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Plastid Engineering in Land Plants: A Conservative Genome is Open to Change

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Plastid engineering in land plants: a conservative genome is open to change

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SUMMARY

We have developed efficient transformation protocols to modify each of the 500–10 000 plastid genome copies in a tobacco cell. The transforming DNA is introduced on the surface of microscopic tungsten particles by the biolistic process. Selection for transplastomes is by spectinomycin resistance based on expression of aminoglycoside-3′′-adenyltransferase from a chimeric *aadA* gene in the transforming DNA. Manipulations that are now feasible include replacement of endogenous plastid genes with DNA sequences modified *in vitro*, targeted gene disruption, and insertion of reporter genes into the plastid genome. Alternative methods for plastid genome manipulations may be developed utilizing an extrachromosomal element which was identified during the transformation studies.

Introduction of foreign genes under control of plastid gene expression elements results in duplication of endogenous regulatory sequences. A sensitive somatic assay to detect deletions via such direct repeats confirmed that these sequence duplications do not result in significant genome instability.

The ability to transform plastids will facilitate the study of plastid gene regulation, and the application of genetic engineering to crop improvement.

1. INTRODUCTION

The circular, 120- to 180-kb genome (ptDNA) is identical in all plastid types, and includes a region that is duplicated in an inverted orientation (Palmer 1985). However, the number of plastids and ptDNA per cell is dependent on the cell type. There are only 10–15 proplastids in a meristematic cell, each containing about 50 ptDNA copies. In a leaf cell, there may be as many as 100 chloroplasts, each with about 100 ptDNAs, giving a total of 10 000 genome copies per cell. There may be significant species-specific deviation from these main values, with a total number of genome copies per leaf cell in the range of 1900–50 000 (Bendich 1987). Plants must have an efficient repair system that constantly monitors the genome copies for sequence alterations and eliminates new mutations, since uniformity of the genome copies is maintained within a species. Transformation of the plastid genome requires that each of the ptDNA copies be uniformly altered, in spite of the operation of the system that maintains uniformity of genome copies. Plastid transformation is achieved through a multi-step process that takes at least 15–20 cell divisions. Transformation is initiated by the integration of foreign DNA into a single genome copy, followed by replication of the transplastome, and sorting of transformed and wild-type genome copies under selection pressure that eventually yields homoplasmic lines (Maliga 1993). Success of transformation depends on the efficiency of genetic markers that are used to identify and selectively amplify the transgenomes. Plastid sorting is facilitated by the lack of a requirement for an exact duplication of the cytoplasm upon cell division. This is in contrast to chromosomal DNA which is precisely duplicated and distributed to the daughter cells during somatic cell division.

Plastid transformation was first achieved in *Chlamydomonas*, a unicellular alga, that has a single chloroplast, and 5–80 ptDNA copies (Boynton *et al.* 1988). Transformation was made feasible by solving the problem of DNA delivery through the chloroplast's double membrane, introducing the DNA on the surface of microscopic tungsten particles with the biolistic acceleration system. Chloroplast transformants were recovered in a randomly bombarded cell population by selection for appropriate genetic markers. For a review of genetic manipulation of *Chlamydomonas* chloroplasts see Rochaix (1992) and Boynton & Gillham (1993).

Chlamydomonas, with a single chloroplast, is genetically less complex than tobacco, a flowering plant, with at least 100 chloroplasts per leaf cell. Still, transformation of chloroplasts in tobacco leaf cells could be achieved by the biolistic protocol (Svab *et al.* 1990) and polyethylene glycol treatment (Golds *et al.* 1993). Recovery of plastid transformants was made feasible by the non-lethal spectinomycin-resistance marker system (below). A review of the problems and applications of plastid transformation in flowering

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plants has been published elsewhere (Maliga 1993). This paper will summarize recent technical advances in tobacco, the model for plastid genome engineering in land plants.

2. MARKER GENES TO MANIPULATE THE PLASTID GENOME

Genes conferring resistance to streptomycin and lincomycin were shown to be non-lethal plastid markers at the cellular level, but selective at the plastid level in cultured tobacco cells (Moll *et al.* 1990). Spectinomycin is similar to these drugs with respect to its selection characteristics (Svab & Maliga 1991). Because spectinomycin resistance is the most frequently used marker in plastid transformation experiments, non-lethal selection will be discussed here through the example of this drug resistance marker. Spectinomycin is an inhibitor of plastid protein synthesis, but not of protein synthesis by the eukaryotic-type, cytoplasmic ribosomes. Sensitive tobacco cells continue to proliferate on a selective culture medium since sucrose in the culture medium obviates the requirement for photosynthesis. As plastid protein synthesis is required for organelle maintenance, spectinomycin-sensitive plastids will gradually degenerate under selection while the transformed, resistant chloroplasts will take their place and gradually dominate the plastid population. It is possible to distinguish between sensitive and resistant cells on the basis of colour: sensitive cells are bleached, whereas resistant cells are green when grown on a selective medium. Given the large number of genome copies per cell, prolonged culture under such non-lethal selection in phenotypically sensitive cells is important for obtaining homoplasmic plastid transformants in the highly polyploid tobacco system.

Plastid transformants were selected by spectinomycin resistance encoded in a mutant 16*S* rRNA gene (Svab *et al.* 1990; Staub & Maliga 1992), or by a chimeric *aadA* gene encoding a spectinomycin detoxifying enzyme aminoglycoside-3″-adenyltransferase (Svab & Maliga 1993). The efficiency of transformation with the 16*S* rRNA genes is relatively low. It takes screening of 50–100 bombarded leaf samples to obtain a single plastid transformant whereas about one transformant per bombardment is obtained with the *aadA* gene as the tranforming marker gene. We assume that the 16*S* rRNA markers are recessive, as in *E. coli*, and therefore there is no phenotypic resistance until essentially all the genome copies in one organelle are transgenic. Based on the observed discrepancy of transformation efficiencies with the recessive and dominant markers, we speculate that repair eliminates the first integration products in 99 out of 100 cases before resistance can be expressed (Svab & Maliga 1993).

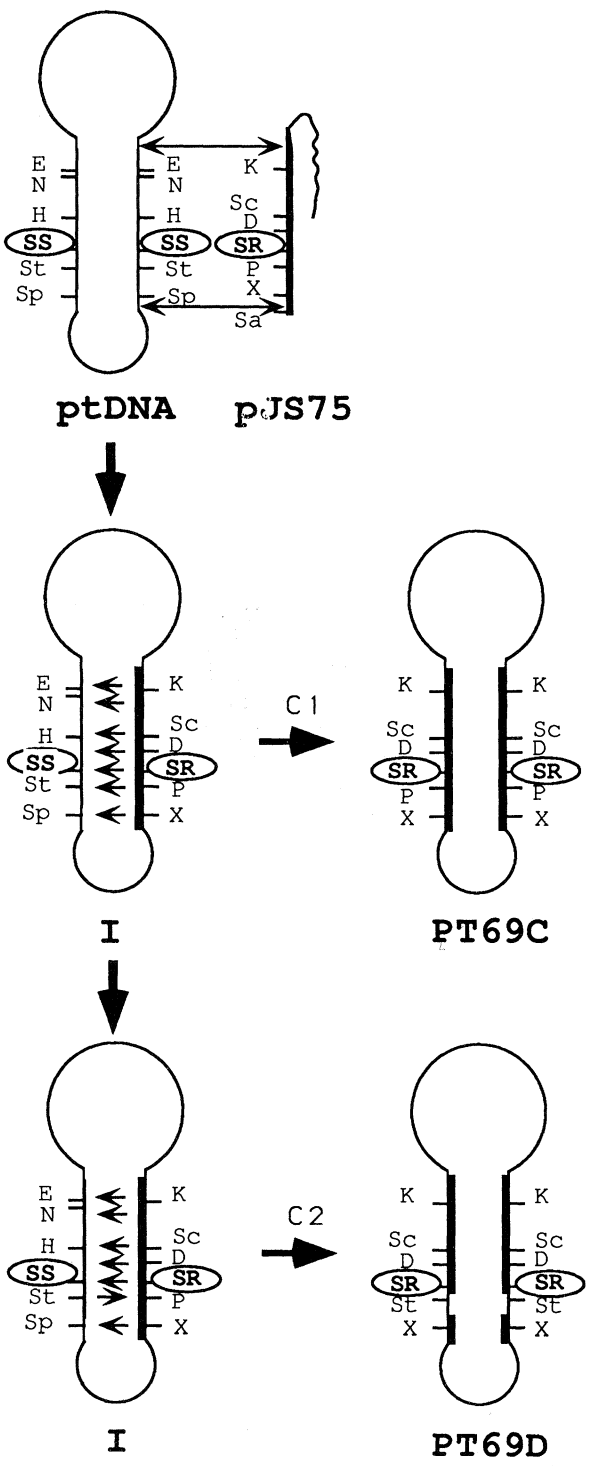

Figure 1. Gene replacement in the tobacco inverted repeat. Transgenic clone PT69 was selected by *16SrDNA*-encoded spectinomycin resistance (**SR**) on plasmid pJS75 that carries a 6.2 kb plastid DNA fragment. Inverted repeat sequences are paired such that large and small single-copy regions form loops. The figure is not drawn to scale. Initial interaction of the recipient wild-type plastid genome (ptDNA) with plasmid pJS75 leads to replacement of wild-type sequences (thin line) carrying the spectinomycin sensitive (**SS**) allele with transforming DNA (bold line) in one of the repeats. The transformation intermediate (I) replicates. Copy correction (C1) in one copy of the I genome stabilizes all the markers yielding genome PT69C. Differential copy correction (C2) in a second I genome removes the transgenic PstI (P) site, but preserves all the other RFLP markers, yielding the genome of the T69D plant. Abbreviation of RFLP markers: D, DraI; E, EcoRV; H, HincII; K, KpnI; N, NciI; P, PstI; Sa, SalI; Sc, ScaI; Sp, SphI; St, StyI; X, XbaI. Modified from Staub & Maliga (1992).

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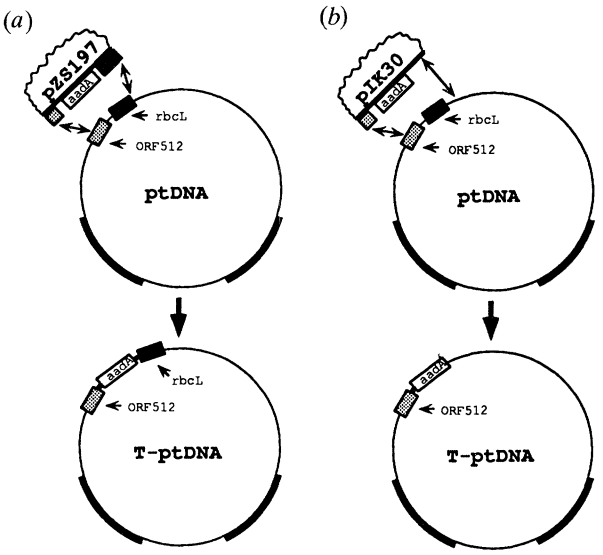

Figure 2. Targeted insertion of heterologous sequences into the plastid genome. (*a*) Insertion of a selectable *aadA* gene between the *rbcL* and *ORF512* genes. Plasmid pZS197, a pBluescriptKS+ vector (wavy line) with the *rbcL*/*aadA*/*ORF512* insert (bold line), was introduced into plastids by the biolistic process. Transplastomic lines were selected by spectinomycin resistance encoded in the *aadA* gene. Two homologous recombination events (arrows) lead to the incorporation of *aadA* between the *rbcL* and ORF512 genes in the recipient ptDNA, yielding the T-ptDNA transplastome (Svab & Maliga 1993). (*b*) Deletion of *rbcL* from the plastid genome. Note that selectable *aadA* gene in transforming plasmid pIK30 replaces the *rbcL* coding region. Selection for spectinomycin resistance, and homologous recombination via targeting sequences introduces mutant plastid structure of pIK30 into ptDNA.

Streptomycin resistance is also a non-lethal plastid marker. Although suitable for direct selection, this marker was used as an unselected marker, to follow the fate of transforming DNAs. Streptomycin resistance in the transplastomic lines was due to incorporation into the recipient plastid genome of a point mutation of the 16*S* rRNA gene (Svab *et al.* 1990) or of the *rps12* ribosomal protein gene (Staub & Maliga 1992).

Resistance to kanamycin was obtained when expressing the *kan* gene encoding neomycin phosphotransferase in tobacco plastids (Carrer *et al.* 1993). Kanamycin is toxic to cells at concentrations that are normally used for selection. Direct selection protocols for kanamycin resistance are currently being tested.

3. GENE REPLACEMENT

Initial transformation experiments involved replacing a segment of the wild-type recipient ptDNA with a DNA fragment cloned from antibiotic resistant tobacco mutants. Because the 16*S* rRNA gene is located in the inverted repeat, probing for RFLPS established that replacement of both 16*S* rRNA gene copies occurs within a short period of time (Svab *et al.* 1990; Staub & Maliga 1992). As transformation is relatively infrequent and the transgenic segments in the repeated regions are identical, the second transgenic copy must be generated by the repair processes that are involved in maintaining sequence identity of the repeated region (figure 1). To account for different transplastomes in one clone we proposed that transformation involves only one of the 16*S* rRNA genes, followed by replication of the transformation intermediate and differential copy correction (figure 1; Staub & Maliga 1992).

4. INSERTION OF HETEROLOGOUS SEQUENCES INTO THE PLASTID GENOME BY TARGETING *IN VITRO*

All characterized recombination events in plastids have been between homologous DNA sequences. However, insertion of foreign genes may be obtained readily by flanking the heterologous sequence with homologous plastid DNA fragments. This homologous ptDNA is referred to as targeting sequence since it directs the insertion of the foreign DNA in the plastid genome. The site of gene insertion into the plastid genome is therefore determined by the site into which it has been cloned *in vitro*. Insertion of *aadA* between the *rbcL* and *ORF512* genes is shown schematically in figure 2*a*.

The *rbcL* and *psbA* coding regions were deleted in the tobacco plastid genome by targeted insertion of the *aadA* gene. As shown in figure 2*b* for the *rbcL* gene, the *aadA* was cloned into a plastid DNA fragment to replace the coding region of the plastid gene, than the mutant structure in plasmid pIK30 was introduced into the plastid genome by selection for spectinomycin resistance. The plants transformed with plasmid pIK30 are now resistant to spectinomycin, but are photosynthetically defective due to the loss of the *rbcL* photosynthetic gene.

For introduction into plastids, the foreign genes must either encode a selectable phenotype, or be linked to a selectable marker gene. Passenger genes have been introduced by linkage to both, the spectinomycin resistant allele of the 16*S* rRNA gene (Staub & Maliga 1992) and *aadA* (unpublished data). A targeting sequence of about 1 kb on either side of the heterologous sequence is sufficient to direct the integration of 1.3–3.7 kb heterologous DNA sequences at an efficiency of about one transgenic clone per bombardment. A 3.7 kb fragment is sufficiently large to include the selectable marker gene, *aadA*, and a linked *uidA* reporter gene (figure 3).

5. MUTAGENESIS OF PLASTID GENES

Mutagenesis of endogenous plastid genes may be obtained by incorporating the mutation within the targeting sequence linked to a selectable *aadA* gene. For linkage, the *aadA* gene should be cloned into intergenic regions so that it does not interfere with the expression of flanking genes. Figure 4 outlines the mutagenesis of *rbcL* by linkage to *aadA* which is cloned downstream of the target gene. Exchange of the *rbcL* 3′ region with the *rps16* 3′ region has been successful in every transplastomic clone. Since the *rps16* 3′ fragment and the chimeric *aadA* gene form a block of heterolo-

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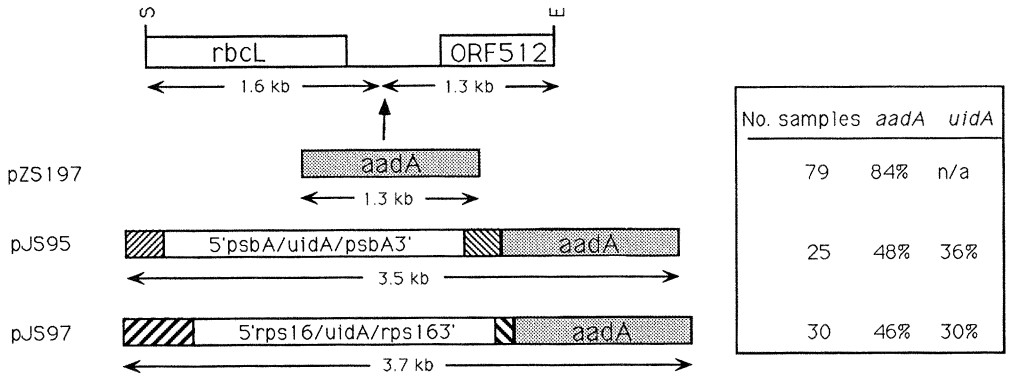


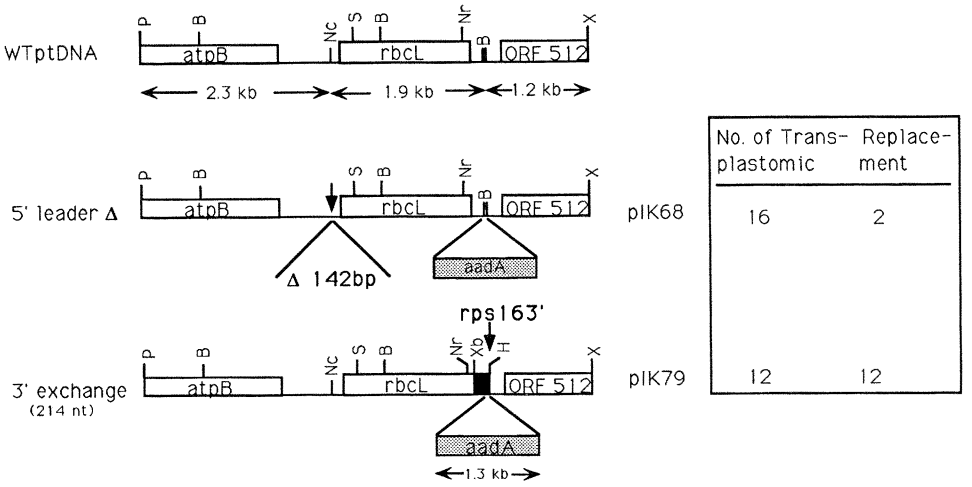
No. samples	*aadA*	*uidA*
79	84%	n/a
25	48%	36%
30	46%	30%

Figure 3. Introduction of chimeric *uidA* reporter genes into the plastid genome by linkage to the selectable *aadA* gene. The *rbcL*/*ORF512* targeting casette is shown on top (E, *Eco*RV; S, *Sac*II restriction endonuclease sites). In the table (boxed) the number of bombarded leaf samples, the % of bombarded leaf samples that yielded transplastomic lines (carry *aadA* in ptDNA), and the % of transplastomic lines with the passenger gene are given for the three constructs. Note, that *uidA* is absent in about one-third of the clones. The *uidA* gene is expressed in casettes derived from the plastid *psbA* and *rps16* genes. Based on Svab & Maliga (1993) and unpublished.

gous DNA sequences, and since recombination is feasible only via homologous sequences, the *rps16* 3′ and *aadA* can not be separated by recombination via sequences between the two. However, the 142 b.p. *rbcL* leader deletion was present only in two of the 16 transplastomic clones. The 1.9 kb of homologous *rbcL* coding sequence between the deletion and *aadA* is apparently sufficiently long for the integration of *aadA* without incorporating the deletion in most transplastomic genomes. It is not clear if the low frequency is due to the physical distance (2 kb) of the deletion from the selectable marker gene, the nature of the mutation itself, or due to linkage to a heterologous sequence, since incorporation of about 6 kb of homologous sequence has been obtained by selection for spectinomycin resistance encoded in a mutant 16*S* rRNA gene (Staub & Maliga 1992).

6. STABILITY OF TRANSGENES IN THE PLASTID GENOME

Short, repeated DNA sequences in the plastid genome have been shown to mediate rearrangements on an evolutionary timescale (Ogihara *et al.* 1992). The transgenic approach involves introduction of chimeric genes under control of DNA sequences regulating expression of endogenous plastid genes. This results in an unavoidable duplication of endogenous DNA sequences. These duplicated DNA sequences could conceivably cause instability of the transgenome. To test the stability of transgenomes experimentally, a selectable *aadA* gene was inserted downstream of the plastid *psbA* gene. As this *aadA* is expressed in a *psbA* cassette, in this line the 5′ and 3′ *psbA* regulatory regions (about 200 b.p.) are duplicated in a tandem arrangement (figure 5).



No. of Trans-plastomic	Replace-ment
16	2
12	12

Figure 4. Targeted mutagenesis of the tobacco *rbcL* plastid gene. The wild-type plastid genome with the coding region of the *rbcL*, *atpB* and *ORF512* genes (open boxes) is shown on top. In plasmid pIK68, a 142 b.p. region has ben deleted from the *rbcL* leader region. In plasmid pIK79, a 214 b.p. DNA segment immediately downstream of the *rbcL* coding region was replaced with the cognate sequence of the *rps16* plastid ribosomal protein gene (black bar). In the table (boxed) the number of transplastomic clones with *aadA* gene, and the number of those with the desired replacement are given. Abbreviation of restriction endonuclease recognition sites: B, *Bam*HI; H, *Hin*dII; Nc, *Nco*I, Nr, *Nru*I, P, *Pvu*II, S, *Sac*II, Xb, *Xba*I; X, *Xho*I.

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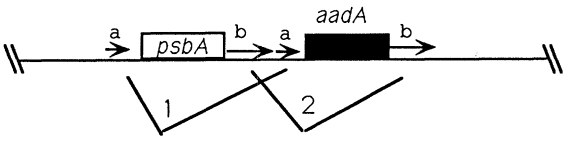

Figure 5. Transgenic assay to test recombination via direct repeat sequences in the plastid genome. The *aadA* gene, expressed from *psbA* regulatory sequences (5′ region is a-repeat; 3′ region is b-repeat), is inserted downstream of the endogenous *psbA* gene. Loss of *psbA* by recombination via 5′ a-repeats is detected as pigment deficient somatic sectors in leaves. Loss of *aadA* by recombination via the 3′ b-repeats is detected in seedlings, by the spectinomycin-sensitive phenotype.

Intramolecular recombination via the duplicated *psbA* 5′-region results in the loss of the *psbA* gene, which is manifested as a pigment deficient somatic leaf sector. Sector formation indicated loss of the *psbA* coding region, on average, once in 17 plants. In every case, the sectors were limited to a small group of cells in one leaf. Loss of the *aadA* gene by intramolecular recombination via the duplicated *psbA* 3′-region was tested by the loss of spectinomycin resistance in the seed progeny. No sensitive seedlings were found in a sample of 12 000 seedlings. Therefore, if *aadA* is lost, it may occur in small somatic sectors as for the *psbA* gene and would not be transmitted to the seed progeny. Our findings confirm that genome rearrangements via short direct repeats are rare in transplastomic lines and that this low frequency does not interfere with applications of plastid engineering.

7. PLASTID EXTRACHROMOSOMAL ELEMENT

Plasmids are extrachromosomal genetic elements that replicate independently from the host genome. Extrachromosomal genetic elements have been described in mitochondria of flowering plants (Pring & Lonsdale 1985) and in plastids of algae (Nass & Ben-Saul 1972; Green 1976; Mazza *et al.* 1980), but not in the plastids of land plants.

We report an extrachromosomal plasmid element that formed in a transplastomic line, by recombination via an imperfect 16 b.p. direct repeat (figure 6). The 868 b.p. extrachromosomal element duplicates sequences in the main plastid genome. The plastid mini-circle is present in a multimeric series, as is the main plastid genome (Deng *et al.* 1989). Probing of DNA gel blots with the cloned minicircle DNA detects up to 8-mers, that yield a unit-length monomer upon digestion with the *Xba*I restriction endonuclease for which there is only a single site in the minicircle DNA. This element is present in the plastids of regenerated plants, but is gradually lost in the absence of selection pressure since the sequences are duplicated in the plastid's main genome. The vector potential of the minicircle is currently being explored by constructing shuttle vectors that are maintained extrachromosomally in both plastids and *E. coli*. The shuttle vectors may be used for marker rescue, mutagenesis, and the study of genetic recombination between a shuttle

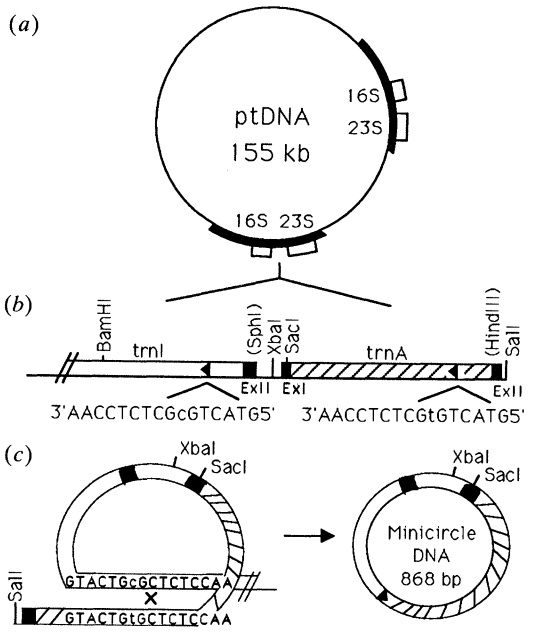

Figure 6. Extrachromosomal minicircle in tobacco plastids. Map position of (*a*) minicircle sequences in the plastid genome (ptDNA) and of (*b*) a 16 b.p. imperfect repeat in plasmid pJS75 (black triangles). (*c*) Formation of the minicircle by intramolecular recombination via the imperfect repeat. Abbreviations: *Bam*HI, *Sca*I, *Sal*I, *Xba*I are restriction enzyme recognition sites; 16*S*, 23*S* are the genes for the 16*S* and 23*S* ribosomal RNAs; *trnI* and *trnA* are genes for tRNAs.

plasmid and the plastid genome, further expanding the technical tools available for the manipulation of the plastid genome.

8. FUTURE PROSPECTS

The tools developed in this laboratory open up the plastid genome of land plants for gene replacement, *in vitro* mutagenesis, and targeted gene disruption. The procedures depend on the efficient homologous recombination system in plastids, and are similar to manipulation of large bacterial plasmids.

The tools of plastid engineering can now be applied to study plastid gene expression and regulation. Encouraging in this regard is our success in expressing in plastids the marker genes *aadA* (Svab & Maliga 1993) and *kan* (Carrer *et al.* 1993), and the *uidA* reporter gene (Staub & Maliga 1993), that may be utilized in these studies.

Application of plastid engineering to crops may now be tried. One potential advantage of incorporating transgenes in the plastid genome is the lack of plastid transmission in most crops. Due to lack of pollen transmission the transplastomes are confined to the crop, unlike transgenes incorporated into nuclear genome. Instantaneous amplification to high copy numbers is the second attractive feature of incorporating genes in the plastid genome. This may be important if a high level of protein accumulation is desired. In this respect it is encouraging that expression of *uidA* and *kan* in plastids results in accumulation of 2.5% β-glucuronidase (GUS) and

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1% neomycin phophotransferase in tobacco leaves, respectively (Staub & Maliga 1993; Carrer *et al.* 1993). Optimization of expression may result in the accumulation of proteins at significantly higher levels.

So far all plastid genome manipulations have been carried out in tobacco. This is due to the availability of plastid genetic markers, the ease with which tobacco can be manipulated in cell culture, and the well characterized plastid genome. Particularly helpful is the availability of the entire ptDNA sequence (Shinozaki *et al.* 1986). Immediate application of plastome engineering to other plant species depends on their suitability for cell culture manipulations, a prerequisite of current plastid transformation technology. After appropriate adjustments of the protocols, applications to other land plants will undoubtedly follow.

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