Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7468–7472, August 1994
Genetics

Extrachromosomal elements in tobacco plastids

(DNA recombination/marker rescue/plastid transformation/shuttle plasmid/transplastomic plants)

JEFFREY M. STAUB AND PAL MALIGA*

Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, NJ 08855-0759

Communicated by Charles S. Levings III, April 26, 1994 (received for review January 13, 1994)

ABSTRACT **The plastid genome of higher plants is a circular double-stranded DNA molecule which is present in multiple identical copies. We report here an 868-bp plastid DNA minicircle, NICE1, that formed in tobacco (*Nicotiana tabacum*) plastids during transformation, as an unexpected product of homologous recombination. Such extrachromosomal elements are normally absent in plastids of higher plants. We have constructed shuttle plasmids containing NICE1 sequences which are maintained extrachromosomally when reintroduced into plastids by particle bombardment. Furthermore, recombination between homologous sequences in the shuttle plasmids and the main plastid genome occurs. Recombination products were characterized after recovery of the shuttle plasmids in *Escherichia coli* and of recombinant plastid genomes in the progeny of transformed plants. Our findings indicate that shuttle plasmids can be used to engineer plastid genes without concomitant integration of foreign DNA and to recover plastid mutations in *E. coli*.**

The plastid genome of higher plants is a circular double-stranded DNA molecule of 120–160 kb (1) which may be present in up to 50,000 identical copies per cell (2). Extrachromosomal genetic elements are absent in plastids of higher plants, although they are found in plastids of a few algae (1). In contrast, several different extrachromosomal elements have been described for the mitochondria of higher plants (3, 4).

We report here a *Nicotiana* plastid extrachromosomal element, NICE1, that formed in a tobacco line during plastid transformation. Transformation of tobacco plastids normally involves integration of the transforming DNA by homologous recombination. Replication of the transplastome and sorting of the transformed genome copies under selection pressure eventually yields homoplasmic lines, with each of the plastid genome copies uniformly altered. The transformation vector is rapidly eliminated and is not detected in the transformed lines (5, 6).

NICE1 was identified in a transplastomic tobacco line, PT69, obtained by transformation with plasmid pJS75 (7). We report here that NICE1 is a subgenomic plastid DNA (ptDNA) circle. We utilized NICE1 sequences to construct shuttle plasmids that are maintained extrachromosomally in both plastids and in *Escherichia coli*. Furthermore, we show the potential of the shuttle plasmids for studying ptDNA recombination and for engineering the plastid genome.

MATERIALS AND METHODS

DNA Manipulations. Cloning and sequencing of DNA was accomplished by established protocols (8). Shuttle vectors are based on a pUC119 plasmid (9) that carries a chimeric *aadA* gene at its *Eco*RI site. In the chimeric *aadA* gene (10) the 3′ region was replaced with that of the plastid *rbcL* gene (213 bp, nucleotides 59,026–59,238 in the plastid genome; ref. 11). Plasmid pJS101 was obtained by cloning the *Sac* I-linearized minicircle into the *Sac* I site of the multiple cloning site of the pUC119 derivative (see Fig. 2). Plasmid pJS103 carries the 1.4-kb *Sal* I–*Bam*HI plastid DNA fragment from pJS75 cloned between the *Sal* I and *Hin*dIII sites in the multiple cloning site of the pUC119 derivative (nucleotides 136,330–137,723 in ref. 11; originally a *Hin*dIII–*Bam*HI ptDNA fragment), which contains NICE1 sequences. During plasmid construction the *Bam*HI and *Hin*dIII sites were destroyed.

Transformation and Regeneration of Transgenic Plants. Tobacco (*Nicotiana tabacum*) plants were grown aseptically on agar-solidified medium containing Murashige–Skoog salts (12) and sucrose (30 mg/ml). For plastid transformation, the DNA was introduced on the surface of microscopic tungsten particles (10) by using the DuPont PDS1000He Biolistic gun. Spectinomycin-resistant calli and shoots were selected on RMOP medium containing spectinomycin dihydrochloride at 500 μg/ml (10). Resistant shoots were regenerated on the same selective medium and rooted on Murashige–Skoog agar medium.

Isolation of ptDNA. ptDNA was prepared from chloroplasts isolated from leaves of greenhouse-grown plants or from leaves of plants grown in sterile culture, as indicated in *Results*. To characterize NICE1, ptDNA from the chloroplasts was isolated by the method of Kolodner and Tewari (13) except that the CsCl gradient purification was omitted and the DNA was purified by extraction with phenol/chloroform followed by precipitation with ethanol. To characterize the shuttle plasmids, ptDNA was isolated by the newer method of Liu and Rose (14).

Southern Blot Analysis. Total cellular DNA was digested with the appropriate restriction enzymes, electrophoresed in 0.7% agarose gel, and transferred to nylon membrane (Amersham) with the PosiBlot Transfer apparatus (Stratagene) (15). Blots were probed using rapid hybridization buffer (Amersham) with ^{32}P-labeled probes generated by random priming (Boehringer Mannheim).

Testing of Seedling Phenotypes. Seedling phenotypes were determined by plating surface-sterilized seeds on Murashige–Skoog salts medium (10). On selective medium with spectinomycin dihydrochloride (500 μg/ml), resistant progeny are green, whereas sensitive progeny are white (10).

RESULTS

Identification of NICE1 in Plastids. We have previously demonstrated the homologous replacement of ptDNA with 6.2 kb of DNA from plasmid pJS75 by restriction fragment length polymorphism (RFLP) analysis (7). In this report we characterize a plastid DNA species derived from the 6.2-kb DNA in the PT69 line (7). In undigested DNA isolated from

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ptDNA, plastid DNA; RFLP, restriction fragment length polymorphism.

*To whom reprint requests should be addressed.

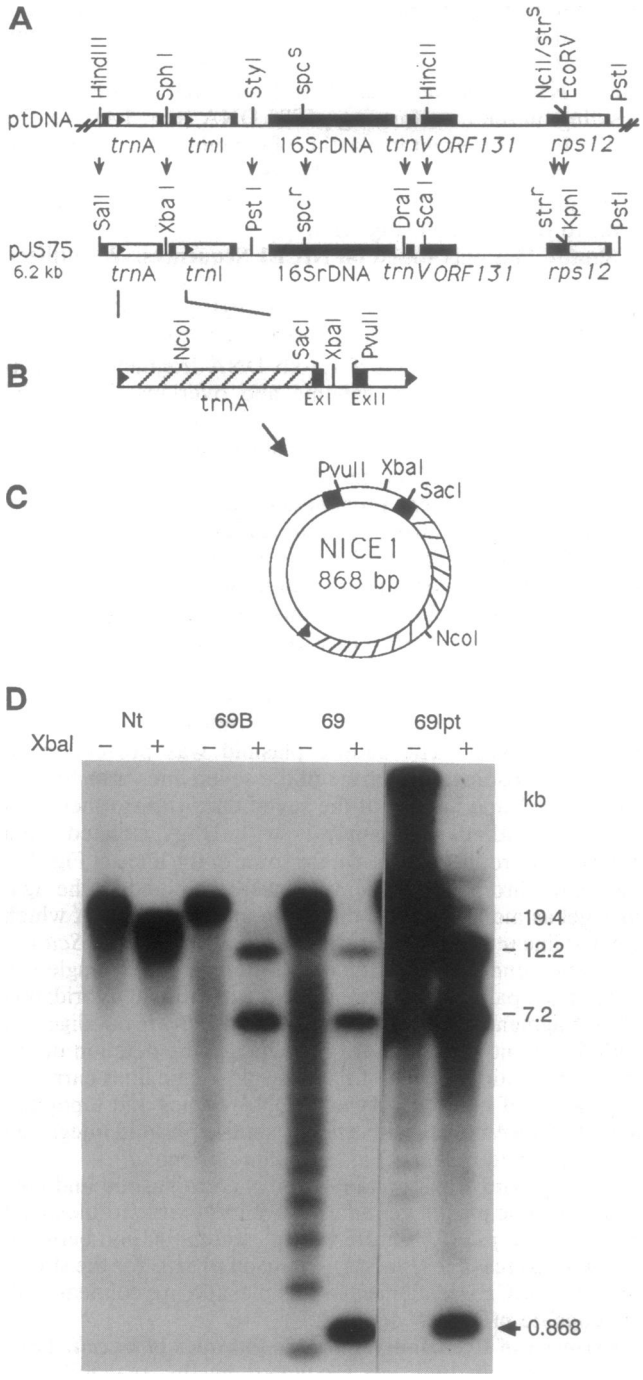

FIG. 1. Formation of NICE1 in tobacco plastids. (*A*) Restriction map of ptDNA in pJS75 and corresponding region in wild-type plastids (ptDNA). Vertical arrows pinpoint restriction site differences used in RFLP analysis (7). For gene designations, see ref. 11. Filled and open boxes are exons and introns, respectively; the 16-bp imperfect repeats are marked by filled triangles. (*B*) NICE1 segment in the pJS75 DNA bordered by the 16-bp imperfect repeats. Note different shading of the intron of *trnA* (hatched) and *trnI* (open) genes and additional restriction sites. (*C*) Formation of NICE1 by intramolecular recombination via the 16-bp imperfect repeat sequence. Note that only one of the repeats (from *trnI*) is present in NICE1. (*D*) NICE1 forms a multimeric series in plastids. Lanes contained total cellular DNA from the recipient wild-type tobacco (Nt), the PT69 callus (69) and subclone (independently regenerated plant) PT69B (69B). Lanes with purified chloroplast DNA from subclone PT69I (69Ipt) are also included. Undigested DNA (−) and DNA digested with the *Xba* I restriction endonuclease (+) were electrophoresed and probed with the 6.2-kb ptDNA fragment (7) from pJS75 containing NICE1 sequences. Note the series of low molecular weight bands in undigested PT69 and PT69Ipt DNA samples and unit-length

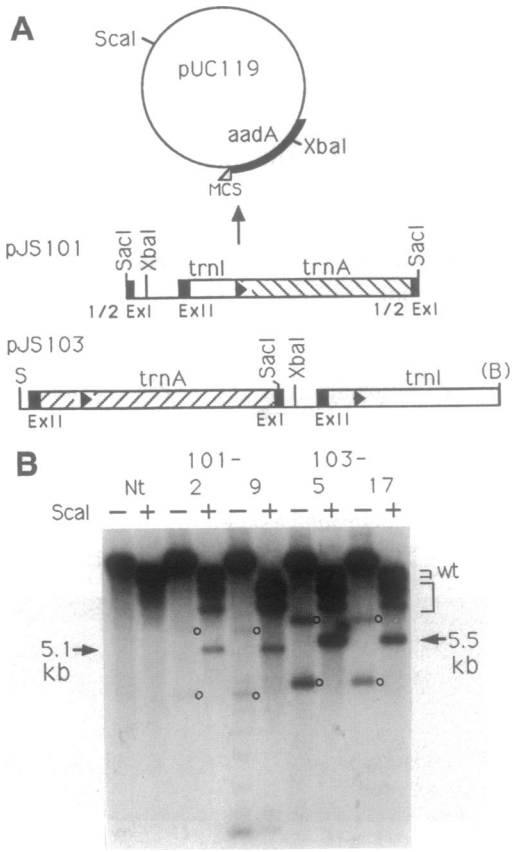

FIG. 2. Plasmids containing NICE1 sequences are maintained extrachromosomally in plastids. (*A*) Shuttle plasmids pJS101 and pJS103. (*Upper*) Plasmid pUC119 with the multiple cloning site (MCS) and chimeric *aadA* gene (black arc). The single *Sca* I site is shown. (*Lower*) Plastid DNA fragments containing NICE1 sequences inserted into the MCS of the pUC119 derivative (vertical arrow; see *Materials and Methods*). B, blunted *Bam*HI site; Ex, exon; S, *Sal* I site. (*B*) Southern blots confirm the presence of extrachromosomal shuttle plasmids in chloroplasts. Undigested (−) and *Sca* I-digested (+) plastid DNA samples were probed with the 1.4-kb *Bam*HI–*Sal* I fragment in pJS103 (Fig. 2*A Lower*). The extrachromosomal plasmids (circles) in the undigested plastid DNA of NtpJS101 lines (101-2, 101-9) and NtpJS103 lines (103-5, 103-17) are linearized to monomeric lengths of 5.1 kb and 5.5 kb, respectively, by *Sca* I digestion. Note wild-type (Nt) restriction fragments (bracket, wt) and ptDNA fragments containing a copy of the shuttle plasmid (bracket, right) integrated via homologous plastid DNA sequences (data not shown). Deletion derivatives lack the *Sca* I site in the NtpJS101-9 line.

chloroplasts, this DNA species is present as a multimeric series (Fig. 1*D*). Digestion with *Xba* I (Fig. 1*D*) or *Sac* I, *Pvu* II, or *Nco* I (data not shown) restriction enzyme converts the multimeric series into a unit-length monomer. Since the mobility of the smallest undigested molecular species is faster than that of the unit-length linear molecule, we infer that the multimeric series is circular. Interestingly, the circular plastid genome itself is present as a multimeric series (16).

Sequencing of the cloned NICE1 fragment revealed that it is 868 bp in size and is derived from the rRNA operon spacer region (Fig. 1 *A–C*). NICE1 sequences are bordered by two 16-bp imperfect direct repeats present in the wild-type plastid genome at nucleotide positions 136,493 (5′-GTACTGtGCTC-

(868 bp) band in the same DNAs digested with *Xba* I; note also the absence of extrachromosomal NICE1 in the Nt and PT69B DNA samples. The new 12.2-kb and 7.2-kb *Xba* I ptDNA fragments are formed from the 19.4-kb wild-type fragment due to incorporation of the *Xba* I site from plasmid pJS75.

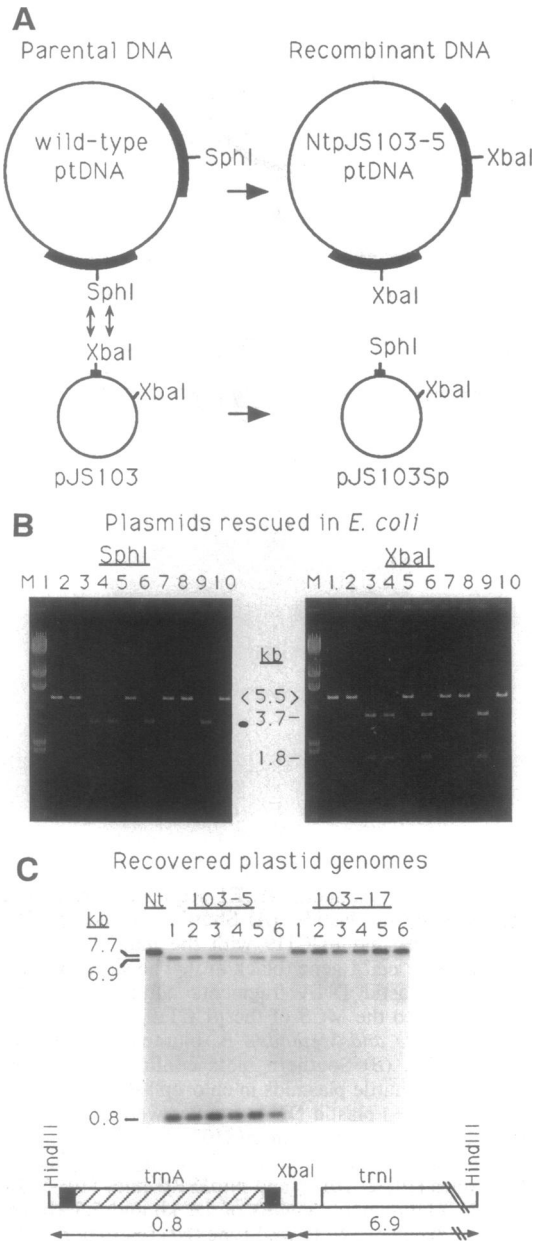

FIG. 3. Recombination between shuttle plasmid pJS103 and the plastid genome. (*A*) Recombination products. The wild-type ptDNA with the *Sph* I site, the recombinant NtpJS103-5 ptDNA with the transgenic *Xba* I site, shuttle plasmid pJS103 (two *Xba* I sites and no *Sph* I site), and its derivative with the acquired *Sph* I site (pJS103Sp; one *Xba* I site) are shown. Recombination between the ptDNA fragment in pJS103 (black box) and wild-type ptDNA is indicated by double-headed arrows. Black arcs mark the repeated region of ptDNA. Drawing is not to scale. (*B*) Recovery in *E. coli* of pJS103 shuttle plasmids with the *Sph* I site from the plastid genome. Shuttle plasmids recovered in *E. coli* by transformation with total cellular DNA from the NtpJS103-5 line were digested with *Xba* I or *Sph* I restriction enzyme, separated according to size by electrophoresis in agarose gels, and stained with ethidium bromide. Plasmids with the pJS103 restriction pattern (two *Xba* I sites yielding 3.7-kb and 1.8-kb fragments; not digested with *Sph* I) are shown in lanes 3, 4, 6, and 9; plasmids with a pJS103Sp restriction pattern (linear 5.5-kb fragment by *Sph* I or *Xba* I digestion) are shown in lanes 1, 2, 5, 7, 8, 10 (dash, *Xba* I fragments; <, linear plasmid; dot, undigested DNA lanes M, size markers). (*C*) Recovery of plastid genomes with the *Xba* I site from shuttle plasmid pJS103. Total cellular DNA from spectinomycin-sensitive seedlings was digested with the *Hin*dIII–*Xba* I restriction endonucleases and probed with the 1.4-kb *Sal* I–*Bam*HI fragment from pJS103 (Fig. 2A *Lower*). Southern blot hybridization detects a 7.7-kb fragment in the wild-type plastid genome, and 6.9-kb and 0.8-kb fragments in the NtpJS103-5 ptDNA with the acquired *Xba* I site (see map for the NtpJS103-5 line). Note lack of the transgenic *Xba* I site in NtpJS103-17 seedlings.

TCCAA-3') and 137,345 (5'-GTACTGcGCTCTCCAA-3'; in inverted repeat A, ref. 11). The repeat in the *trnI* gene intron, 5'-GTACTGcGCTCTCCAA-3', is also present in the element. NICE1 contains an *Xba* I site, the RFLP marker residing on the transforming pJS75 DNA (Fig. 1*A*).

NICE1 was present in plastids of greenhouse-grown PT69I plants even after several months. However, it was absent in other plants—for example, PT69B—due to random sorting prior to regeneration (Fig. 1*D*).

Shuttle Plasmids Based on NICE1 Sequences. We utilized NICE1 sequences to construct vectors that can shuttle between plastids and *E. coli*. Plasmid pJS101 (5.1 kb) includes the 868-bp NICE1 DNA as a *Sac* I DNA fragment. Plasmid pJS103 (5.5 kb) contains a 1.4-kb DNA fragment which is colinear with the main genome and overlaps the NICE1 region. To facilitate the recovery of clonal transplastomic lines, *aadA*, a spectinomycin-resistance gene, was incorporated in the constructs (Fig. 2).

Plasmid DNAs were precipitated onto microscopic tungsten particles and introduced into leaf cells with the DuPont PDS1000He Biolistic gun. Leaf segments were cultured on a selective medium containing spectinomycin dihydrochloride (500 μg/ml). Plastid transformants were identified by spectinomycin resistance due to expression of the *aadA* gene, which allows formation of green callus and shoots on the selective medium, on which sensitive cells are white (10).

Seven transplastomic lines were studied for each of the shuttle plasmids. The shuttle plasmid was present in an extrachromosomal form in six of the seven lines transformed with pJS101 and in each of the seven lines transformed with pJS103. Southern blot analysis with DNA isolated from purified chloroplasts is shown for some of the lines in Fig. 2*B*. The extrachromosomal elements were resolved in the agarose gel as monomers and dimers in undigested DNA which yielded linear monomeric forms upon digestion with *Sca* I, a restriction endonuclease for which there is only a single site in the pUC part of the plasmids. Note additional hybridizing DNA fragments in the NtpJS101-9 line which are not digested with *Sca* I and therefore are presumed to be deletion derivatives. In addition, each of the transplastomic lines carried a population of nontransformed ptDNA copies and a population of ptDNA copies with a single shuttle plasmid integrated in the inverted repeats (Fig. 2*B*; data not shown).

Since plastid transformants were heteroplasmic and carried wild-type plastid genomes as well as extrachromosomal copies of the pJS103 shuttle vector, recombination between the two was feasible (Fig. 3*A*). We used pJS103 for the study, since the NICE1 sequences in this plasmid are colinear with the cognate ptDNA sequences.

Recovery of Recombinant Shuttle Plasmids in *E. coli*. Testing of recombination was facilitated by the availability of RFLP markers: an *Sph* I site in the wild-type recipient plastid genome and an *Xba* I site in the shuttle plasmid at the same position (Fig. 3*A*).

Shuttle plasmids from four of the transplastomic lines were recovered in *E. coli* and examined for the RFLP marker. Plasmids recovered from the NtpJS103-11 and NtpJS103-17 lines (19 each) carried exclusively the *Sph* I marker from the plastid genome. Shuttle plasmids from lines transformed with plasmid pJS103 carried either the transgenic *Xba* I or the wild-type *Sph* I marker (4 *Xba* I and 12 *Sph* I plasmids rescued from the NtpJS103-1 line; 5 *Xba* I and 11 *Sph* I plasmids rescued from the NtpJS103-5 line). Examples of such recombination products are shown in Fig. 3*B*.

Recovery of the Transgenic *Xba* I Site in Plastids Lacking the Shuttle Vector. We screened for the second product of recip-

rocal recombination, spectinomycin-sensitive ptDNA with the transgenic *Xba* I site (Fig. 3*A*). This was accomplished by analyzing the seed progeny of plastid transformants which lost the shuttle plasmid, indicated by the loss of the *aadA* gene. Resistant and sensitive seedlings were identified by their green or white color on selective spectinomycin medium, respectively. The seed progeny of line NtpJS103-5 were predominantly sensitive, whereas those of the NtpJS103-17 line were predominantly resistant to spectinomycin. Six sensitive seedlings were tested from one seed capsule of each line. The sensitive NtpJS103-5 seedlings carried the transgenic *Xba* I site, whereas the sensitive NtpJS103-17 progeny had the wild-type *Sph* I RFLP marker (Fig. 3*C*).

Transmission of the Extrachromosomal Elements Through Seed. The regenerated NtpJS103 plants carried a mixed population of wild-type and transformed ptDNA copies (Fig. 2). It was of great interest, therefore, to determine whether integrated and/or extrachromosomal copies of the shuttle plasmid would be transmitted through seed. For this reason, total cellular DNA from selfed spectinomycin-resistant seedlings was analyzed. Southern blot analysis of undigested DNA, probed with NICE1 sequences, revealed significant amounts of extrachromosomal shuttle plasmid in a progeny of the NtpJS103-5 line and barely detectable amounts in a NtpJS103-17 seedling (Fig. 4*A*). The ptDNA of the seedlings contains a single copy of the shuttle plasmid integrated in the homologous region (6.9 kb) along with wild-type DNA fragments (1.4 kb; Fig. 4*B*). In addition, DNA fragments with an unpredicted size are present that are likely to be products of homologous recombination via the ptDNA sequences controlling *aadA* expression (10).

DISCUSSION

The 868-bp NICE1 minicircle was identified in the PT69 transplastomic line and contains only ptDNA sequences. Since NICE1 carries the transgenic *Xba* I RFLP marker that was engineered into plasmid pJS75, we conclude that the element is excised from this plasmid. Excision of NICE1 (Fig. 1 *B* and *C*) from the transforming DNA by homologous recombination via the 16-bp repeat is apparently linked to the process of transformation, since such extrachromosomal elements are not observed in wild-type tobacco plastids whose genome also carries the 16-bp repeats. Extrachromosomal maintenance of NICE1 is not indefinite due to the lack of a selection pressure. Once the element was lost, it did not reappear in the PT69B plastid transformant (Fig. 1*D*; data not shown). However, NICE1 was maintained in some of the regenerated plants for several months. We believe, therefore, that maintenance of NICE1 was by replication (see below).

Shuttle vectors were developed for transformation of tobacco plastids by incorporating NICE1 sequences and a selectable marker gene, *aadA*, into the *E. coli* plasmid pUC119. After bombardment and selection for the vector DNA, plastid transformants containing extrachromosomal elements were obtained (Fig. 2*B*). This suggests that the engineered elements replicate as did the original NICE1 element. Transient maintenance of the transforming plasmid was also found in a *Chlamydomonas* plastid transformation experiment (17). However, localization of the plasmids in chloroplasts was not shown.

ptDNA initially replicates bidirectionally, from two closely spaced D-loops, followed by a rolling-circle mechanism of replication (18). It is unknown which, if any, of these mechanisms is involved in the autonomous replication of the extrachromosomal elements. Interestingly, one of the D-loops implicated in ptDNA replication was mapped to the region containing NICE1 sequences in other plant species (19, 20), although not in tobacco (21). Alternatively, the engineered elements may replicate as part of the plastid

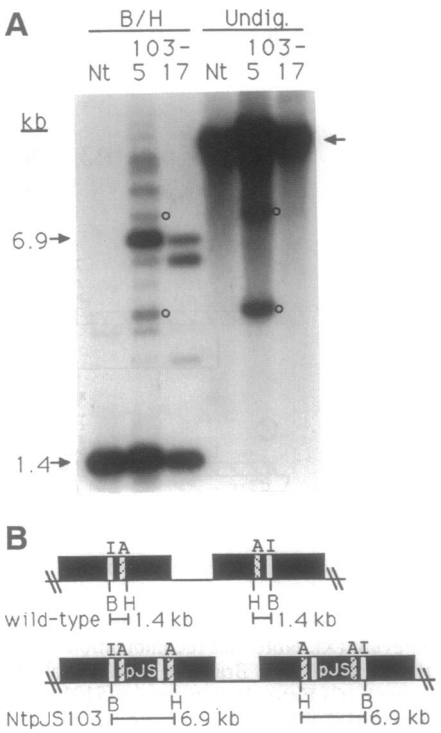

FIG. 4. The pJS103 plasmid is transmitted through seed. (*A*) Southern blot analysis of total cellular DNA from spectinomycin-resistant NtpJS103-5 (103-5) and NtpJS103-17 (103-17) seedlings. Undigested (Undig.) DNA or DNA digested with *Bam*HI/*Hin*dIII (B/H) was probed with the 1.4-kb NICE1 DNA fragment in pJS103 (Fig. 2*A Lower*). Plasmid pJS103 has no sites for *Bam*HI or *Hin*dIII. Note multimeric shuttle plasmids (open circles), the 1.4-kb wild-type band, and undigested ptDNA (arrow at right). Note also the 6.9-kb fragment containing a single copy of pJS103 and hybridizing fragments of unexpected size. (*B*) Partial map of the wild-type (Nt) ptDNA and NtpJS103 ptDNA with an integrated copy of plasmid pJS103. The ptDNA inverted repeats are marked by the boxes; I and A mark the location of the *trnI* and *trnA* genes, respectively; and pJS designates the plasmid backbone (pUC119, *aadA*). Restriction sites for *Bam*HI (B) and *Hin*dIII (H) are also marked. Bars indicate position of predicted restriction fragments in ptDNA. Drawing is not to scale.

genome and the extrachromosomal copies may be generated by excision of the shuttle plasmid from the main genome at any location of the 1.4-kb duplicated ptDNA sequence (Fig. 5). Integration of a copy of the shuttle vector in each of the 14 plastid transformants is consistent with this explanation.

Recombinant shuttle plasmids and ptDNA were recovered in *E. coli* and plants, respectively. Given the uncertainty by which the extrachromosomal elements are maintained, we can only speculate about the mechanism that generates the recombinant DNA molecules. If the element is maintained by autonomous replication, recombinant DNA molecules may arise by gene conversion, a mechanism proposed for the large ptDNA inverted repeats (22), or by reciprocal recombination (Fig. 3). Alternatively, the recombinant products may be generated by a mechanism involving integration and excision of the shuttle plasmid (Fig. 5).

Recovery in *E. coli* of pJS103 shuttle plasmids with the plastid *Sph* I marker (Fig. 3*B*) confirms the shuttle's potential to rescue mutant plastid genes. This application would be analogous to the use of the 2-μm plasmid-based vectors in yeast (23). Furthermore, our ability to recover plastid genomes with the *Xba* I marker from the pJS103 shuttle plasmid (Fig. 3*C*) demonstrates that the shuttle vectors are suitable for the engineering of plastid genes without concomitant integration of a foreign selectable marker gene. Since incorporation of novel useful genes into plastids is now a reality

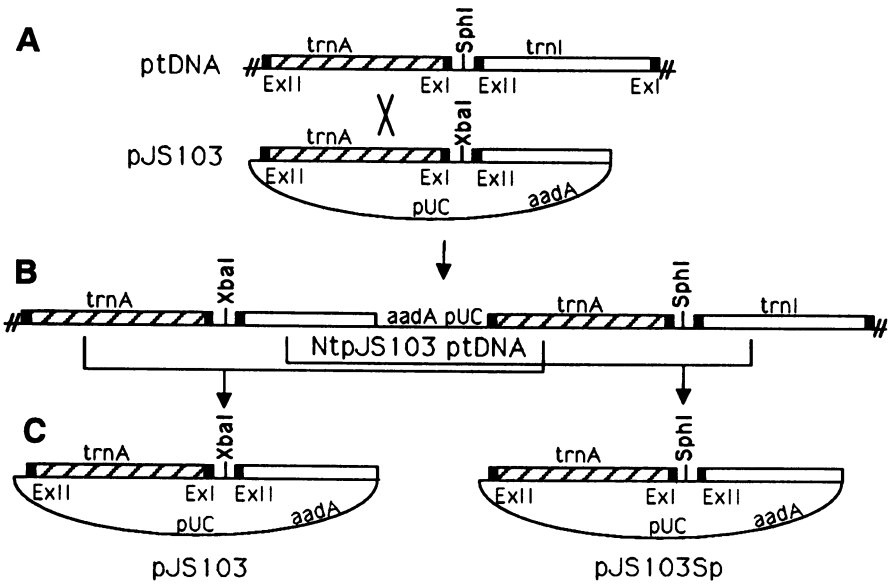

FIG. 5. Integration and excision of shuttle plasmid pJS103 into ptDNA. (*A*) Integration of plasmid pJS103 into wild-type ptDNA by single recombination event (X). Note that recombination may occur at any location between homologous plastid sequences. (*B*) Cointegrate ptDNA structure in the NtpJS103 line. Brackets mark homologous sites through which recombination generates different extrachromosomal copies of the shuttle plasmid. (*C*) Extrachromosomal pJS103 plasmids generated by excision at sites shown in *B*. Note original plasmid pJS103 with *Xba* I site and plasmid pJS103Sp with *Sph* I site. Ex, exon.

(5), eliminating the need to incorporate selectable marker genes will alleviate the risk of unnecessarily disseminating transgenes in the environment.

J.M.S. is a recipient of a Johanna and Charles Busch Predoctoral Fellowship Award. This research was supported by National Science Foundation Grants DMB 90-04054 and MCB 93-05037 to P.M.

1. Palmer, J. D. (1991) in *The Molecular Biology of Plastids, Cell Culture and Somatic Cell Genetics of Plants*, eds. Bogorad, L. & Vasil, I. K. (Academic, San Diego), Vol. 7A, pp. 5–53.
2. Bendich, A. J. (1987) *BioAssays* **6**, 279–282.
3. Pring, D. R. & Lonsdale, D. M. (1985) *Int. Rev. Cytol.* **97**, 1–46.
4. Newton, K. J. (1988) *Annu. Rev. Plant Phys. Plant Mol. Biol.* **39**, 503–532.
5. Maliga, P. (1993) *Trends Biotechnol.* **11**, 101–106.
6. Maliga, P., Carrer, H., Kanevski, I., Staub, J. & Svab, Z. (1993) *Philos. Trans. R. Soc. London B* **342**, 203–208.
7. Staub, J. M. & Maliga, P. (1992) *Plant Cell* **4**, 39–45.
8. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
9. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153**, 3–11.
10. Svab, Z. & Maliga, P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 913–917.
11. Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torozawa, K., Meng, B. Y., Sugita, M., Deno, H., Kamoyashira, T., Yamada, K., Kusuda, J., Takawa, F., Kato, A., Tohdoh, N. & Sugiura, M. (1986) *EMBO J.* **5**, 2043–2049.
12. Murashige, T. & Skoog, F. (1962) *Physiol. Plant* **15**, 493–497.
13. Kolodner, R. & Tewari, K. K. (1975) *Biochim. Biophys. Acta* **402**, 372–390.
14. Liu, J. W. & Rose, R. J. (1993) *Plant Mol. Biol. Rep.* **11**, 48–53.
15. Staub, J. & Maliga, P. (1993) *EMBO J.* **12**, 601–606.
16. Deng, X.-W., Wing, R. A. & Gruissem, W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4156–4160.
17. Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B. & Sanford, J. C. (1988) *Science* **240**, 1534–1538.
18. Kolodner, R. D. & Tewari, K. K. (1975) *Nature (London)* **256**, 708–711.
19. Meeker, R., Nielsen, B. & Tewari, K. K. (1988) *Mol. Cell. Biol.* **8**, 1216–1223.
20. Chiu, W. L. & Sears, B. B. (1992) *Mol. Gen. Genet.* **232**, 33–39.
21. Takeda, Y., Hirokawa, H. & Nagata, T. (1992) *Mol. Gen. Genet.* **232**, 191–198.
22. Bedbrook, J. R., Kolodner, R. & Bogorad, L. (1977) *Cell* **11**, 739–749.
23. Rothstein, R. (1991) *Methods Enzymol.* **194**, 281–301.