Plant Physiol. (1993) 102: 145–153

DNA Strand-Transfer Activity in Pea (*Pisum sativum* L.) Chloroplasts[1]

Heriberto Cerutti[2], and André T. Jagendorf*

Section of Plant Biology, Cornell University, Ithaca, New York 14853

The occurrence of DNA recombination in plastids of higher plants is well documented. However, little is known at the enzymic level. To begin dissecting the biochemical mechanism(s) involved we focused on a key step: strand transfer between homologous parental DNAs. We detected a RecA-like strand transfer activity in stromal extracts from pea (*Pisum sativum* L.) chloroplasts. Formation of joint molecules requires Mg^{2+}, ATP, and homologous substrates. This activity is inhibited by excess single-stranded DNA (ssDNA), suggesting a necessary stoichiometric relation between enzyme and ssDNA. In a novel assay with Triton X-100-permeabilized chloroplasts, we also detected strand invasion of the endogenous chloroplast DNA by ^{32}P-labeled ssDNA complementary to the 16S rRNA gene. Joint molecules, analyzed by electron microscopy, contained the expected displacement loops.

Genetic recombination is an essential cellular function common to all organisms (Bernstein et al., 1985; Hotta et al., 1985; Roca and Cox, 1990; Eggleston and Kowalczykowski, 1991). It has been argued that its primary biological role is the repair of damaged DNA rather than the production of variation by the exchange of genetic information (Bernstein et al., 1985; Roca and Cox, 1990). In higher plants, DNA recombination occurs in the three genetic systems of the cell, namely, nuclear, mitochondrial, and chloroplast genomes (Hotta et al., 1985; Medgyesy et al., 1985; Levings and Brown, 1989).

Chloroplast DNA recombination has been demonstrated in the unicellular green alga *Chlamydomonas* (Boynton et al., 1991). Several lines of evidence suggest that recombination and related processes, such as gene conversion, also occur in the chloroplast genome (plastome) of higher plants. The existence of multimeric forms of the plastome has been interpreted as resulting from intermolecular recombination (Kolodner and Tewari, 1979; Deng et al., 1989). Several inversions, deletions, and other chromosomal rearrangements occurring during chloroplast DNA evolution are the likely products of recombination events involving repeated elements (Palmer, 1985; Howe et al., 1988; Hiratsuka et al., 1989). Albino wheat and barley plants, regenerated from microspores by another culture, contain deleted forms of the chloroplast genome apparently generated by intramolecular recombination at direct repeats (Day and Ellis, 1984). Intramolecular (flip-flop) recombination in the large inverted repeats is also thought to maintain equimolar populations of two chloroplast DNA isomers, differing only in the relative orientation of their single-copy regions (Palmer, 1985). Moreover, an active mechanism of copy correction is suggested by the sequence conservation of the large inverted repeats, where silent substitutions are less frequent than in single-copy regions and, when present, are fixed symmetrically in both repeats (Palmer, 1985; Fromm et al., 1989; Clegg et al., 1991; Staub and Maliga, 1992).

Direct evidence for chloroplast DNA recombination in higher plants was obtained using selectable markers. Plants with recombinant chloroplast genomes were recovered by efficient selection following interspecific protoplast fusion (Medgyesy et al., 1985; Thanh and Medgyesy, 1989; Fejes et al., 1990). Moreover, transformation of the chloroplast genome apparently occurs by homologous integration of donor DNA (Staub and Maliga, 1992). Little is known about the enzymic mechanism(s) involved. However, considering the endosymbiotic origin of plastids (Gray, 1989), the chloroplast recombination system(s) is probably related to the eubacterial counterpart.

The RecA protein of *Escherichia coli* is a multifunctional enzyme required for homologous recombination and a variety of cellular responses to DNA damage (Roca and Cox, 1990; Sassanfar and Roberts, 1990; Kowalczykowski, 1991; Radding, 1991; West, 1992). The role of the RecA protein in genetic recombination in vivo is inferred from its enzymic activities in vitro. *E. coli* RecA promotes homologous pairing and strand exchange between DNA molecules in an ATP-dependent reaction. The mechanism is complex and proceeds in three distinct steps: presynapsis, synapsis, and branch migration (for reviews, see Roca and Cox, 1990; Kowalczykowski, 1991; Radding, 1991; West, 1992). In the presence of ATP, RecA polymerizes cooperatively on ssDNA to form a presynaptic nucleoprotein filament. The optimal stoichiometry of binding is approximately one RecA monomer per

[1] Partial support was provided by Hatch grant 0155928 and by grant 91-37301-6421 from the U.S. Department of Agriculture/National Research Initiative Competitive Grants Program. H.C. was supported by a predoctoral fellowship from the Cornell National Science Foundation Plant Science Center, a unit in the U.S. Department of Agriculture-Department of Energy-National Science Foundation Plant Science Centers Program and a unit of the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the U.S. Army Research Office.

[2] Present address: Department of Botany, Duke University, Durham, NC 27706.

* Corresponding author; fax 1–607–255–5407.

Abbreviations: D-loop, displacement loop; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

three to four nucleotide residues. During synapsis, the nucleoprotein filament binds and eventually pairs with homologous dsDNA to form joint molecules. The first contacts are between nonhomologous regions, resulting in the formation of a large network of molecules that contributes to acceleration of pairing. This is followed by homologous alignment and the formation of joint molecules, with limited unwinding of the dsDNA. In the final step, homologously aligned molecules undergo a unidirectional, RecA-mediated extension of the heteroduplex region that results in strand exchange.

The key role played by RecA homologs in general recombination in every eubacterium studied in some detail (Roca and Cox, 1990; Eggleston and Kowalczykowski, 1991), and the endosymbiotic origin of chloroplasts (Gray, 1989), made a search for a RecA-like activity a reasonable first step in understanding the biochemical mechanism(s) involved in plastid DNA recombination. In addition, we have recently cloned an *Arabidopsis thaliana* cDNA encoding a protein highly homologous to *E. coli* RecA and containing a predicted chloroplast transit peptide at its amino terminus (Cerutti et al., 1992). By using assays devised to study the strand exchange catalyzed by *E. coli* RecA, we have found a strand transfer activity in pea (*Pisum sativum* L.) chloroplasts. As with the *E. coli* protein, formation of joint molecules requires homologous DNA substrates, Mg^{2+}, and ATP. These results suggest, for the first time, the involvement of a RecA-like activity in chloroplast DNA recombination.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. Calf intestinal alkaline phosphatase and BSA were from Boehringer-Mannheim. Proteinase K, creatine kinase, creatine phosphate, and ATP were from Sigma. *Escherichia coli* RecA protein was from Pharmacia. Exonuclease III, mung bean nuclease, and pBluescript II were obtained from Stratagene. Replicative form I M13mp19 and pUC 8 were from Life Technologies Inc. ^{32}P-labeled nucleotides were obtained from Amersham.

Pea seedlings (*Pisum sativum* L. cv Progress No. 9) were grown as described previously (Nivison et al., 1986). Seven-day-old seedlings were partially etiolated by growing them in the dark for 2 d. Then they were irradiated with 7500 J m^{-2} of UV light from a germicidal lamp (Phillips TL 40W) and kept in the dark for 12 to 15 h before chloroplast isolation.

Preparation of DNA Substrates for Strand-Transfer Assays

Linear [^{32}P]dsDNA and Circular M13 ssDNA

This method was modified from that of McCarthy et al. (1988). A 1.87-kb *Hae*II fragment from pUC8 was blunt ended with mung bean nuclease and cloned into M13mp19. ssDNA was purified by standard procedures (Sambrook et al., 1989). ^{32}P-labeled homologous dsDNA was prepared by digesting supercoiled pUC8 with *Hae*II, followed by treatment with mung bean nuclease. After separation on 1% agarose gels, the 1.87-kb segment was isolated as described by Tautz and Renz (1983) and labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al., 1989).

Linear ^{32}P-ssDNA and Closed Circular Duplex DNA

This method was modified from that of Konforti and Davis (1987). Supercoiled pUC8 was isolated by CsCl/ethidium bromide isopyknic centrifugation (Sambrook et al., 1989). Homologous linear ssDNA was prepared by digesting pUC8 with *Pst*I and *Eco*RI. Then, one strand was digested with exonuclease III (Henikoff, 1984) and the remaining strand purified by agarose gel electrophoresis (Tautz and Renz, 1983). The ssDNA was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al., 1989).

Linear ^{32}P-ssDNA Homologous to the Plastid 16S rRNA Gene

The pea chloroplast 16S rDNA was cloned into the pBluescriptII KS$^+$ vector (Cerutti and Jagendorf, 1991). A 0.45-kb *Eco*RI/*Dra*II fragment was excised by standard procedures and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al., 1989). Labeled DNA was denatured with urea and moderate heat and separated on 5% nondenaturing polyacrylamide gels (James and Bradshaw, 1984). The two bands of ssDNA were cut out of the gel and DNA purified by a crush-and-soak procedure (Dybczynski and Plucienniczak, 1988).

Linear ssDNA with Ends Homologous to Plastid DNA (for EM Analysis)

The pBluescriptII KS$^+$ vector containing a 2.0-kb *Sac*I/*Hin*cII chloroplast fragment, comprising the 16S rRNA gene (Cerutti and Jagendorf, 1991), was linearized with *Eco*RI. This leaves 0.5 and 1.5 kb of sequences homologous to chloroplast DNA (Fig. 6) flanking vector sequences. The DNA was denatured (James and Bradshaw, 1984) and separated on 1.3% agarose gels, and the ssDNA bands were purified as described by Tautz and Renz (1983).

Strand-Transfer Assays

Stromal Extract

Chloroplasts were isolated from pea leaves as described by Nivison et al. (1986). They were resuspended in incubation buffer (25 mM Hepes-KOH, pH 7.5; 330 mM sorbitol; 20 mM KCl; 1 mM DTT; 0.05% BSA) at approximately 1.5 to 2.0 mg of Chl mL^{-1} and broken by adding Triton X-100 to a final concentration of 0.1%. Membranes were pelleted by centrifugation in a microfuge for 5 min at 4°C. Eighty-microliter aliquots of the supernatant were used for the reactions. When needed, ATP and $MgCl_2$ were added to 10 mM final concentration. The standard reactions contained 100 ng of ssDNA and 750 ng of dsDNA and were adjusted to a final volume of 100 μL with incubation buffer. The dsDNA was added last, followed, after 5 min, by SDS to a 0.5% final concentration. Samples were incubated in the dark for 40 min at 25°C. As reported before (McKown and Tewari, 1984), the stromal

extract has significant nuclease activity, and the addition of SDS was found to improve the signal-to-noise ratio. Presumably, SDS inactivates the nuclease(s) much faster than the recombinase(s). However, the effective concentration of SDS is unknown because it depends on the amount of interacting proteins, membranes, etc. present in the extract. This caused some variability in the experiments and, occasionally, complete loss of the strand-transfer activity.

Reactions were stopped by adding SDS, sodium sarcosinate, and EDTA to 1.5%, 1.5%, and 50 mM final concentration, respectively. All subsequent manipulations were done at 4°C to minimize branch migration. DNA was purified by standard phenol/chloroform extractions (Sambrook et al., 1989) and separated by agarose gel electrophoresis. Gels were dried as described by Silhavy et al. (1984) and exposed to Kodak XAR-5 film.

Permeabilized Chloroplasts

Chloroplasts were isolated and broken with Triton X-100 as described above. Eighty-microliter aliquots of the permeabilized chloroplasts were used for the reactions. The substrates were the endogenous chloroplast DNA (presumably dsDNA) and 100 ng of ^{32}P-ssDNA homologous to the plastid 16S rRNA gene. Incubation conditions and subsequent DNA isolation were as described for stromal extract. Purified DNA was digested with *PstI* at 20°C for 1 to 2 h, using a 30-fold excess of enzymic activity. Under these conditions the digestion was found to proceed to completion while minimizing loss of D-loop structures by branch migration. Agarose gel electrophoresis and autoradiographic detection were as described above.

Control Assays with E. coli RecA

When using as substrates linear [^{32}P]-dsDNA from pUC8 and circular M13 ssDNA, strand-transfer reactions were carried out as described by McCarthy et al. (1988) for 5 min, to allow formation of stable joint molecules. In the assays with linear ^{32}P-ssDNA and closed circular dsDNA from pUC8, we used the conditions described by Konforti and Davis (1987) with 10 min of incubation without adding *E. coli* ssDNA-binding protein. All subsequent manipulations were as described above.

EM Analysis

The strand-transfer reactions were performed using permeabilized chloroplasts and, as substrates, the endogenous chloroplast DNA and unlabeled ssDNA with chloroplast rDNA sequences flanking vector sequences (see above). After purification, the DNA was digested with *BamHI* and separated on 0.8% agarose gels. A broad band containing the joint molecules, previously identified by using labeled ssDNA, was cut out of the gel, and DNA was isolated as described by Tautz and Renz (1983). Purified DNA was spread for EM using the formamide/Cyt *c* technique (Inman and Schnös, 1970). Grids were rotary shadowed with Pt/Pd (4:1) and examined in a Phillips 300 electron microscope. Micrographs were projected onto a digitizing tablet to determine the contour length of the DNA molecules (Zidas Image Analysis; Carl Zeiss).

RESULTS

Strand-Transfer Activity in Chloroplast Extracts

E. coli RecA promotes the exchange of DNA strands between a variety of cDNA substrates. Gel electrophoresis has been used extensively to identify the products of this reaction by their mobility (Konforti and Davis, 1987; Griffith and Harris, 1988; McCarthy et al., 1988). However, in crude cellular extracts, other enzymic activities can lead to artifactual products as discussed in detail by Griffith and Harris (1988). Therefore, we used several assays to identify a true strand-transfer activity in chloroplasts of pea.

In one assay, modified from that of McCarthy et al. (1988), a recombinase catalyzes strand transfer between a ^{32}P-labeled linear dsDNA fragment and a complementary segment cloned into the ssDNA *E. coli* phage M13mp19. A scheme for such a reaction, consistent with the mechanism of strand transfer by RecA (Roca and Cox, 1990; Kowalczykowski, 1991; Radding, 1991), is shown in Figure 1A. The DNA

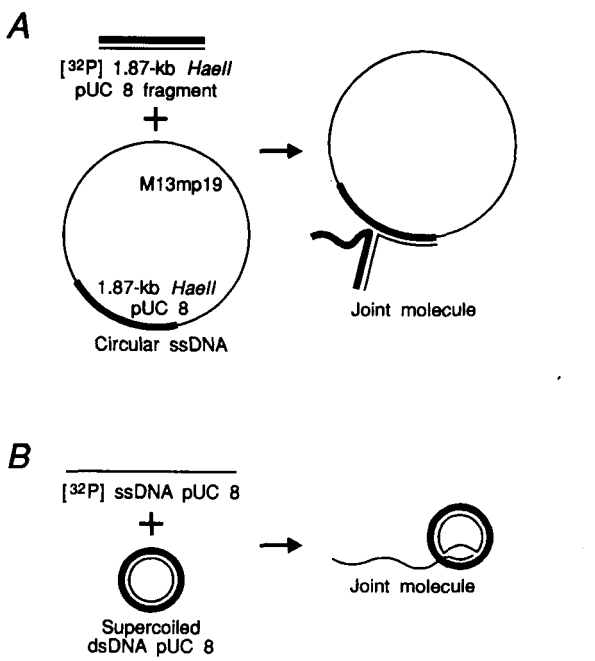

Figure 1. DNA substrates used to assay for a chloroplast strand-transfer activity. A, Formation of joint molecules by strand transfer between the 1.87-kb *HaeII* fragment from pUC8 (linear dsDNA) labeled with ^{32}P and the unlabeled M13mp19 vector into which this fragment has been cloned (circular ssDNA). In the linear dsDNA, thin and thick lines indicate complementary strands. In the circular ssDNA, the thick line indicates the sequence corresponding to the *HaeII* fragment (complementary to the strand indicated by a thin line in the linear dsDNA). The M13mp19 vector sequence is indicated by a thin line in the circular molecule. B, Formation of joint molecules by strand transfer between ^{32}P-labeled linear ssDNA and unlabeled supercoiled dsDNA. The thick and thin lines indicate complementary strands.

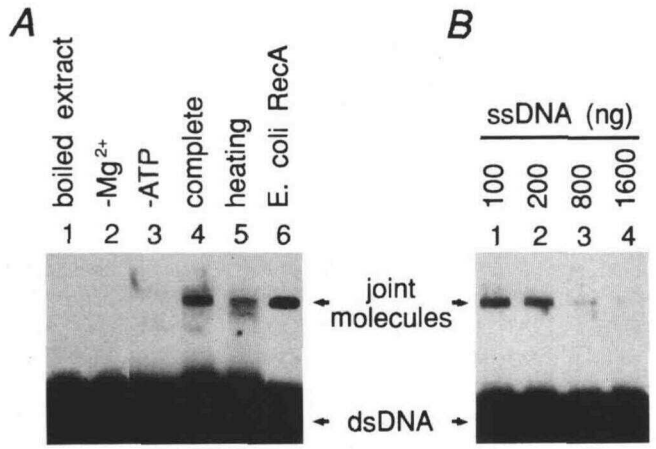

Figure 2. The formation of joint molecules requires a heat-sensitive factor, Mg^{2+}, and ATP, and it is inhibited by excess of ssDNA. A, Strand-transfer assays were carried out as described in "Materials and Methods" using the substrates depicted in Figure 1A. Lane 1, DNA substrates incubated with boiled stromal extract; lane 2, DNA substrates incubated with native stromal extract in the absence of Mg^{2+}; lane 3, DNA substrates incubated with native stromal extract in the absence of ATP; lane 4, complete reaction; lane 5, DNA from a complete reaction was purified and heated to 65°C for 5 min before loading the gel; lane 6, control reaction with purified *E. coli* RecA. B, Inhibition of the chloroplast strand-transfer activity by excess homologous ssDNA. Lanes 1 to 4, Reactions carried out in the presence of 100, 200, 800, and 1600 ng of ssDNA substrate, respectively.

substrates were incubated with a stromal extract from pea chloroplasts, under the conditions described in "Materials and Methods." As previously reported (McKown and Tewari, 1984), this stromal extract contains a significant level of nuclease and/or phosphatase activity (data not shown). However, a product with the same mobility as that generated by purified *E. coli* RecA was clearly detected (Fig. 2A). On the other hand, this activity was too low to generate a visible signal in conventional ethidium bromide-stained gels (data not shown).

The formation of joint molecules requires a heat-sensitive factor(s), because boiling of the stromal extract abolished detection of any product (Fig. 2A). Incubation of the dsDNA with nonhomologous ssDNA also failed to generate a product (data not shown). The strand-transfer activity was dependent on Mg^{2+} and ATP (Fig. 2A) and, as predicted by the strand-transfer scheme shown in Figure 1A, the reaction products were partially unstable to heating at 65°C for 5 min (Fig. 2A). However, they were stable to treatment with 1.5% SDS, 1.5% sodium sarcosinate, and phenol/chloroform, routinely used for DNA purification before gel electrophoresis.

In this assay, the conversion of the labeled dsDNA substrate to a partially or completely single-stranded molecule by a nuclease or helicase, followed by annealing to the complementary ssDNA substrate, would generate an artifactual product. As a control, we incubated labeled linear dsDNA and circular ssDNA separately with the stromal extract, under the conditions used for the strand-transfer reaction. These DNA substrates were purified by phenol/chloroform extractions, combined, and incubated in a standard reaction lacking the protein extract. The amount of annealed product detected was less than 10% of that generated by the recombinase activity in the stromal extract (data not shown).

A further indication that the activity forming most of the joint DNA molecules is not a nuclease or helicase is given by the fact that an excess of homologous ssDNA substrate inhibited the reaction (Fig. 2B). This rules out the mechanism in which a nuclease or helicase generates ssDNA, which can anneal with the added ssDNA. We are left with a very high probability of a chloroplast enzyme which, like *E. coli* RecA, accomplishes strand transfer by binding to ssDNA with an optimal stoichiometry to form a nucleoprotein filament (Roca and Cox, 1990; Kowalczykowski, 1991; Radding, 1991; West, 1992).

A second strand-transfer assay involved ^{32}P-labeled linear ssDNA and homologous closed circular dsDNA (Shibata et al., 1979; Konforti and Davis, 1987), as depicted in Figure 1B. These substrates are more representative of recombinogenic DNA existing in a cell (Siddiqi and Fox, 1973; Konforti and Davis, 1987; Sun et al., 1991). In addition, this assay serves as a control against a role of DNA ligases in product formation, because these enzymes are unable to link free ssDNA molecules (Lehman, 1974).

Incubation of the substrates with the stromal extract resulted in the formation of a product comigrating with that

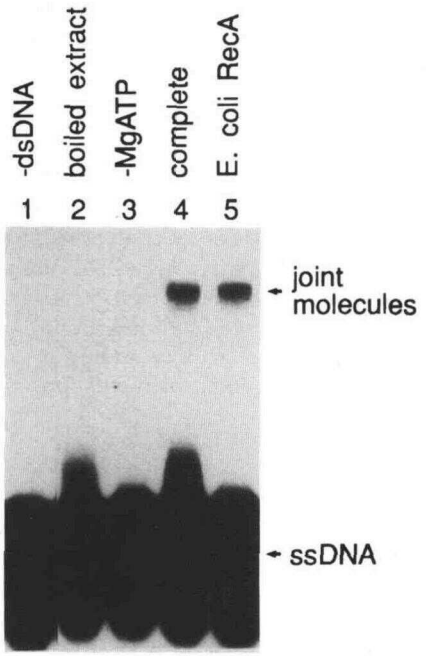

Figure 3. Formation of joint molecules by strand transfer between ^{32}P-labeled linear ssDNA and unlabeled supercoiled dsDNA, as depicted in Figure 1B. Lane 1, ssDNA incubated with stromal extract in the absence of complementary dsDNA; lane 2, DNA substrates incubated with boiled stromal extract; lane 3, DNA substrates incubated with native stromal extract in the absence of MgATP; lane 4, complete reaction; lane 5, control reaction with purified *E. coli* RecA.

generated by *E. coli* RecA (Fig. 3). As before, this activity was abolished by boiling the extract. The formation of joint molecules also exhibits an absolute dependence on MgATP (Fig. 3). The joint molecules migrate slightly slower than a relaxed circle of dsDNA. This argues against the involvement of a protein covalently binding to the ends of the ssDNA and forming a circle (Griffith and Harris, 1988) or circularization of the ssDNA by RNA ligase (Tessier et al., 1986), because these products would migrate faster on an agarose gel.

Strand-Transfer Activity in Permeabilized Chloroplasts

The assays with naked exogenous DNA substrates indicated the existence of a strand-transfer activity in chloroplasts of pea. However, in vivo chloroplast DNA is organized in nucleoids, interacting with several proteins (Nemoto et al., 1988; Kuroiwa, 1991). Thus, we were interested in finding whether this activity can operate on exogenous naked DNA and the endogenous chloroplast DNA, the predicted substrates in experiments of chloroplast genome transformation by recombination (Boynton et al., 1991; Staub and Maliga, 1992).

We used Triton X-100-permeabilized chloroplasts in a reaction equivalent to that depicted in Figure 1B. The ^{32}P-ssDNA was a 450-nucleotide segment internal to the plastid 16S rRNA gene (Cerutti and Jagendorf, 1991), and the complementary substrate was the 120-kb polyploid chloroplast genome (Palmer and Thompson, 1981; Palmer, 1985). After incubation, the DNA was purified and digested with *Pst*I, under conditions that minimize branch migration. The fragments were separated by agarose gel electrophoresis, and the radioactive signal was detected by autoradiography.

The 16S rRNA gene is located in a 12.5-kb *Pst*I fragment in the chloroplast genome of pea (Palmer and Thompson, 1981; Meeker et al., 1988). Consistent with the interaction of the exogenous ^{32}P-ssDNA with the chloroplast 16S rDNA, a band of approximately 12.3 kb was detected in the complete reaction (Fig. 4). The formation of this product was dependent on MgATP and the presence of ^{32}P-ssDNA homologous to the chloroplast DNA (Fig. 4). As before, the reaction was inhibited by the addition of excess unlabeled nonhomologous ssDNA (Fig. 4).

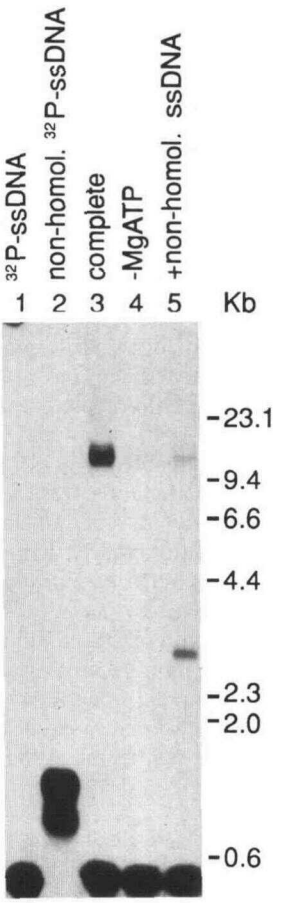

Figure 4. Detection of strand-transfer activity in permeabilized chloroplasts using the endogenous chloroplast DNA and labeled homologous ssDNA as substrates. After the reaction, the DNA was digested with *Pst*I and separated on agarose gels. Lane 1, Labeled ssDNA substrate. Lane 2, Permeabilized chloroplasts incubated with labeled nonhomologous ssDNA (from pUC8); the lower molecular mass band seems to be a degradation product of the isolated ssDNA. This interpretation is supported by the fact that no renaturation to dsDNA was observed under any conditions, as would be expected if the complementary strand were present. Lane 3, Permeabilized chloroplasts incubated with labeled ssDNA homologous to a region internal to the 16S rRNA gene (complete reaction). Lane 4, As in lane 3 but in the absence of MgATP. Lane 5, As in lane 3 with the addition of 1800 ng of ssDNA (M13mp19) nonhomologous to the chloroplast DNA. The approximately 3.0-kb band in this treatment comigrates with the unlabeled circular M13mp19 ssDNA and presumably results from annealing of the labeled ssDNA, although the two ssDNA molecules are not expected to be homologous.

Characterization of the Joint Molecules by EM

To obtain more direct evidence for the formation of joint molecules, we analyzed by EM the products of the assay with permeabilized chloroplasts. In this case, we used as ssDNA substrate the pBluescript vector flanked by sequences homologous to chloroplast rDNA, as described in "Materials and Methods." Thus, the strand-transfer reaction is expected to stop at the region of nonhomology (vector sequences), generating a D-loop with a tail of ssDNA (Shibata et al., 1979; Konforti and Davis, 1987; Roca and Cox, 1990; Kowalczykowski, 1991).

In pea chloroplast DNA, there are two replication origins that have been mapped as D-loops by EM analysis. They are located in the spacer region between the 16S and 23S rRNA genes and downstream from the 23S rRNA gene (Meeker et al., 1988). To avoid artifactual results due to these structures, the DNA was digested with *Bam*HI. The 16S rRNA gene is contained in an 8-kb *Bam*HI fragment that has no replication D-loops and is not active as a template for chloroplast DNA polymerase in vitro (Meeker et al., 1988). In preliminary experiments using ^{32}P-ssDNA, we determined that the joint molecules migrated as an approximately 8.9- to 9.0-kb band. Thus, a wide agarose slice comprising the region from 8.0 to 9.1 kb was cut out from the gel. The DNA was purified and subject to EM analysis, as described in "Materials and Meth-

ods." The linear 8-kb fragments isolated with the joint molecules were used as internal standards for size determination.

We found several molecules with the expected D-loop structures and ssDNA tails (Fig. 5). Under the spreading conditions used, ssDNA is thinner and kinkier than dsDNA. Thus, one side of the D-loops seems single stranded, whereas the other side appears to be double stranded, with a protruding tail of ssDNA (Fig. 5). In all cases, the D-loops were found in the corresponding region of homology, and the joint molecules were of the expected size (Fig. 6). However, the ssDNA tails were shorter than expected, and in a few cases they were missing. Presumably, this is due to breakage during the DNA manipulations (McEntee et al., 1979) and/or digestion by nucleases in the chloroplast extract (McKown and Tewari, 1984).

Other structures, presumably formed by annealing of the added ssDNA to complementary ssDNA gaps in the chloroplast DNA, were also detected (data not shown). Because the plants were irradiated with UV light before chloroplast isolation, noncoding lesions (such as pyrimidine dimers) could have caused DNA synthesis to become discontinuous, generating ssDNA gaps when replication reinitiates downstream from the lesions (McLennan, 1988; Sassanfar and Roberts, 1990). We also observed long, free ssDNA fragments (Fig. 5 and data not shown), presumably resulting from breakage and protein-independent branch migration. Such a phenomenon has been observed before for the strand-transfer products of RecA (Kahn et al., 1981).

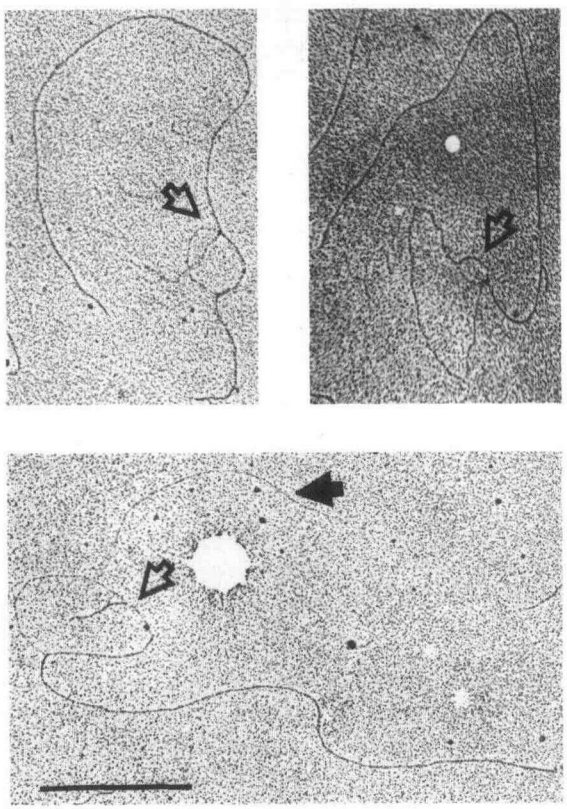

Figure 5. Electron micrographs of joint molecules formed in the assay with permeabilized chloroplasts. Note the presence of D-loops with the expected ssDNA tails (open arrows). Long free pieces of ssDNA were also found (closed arrow). Bar, 0.5 μm.

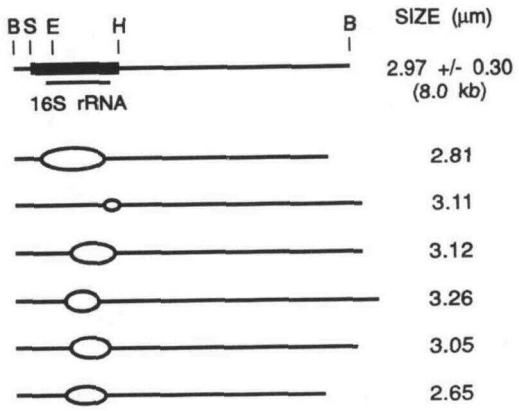

Figure 6. In the molecules analyzed by EM, the D-loops were found in the expected region of homology with the chloroplast DNA. The 8-kb *Bam*HI fragment containing the 16S rRNA gene in pea chloroplast DNA is shown at the top. The thick line indicates the 2-kb *Sac*I/*Hin*cII chloroplast DNA fragment cloned into the pBluescript vector. The positions of the D-loops and contour lengths of observed individual molecules are indicated below. The size of the standard 8-kb fragment is expressed as the mean ± SD (n = 30). Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*cII; S, *Sac*I.

As a control we incubated permeabilized chloroplasts, under standard conditions, in the absence of exogenous ssDNA. Chloroplast DNA was purified and complementary ssDNA added before the digestion with *Bam*HI. After separation by agarose gel electrophoresis, DNA was isolated from the 8.0- to 9.1-kb region and analyzed by EM. However, we were unable to find any molecule with a D-loop structure or ssDNA hybridizing to a ssDNA gap. We could not detect free ssDNA fragments either (data not shown).

DISCUSSION

There is convincing evidence that DNA recombination takes place in chloroplasts of higher plants (Medgyesy et al., 1985; Thanh and Medgyesy, 1989; Fejes et al., 1990; Staub and Maliga, 1992). However, our understanding of the biochemical mechanism(s) involved is still extremely limited. Current models of general recombination require initiation of the reaction by strand exchange between ssDNA from one molecule and a homologous region of dsDNA from the other (Holliday, 1964; Meselson and Radding, 1975; Szostak et al., 1983). This strand exchange is uniquely catalyzed by recombinases, like *E. coli* RecA (Roca and Cox, 1990; Dykstra et al., 1991; Eggleston and Kowalczykowski, 1991; Johnson and Kolodner, 1991; Sanders et al., 1991; West, 1992).

Several assays have been devised to analyze the strand-exchange activity of RecA (Shibata et al., 1979; McEntee et al., 1980; Konforti and Davis, 1987; Griffith and Harris, 1988; McCarthy et al., 1988). During this reaction, invading ssDNA from one molecule pairs with its complementary recipient in dsDNA, thereby displacing the noncomplementary strand of the acceptor DNA molecule and forming a joint molecule.

Using several assays based on this general mechanism, we have found a strand-transfer activity in pea chloroplasts.

The formation of joint molecules by chloroplast extracts requires DNA homology, Mg^{2+}, and ATP. One can envision several alternative pathways whereby two homologous DNA substrates become joined by heteroduplex regions or artifactual products with similar migration are formed (Griffith and Harris, 1988; McCarthy et al., 1988). However, as already discussed, results of several control experiments argue against a role of nucleases, ligases, or a DNA-binding protein in product formation. Perhaps the most convincing evidence for strand transfer is the direct visualization by EM of molecules with the expected D-loop structures. We cannot exclude the possibility that these molecules are formed by a helicase, which unwinds the dsDNA, allowing annealing of one strand to the complementary ssDNA. However, recent experiments have shown that DNA helicases either have no effect or inhibit the homologous pairing step in bacteriophage T4 UvsX-mediated and *E. coli* RecA-mediated strand-exchange reactions (Kodadek, 1991). Moreover, the formation of joint molecules in chloroplasts is inhibited by excess ssDNA, which is not consistent with a mechanism involving a helicase.

Proteins able to carry out DNA strand transfer have been isolated from a number of eukaryotes (Hotta et al., 1985; McCarthy et al., 1988; Roca and Cox, 1990; Dykstra et al., 1991; Eggleston and Kowalczykowski, 1991; Johnson and Kolodner, 1991; Sanders et al., 1991; West, 1992). Two genes involved in DNA repair and recombination in *Saccharomyces cerevisiae* have recently been isolated and shown to encode proteins structurally similar to eubacterial RecA (Bishop et al., 1992; Shinohara et al., 1992; West, 1992). However, the actual mechanism of action of these proteins is still unknown. Several other eukaryotic proteins promote DNA strand exchange without the need for a nucleoside triphosphate cofactor and can function at nearly catalytic concentrations (McCarthy et al., 1988; Dykstra et al., 1991; Eggleston and Kowalczykowski, 1991; Johnson and Kolodner, 1991; Sanders et al., 1991; West, 1992). Moreover, these strand-exchange proteins also contain an intrinsic DNA exonuclease (Dykstra et al., 1991; Johnson and Kolodner, 1991; Sanders et al., 1991; West, 1992). None of these proteins has been shown yet to form a nucleoprotein filament, and they apparently act by a mechanism different from that of *E. coli* RecA (Eggleston and Kowalczykowski, 1991; Radding, 1991; West, 1992).

In contrast, the activity detected in chloroplasts of pea appears to be similar to that of *E. coli* RecA. The requirement for ATP and the inhibition by excess ssDNA, suggesting a required stoichiometric relation between the enzyme and ssDNA, are consistent with the mechanism of strand transfer that has been demonstrated for RecA. In further support, we have found in chloroplasts of pea a 39-kD protein, immunologically related to *E. coli* RecA, that is induced by DNA-damaging agents (H. Cerutti et al., 1993). A similar protein, with an apparent molecular mass of 40.5 kD, was also detected in *A. thaliana* chloroplasts (Cerutti et al., 1992). Moreover, a gene encoding a RecA-like protein, including a predicted chloroplast transit peptide, was cloned from an *A. thaliana* cDNA library (Cerutti et al., 1992). The combination of these four lines of evidence strongly suggests that the chloroplast strand-transfer activity is due to a RecA protein homolog.

A role of ssDNA in initiating DNA strand exchange has been proposed in several models of recombination (Holliday, 1964; Meselson and Radding, 1975; Szostak et al., 1983). In *S. cerevisiae* an early event in meiosis is the formation at or near meiotic hot spots for recombination of double-strand breaks having 3′ single-stranded tails. Results of biochemical and genetic analyses strongly suggest that molecules with ssDNA ends are early intermediates in yeast meiotic recombination (Padmore et al., 1991; Sun et al., 1991; Bishop et al., 1992; Shinohara et al., 1992). Moreover, in studies of *E. coli* Hfr conjugation, Siddiqi and Fox (1973) found that donor DNA integrates as a single strand, preferentially into the newly synthesized recipient strands. Because Triton X-100 apparently does not affect the structure of plastid nucleoids (Nemoto et al., 1988; Kuroiwa, 1991), our results with permeabilized chloroplasts suggest that ssDNA can also act as an efficient substrate for homologous invasion of the chloroplast DNA. This reaction probably mimics the initial events in the integration of homologous donor DNA during chloroplast transformation (Boynton et al., 1991; Staub and Maliga, 1992).

The RecA-like activity shown here may function in chloroplast DNA recombination and the integration of homologous donor DNA during chloroplast transformation. Further research on the biochemical mechanism(s) involved in chloroplast recombination is needed and may provide insights into a wide range of problems such as chloroplast DNA evolution, chloroplast DNA repair, sequence conservation of plastid DNA, and uniparental inheritance of the chloroplast genome. From a practical point of view, this information might be useful for improving the current technology of chloroplast genome transformation.

ACKNOWLEDGMENT

We would like to thank M.V. Parthasarathy for generously allowing the use of the electron microscope facilities.

Received October 12, 1992; accepted January 29, 1993.
Copyright Clearance Center: 0032-0889/93/102/0145/09.

LITERATURE CITED

Bernstein H, Byerly HC, Hopf FA, Michod RE (1985) Genetic damage, mutation, and the evolution of sex. Science **229:** 1277–1281

Bishop DK, Park D, Xu L, Kleckner N (1992) DMC1: a meiosis-specific yeast homolog of *E. coli* recA required for recombination, synaptonemal complex formation, and cell cycle progression. Cell **69:** 439–456

Boynton JE, Gillham NW, Newman SM, Harris EH (1991) Organelle genetics and transformation of *Chlamydomonas*. *In* R Herrmann, ed, Cell Organelles—Advances in Plant Gene Research, Vol 6. Springer-Verlag, Vienna, Austria, pp 1–48

Cerutti H, Ibrahim HZ, Jagendorf AT (1993) Treatment of pea protoplasts with DNA-damaging agents induces a 39-kilodalton chloroplast protein immunologically related to *Escherichia coli* RecA. Plant Physiol **102:** 155–163

Cerutti H, Jagendorf AT (1991) Nucleotide sequence of the chloroplast 16S rRNA gene from pea (*Pisum sativum* L.). Plant Mol Biol **17:** 125–126

Cerutti H, Osman M, Grandoni P, Jagendorf AT (1992) A homolog

of *Escherichia coli* RecA protein in plastids of higher plants. Proc Natl Acad Sci USA **89:** 8068–8072

Clegg MT, Learn GH, Golenberg EM (1991) Molecular evolution of chloroplast DNA. *In* R Selander, A Clark, T Whittam, eds, Evolution at the Molecular Level. Sinauer Associates, Sunderland, MA, pp 135–149

Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible bases for maternal inheritance of chloroplasts. Cell **39:** 359–368

Deng XW, Wing RA, Gruissem W (1989) The chloroplast genome exists in multimeric forms. Proc Natl Acad Sci USA **86:** 4156–4160

Dybczynski I, Plucienniczak A (1988) A protocol for DNA fragment extraction from polyacrylamide gels. Biotechniques **6:** 924–926

Dykstra CC, Kitada K, Clark AB, Hamatake RK, Sugino A (1991) Cloning and characterization of DST2, the gene for DNA strand transfer protein β from *Saccharomyces cerevisiae*. Mol Cell Biol **11:** 2583–2592

Eggleston AK, Kowalczykowski SC (1991) An overview of homologous pairing and DNA strand exchange proteins. Biochimie **73:** 163–176

Fejes E, Engler D, Maliga P (1990) Extensive homologous chloroplast DNA recombination in the pt14 *Nicotiana* somatic hybrid. Theor Appl Genet **79:** 28–32

Fromm H, Galun E, Edelman M (1989) A novel site for streptomycin resistance in the "530 loop" of chloroplast 16S ribosomal RNA. Plant Mol Biol **12:** 499–505

Gray MW (1989) The evolutionary origin of organelles. Trends Genet **5:** 294–299

Griffith JD, Harris LD (1988) DNA strand exchanges. CRC Crit Rev Biochem **23:** S43–S86

Henikoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:** 351–359

Hiratsuka J, Shimada H, Whittier R, Ishibashi T, Sakamoto M, Mori M, Kondo C, Honji Y, Sun CR, Meng BY, Li YQ, Kanno A, Nishizawa Y, Hirai A, Shinozaki K, Sugiura M (1989) The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol Gen Genet **217:** 185–194

Holliday R (1964) A mechanism for gene conversion in fungi. Genet Res **5:** 282–304

Hotta Y, Tabata S, Bouchard RA, Piñon R, Stern H (1985) General recombination mechanisms in extracts of meiotic cells. Chromosoma **93:** 140–151

Howe CJ, Barker RF, Bowman CM, Dyer TA (1988) Common features of three inversions in wheat chloroplast DNA. Curr Genet **13:** 343–349

Inman RB, Schnös M (1970) Partial denaturation of thymine- and 5-bromouracil-containing DNA in alkali. J Mol Biol **49:** 93–98

James R, Bradshaw RA (1984) Strand separation of DNA fragments and their isolation from nondenaturing polyacrylamide gels. Anal Biochem **140:** 456–458

Johnson AW, Kolodner RD (1991) Strand exchange protein 1 from *Saccharomyces cerevisiae*. A novel multifunctional protein that contains DNA strand exchange and exonuclease activities. J Biol Chem **266:** 14046–14054

Kahn R, Cunningham RP, DasGupta C, Radding CM (1981) Polarity of heteroduplex formation promoted by *Escherichia coli* RecA protein. Proc Natl Acad Sci USA **78:** 4786–4790

Kodadek T (1991) Inhibition of protein-mediated homologous pairing by a DNA helicase. J Biol Chem **266:** 9712–9718

Kolodner R, Tewari KK (1979) Inverted repeats in chloroplast DNA from higher plants. Proc Natl Acad Sci USA **76:** 41–45

Konforti BB, Davis RW (1987) 3′ Homologous free ends are required for stable joint molecule formation by the RecA and single-stranded binding proteins of *Escherichia coli*. Proc Natl Acad Sci USA **84:** 690–694

Kowalczykowski SC (1991) Biochemistry of genetic recombination: energetics and mechanism of DNA strand exchange. Annu Rev Biophys Chem **20:** 539–575

Kuroiwa T (1991) The replication, differentiation, and inheritance of plastids with emphasis on the concept of organelle nuclei. Int Rev Cytol **128:** 1–62

Lehman IR (1974) DNA ligase: Structure, mechanism, and function. Science **186:** 790–797

Levings CS, III, Brown GG (1989) Molecular biology of plant mitochondria. Cell **56:** 171–179

McCarthy JG, Sander M, Lowenhaupt K, Rich A (1988) Sensitive homologous recombination strand-transfer assay: partial purification of a *Drosophila melanogaster* enzyme and detection of sequence effects on the strand-transfer activity of RecA protein. Proc Natl Acad Sci USA **85:** 5854–5858

McEntee K, Weinstock GM, Lehman IR (1979) Initiation of general recombination catalyzed *in vitro* by the RecA protein of *Escherichia coli*. Proc Natl Acad Sci USA **76:** 2615–2619

McEntee K, Weinstock GM, Lehman IR (1980) RecA protein-catalyzed strand assimilation: stimulation by *Escherichia coli* single-stranded DNA-binding protein. Proc Natl Acad Sci USA **77:** 857–861

McKown RL, Tewari KK (1984) Purification and properties of a pea chloroplast DNA polymerase. Proc Natl Acad Sci USA **81:** 2354–2358

McLennan AG (1988) DNA damage, repair, and mutagenesis. *In* J Bryant, V Dunham, eds, DNA Replication in Plants. CRC Press, Boca Raton, FL, pp 135–186

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast DNA recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA **82:** 6960–6964

Meeker R, Nielsen B, Tewari KK (1988) Localization of replication origins in pea chloroplast DNA. Mol Cell Biol **8:** 1216–1223

Meselson MS, Radding CM (1975) A general model for genetic recombination. Proc Natl Acad Sci USA **72:** 358–361

Nemoto Y, Kawano S, Nakamura S, Mita T, Nagata T, Kuroiwa T (1988) Studies on plastid-nuclei (nucleoids) in *Nicotiana tabacum* L. I. Isolation of proplastid-nuclei from cultured cells and identification of proplastid-nuclear proteins. Plant Cell Physiol **29:** 167–177

Nivison HT, Fish LE, Jagendorf AT (1986) Translation by isolated pea chloroplasts. Methods Enzymol **118:** 282–295

Padmore R, Cao L, Kleckner N (1991) Temporal comparison of recombination and synaptonemal complex formation during meiosis in *S. cerevisiae*. Cell **66:** 1239–1256

Palmer JD (1985) Comparative organization of chloroplast genomes. Annu Rev Genet **19:** 325–354

Palmer JD, Thompson WF (1981) Rearrangements in the chloroplast genomes of mung bean and pea. Proc Natl Acad Sci USA **78:** 5533–5537

Radding CM (1991) Helical interactions in homologous pairing and strand exchange driven by RecA protein. J Biol Chem **266:** 5355–5358

Roca AI, Cox MM (1990) The RecA protein: structure and function. CRC Crit Rev Biochem Mol Biol **25:** 415–456

Sambrook J, Fritsch EG, Maniatis T (1989) Molecular Cloning—A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanders M, Lowenhaupt K, Lane WS, Rich A (1991) Cloning and characterization of Rrp1, the gene encoding *Drosophila* strand transferase: carboxy-terminal homology to DNA repair endo/exonucleases. Nucleic Acids Res **19:** 4523–4529

Sassanfar M, Roberts JW (1990) Nature of the SOS-inducing signal in *Escherichia coli*. The involvement of DNA replication. J Mol Biol **212:** 79–96

Shibata T, DasGupta C, Cunningham RP, Radding CM (1979) Purified *Escherichia coli* RecA protein catalyzes homologous pairing of superhelical DNA and single-stranded fragments. Proc Natl Acad Sci USA **76:** 1638–1642

Shinohara A, Ogawa H, Ogawa T (1992) Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. Cell **69:** 457–470

Siddiqi O, Fox MS (1973) Integration of donor DNA in bacterial conjugation. J Mol Biol **77:** 101–123

Silhavy TJ, Berman ML, Enquist LW (1984) Experiments with Gene Fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Staub JM, Maliga P (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. Plant Cell **4:** 39–45

Sun H, Treco D, Szostak JW (1991) Extensive 3′-overhanging, single-stranded DNA associated with the meiosis-specific double-strand breaks at the ARG4 recombination initiation site. Cell **64:** 1155–1161

Szostak J, Orr-Weaver T, Rothstein R, Stahl F (1983) The double-strand break repair model for recombination. Cell **33:** 25–35

Tautz D, Renz M (1983) An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal Biochem **132:** 14–19

Tessier DC, Brousseau R, Vernet T (1986) Ligation of single-stranded oligodeoxyribonucleotides by T4 RNA ligase. Anal Biochem **158:** 171–178

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol **12:** 87–93

West SC (1992) Enzymes and molecular mechanisms of genetic recombination. Annu Rev Biochem **61:** 603–640