNA (1 µg/µl), 50 µl 2.5M $CaCl_2$ and 20 µl 0.1M spermidine free base (Sigma S4139). Make sure to add in the order described and that the contents are thoroughly mixed before adding next component.

3. Shake tubes on Vortex at setting 3 for 5 minutes.

4. Sediment gold by spinning in microcentrifuge at 3000 rpm (600*g*) for 1 minute.

5. Discard the supernatant and add 140 µl 70% ethanol to each tube.

6. Tap tube lightly until the pellet just starts to come into solution to make sure pellet is not tightly packed. If gold does not go into solution by gently tapping the tube, break up pellet by pipetting up and down.

7. Sediment gold by spinning in a microcentrifuge at 3000 rpm (600*g*) for 1 minute.

8. Remove supernatant and add 140 µl ice-cold 100% ethanol to each tube.

9. Lightly tap tube until the pellet just starts to come into solution.

10. Sediment gold by spinning in a microcentrifuge at 5000 rpm (1,700*g*) for 15 seconds.

11. Resuspend coated gold pellet in 50 µl 100% ethanol by gently tapping tube. Pellet should easily enter solution. Shake tubes at setting 3 while waiting to use them for bombardment. If tubes are sitting for a long period of time before bombardment, replace ethanol in tube with fresh 100% ethanol.

Introduction of DNA into Chloroplasts by the Biolistic Process

The protocol we described here is for transformation of tobacco leaves, collected from plants grown on RM medium in Magenta boxes under sterile conditions. Plastid transformation can also be accomplished using surface sterilized leaves of greenhouse-grown plants. The protocol we describe here is for biolistic transformation with the Bio-Rad PDS1000/He biolistic gun (Catalog No. 165-2257). A suitable vacuum pump for the gun is ThermoSavant VLP285 (ThermoSavant, Holbrook, NY). With this gun, 20 to 30 leaf samples are bombarded per DNA construct. We expect to obtain one transplastomic clone per plate (range 5-0.5). The Hepta-adaptor version of the gun (which is simultaneously using seven macrocarriers) is more efficient; bombardment of five plates is sufficient to obtain a similar number of transformants.

1. Place a tobacco leaf for biolistic transformation abaxial side up on two sterile Whatman No. 4 filter papers on a Petri plate (10 cm) of solid RMOP medium (20 ml) (Fig. A3-1A). Use more than one leaf if necessary to cover the central area. (If you are using greenhouse leaves, you need to cut out a segment that covers the plate.)

2. Leaf bombardment with the gun is carried out in a sterile laminar flow hood. Before bombardment sterilize gun main chamber, rupture disk retaining cap, microcarrier launch assembly and target shelf by wiping off with a cloth soaked in 70% ethanol.

3. Sterilize rupture disks (1100 psi), macrocarriers, macrocarrier holders and stopping screens by soaking in 100% ethanol (5 minutes) then air dry them in tissue culture hood in open container.

4. Turn on helium tank and set delivery pressure in regulator (distal to tank) for 1300 psi (200 to 300 psi above rupture disk value).

5. Turn on vacuum pump and gene gun. Set the vacuum rate on the gene gun to 7 and the vent rate to 2.

6. Prepare DNA-coated gold particles as described above. Pipette 10 µl of DNA-coated gold onto one flying disk (placed in holder) and let air dry for 5 minutes. 5 samples may be made up at one time.

7. Place rupture disk into retaining cap and screw in tightly.

8. Put stopping screen and flying disk (face down) in microcarrier launch assembly and place in chamber just below rupture disk. For description see Bio-Rad Bulletin 9075.

9. Place leaf on thin RMOP plate into chamber 9 cm (4^{th} shelf from top) below the microcarrier launch assembly and close the door.

10. Press vacuum button to open valve. When vacuum reaches 28 in. Hg hold down fire button until the pop from the gas breaking the rupture disk is heard. If gun is fired at lower pressure, DNA coated particles will lack momentum to penetrate cells and no transplastomic lines will be obtained. If you have no experience with biolistic transformation, we recommend that you test particle coating and DNA delivery using transient expression of nuclear *uidA* gene, that encodes β-glucuronidase, an enzyme the activity of which can be readily detected by histochemical staining (Jefferson et al., 1987; Gallagher, 1992).

11. Immediately release the vacuum and remove the leaf sample.

12. Repeat steps 6-11 until all leaf samples are bombarded. When finished, turn off helium tank, and release pressure by holding down the fire button while vacuum is on. Turn off vacuum pump.

13. Place plastic bag over plates containing bombarded leaf samples and incubate in culture room. Incubation allows time for marker gene expression before selection is started.

14. After 2 days, cut bombarded leaves into small (1 cm square) pieces and place abaxial side up in deep RMOP-Spectinomycin (500 mg/l) plates (Fig. A3-1B). Place only 7 pieces per plate as the leaf pieces will grow and expand. Typically leaf pieces from 1 leaf bombardment fit on 3-5 thick plates. If leaf sections are too large, there will be insufficient nutrient in medium to support growth for up to 12 weeks, the time frame within which transplastomic clones appear. Diagnostic sign of overcrowding is absence of spontaneous spectinomycin resistant mutants and transplastomic clones. Overcrowding may be also caused by less than the desired 50 ml culture medium in a deep plate.

15. Individually seal each plate on the side with a strip of plastic wrap that is permeable to gas exchange (Glad ClingWrap, The Glad Products Co., Oakland, CA) and incubate plates in culture room for 4-12 weeks.

Identification of Transplastomic Plants

1. Green, spectinomycin resistant shoots appear on the bleached leaf sections between 4-12 weeks after bombardment (Fig. A3-1B). Spectinomycin resistance may be due to expression of the *aadA* gene or to a spontaneous mutation in the plastid small ribosomal RNA (*rrn16*) gene (Svab and Maliga, 1993). Each shoot at a distinct location derives

from an independent transformation event, therefore is an independently derived clone. The clones are identified by the plasmid name and a serial number.

2. Transgenic clones are resistant to both spectinomycin and streptomycin, whereas spontaneous spectinomycin resistant mutants are resistant only to spectinomycin (Svab and Maliga, 1993). To distinguish transgenic clones from mutants, test each regenerated shoot for resistance to streptomycin by inoculating small callus or small leaf sections in deep plates on selective RMOP streptomycin-spectinomycin (500 mg/l each) media. Putative transplastomic clones are resistant to both antibiotics and form green calli in three to six weeks, whereas plastid mutants will be sensitive and bleach on streptomycin-containing medium (Fig. A3-1C). Since streptomycin delays shoot regeneration, simultaneously regenerate new shoots on RMOP spectinomycin medium (500 mg/l) to obtain homoplastomic shoots (Fig. A3-1D). The *aadA* gene rarely inserts and expresses in the nuclear genome. Transplastomic clones are positively identified by confirming incorporation of *aadA* in the plastid genome by DNA gel blot analysis.

3. Identify clones that are resistant to streptomycin and spectinomycin. Take ten shoots of the same clone regenerating on RMOP spectinomycin plates and root them in RM deep plates, because not all shoots develop into a plant. Test plants from three or four clones because ~10% of plants regenerated in tissue culture are sterile due to somaclonal variation. The shoots will root and form multiple leaves in ~3 weeks (Fig. A3-1E,F). Shoots regenerated from the same clone (initial shoot) are considered subclones and are distinguished with letters. Normally, we perform DNA gel blot analysis on two shoots

from three to four clones. Southern analysis will show that some of the plants are homoplastomic (all ptDNA copies transformed), some are heteroplastomic (contain transformed and wild-type ptDNA copies) and some contain only wild-type ptDNA copies.

4. Although Southern analysis indicates the homoplastomic state, a small number of wild-type ptDNA copies may remain undetected. Repeating plant regeneration on RMOP spectinomycin medium (500 mg/l) from the leaves of homoplastomic plants identified in Step 3 ensures that no wild-type ptDNA is retained.

5. Transfer homoplastomic plants to soil in the greenhouse after gently breaking up the agar, and washing off the agar-solidified RM medium.

6. Cover pots with household plastic foil (Glad ClingWrap, The Glad Products Co., Oakland, CA) to prevent desiccation. Grow plants in shade for about a week, then remove plastic foil and expose plants to full sunlight.

7. Collect mature seed pods from transplastomic plants and let dry on lab bench at room temperature for one week.

8. Germinate surface-sterilized seeds (Lutz et al., 2006a) on RM-spectinomycin (500 mg/l) medium. Homoplastomic seedlings will be dark green, whereas sensitive seedlings

will be white. One hundred percent green seedlings confirm homoplasmic state of the plant; no segregation for spectinomycin resistance should be seen.

Protocols for Testing RNA Editing in Tobacco Chloroplasts

Study of cDNA is the most reliable method to identify RNA editing sites in plants. Occasionally digestion of cDNA at fortuitous restriction endonuclease sites can be used to determine if RNA editing has occurred, but the creation/absence of these sites by RNA editing is rarely feasible. The most common in vivo approach is sequencing of reverse transcribed PCR amplified cDNA. RNA isolation can be performed using the TRIzol ((Chomczynski and Sacchi, 1987) (Invitrogen, Carlsbad, CA) or lithium chloride (Stiekema et al., 1988) methods or with the Qiagen kit (Qiagen, Valencia, CA). We found that good quality RNA can be obtained in a short time from 100 mg tobacco leaf tissue using Qiagen RNeasy Plant Mini Kit Catalog # 74903. The TRIzol and lithium chloride methods yield more RNA, but contain more contaminating DNA. We describe here a protocol for reverse transcription of RNA and PCR amplification of cDNA but not DNA sequencing, because there are no plant-template specific protocols.

Reverse Transcription of RNA and PCR Amplification of cDNA

Due to the sensitivity of PCR it is essential to remove all traces of DNA before PCR amplification of the cDNA with DNaseI using the QIAGEN RNase-Free DNase Set protocol (Catalog # 79254). To verify that no contaminating DNA is present, a control PCR experiment should be performed using the RNA as template: the absence of PCR

product in the control indicates the absence of contaminating DNA. The protocol to obtain cDNA is as follows.

1. Resuspend 0.5 μg RNA in RNase-free water to a total volume of 33.5 μl. Add 3 μl of random primers (Promega, Madison, WI) and incubate at 95°C for 1 minute and immediately place on ice to anneal the primers.

2. To perform the reverse transcription reaction, add to the tube 5 μl 10X PCR buffer (100 mM Tris-HCl pH 8.4, 500 mM KCl, 15 mM $MgCl_2$, 0.1% w/v gelatin), 4 μl 2.5 M dNTPs, 0.5 μl RNAguard (GE Healthcare, Piscataway, NJ), 1.5 μl AMV reverse transcriptase (USB Corporation, Cleveland, OH). Place the tube at room temperature for 10 minutes and then transfer to 42°C for 50 minutes.

3. To PCR amplify the cDNA, add 1 μl of each of the gene-specific oligonucleotide pair (100 pmol/μl each) and 0.5 μl of Ampiltaq polymerase to tube and run PCR program: 3 minutes at 92°C, 30 cycles of 1 minute at 92°C, 1 minute at 55°C, 1 minute at 72°C, 1 cycle of 11 minutes at 72°C, then keep tube at 4°C. Run 5 μl of PCR reaction on a 0.8% agarose gel to check products.

4. To determine if all DNA has been removed from the RNA sample perform a control PCR reaction with RNA that has not undergone reverse transcription. Resuspend 0.5 μg DNaseI-treated RNA in RNase-free water to a final volume of 38.5 μl. Add 5 μl 10X PCR buffer, 4 μl 2.5 M dNTP's, 1 μl each of the oligonucleotides (100 pmol/μl) and 0.5

μl of Ampiltaq polymerase. Perform PCR reaction as described in Step 3. Run 5 μl of PCR reaction on a 0.8% agarose gel to check products. If gene-specific PCR fragment is obtained, the RNA sample is contaminated with DNA. The cDNA prepared with the contaminated template should be discarded and DNaseI treatment of the RNA should be repeated.

5. PCR amplify a DNA sample to obtain template for the reference sequence by mixing: 1 μl (20ng/μl) of tobacco total leaf DNA prepared with the CTAB protocol (Lutz et al., 2006a), 5 μl Amplitaq 10X AmpliTaq PCR Buffer (Applied Biosystems, Foster City, CA), 1 μl each of the gene-specific primers (100 pmol/μl), 4 μl 2.5 M dNTP's, 0.5 μl AmpliTaq (Applied Biosystems, Foster City, CA), 37.5 μl H_20. Use PCR program of Step 3. Run 5 μl of reaction on a 0.8% agarose gel to check PCR products.

Conclusions and Future Directions

Progress in understanding RNA editing in plastids has been driven by technical innovation. Plastid transformation reviewed here provided the first experimental tool to study *cis*-sequences and *trans*-factors involved in editing. The toolkit available to study plastid RNA editing has recently been expanded to include *in vitro* editing systems (Hirose and Sugiura, 2001; Hegeman et al., 2005a; Sasaki et al., 2006) reviewed elsewhere in this volume. The components of the plastid RNA editing machinery still elude identification. A breakthrough in this area may be achieved by purification and identification of the components by a proteomics approach or by genetic screens to identify mutations in nuclear genes encoding components of the editing machinery.

Future studies will focus on understanding the role of RNA editing in plastids. RNA editing is a corrective mechanism when it restores a functionally important, conserved amino acid, as reported for the *psbF* (Bock et al., 1994), *accD* (Sasaki et al., 2001), *atpA* (Schmitz-Linneweber et al., 2005) and *ndhD* (Hirose and Sugiura, 1997) (Kotera et al., 2005) genes. Since both edited and non-edited mRNAs are translated, RNA editing may serve as a regulatory mechanism yielding multiple proteins from the same gene. The plastid *ndh* genes encode a significant number of editing sites; at least one of these is differentially edited in light-grown and dark-grown tissues establishing a possible link between photosynthesis, light-induced chloroplast development and RNA editing (Karcher and Bock, 2002). Interestingly, the editing sites in the plastid RNA polymerase β subunits are clustered adjacent to the dispensable region (Corneille et al., 2000). This means that multiple, catalytically active RNA polymerase β subunit polypeptides may be obtained from edited and non-edited mRNAs. Identification of the components of the RNA editing machinery will open the way to study the role of editing in plastid metabolism and in regulation of plastid gene expression.

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Appendix 4

Steroid-inducible BABY BOOM Facilitates Recovery of Fertile, Spectinomycin Resistant Mutant in *Arabidopsis thaliana*

Introduction

Small size, rapid life cycle and DNA sequence information on its nuclear (Arabidopsis Genome Initiative, 2000), plastid (Sato et al., 1999) and mitochondrial (Unseld et al., 1997) genomes made *Arabidopsis thaliana* the most advanced plant model species. Because of the available genomic resources, rapid progress is being made to understand plastid function and the interaction of plastid and nuclear genes (Nott et al., 2006). Plastid transgenes would be useful complements as experimental tools to identify nuclear genes controlling plastid gene transcription, mRNA translation and RNA editing.

Transplastomic plants have already been obtained in Arabidopsis using leaf cells as recipients for plastid transformation (Sikdar et al., 1998). However, the transplastomic clones were sterile. Possible reasons for the sterility of the plants could be polyploidy or aneuploidy caused by 2,4D treatment necessary to trigger uniform leaf cell division; somaclonal variation caused by prolonged selection in culture required to obtain a uniform population of transformed plastid genome (ptDNA) copies; and natural polyploidy of mature Arabidopsis leaf cells that were the recipients of transforming DNA (Galbraight et al., 1991; Melaragno et al., 1993; Zoschke et al., 2007). Therefore, we were looking for a tissue culture system that enables induction of uniform cell division, sustained capacity for plant regeneration and maintenance of diploid state. Meristematic cells in a shoot apex or cells of a developing embryo meet these criteria. We decided to

test whether or not embryogenic cultures obtained by ectopic expression of transcription factors are suitable for regeneration of fertile plants after prolonged tissue culture selection.

Constitutive expression of BABY BOOM (BBM), a member of the AP2/ERF family of transcription factors (Riechmann et al., 2000; Nole-Wilson et al., 2005), was reported to sustain spontaneous production of somatic embryos but did not yield fertile plants (Boutilier et al., 2002). Therefore we decided to regulate BABY BOOM function by fusing BBM with the glucocorticoid receptor steroid-binding domain. Direct fusion of plant transcription factors with a steroid nuclear receptor has been successfully employed to regulate expression, for example, of the maize transcriptional regulator R (Lloyd et al., 1994), APETALA1 (Wagner et al., 1999), SHOOT MERISTEMLESS (Gallois et al., 2002) KNOTTED1 (Hay et al., 2003); and recently BABY BOOM (Srinivasan et al., 2007). Although the fusion proteins are constitutively expressed, transcription of the downstream target genes is dependent on the supply of steroid hormones in the culture medium. In the absence of the steroid hormone, the receptor associates with cellular regulatory proteins, including Hsp90, and becomes anchored in the cytosol as a monomer. Association of steroid with the hormone-binding domain leads to the release of HSP90 from the receptor. The receptor subsequently dimerizes, translocates into the nucleus and binds to the target DNA to activate transcription (Zuo and Chua, 2000).

Here we report on a translational fusion of the BABY BOOM (BBM) transcription factor with the green fluorescent protein (GFP) and the glucocorticoid receptor (GR) steroid-binding domain that enables regeneration of fertile plants after extended periods of tissue culture. Plants carrying the fusion protein were

indistinguishable from non-transformed plants in the absence of the inducer. However, when the synthetic steroid dexamethasone was included in the culture medium, prolific shoot regeneration on Arabidopsis leaf segments and formation of somatic embryos on seedlings was obtained. The utility of the system was shown by recovery of fertile plants of the Ler-SPC2 line carrying a plastid-encoded spectinomycin-resistant mutation in the 16S rRNA.

Results

Construction of Arabidopsis plants with an inducible BBM *gene*

To construct a steroid-inducible BBM, the *BBM* coding region was translationally fused with the coding region of *gfp*, the *Aequorea victoria* green fluorescent protein (GFP) and the ligand-binding domain of the rat glucocorticoid receptor (GR). The BBM:GFP:GR fusion protein was cloned into a Cauliflower Mosaic Virus 35S expression cassette in a derivative of the pPZP200 binary plasmid carrying gentamycin resistance as a plant marker gene to create plasmid pKO216 (Figure A4-1). *Arabidopsis thaliana* RLD and Landsberg erecta (Ler) plants were transformed with Agrobacterium strain EHA101 carrying binary plasmid pKO216 by the floral dip protocol. Eight independent insertion events were identified by selection of seedlings on medium containing 25 mg l^{-1} gentamycin. The gentamycin resistant seedlings were transferred to pots where they developed into normal plants, flowered and produced seed. Lines were designated by the ecotype, the plasmid name and a serial number, for example Ler-pKO216-2.

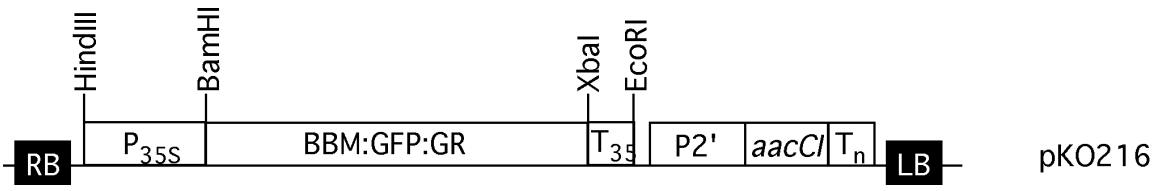

Figure A4-1. The map of the Agrobacterium binary vector pKO216 T-DNA region. Shown are the BBM:GFP:GR coding region expressed in a 35S promoter-terminator (P_{35S}/T_{35S}) cassette, the *aacC1* gentamycin resistance gene expressed in the P_2/T_{nos} cassette, and the T-DNA left (LB) and right (RB) border regions.

Induction of somatic embryos from seed in the absence of plant hormones

BBM was shown to induce formation of somatic embryos in the absence of plant hormones (Boutilier et al., 2002). To test if the BBM:GFP:GR construct is biologically active, we germinated seed on a hormone-free plant maintenance medium (ARM) containing dexamethasone (DEX) (5 μM) and gentamycin (50 mg l^{-1}). We have found that seedlings in six out of the seven independently transformed lines tested formed clusters of somatic embryos and cotyledons indicating that the fusion proteins are biologically active (Figure A4-2a). Interestingly, not all gentamycin resistant seedlings responded by forming embryos indicating that the transgene phenotype does not have 100% penetrance. We therefore decided to test if a rapid, uniform tissue culture response can be obtained in BBM:GFP:GR plants in the absence of 2,4-D. The experiments were carried out with non-segregating, gentamycin resistant progenies identified in two of the lines, Ler-pKO216-2-31 (Ler-216-2) and RLD-pKO216-22-2 (RLD-216-22).

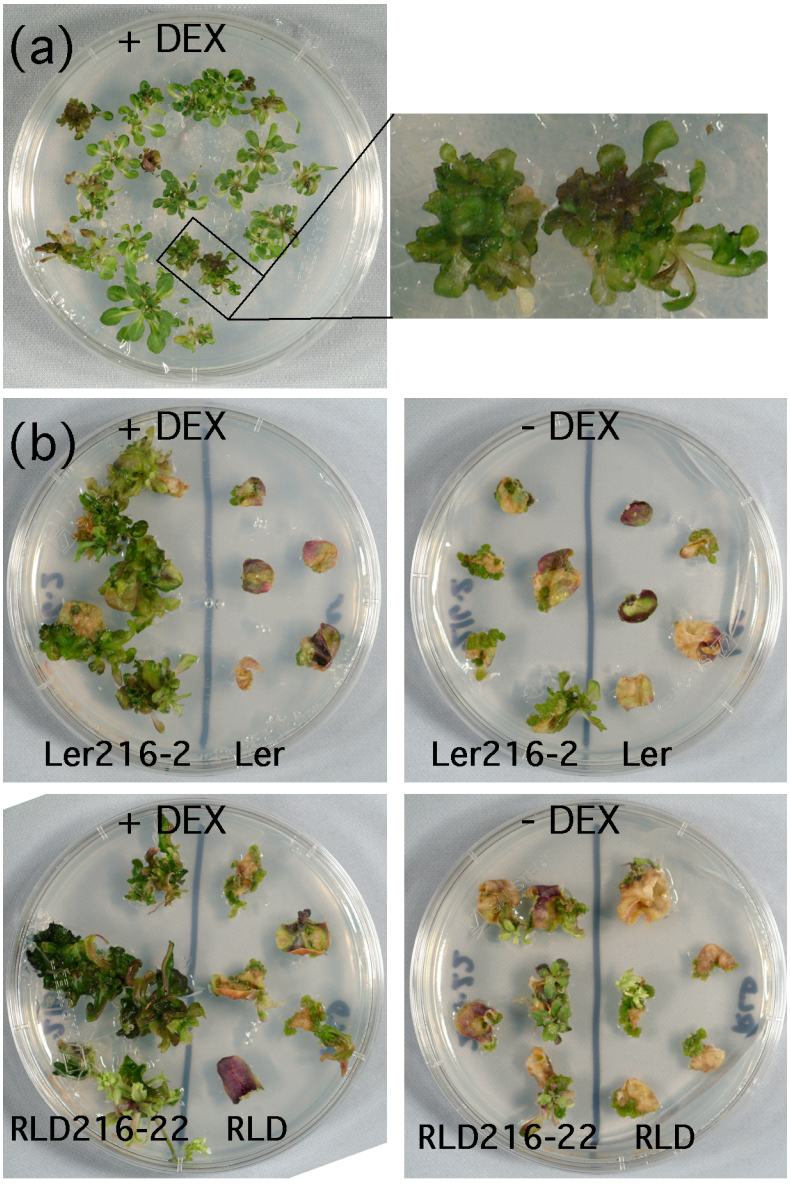

Figure A4-2. Dexamethasone-inducible plant regeneration in *A. thaliana* transformed with the BBM:GFP:GR gene in plasmid pKO216. (a) Formation of embryogenic clusters from Ler-pKO216-2 seedlings germinated on hormone-free ARM medium containing dexamethasone. (b) DEX (5 μM) induces shoot regeneration from BBM:GFP:GR-transformed Ler-216-2 and RLD-216-22 Arabidopsis leaves on ARM-B medium.

BBM potentiates shoot regeneration from leaves

Capacity for plant regeneration in *Arabidopsis thaliana* is ecotype dependent and depends on explant type: ecotype RLD is easier to regenerate than Landsberg erecta

(Ler); roots regenerate plants faster than leaves (Valvekens et al., 1988; Marton and Browse, 1991). Cell division and efficient shoot regeneration from both leaves (Feldmann and Marks, 1986) and roots (Valvekens et al., 1988; Marton and Browse, 1991) was dependent on short-term cultivation of explants on a medium containing 2,4-D, a synthetic auxin. Since BBM is known to induce somatic embryogenesis in the absence of plant hormones (Boutilier et al., 2002), we cultured leaf sections with and without the inducer, 5 μM dexamethasone, on ARM I medium lacking 2,4-D (ARM-B medium) to test if BBM expression can replace the short-term 2,4-D treatment. In one month most leaf sections of wild-type Ler turned brown and died on the ARM-B medium (ARM medium containing IAA, 2IP and BA, but not 2,4-D), whether or not the inducer was included in the culture medium (Figure A4-2b). In dramatic contrast, Ler-pKO216-2 leaf sections produced prolific shoots and some callus in the presence of DEX. Activation by DEX is somewhat leaky, since some shoot regeneration is also seen in the absence of the inducer (-DEX, Ler-216-2; Figure A4-2b). The BBM response in the RLD background is similar, but reflects an overall better potential for shoot regeneration from leaf sections. Thus, significantly enhanced, uniform shoot regeneration could be obtained in both ecotypes in the presence of the inducer indicating that BBM expression could replace induction by 2,4-D.

Identification of spectinomycin resistant mutants in embryogenic cultures

Selection of transplastomic clones by spectinomycin resistance is anticipated in one of two systems: in cultures initiated from seed or in embryogenic roots. We tested the utility

of these systems by selection of spectinomycin resistant mutants using the steroid inducible *BBM:GFP:GR* gene. Spectinomycin resistant mutants in seedling-derived cultures were selected in mutagenised seed populations (2.5 mM NMU). Seed were selected on agar-solidified ARM-B medium containing 5 μM DEX and spectinomycin (25 and 100 mg l^{-1}). The resistant mutants were identified as green sectors on ~ 0.5 % of the seedling calli (Table A4-1), which were white due to inhibition of protein synthesis on the prokaryotic-type plastid ribosomes (Figure A4-3a).

Selection for spectinomycin resistance was also carried out in embryogenic Arabidopsis root cultures in a liquid ARM-B medium containing 5 μM DEX and spectinomycin (25 mg l^{-1} and 100 mg l^{-1}). One of the cultures contained a large green embryogenic callus (Figure A4-3b), which was transferred to DEX-free medium to facilitate shoot regeneration. The At-Ler-SPC2 shoots were transferred to hormone-free ARM medium where they flowered and set seed in a Magenta box. The seed was sown on a hormone-free ARM medium in the presence of spectinomycin (100 mg l^{-1}) (Figure A4-3c). Spectinomycin resistant green Ler-SPC2 seedlings confirmed inheritance of the plastid-encoded spectinomycin resistance mutation (see below).

Spectinomycin resistance in tobacco and *Solanum nigrum* was shown to be due to mutations in the plastid small 16S rRNA subunit (Fromm et al., 1987; Svab and Maliga, 1991; Kavanagh et al., 1994). We therefore PCR-amplified the *rrn16* gene (encoding 16S rRNA) from four mutants derived from the mutagenized seedlings and the Ler-SPC2 spontaneous mutant. Direct sequencing of PCR-amplified DNA identified three point mutations. Two of the mutations are known to confer spectinomycin resistance in tobacco: C to T at nucleotide position 102,151 and G to A at position 102,152 in the *A.*

thaliana ptDNA, and eliminate an *AatII* restriction site. A third mutation, a C to T exchange at nucleotide position 102,026, has been described in *E. coli* (position 1066)(Johanson and Hughes, 1995) but not yet in plastids. This mutant carries a second point mutation, a G to A exchange at position 102,217, which is not known to be involved in spectinomycin resistance. Spectinomycin resistance mutations identified in this study are listed in Figure A4-4. We maintain the Ler-SPC2 spontaneous mutant by seed. No attempt was made to obtain plants from the mutagenised seedlings since NMU mutagenesis normally yields plants with fertility problems.

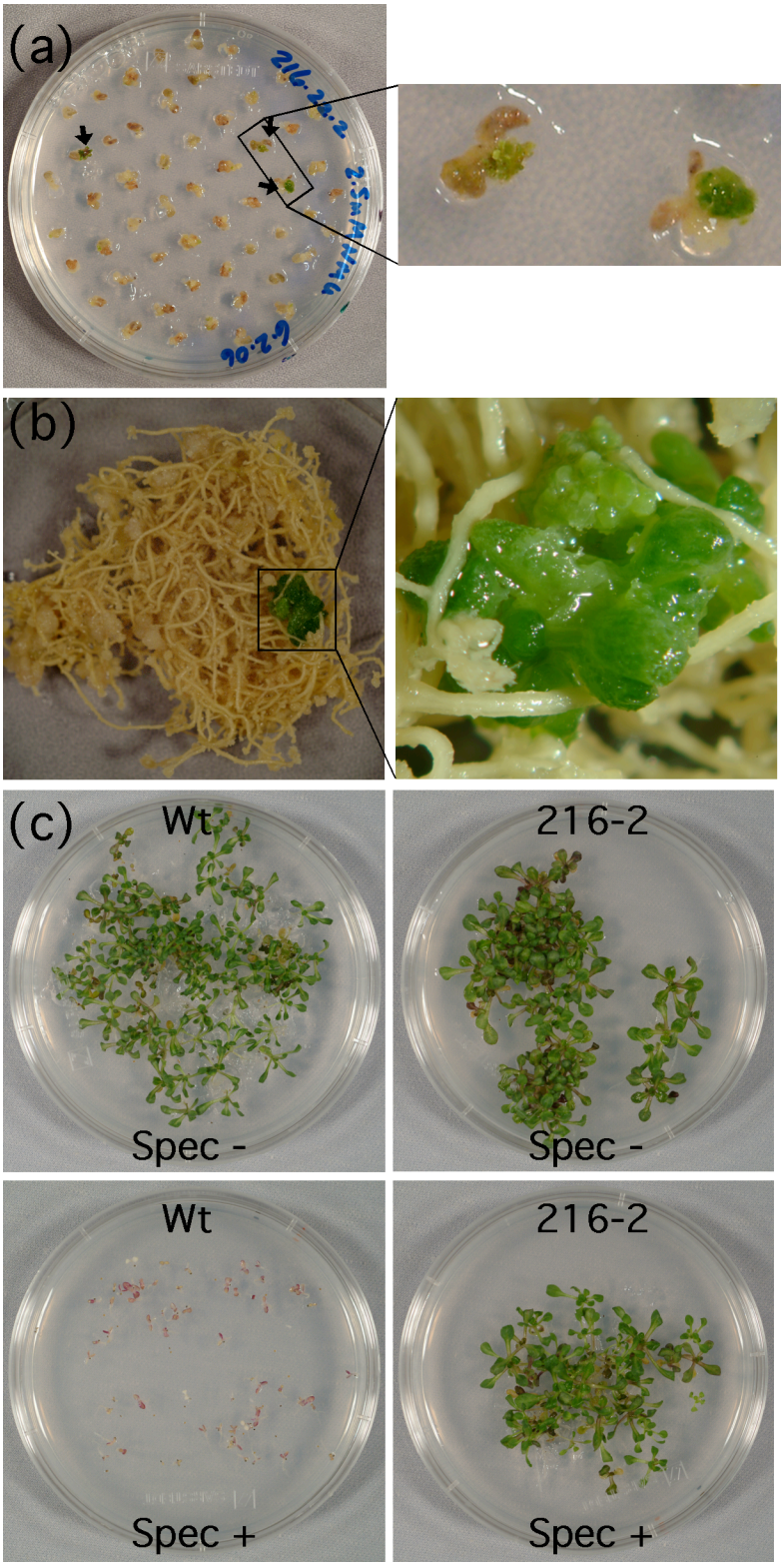

Figure A4-3. Isolation of spectinomycin resistant mutants in *Arabidopsis thaliana*. (a) Identification of spectinomycin resistant mutants in mutagenized Arabidopsis seed culture. The seedlings were germinated on ARM-B medium containing 5 μM DEX and

spectinomycin (25 mg l^{-1}) and selected on ARM-B medium containing 100 mg l^{-1} spectinomycin. Note mutants forming green embryogenic callus. (b) The Ler-SPC2 spontaneous spectinomycin resistant mutant in root culture. (c) Inheritance of spectinomycin resistance in the Ler-SPC2 seed progeny.

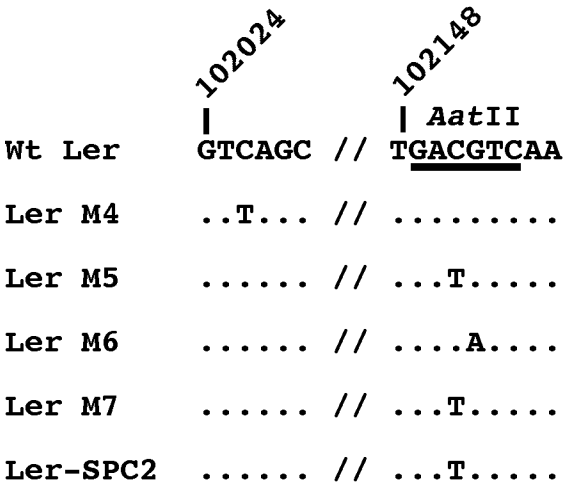

Figure A4-4. Mutations conferring spectinomycin resistance in the plastid *rrn16* gene encoding the 16S rRNA ribosomal subunit.

Discussion

We report here posttranscriptionally regulated plant regeneration in *Arabidopsis thaliana* based on a BBM:GFP:GR fusion protein. Plants expressing the *BBM:GFP:GR* gene from a constitutive promoter are normal in the absence of DEX, the GR ligand. BBM:GFP:GR biological activity in the presence of the inducer DEX was demonstrated in two systems: in seed culture and in leaf culture. Seeds carrying a *BBM:GFP:GR* gene formed somatic embryos in the absence of plant hormones as seen in seedlings constitutively expressing BBM (Boutilier et al., 2002). The response was not uniform as only some of the seeds formed embryo clusters indicating less than a 100% phenotypic penetrance of the *BBM:GFP:GR* gene. Interestingly, enhanced shoot regeneration on hormone-containing

medium in BBM:GR:GFP leaves was uniform, enabling plant regeneration. Different Arabidopsis ecotypes regenerate plants at different efficiencies (Schmidt and Willmitzer, 1988; Luo and Koop, 1997). Thus, one use of the BBM system will be boosting morphogenic response in ecotypes which are recalcitrant to plant regeneration.

Experiments reported here were carried out with a protein obtained by fusing three proteins: BBM, GFP and GR. Although the protein contained GFP, no GFP fluorescence was detectable in the cells (data not shown). Plants expressing a BBM-GR fusion protein obtained by transformation with vector pKO214 behaved similarly: had no phenotype in the absence of the inducer and formed embryogenic clusters in the absence of hormones from seed (data not shown). While this manuscript was in preparation, a similar BBM:GR fusion protein was described, and shown to enhance plant regeneration in tobacco (Srinivasan et al., 2007).

BBM is not the only regulated plant regeneration system in Arabidopsis. The WUSCHEL gene, when expressed in a transcriptionally regulated system, promoted vegetative-to-embryonic transition and yielded fertile plants after the removal of the inducer (Zuo et al., 2002). LEC1 and LEC2 are two additional seed-expressed transcription factor genes which, when expressed constitutively, promoted spontaneous embryo formation on vegetative tissues (Lotan et al., 1998; Stone et al., 2001). Interestingly, regulated over-expression of LEC1 cited in ref. (Zuo et al., 2002) and induction of a LEC2:GR fusion (Santos Mendoza et al., 2005) did not result in formation of embryo-like structures.

When we transferred embryos from the embryogenic clusters to inducer-free medium, the embryos grew into fertile plants and set viable seed in Magenta boxes on

ARM medium (data not shown). This system was useful to obtain fertile plants from the spontaneous spectinomycin resistant mutant Ler-SPC2, which was grown in a selective spectinomycin medium for three months in the presence of the inducer. Since the Arabidopsis ecotypes have distinct plastid DNA markers (Azhagiri and Maliga, 2007), this mutant represents a valuable resource that enables tracking of plastids in crosses of different ecotypes. The new steroid-inducible BBM system will facilitate the recovery of fertile transplastomic plants.

Experimental procedures

Construction of BBM:GFP:GR plants

To construct a steroid-inducible BBM, the *BBM* coding region was translationally fused with the coding region of *gfp*, the *Aequorea victoria* green fluorescent protein, and the ligand-binding domain of the rat glucocorticoid receptor (GR). The fusion protein is contained in a *Bam*HI-*Xba*I fragment. The BBM coding region was PCR amplified from cDNA (Genbank Accession Number AF317907). The *gfp* gene is a derivative of plasmid *psmGFP*, encoding the soluble modified version of GFP (GenBank Accession No. U70495) obtained under order number CD3-326 from the Arabidopsis Biological Resource Center, Columbus, OH (Davis and Vierstra, 1998). The ligand-binding domain of the rat glucocorticoid receptor (508-795) derives from plasmid pBI-ΔGR constructed by Alan Lloyd (Lloyd et al., 1994). Plasmid pKO216 is a pPZP200 binary plasmid derivative (Hajdukiewicz et al., 1994) in which the plant gentamycin resistance marker is expressed in a 2' promoter and *nos* terminator cassette, and the BBM:GFP:GR coding region is cloned as a *Bam*HI-*Xba*I fragment in the Cauliflower Mosaic Virus 35S

promoter-terminator cassette derived from plasmid pFF19G (Timmermans et al., 1990). Plasmid pKO216 was introduced into the RLD and Ler ecotypes of *Arabidopsis thaliana* by the floral dip protocol (Clough and Bent, 1998). Eight independent insertion events were identified by selection of seedlings on medium containing 25 mg l^{-1} gentamycin, a relatively low concentration because in this vector variant the *aacC1* gene is poorly expressed.

Tissue culture media

The tissue culture protocols were adopted from (Marton and Browse, 1991; Czako et al., 1993). The *Arabidopsis* tissue culture media (ARM) are derivatives of the Murashige & Skoog MS medium (Murashige and Skoog, 1962). ARM medium: MS salts, 3% sucrose, 0.8% agar (A7921; Sigma, St. Louis, MO), 200 mg myo-inositol, 0.1 mg l^{-1} biotin (1 ml l^{-1} of 0.1 mg m l^{-1} stock), 1 ml vitamin solution (10 mg vitamin B1, 1 mg vitamin B6, 1 mg nicotinic acid, 1 mg glycine per ml), pH 5.8. ARM-B medium: ARM medium containing 3 mg indolacetic acid (IAA), 0.6 mg benzyladenine (BA) and 0.3 mg isopentenyladenine (IPA) per liter. The *Arabidopsis* shoots were rooted on ARM medium. *Arabidopsis* seed culture (ARM5) medium: ARM medium supplemented with 5% sucrose (Sikdar et al., 1998). The stocks of plant hormones were filter sterilized, and added to media cooled to 45ºC after autoclaving. Dexamethasone (D4902) purchased from Sigma, St. Louis, MO, was used at 5 μM final concentration in the culture medium. The 10 mM stock solution was prepared by dissolving 4 mg DEX in 1 ml DMSO and filter sterilized using DMSO Safe Acrodisc Syringe Filter (Pall Corporation, Ann Arbor, MI).

Selection of spectinomycin resistant mutants

Seed were vapor sterilized in a 1.5 ml eppendorf tube overnight in a desiccator with a flask containing 100 ml bleach and 3 ml of concentrated (37.8%) HCl. Flasks should be placed in desiccator immediately after bleach and HCl are mixed. Seed were sprinkled onto ARM plates, cold treated by incubating plates in cold (4 ºC) for three days, then transfered to the culture room (16 hrs light cycle; 25 ºC).

Mutagenesis of sterile seed was carried out by incubating the seed for 6 hrs at 25ºC in 2.5 mM nitrosomethyl urea (NMU) (Sigma, St. Louis, MO) dissolved in citric acid –Na_2PO_4 buffer, pH 5.0 (Hagemann, 1982). The seeds were rinsed in sterile distilled H_2O, and plated on selective (25 mg l^{-1} spectinomycin HCl) ARM I (Ler-216-2) or ARM-B (Ler; RLD, RLD-216-22) medium containing 5 μM DEX, cold treated, then germinated. The seedlings were then transferred twice (for two weeks each) onto a selective ARM-B medium containing spectinomycin and 5 μM DEX. During the 1st and 2nd selection cycles the medium contained 25 mg l^{-1} and 100 mg l^{-1} spectinomycin HCl, respectively. Spectinomycin resistant mutants were identified during the second selection cycle.

The spontaneous mutant Ler-SPC2 was identified in Ler-pKO216-2 root culture. Sterilized seed were sprinkled onto SCM plates (Zuo et al., 2002) supplemented with 1 ml vitamin stock solution, 2 % glucose, 1 mg l^{-1} IAA, 0.3 mg l^{-1} IPA and 5 μM Dex. One week after germination the hypocotyls with the roots were transferred into liquid ARM-B medium containing 5 μM DEX. After ~1.5 months of incubation on a gyratory shaker (90 rpm) in the light (16 hrs cycle) roots were transferred to a fresh ARMB medium containing spectinomycin (25 mg l^{-1}) and 5 μM DEX. After two weeks the medium was

replaced with a new batch of the same selective medium; during the 3rd selection cycle the spectinomycin concentration was increased to 100 mg l^{-1}. The spontaneous Ler-SPC2 mutant was identified three months after starting the spectinomycin selection.

Sequencing of rrn16 *genes*

A 958 bp fragment of the plastid *rrn16* gene was PCR amplified using primers Lf1 5'-GGAGGAGTACGCCATGCTAATGTG-3' and Lf2 5'-CGCCTGTGTCCACCACGTACCGAC-3' with the following PCR program: 3 min at 94ºC, followed by 30 cycles of 30 seconds at 94ºC, 1 minute at 55ºC and 1 min at 72ºC; and 1 cycle of 11 minutes at 72ºC. The PCR fragment was excised from an agarose gel and the DNA was isolated with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Direct sequencing of the PCR fragment was performed by Genewiz (South Plainfield, NJ) using primer Lf3 5'-GCAGCCTGCAATCCGAACTG-3'.

Acknowledgements

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Table A4-1 Selection of spectinomycin resistant mutants on ARM-B medium in mutagenized (2.5 mM NMU) Arabidopsis seedling culture

Line	**No. of seed**	**No. mutants**
RLD	200	11
RLD-216-22	200	7
Ler	200	1
Ler-216-2	410	5

Author contributions to manuscript on which Appendix 4 is based: Kerry Lutz, Arun Azhagiri and Pal Maliga designed research. KL performed experiments; KL, AA and PM analysed data and KL and PM wrote the manuscript.

Curriculum Vitae for Kerry LUTZ

Education

Rutgers, the State University of New Jersey, New Brunswick, NJ
2003-2007 Ph.D. Plant Biology
Thesis Title: Site-Specific Recombinases to Manipulate the Plastid Genome

Binghamton University, Binghamton, NY
1995-1997 M.A. Biological Sciences
Thesis Title: Characterization of two marine *Cyanothece* sp. capable of aerobic nitrogen fixation.

Rutgers, the State University of New Jersey, New Brunswick, NJ
1991-1995 B.A. Biology

Professional Experience

1997-2003 Laboratory Researcher, Rutgers University, Dr. Pal Maliga

Publications

Lutz, K.A. and Maliga P. (2007) Transformation of the plastid genome to study RNA editing. Methods Enzymol. **23:**502-516.

Lutz, K.A. and Maliga P. (2007) Construction of marker-free transplastomic plants. Curr. Opin. Biotecnol.**18:**107-114.

Kittiwongwattana, C., Lutz, K.A., Clark, M. and Maliga, P. (2007) Plastid marker excision by the phiC31 phage site-specific recombinase. Plant Mol. Biol.**64:**137-143.

Chakrabarti, S.K., Lutz, K.A., Lertwiriyawong, B., Svab, Z. and Maliga, P. (2006) Expression of the *cry9Aa2 B.t.* gene in tobacco chloroplasts confers resistance to potato tuber moth. Transgenic Res. **15:**481-488.

Lutz, K.A., Svab, Z. and Maliga P. (2006) Construction of marker-free transplastomic tobacco using the CRE-*loxP* site-specific recombination system. Nat. Prot. 10.1038/nprot.2006.118.

Lutz, K.A., Bosacchi, M.H., and Maliga, P. (2006) Plastid marker-gene excision by transiently expressed CRE recombinase. Plant J. **45:**447-456.

Lutz, K.A., Corneille, S., Azhagiri, A.K., Svab, Z. and Maliga P. (2004) A novel approach to plastid transformation utilizes the phiC31 phage integrase. Plant J. **37:**906-913.

Corneille, S., Lutz, K.A., Azhagiri, A.K. and Maliga P. (2003) Identification of functional *lox* sites in the plastid genome. Plant J. **35:**753-762.

Lutz, K.A., and Maliga, P. (2001) Lack of conservation of editing sites in mRNAs that encode subunits of the NAD(P)H dehydrogenase complex in plastids and mitochondria of *Arabidopsis thaliana*. Curr. Genet. **40:**214-219.

Corneille, S., Lutz, K., Svab, Z. and Maliga P. (2001) Efficient elimination of selectable marker genes from the plastid genome by the CRE-*lox* site-specific recombination system. Plant J. **27:**171-178.

Lutz, K.A., Knapp, J.E. and Maliga, P. (2001) Expression of *bar* in the plastid genome confers herbicide resistance. Plant Physiol. **125:**1585-1590.

Corneille, S., Lutz, K. and Maliga, P. (2000) Conservation of RNA editing between rice and maize plastids: are most editing events dispensable? Mol. Gen. Genet. **264:**419-424.

Patents

WO 2004/078935 entitled “Removal of plastid sequences by transiently expressed site-specific recombinases”. Inventors: P. Maliga and K. Lutz.

WO 2002/079409 entitled “Integrases for the insertion of heterologous nucleic acids into the plastid genome”. Inventors: P. Maliga, S. Corneille and K. Lutz.

WO 2001/021768 entitled "Site-specific recombination system to manipulate the plastid genome of higher plants". Inventors: P. Maliga, S. Corneille and K. Lutz.