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Rearrangements in the chloroplast genomes of mung bean and pea

(inverted repeat/restriction endonuclease maps/ribosomal genes/sequence arrangement)

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ABSTRACT **We have mapped all the cleavage sites for the restriction endonucleases *Bst*EII, *Kpn* I, *Pst* I, *Pvu* II, *Sac* I, *Sal* I, *Sma* I, and *Xho* I on the circular chloroplast chromosomes from mung bean and pea. The mung bean chloroplast genome measures 150 kilobase pairs (kb) in length; it includes two identical sequences of 23 kb that contain the ribosomal genes and are arranged as an inverted repeat separated by single-copy regions of 21 and 83 kb. The pea chloroplast genome is only 120 kb in size, has only one set of ribosomal genes, and does not possess any detectable repeated sequences. The mung bean inverted repeat structure is common to all other nonleguminous higher plant chloroplast genomes studied, whereas the pea structure has been found only in the closely related legume *Vicia faba*. We conclude from these data that loss of one copy of the inverted repeat sequence has occurred only rarely during the evolution of the Angiosperms, and in the case of the legumes after the divergence of the mung bean line from the pea–*Vicia* line. We present hybridization data indicating that rearrangements that change the linear order of homologous sequences within the chloroplast genome have been quite frequent during the course of legume evolution.**

The great majority of higher plant chloroplast genomes studied consist of a circular molecule 134 kilobase pairs (kb) to 150 kb in length, which contains a large inverted repeat sequence of 20–25 kb. This pattern has been demonstrated in corn, spinach, and lettuce by intramolecular homoduplex formation (1) and in corn, wheat, spinach, *Oenothera*, tobacco, petunia, and *Spirodela* by restriction endonuclease mapping (2–7). The only exceptions to this pattern so far reported are pea and broad bean (*Vicia faba*), both members of the legume family. Pea chloroplast DNA lacks the inverted repeat structure as judged by electron microscopy (1) but is reported to possess two copies of the ribosomal RNA genes (8, 9), which may be repeated in tandem (10). Electron microscopy and restriction mapping reveal the absence of an inverted repeat in broad bean, as well as the presence of only one copy of the ribosomal RNA genes (11).

We have constructed detailed restriction maps of the chloroplast genomes of mung bean and pea. The mung bean genome, unlike that of pea and broad bean, possesses the inverted repeat structure common to all other higher plant chloroplast genomes. We confirm that the pea genome lacks an inverted repeat and demonstrate that it contains only one set of ribosomal genes and no detectable repeated sequences. From these data we conclude that deletion of one segment of the highly conserved inverted repeat has occurred during the evolution of the legumes. In addition, we have mapped a number of rearrangements of sequences common to the mung bean and pea chloroplast genomes.

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MATERIALS AND METHODS

Chloroplast DNA was isolated as described by Kolodner and Tewari (12). Recombinant plasmids containing mung bean and pea chloroplast DNA restriction fragments (13) were isolated by a modification of the alkaline procedure of Birnboim and Doly (14). Recombinant DNA was handled in accordance with National Institutes of Health guidelines. Restriction enzymes were purchased from Bethesda Research Laboratories (*Bst*EII, *Kpn* I, *Pst* I, *Sal* I, *Sma* I, and *Xho* I) and New England BioLabs (*Pvu* II and *Sac* I) and digestions were performed according to the supplier's instructions. Electrophoresis was on 0.5–1.5% horizontal agarose slab gels of 0.4 × 20 × 22–40 cm in 100 mM Tris–acetate (pH 8.1)/1 mM EDTA. Nitrocellulose filters were prepared by transferring chloroplast DNA restriction fragments from agarose gels according to Wahl *et al.* (15). Recombinant plasmids containing chloroplast DNA inserts were labeled with [α-^{32}P]dGTP (Amersham) by the nick-translation reaction (16) and hybridized to the filters in 0.6 M NaCl/60 mM trisodium citrate/0.1% sodium dodecyl sulfate/0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone and calf thymus DNA at 50 μg/ml for 48 hr at 65°C. The filters were washed in several changes of 75 mM NaCl/7.5 mM trisodium citrate/0.1% sodium dodecyl sulfate over a period of 4 hr at 65°C and exposed to Kodak X-Omat film, using a Du Pont Lightning Plus intensifying screen for 1–12 days at −70°C.

RESULTS

The fragments produced by digestion of total mung bean and pea chloroplast DNA with the eight restriction enzymes chosen for the mapping study are displayed in Fig. 1. Summation of restriction fragments yields a genome size for mung bean chloroplast DNA of 150 kb, while the pea genome is significantly smaller at 120 kb (Fig. 2). The strategy for mapping these fragments was to divide the relatively large circular chloroplast molecules into smaller cloned pieces that could easily be mapped as separate entities by straightforward multiple digestions. The cloned mapped fragments were then ordered by Southern hybridizations (17). All pea and mung bean chloroplast DNA *Pst* I fragments larger than 1 kb, with the two exceptions of a 12.2-kb pea fragment and a 34-kb mung bean fragment, have been inserted into pBR322 and cloned (13). The mung bean *Sal* I fragments 13.3, 16.5, and 20.5 kb long, which were found to cover the mung bean 34-kb *Pst* I region, as well as a fourth mung bean *Sal* I fragment 21.5 kb long, have also been inserted into pBR322 and cloned (13).

The cleavage sites for the eight enzymes were mapped in the above recombinant plasmids by a two-step procedure. Double digestions of each plasmid with the enzyme used for cloning together with each of the other seven enzymes showed which

Abbreviation: kb, kilobase pair(s).

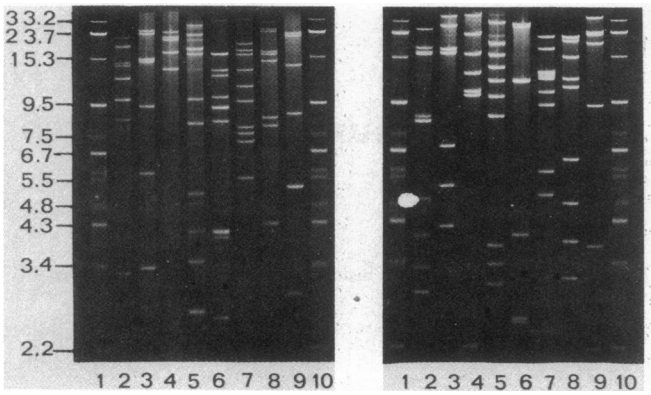

FIG. 1. Restriction fragments of mung bean (*Left*) and pea (*Right*) chloroplast DNA separated on 0.7% agarose gels. Lanes 1 and 10, *Eco*RI, *Hin*dIII, and *Sal* I restriction fragments of phage λ DNA. Sizes are indicated in kb. Lanes 2–9, chloroplast DNA digested with *Xho* I (2), *Sma* I (3), *Sal* I (4), *Sac* I (5), *Pvu* II (6), *Pst* I (7), *Kpn* I (8), and *Bst*EII (9). Several of the bands in the *Sac* I profiles represent partial digestion products by comparison with a number of complete *Sac* I digestions (data not shown). The mung bean DNA was significantly degraded and thus the highest molecular weight bands in several of the digests are relatively faint. The largest *Bst*EII fragment of 68 kb is not even discernible, although we have seen this band in other preparations of DNA.

enzymes had cleavage sites on any particular cloned fragment. Those sites were then unambiguously mapped by triple digestions in which each plasmid was again cut with the enzyme used for cloning and subsequently digested with every pairwise combination of the enzymes that had been identified as cleaving the chloroplast DNA insert. The order of the cloned fragments in the chloroplast genome was established by hybridizing each clone to a blot containing single digests of total mung bean or pea chloroplast DNA with each of the eight enzymes.

The only uncloned region of these genomes is the 12.2-kb *Pst* I fragment of pea. Hybridization of this fragment (isolated from an agarose gel) with single-enzyme digests of total pea chloroplast DNA allowed the placement of the 12.2-kb *Pst* I fragment between the 6.1- and 11.7-kb *Pst* I fragments. The double digestion products of the 12.2-kb *Pst* I fragment were deduced by subtracting the known double digest products of all the cloned pea *Pst* I fragments from the products of digestion of total pea chloroplast DNA with *Pst* I and each of the other seven enzymes.

The outstanding difference between the mung bean and pea chloroplast DNA restriction maps is the presence in mung bean of a large sequence of 23.1 kb that is repeated twice in an inverted arrangement, whereas pea contains no apparent repeated sequences (Fig. 2). These restriction maps can be used to demonstrate the presence or absence of only fairly large re-

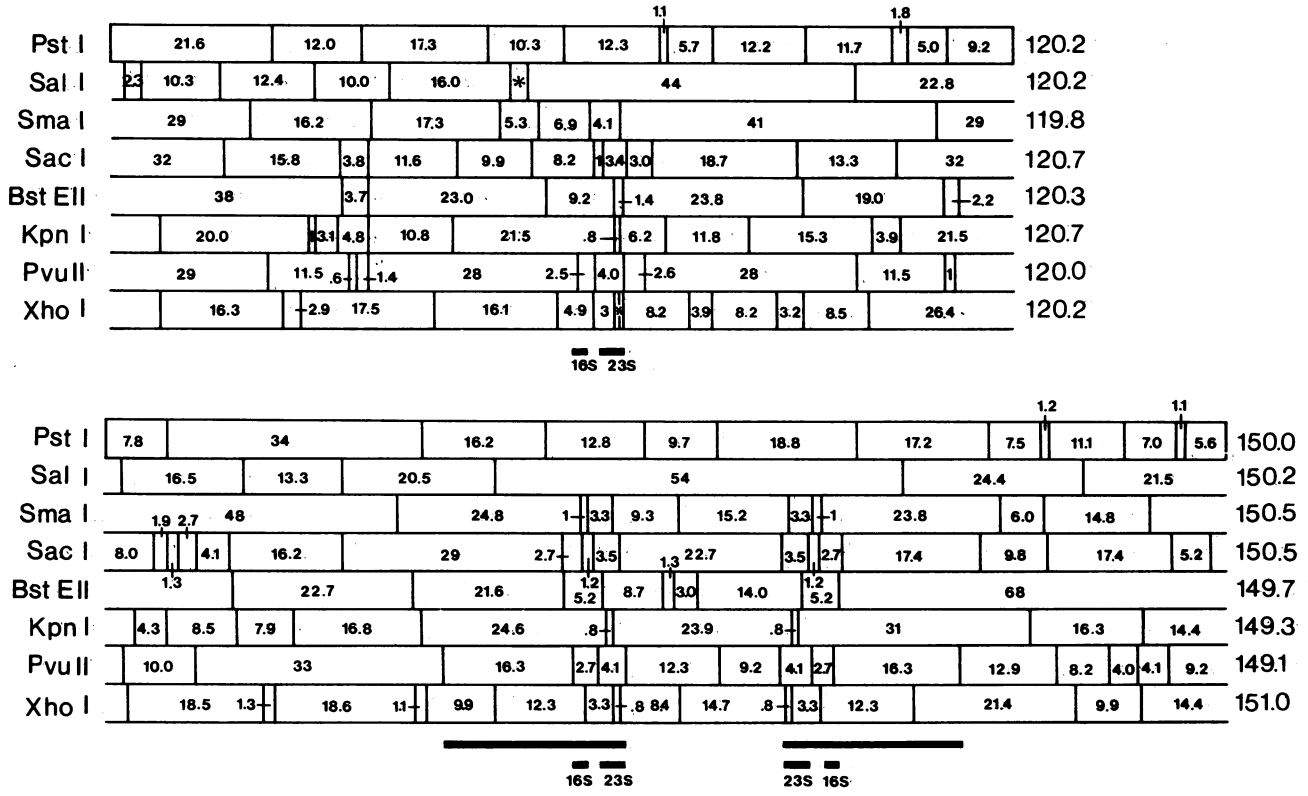

FIG. 2. Restriction maps of mung bean (*Lower*) and pea (*Upper*) chloroplast DNA. The circular maps have been linearized at the *Pst* I sites separating the 5.6- and 7.8-kb (mung bean) and the 9.2- and 21.6-kb (pea) *Pst* I fragments. The size of each restriction fragment is given in kb. The asterisk in the pea *Sal* I map denotes a 2.4-kb region that is composed of three separate, but unordered, fragments, 1.3, 0.6, and 0.5 kb long. The asterisk in the pea *Xho* I map denotes two adjacent 0.6-kb fragments. The two long, heavy black lines beneath the mung bean map represent the extent of the inverted repeat. The boundaries of the inverted repeat as defined by these mapping data are the sites between the 4.1- and 12.3- (9.2)-kb *Pvu* II fragments and the 16.3- and 12.9- (33)-kb *Pvu* II fragments. Including the 2.7-kb *Pvu* II fragment internal to the repeat, the minimal length of the inverted repeat is probably 23.1 kb (4.1 + 2.7 + 16.3 kb). The mapping of the 16S and 23S ribosomal RNA genes, plus the additional mapping required to situate all the restriction sites within these genes that are shown here, will be presented elsewhere. The numbers at the right-hand end of the restriction maps represent the summation of the restriction fragments for each of the enzymes marked at the left-hand end. These numbers represent the average of three separate summations derived by densitometric scanning of negatives of gels such as those shown in Fig. 1.

peated sequences. The Southern hybridizations used to order the cloned fragments provide a more sensitive test for repeated sequences. Aside from fragments that lie on the inverted repeat, no other mung bean *Pst* I fragment and none of the pea *Pst* I fragments showed cross-hybridization to any restriction fragments (including all other *Pst* I fragments) that did not directly overlap them on the maps.

This analysis does not rule out the possibility that there are repeated sequences that lie entirely within an individual *Pst* I fragment. This question is most crucial with respect to the number of ribosomal genes on the pea chloroplast chromosome. Published reports claim that there are two sets of pea chloroplast ribosomal RNA genes (8, 9). Hybridization of 16S and 23S chloroplast ribosomal RNA to Southern blots of *Pst* I-digested mung bean and pea chloroplast DNA reveals that two mung bean fragments of 12.8 kb and 18.8 kb that lie on the inverted repeat code for rRNA, while in pea chloroplast DNA only a single *Pst* I fragment of 12.3 kb codes for rRNA (Fig. 3). We have asked whether there are regions of internal homology within the pea *Pst* I 12.3-kb fragment, as would be expected if it contained two or more copies of the ribosomal genes. The experiment is a two-dimensional version (19) of Southern's (17) blot technique. The plasmid containing the 12.3-kb fragment was digested with *Eco*RI and *Pst* I and blotted onto a sheet of nitrocellulose paper. The blot was then hybridized at right angles to a gel containing precisely the same pattern of fragments labeled with ^{32}P (Fig. 3). Each fragment hybridizes to itself, giving rise to a series of spots on the diagonal. Hybridization between different fragments would produce spots off the diagonal and thus the absence of any such off-diagonal spots indicates the absence of any significant homologies between any of the seven *Eco*RI subfragments of the 12.3-kb pea *Pst* I fragment. This experiment has been repeated with the eight *Hin*dIII subfragments of the 12.3-kb pea *Pst* I fragment and again no cross-hybridization was seen (data not shown). The largest possible repetitive sequence within the 12.3-kb fragment is only 1.7 kb (if the 3.4-kb *Eco*RI fragment represents two repeats of 1.7 kb), which is clearly not sufficient to encode a set of ribosomal genes [4.6 kb (21)]. Therefore we conclude that the 12.3-kb *Pst* I fragment, and by extension the whole pea chloroplast genome, contains only one set of ribosomal genes.

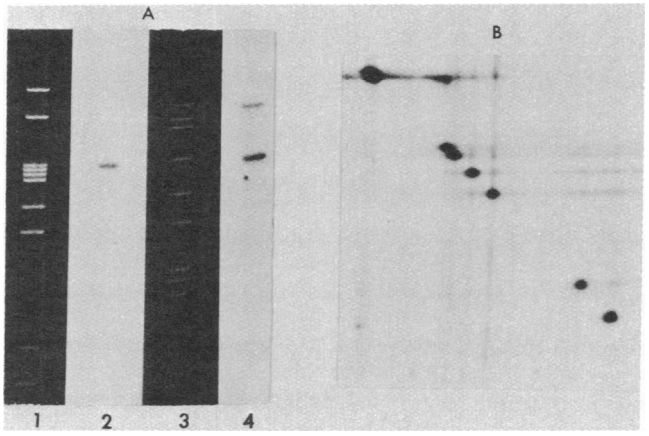

FIG. 3. Location of ribosomal RNA genes in pea and mung bean chloroplast DNA. (*A*) Hybridization of ribosomal RNA to pea (lanes 1 and 2) and mung bean (lanes 3 and 4) chloroplast DNA *Pst* I restriction fragments. The 16S and 23S chloroplast ribosomal RNA from tobacco was hydrolyzed by alkali to 200 bases and labeled at the 5′ end with [γ-^{32}P]ATP by using polynucleotide kinase (18). The ^{32}P-labeled ribosomal RNA was hybridized to pea and mung bean chloroplast DNA *Pst* I fragments separated on a 0.6% agarose gel and transferred to nitrocellulose (17). In a similar experiment, ribosomal RNA failed to hybridize to the pea and mung bean *Pst* I fragments smaller than 2 kb, which had been blotted from a 1.0% gel (data not shown). (*B*) Search for sequence repetition within the pea *Pst* I fragment coding for ribosomal RNA. The *Pst* I/*Eco*RI restriction fragments from the clone containing the 12.3-kb pea *Pst* I fragment were tested for all possible combinations of sequence homology by the "Southern Cross" technique performed exactly as described by Wensink *et al.* (20). The fragments from left to right and from top to bottom are 3.6, 3.4, 2.1, 2.0, 1.8, 1.6, 0.9, and 0.75 kb in size. The 3.6- and 0.75-kb fragments contain only pBR322 vector sequences, while all other fragments derive entirely from the chloroplast DNA insert. Insert fragments of 0.3 and 0.2 kb were not tested.

The one prominent difference in genome organization that we have demonstrated in this study so far is the deletion in pea of one copy of the inverted repeat sequence present in mung bean. We have conducted experiments designed to detect other rearrangements of sequences common to these two genomes. We have probed Southern blots of mung bean restriction fragments with 14 different cloned mung bean fragments, which represent 99% of the mung bean genome. In Fig. 4 we show a representative experiment in which the mung bean *Pst* I 9.7-kb fragment was used as a hybridization probe against several different restriction digests of mung bean and pea chloroplast DNA. The only mung bean *Pst* I fragment that is hybridized is the 9.7-kb fragment used as probe. In contrast, the mung bean probe hybridizes to four pea *Pst* I fragments, indicating the presence of sequences homologous to one or more portions of the probe in at least three separated regions of the pea chloroplast genome (Fig. 5). In control mung bean–mung bean hybridizations (data not shown) each of the other 13 mung bean probe sequences also hybridized only to itself (with the obvious exceptions of probes from the inverted repeat). In general, therefore, hybridization of a mung bean fragment to two widely separated pea fragments can best be interpreted in terms of rearrangement events rather than the more trivial repetition of sequences common to the two genomes. These hybridization experiments (Fig. 5) indicate that a number of rearrangements have occurred that have scrambled homologous sequences in the mung bean and pea chloroplast genomes.

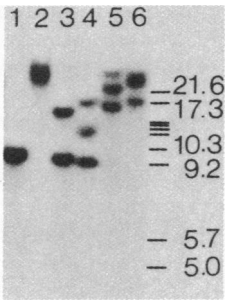

FIG. 4. Hybridization of the cloned mung bean *Pst* I 9.7-kb fragment to a nitrocellulose filter containing mung bean (lanes 1–3) and pea (lanes 4–6) chloroplast DNA *Pst* I (lanes 1 and 4), *Sal* I (lanes 2 and 5), and *Sma* I (lanes 3 and 6) and restriction fragments. Sizes at right (kb) represent the position of hybridization of total pea chloroplast DNA to a marker lane of pea chloroplast DNA *Pst* I fragments. The four unmarked lines represent fragments 12.3, 12.2, 12.0, and 11.7 kb long. The mung bean fragments that hybridize are a 9.7-kb *Pst* I fragment (lane 1), a 43-kb *Sal* I fragment (lane 2), and 15.2- and 9.3-kb *Sma* I fragments (lane 3). The pea fragments that hybridize to the same probe are *Pst* I fragments 17.3, 12.3, 11.7, and 9.2 kb long (lane 4), *Sal* I fragments 43.9, 23.0, and 16.0 kb long (lane 5) and *Sma* I fragments 42.0, 29.0, and 17.3 kb long (lane 6). The signals of the 12.3- and 11.7-kb pea *Pst* I fragments are not resolved in the experiment shown here. These signals were clearly separated and identified in a second experiment in which a lower percentage (0.6%) gel was used to achieve better resolution.

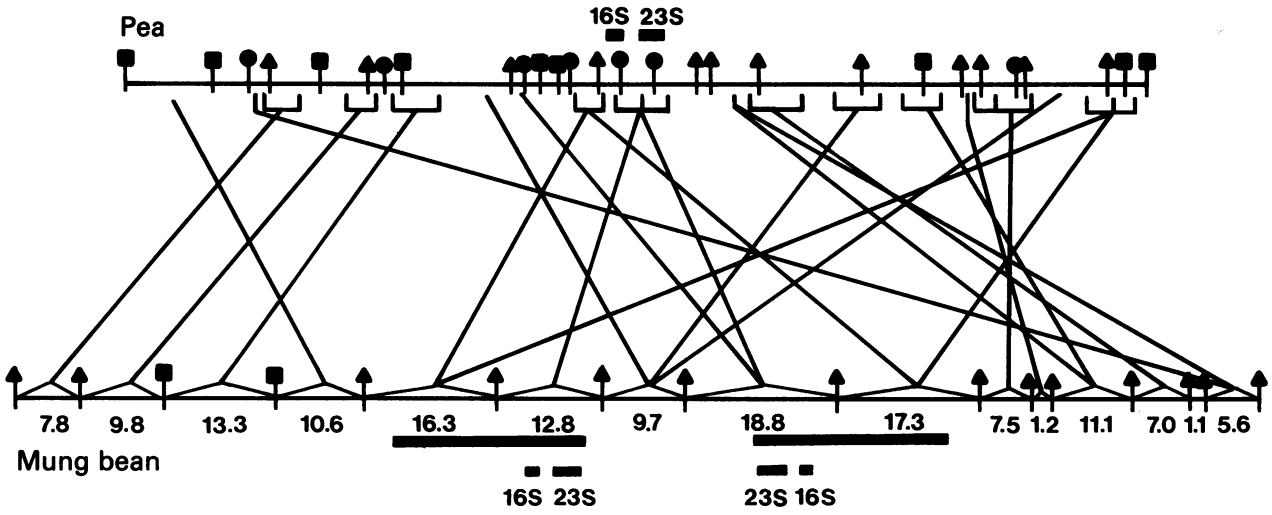

FIG. 5. Arrangement of homologous sequences in the mung bean and pea chloroplast genomes. Fourteen nonoverlapping, cloned mung bean restriction fragments, which represent 99% of the genome, were each hybridized to replica nitrocellulose filters containing both pea and mung bean *Pst* I, *Sal* I, and *Sma* I fragments separated on a 0.7% agarose gel. The extent of the mung bean fragments used as probes is indicated by the two lines that converge above the fragments, while the size of the fragments in kb is given below. The pea fragments to which the mung bean probes hybridize are indicated by the lines leading from the mung bean fragments to the pea fragments. Wherever two different mung bean fragments hybridized to the same pea fragment it was usually possible to differentiate which portion of the pea fragment hybridized to a given probe on the basis of which adjacent pea fragment(s) hybridized to the same probe. Restriction sites are represented by ▲ (*Pst* I), ■ (*Sal* I), and ● (*Sma* I).

DISCUSSION

Our estimate of 120 kb for the size of the pea chloroplast genome differs considerably from published data. Kolodner and Tewari (12) estimated a genome size for pea chloroplast DNA of 136 kb by reassociation kinetics and 143 kb by contour length determinations in the electron microscope [these values are our recalculations based on more recent determinations of the size of the phage T4 (22) and ϕX174 (23) DNA molecules used as internal standards in their experiments]. We do not consider measurements by reassociation kinetics to be as accurate as those obtained by electron microscopy or restriction analysis. In these latter two techniques the larger the molecule the more error there is in its size measurement. Therefore, by cutting the chloroplast genome into 10 or 15 small fragments we greatly decrease the relative error of any measurement; the use of multiple size standards of known molecular weight enables the whole range of fragments from 0.2 to 50 kb to be accurately measured. Moreover, the use of multiple restriction enzymes allows several independent estimates of genome size, and Fig. 2 shows that results with eight different enzymes are in close agreement. For these reasons we believe that our estimate of the pea chloroplast genome size using restriction analysis is more accurate than the estimates made by other means.

Thomas and Tewari (8, 9) concluded that there are two copies of the ribosomal RNA genes per pea chloroplast DNA molecule on the basis of a detailed series of saturation hybridizations of labeled pea chloroplast ribosomal RNA to filter-bound pea chloroplast DNA. In addition, while no data have been published, it has been suggested (10) that the two copies of the ribosomal genes are repeated in tandem. In sharp contrast, we find only one set of ribosomal genes in pea chloroplast DNA. We can see no easy way to reconcile this conflict. We simply point out the detail of our restriction mapping and that every fragment has been accounted for. Similarly, broad bean, which like pea has lost the inverted repeat and has a genome size of approximately 120 kb, has also been shown to have a single set of ribosomal genes (12). Finally, we note that Chu *et al.* (24) have recently studied the pea chloroplast chromosome and also find a single set of ribosomal RNA genes.

Our finding that the mung bean chloroplast genome retains the inverted repeat structure found in all other higher plant families studied, whereas two other legumes, pea and broad bean (11), have lost the inverted repeat, suggests that deletion of the inverted repeat has occurred since the mung bean line diverged from the pea–broad bean line. This interpretation is consistent with standard taxonomic schemes for the family *Leguminosae*, which place both pea and broad bean in the tribe *Vicieae* and mung bean in the separate tribe *Phaesoleae* (25).

Clearly the arrangement of a part of the chloroplast genome as an inverted repeat is not indispensable for chloroplast function, because the loss of this structure has been demonstrated in the legumes. However, the inverted repeat arrangement is highly conserved, being present in all higher plant families so far examined. An evolutionary framework for viewing such a highly conserved, yet dispensable structure, is to consider the inverted repeat as an evolutionary relic, which originated early in plant evolution and remains widespread not because the arrangement confers a selective advantage upon the plant but simply because the structure itself is physically resistant to recombinational loss. The idea that the chloroplast inverted repeat should be resistant to deletion through intramolecular recombination was proposed by Bedbrook and Bogorad (2) on the basis of a model proposed by Adelberg and Bergquist (26). In light of this model the observed deletion of the inverted repeat during legume evolution can be viewed as the chance occurrence of a mechanistically very difficult and rare molecular event. One selective advantage that duplication of chloroplast genes might be expected to confer is increased evolutionary flexibility through sequence divergence and creation of functionally different gene products (27). In fact, quite the opposite has occurred in the evolution of the inverted repeat. Restriction maps of the mung bean (Fig. 2), corn (2), spinach (4), *Oenothera* (4), tobacco (5), petunia (6), and *Spirodela* (7) chloroplast genomes indicate that the two segments of the inverted repeat are always identical for a given species. This situation may also be a consequence of the physical nature of the inverted repeat—i.e., an inverted arrangement should permit continual recombination leading to homogeneity between two repeats (10).

The loss of the inverted repeat structure is clearly just one of many organizational changes that differentiate the pea chloroplast genome from that of mung bean. The experiment summarized in Fig. 5 indicates that sequences common to these two genomes have become extensively rearranged. It is important to realize that Fig. 5 probably represents a minimal estimate of the number of rearrangements that have occurred in these two genomes since the divergence of the mung bean and pea lines. Rearrangements of sequences that have accumulated enough base pair mismatch such that they cannot form stable duplexes at the criterion used in this experiment will not be detected. In addition, small-scale rearrangement events—e.g., deletions/insertions or inversions of a few kb or less—will probably also go undetected as a result of the use of relatively large (10- to 15-kb) fragments as probes.

This latter point is especially relevant because a number of other examples in the literature (28–30) suggest that rearrangement events, particularly small deletions/insertions, occur at a fairly high frequency during chloroplast genome evolution, even though the genomes of distantly related plants retain high levels of base sequence homology (31, 32). Most notably, restriction maps of the five principal chloroplast genomes of the genus *Oenothera* indicate that most of the differences in restriction fragment sizes reflect small deletions/insertions (28). These events are fairly easily detected when comparing very closely related plants, such as those in the same species or genus, whose chloroplast DNA restriction patterns are sufficiently similar as to permit identification of such events by direct inspection. On the other hand, such analysis cannot be performed with plants such as mung bean and pea, which are at opposite ends of the legume family (25), and whose restriction patterns show no similarities at all (Fig. 1). More laborious techniques such as measuring heteroduplex formation will be necessary to analyze such distantly related genomes.

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