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Evidence for homologous recombination between repeated sequences containing 18S and 5S ribosomal RNA genes in wheat mitochondrial DNA

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Closely linked genes for 18S and 5S rRNAs have been located on four different cloned *Sal*I restriction fragments of wheat (*Triticum aestivum* L.) mitochondrial DNA. Restriction analysis has revealed that in each of the cloned fragments, the 18S and 5S rRNA genes are contained within the same basic structural unit, *R*, which is at least 4 kbp long. This unit is flanked by sequences designated *u* (0.8 kbp), *v* (13.7 kbp), *w* (0.7 kbp), and *y* (1.4 kbp), in the orientations *v-R-w*, *v-R-y*, *u-R-w*, and *u-R-y* in the four different *Sal*I fragments. We conclude that 18S + 5S rRNA genes are located at several distinct sites in the wheat mitochondrial genome, and suggest that reciprocal intra- and/or intermolecular recombination between such repeated sequences could promote extensive genomic rearrangement and thus contribute to the physical heterogeneity that is a hallmark of most plant mitochondrial DNAs.
Key words: plant mitochondrial DNA/repeated sequences/ribosomal RNA genes/site-specific recombination/wheat

Introduction

Mitochondrial genomes vary tremendously in size, those in higher plants being the largest (Leaver and Gray, 1982; Gray, 1982; Wallace, 1982). Recent estimates of genome size range from ~ 160 kbp for *Brassica oleracea* (cabbage) mtDNA (Lebacq and Vedel, 1981) to ~ 2400 kbp for *Cucumis melo* (muskmelon) mtDNA (Ward *et al.*, 1981), making plant mtDNA some 10–150 times larger than animal mtDNA. With few exceptions (e.g., Lebacq and Vedel, 1981), restriction patterns of plant mtDNA are very complex (Levings and Pring, 1976; Quetier and Vedel, 1977; Bonen and Gray, 1980; Brennicke, 1980; Ward *et al.*, 1981; Borck and Walbot, 1982) and are characterized by the presence of certain fragments in non-stoichiometric amounts (Bonen and Gray, 1980; Spruill *et al.*, 1980; Ward *et al.*, 1981; Borck and Walbot, 1982). These observations suggest the existence of sequence heterogeneity in plant mtDNA (Quetier and Vedel, 1977).

As isolated from whole tissues, plant mtDNA consists of a highly heterogeneous collection of linear molecules, with varying (but always quite low) proportions of circular molecules (Levings and Pring, 1978; Leaver and Gray, 1982). Although the circular population is itself heterogeneous (e.g., Sparks and Dale 1980; Fontarnau and Hernández-Yago, 1982; but cf., Brennicke, 1980), it is possible to discern discrete size classes (Levings *et al.*, 1979; Synenki *et al.*, 1978; Brennicke and Blanz, 1982) that not only differ among plants (Dale, 1981) but may also differ between individual lines of the same plant species (Dale *et al.*, 1981). Curiously, the proportion of circular molecules is markedly higher in mtDNA prepared from suspension cell cultures than in mtDNA isolated from whole tissues of the same plant (Dale *et al.*, 1981). The molecular basis of this physical heterogeneity and variability remains to be established.

Evidence that molecular heterogeneity in plant mitochondrial genomes may exist at the level of individual genes has come from studies of the arrangement of mitochondrial rRNA genes in wheat (*Triticum aestivum* L.) (Bonen and Gray, 1980). In restriction digests of wheat mtDNA, multiple fragments were found to hybridize with individual mitochondrial rRNA probes (26S, 18S or 5S). Moreover, there appeared to be unequal labelling of fragments hybridizing with a particular rRNA probe in a given restriction digest, suggesting that these fragments are not present in equimolar amounts. These results have been interpreted as suggesting the presence of several distinct rRNA cistrons in the wheat mitochondrial genome (Gray *et al.*, 1982, 1983).

To examine further the structural relationships among multiple *Sal*I restriction fragments that contain closely linked wheat mitochondrial 18S and 5S rRNA genes, we have cloned these fragments and subjected them to detailed restriction analysis. The results reported here indicate that a basic structural unit encompassing 18S and 5S rRNA genes is indeed present at several distinct sites in wheat mtDNA. The distribution of sequences flanking the repeated 18S-5S rRNA coding unit suggests that intra- and/or intermolecular recombination occurs through repeat units located at different sites in wheat mtDNA. We suggest that such a process may play a role in generating both the physical and sequence diversity that characterizes wheat and other plant mtDNAs.

Results

Selection of clones

In initial Southern hybridization experiments with wheat mtDNA (Bonen and Gray, 1980), three different *Sal*I fragments each hybridized specifically with both 18S and 5S rRNA from wheat mitochondria. Two of the fragments (#21 and #19) had lengths estimated to be ~5–6 kbp, while the third (#5,6) was >15 kbp in size. For detailed restriction analysis in the present study, recombinant plasmids pTam-S21 and pTam-S19, carrying *Sal*I fragments #21 and #19, respectively, were selected from among several independent isolates of each. Characterization of clones presumed to contain fragment #5,6 revealed two related but distinct plasmids, designated pTam-S5,6a and pTam-S5,6b. The inserts in these large plasmids correspond to two high mol. wt. fragments that were not resolved previously during agarose gel electrophoresis of *Sal*I digests of wheat mtDNA (Bonen

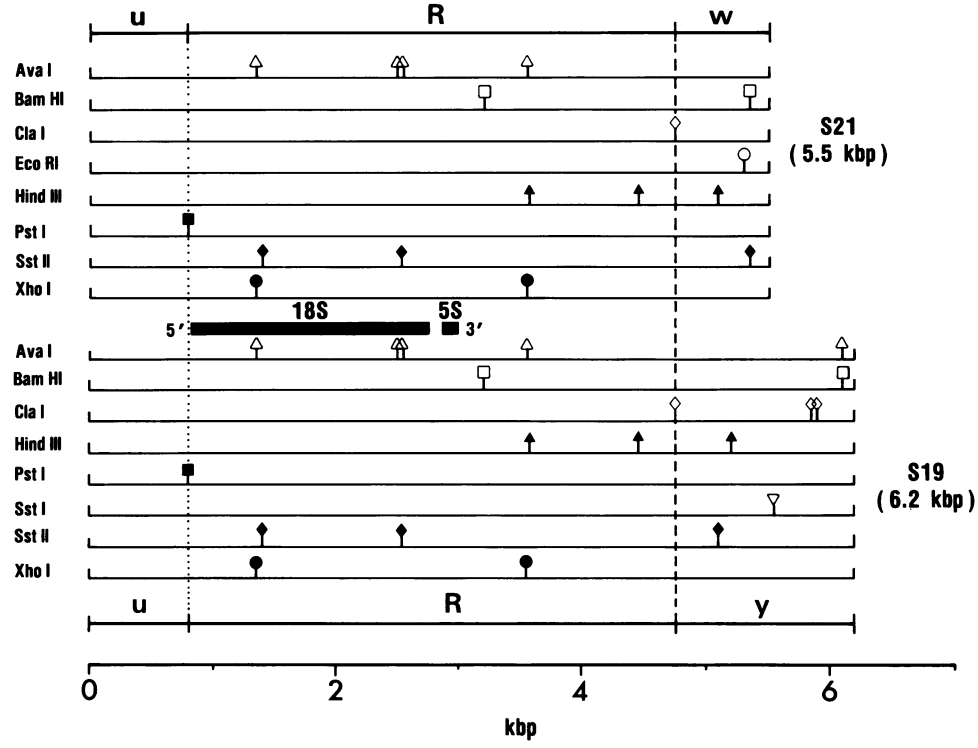

Fig. 1. Restriction maps of cloned fragments S21 and S19. The *R* region encompassing the 18S-5S rRNA coding region is defined by a leftward *PstI* site (dotted line) and a rightward *ClaI* site (dashed line) that are also common to cloned fragments S5,6a and S5,6b (see Figure 2). S21 and S19 start at the same leftward *SalI* site and share identical restriction sites through the *u* and *R* regions (a total of 4.75 kbp); they diverge in the *w/y* region and terminate at different rightward *SalI* sites.

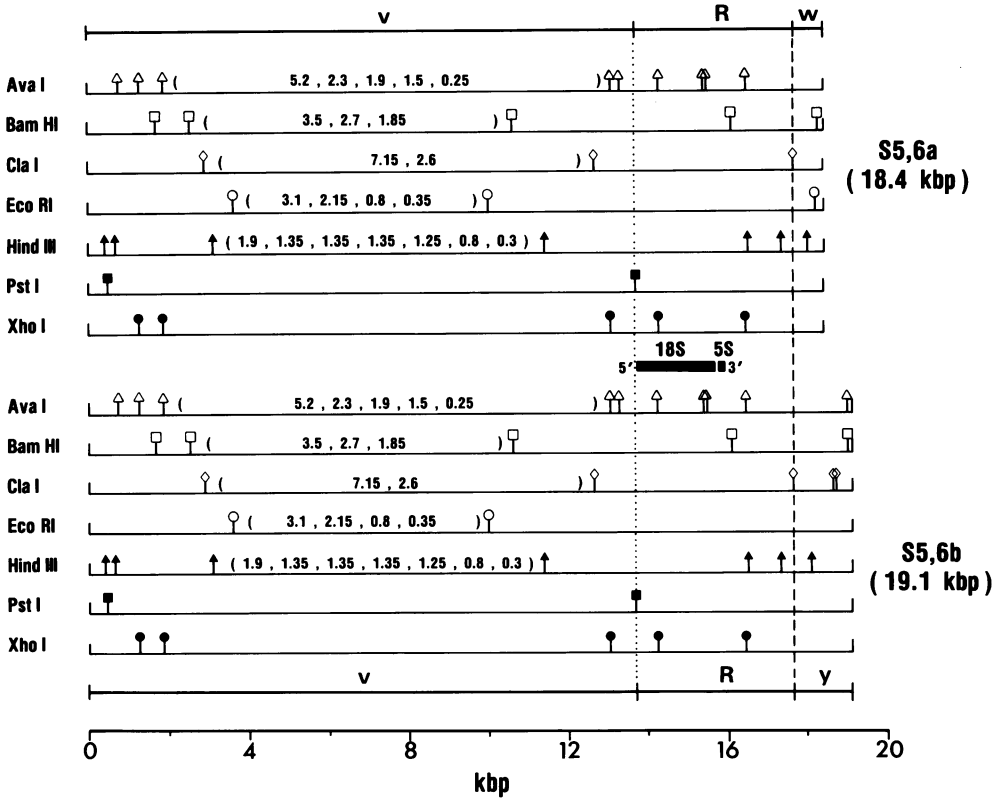

Fig. 2. Restriction maps of cloned fragments S5,6a and S5,6b. The *R* region is defined in Figure 1. Starting at the common leftward *SalI* site and through the *v* and *R* regions (17.65 kbp in total), S5,6a and S5,6b have identical restriction sites; as in the case of S21 and S19 (Figure 1), they diverge in the *w/y* region and terminate at different rightward *SalI* sites.

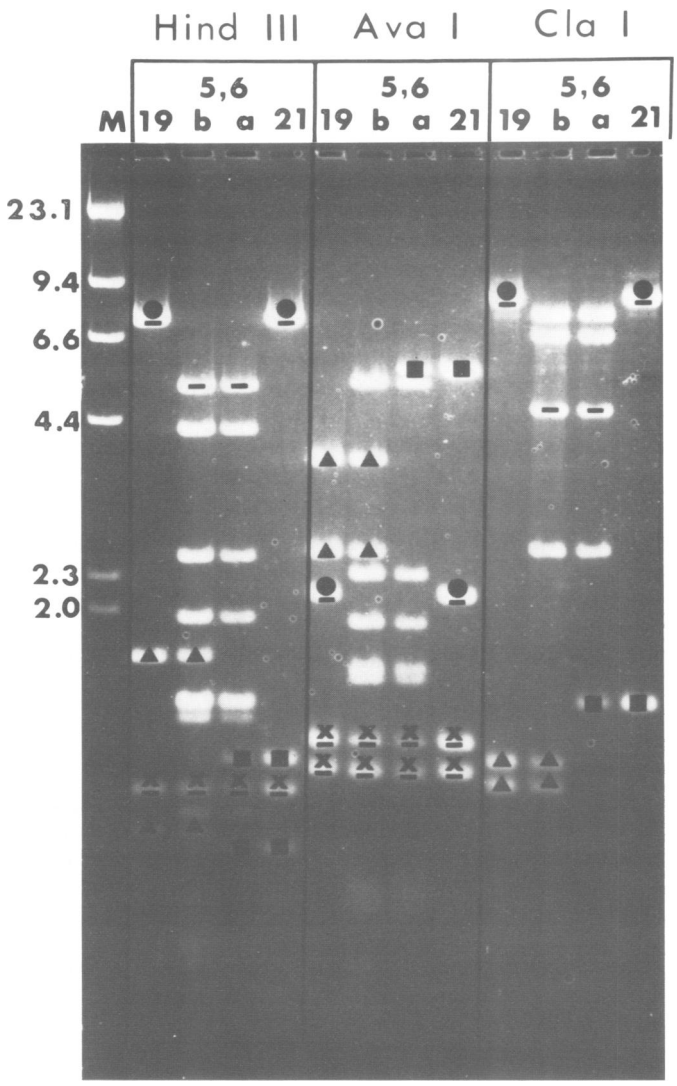

Fig. 3. Comparative restriction profiles of recombinant plasmids containing *Sal*I fragments 19, 5,6a, 5,6b and 21, digested with *Hind*III (left), *Ava*I (center), and *Cla*I (right). Closed circles (●), bands common to 19 and 21; closed triangles (▲), bands common to 19 and 5,6b; closed squares (■), bands common to 21 and 5,6a. Bands marked with an 'x' are *Hind*III and *Ava*I fragments common to all four cloned inserts. Bands marked with a dash (–) are those hybridizing with a wheat mitochondrial 18S rRNA probe. The marker (M) is a *Hind*III digest of λ DNA, with the sizes of the fragments (in kbp) indicated in the left-hand margin.

and Gray, 1980). The four *Sal*I fragments described here (S5,6a, S5,6b, S19, and S21) are the only ones containing the 18S-5S rRNA gene region of wheat mtDNA.

Restriction analysis of S21 and S19

Detailed restriction maps of S21 and S19 are shown in Figure 1. The localization of rRNA cistrons was based on parallel Southern hybridization experiments with wheat mitochondrial rRNA probes, which indicated that the 3′ end of the 18S gene and the 5′ end of the 5S gene were no more than 130 bp apart. Sequence analysis has since established that this distance is 114 bp (D.F.Spencer and M.W. Gray, in preparation). Chain lengths of the two fragments (5.5 kbp for S21, 6.2 kbp for S19) were estimated by summing the measured chain lengths of fragments in various restriction digests. Determination of the entire nucleotide sequence of S19 (D.F.Spencer, unpublished data) has verified all of the cleavage sites identified by restriction mapping and clarified the number and position of previously ambiguous (cf., Gray *et al.*, 1982, 1983) *Cla*I sites near the rightward end of S19. With few exceptions, distances between mapped restriction sites were found to be accurate to within 5% of the true distances established by sequence analysis.

It can be seen that S21 and S19 share identical restriction sites over most of their lengths, starting from the leftward *Sal*I site and extending as far as a common *Cla*I site (dashed line, Figure 1) 4.8 kbp away. The region of shared sequence encompasses the 18S-5S rRNA coding region. Between the last common *Cla*I site and the rightward *Sal*I site, the two fragments appear to be completely different in sequence.

Restriction analysis of S5,6a and S5,6b

Due to their large sizes, the restriction maps of S5,6a (18.4 kbp) and S5,6b (19.1 kbp) (Figure 2) are not as completely defined as those of S21 and S19. Nevertheless, it is clear that S5,6a and S5,6b bear the same structural relationship to each other as do S21 and S19; that is, they share identical restriction sites starting at the same leftward *Sal*I site (different from the common leftward *Sal*I site in S21 and S19) and proceeding as far as a *Cla*I site (dashed line, Figure 2) 17.6 kbp away. As in S21 and S19, the region of shared sequence includes the 18S and 5S rRNA genes.

Structural relationships among the four SalI fragments carrying 18S and 5S rRNA genes

Restriction profiles of plasmids carrying the four *Sal*I fragments are shown in Figure 3. With every enzyme tested, each of the larger fragments proved to be identical to one of the smaller fragments (S5,6a to S21; S5,6b to S19) in the divergent region between the common *Cla*I site and the rightward *Sal*I site. These structural relationships are summarized in the *Xho*I restriction maps and restriction profiles shown in Figure 4, which demonstrate that each *Sal*I fragment is related to two of the other *Sal*I fragments through sequences flanking the 18S-5S rRNA coding region. Only one *Xho*I subfragment (c), which contains the complete 5S rRNA gene and ~75% of the 18S gene, is common to all four clones. This 2.2-kbp subfragment was previously identified as the sole site of hybridization with wheat mitochondrial 5S rRNA, and the major site of hybridization with mitochondrial 18S rRNA, in *Xho*I digests of wheat mtDNA (Bonen and Gray, 1980).

Based on restriction sites present in all four *Sal*I fragments, the region of shared sequence, designated *R*, is at least 4 kbp long, extending from a common *Pst*I site (dotted line, Figures 1 and 2) to the common *Cla*I site beyond which restriction sites begin to diverge in S21 and S19 and in S5,6a and S5,6b (dashed line, Figures 1 and 2). Comparison of the *Ava*I and *Hind*III maps indicates that the precise boundaries of *R*, which includes the 18S + 5S rRNA genes, must lie within ~400 bp upstream of the *Pst*I site and ~300 bp downstream of the *Cla*I site. These boundaries are presently being determined by sequence analysis. As discussed elsewhere (Gray and Spencer, 1983), the *Pst*I cleavage site is 23 bp upstream of the 5′ end of the 18S rRNA gene, and within the gene for an initiator methionine tRNA. Sequences common to only two of the four clones *(u, v, w, y)* flank the *R* region, as shown in Figure 5.

Discussion

The restriction site maps of the four *Sal*I fragments charac-

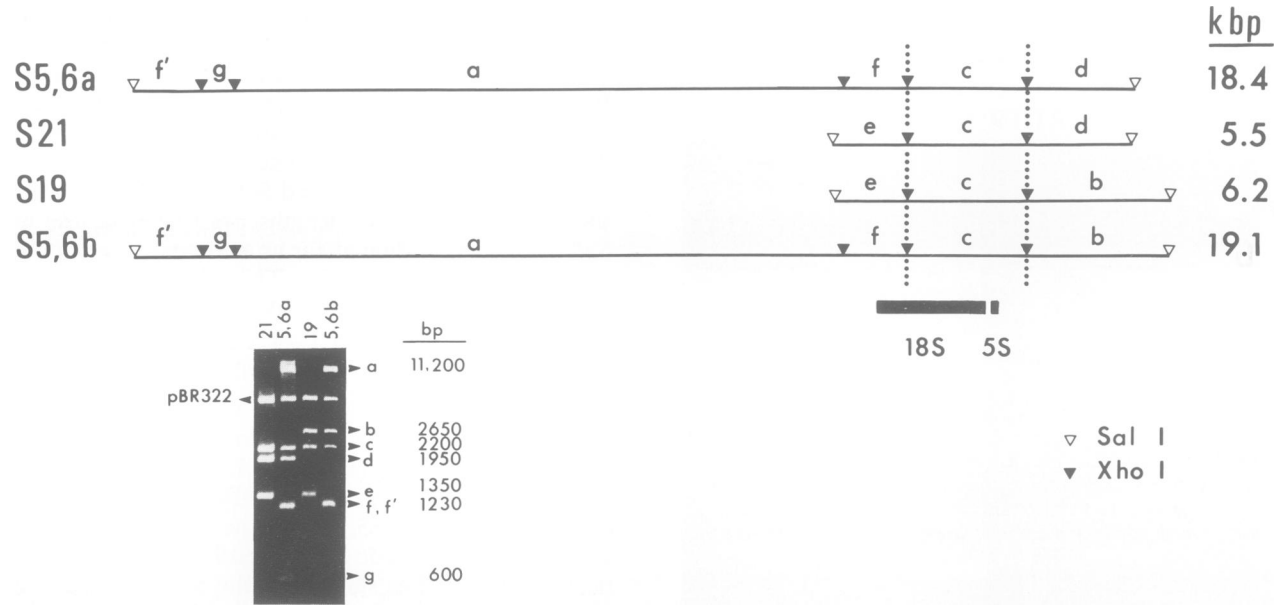

Fig. 4. *Xho*I restriction profiles (bottom) and maps (top) illustrating the structural relationships among cloned *Sal*I fragments S5,6a, S5,6b, S21, and S19. The dotted lines delineate subfragment c (2.2 kbp), the only fragment common to all four cloned inserts. This subfragment carries the complete 5S rRNA gene and all except the 5′-terminal 25% of the 18S rRNA gene.

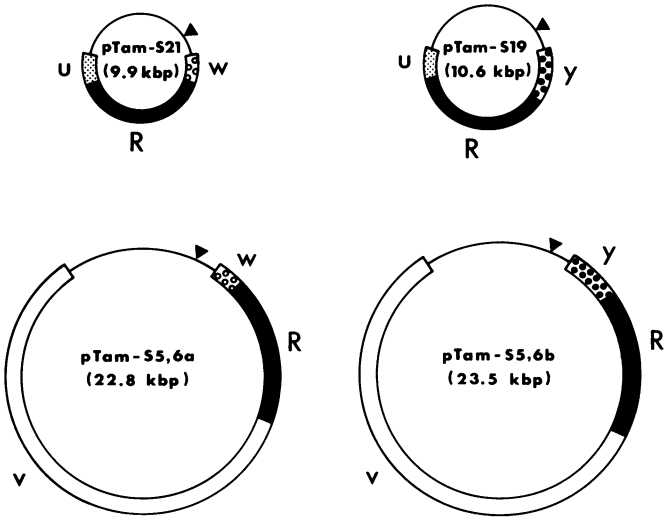

Fig. 5. Structural inter-relationships of recombinant plasmids pTam-S21, -S19, -S5,6a, and -S5,6b. The sizes (in kbp) of the various regions are: *u*, 0.8; *v*, 13.7; *w*, 0.75; *y*, 1.45; *R*, 3.95. The thin line denotes the vector (pBR322). As indicated by the location of the *Eco*RI site (closed triangle) in pBR322, the orientation of the insert is the same in each of the four recombinant plasmids characterized here.

terized here strongly suggest that these fragments are related by homologous recombination within a common repeated element, the *R* region. Two models that could account for the observed structural relationships are outlined in Figure 6. In the first model (Figure 6a), reciprocal crossing-over takes place anywhere with the *R* region, converting one pair of *Sal*I fragments to the other pair. In the second model (Figure 6b), recombination is confined to a sub-region of *R* designated *r*, which is upstream of the 18S rRNA gene. Recombination between an *r* element in S21 or S19 and an *r* element elsewhere in the genome would translocate the 18S-5S rRNA gene region to new sites in the mtDNA. In the example of Figure 6b, an *r* element is shown flanked by *v* and *z* blocs in the arrangement *v-r-z*, so that recombination between this *r* element and one in S21 or S19 would generate S5,6a or S5,6b, respectively. The latter model focuses attention on the extreme left-hand end of the *R* region (which could not exceed ~350 bp in the case presented here) as a possible important determinant in site-specific recombination involving wheat mitochondrial 18S rRNA genes.

In both of the models presented in Figure 6, two copies of *R* per genome could account for the four different *Sal*I fragments observed. In both cases, the relative stoichiometries of the fragments would depend on the equilibrium between alternative combinations. However, the two models do make different predictions regarding the expected relative amounts of the four fragments. According to the first model (Figure 6a), S19 and S5,6a should be present in equimolar amounts, as should S21 and S5,6b. In the second model (Figure 6b), these equivalencies need not hold, since the interconversion of S19 and S5,6b is independent of that of S21 and S5,6a. In fact, we have previously found that S5,6a and S5,6b together account for only ~25% of the total labelling when a *Sal*I restriction digest of wheat mtDNA is probed with ^{32}P-labelled 18S rRNA (see Figures 3C and 4C of Bonen and Gray, 1980). These hybridization results favour the second model (Figure 6b), although it is possible that larger *Sal*I fragments such as S5,6a and S5,6b are systematically underrepresented as a result of random preparative degradation of wheat mtDNA. The model of Figure 6b makes several additional, readily testable predictions, among them that wheat mtDNA should contain copies of the putative *r* element that are not linked to 18S rRNA genes.

Although further studies will be required to elucidate the mechanism of site-specific recombination in wheat mtDNA, it is clear that the process itself, regardless of the mechanistic details, could underly much of the physical heterogeneity seen in plant mtDNA. Further possible examples of site-specific recombination that could contribute to this heterogeneity are shown in Figure 7. Recombination within the *R* regions of two different copies of the same *Sal*I fragment (Figure 7a)

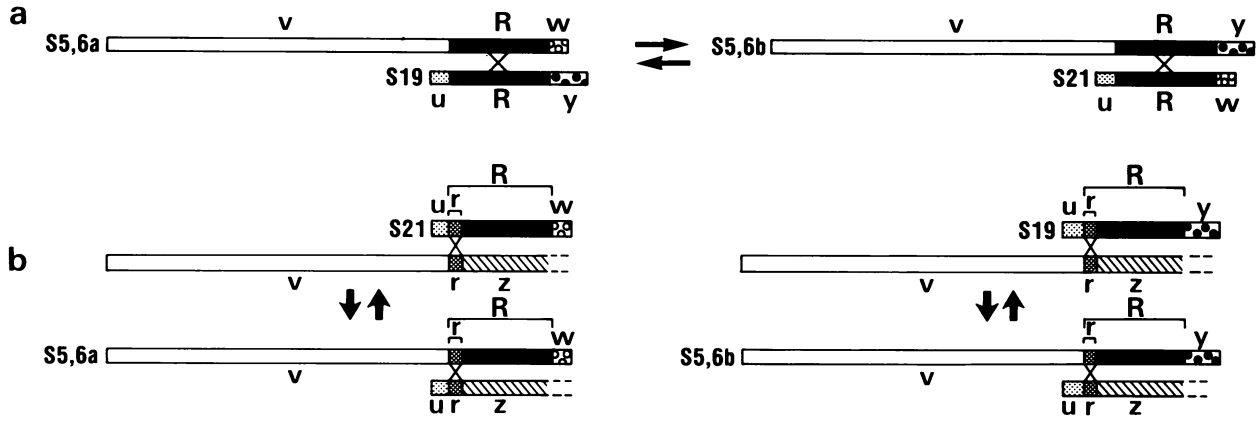

Fig. 6. Models for site-specific interconversion of *SalI* fragments containing the 18S-5S rRNA coding region. **(a)**, reciprocal recombination within the 4 kbp *R* region; **(b)** reciprocal recombination within a much smaller (<0.4 kbp) *r* region. The latter model assumes that the *r* region is contained within *R*, as indicated in the figure, and that it is also found elsewhere in the genome, not associated with the 18S-5S rRNA coding unit. Note that movement of rRNA genes through recombination is symmetric in model **(a)** but asymmetric in model **(b)**.

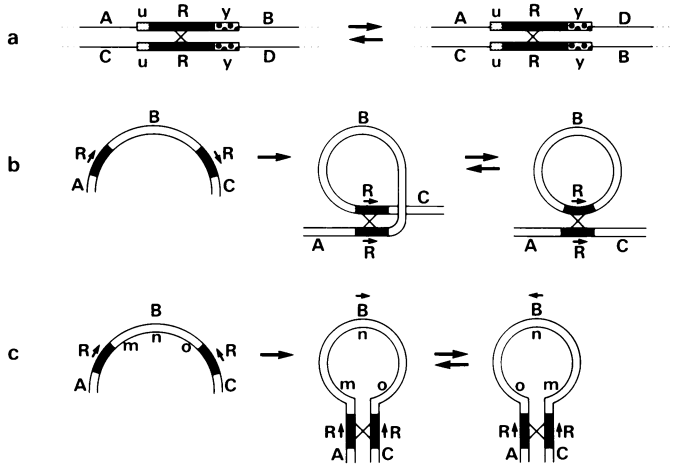

Fig. 7. Additional possible consequences of site-specific recombination between repeated sequences *(R)* in plant mtDNA. **(a)**, rearrangement of flanking sequences by recombination between two copies of the same unit (in this case, S19) located at different sites in the genome; **(b)**, generation of circular molecules by intramolecular recombination between direct repeats; **(c)**, sequence inversion by intramolecular recombination between inverted repeats.

could lead to rearrangement of flanking sequences. Indeed, there is evidence from cosmid mapping that S21 itself is present at, at least, three different sites in wheat mtDNA (S.Delorme, B.Lejeune and F.Quetier, unpublished results). Also, discrete circular classes could be produced by intramolecular recombination between two directly repeated *R* units (Figure 7b), with the size of these circles depending on the distance separating the two repeats. This would be analogous to the site-specific recombination that leads to the excision of integrated prophage (such as lambda) from bacterial genomes (Nash, 1981) and to the production of petite genomes in yeast mitochondria (de Zamaroczy *et al.*, 1983). Intramolecular recombination between inverted *R* units in the same molecule would not change the positions of these units but would re-orient the region between them (Figure 7c). Such a mechanism appears to operate on the inverted rRNA gene repeats in the cyanelle of *Cyanophora paradoxa* (Bohnert and Löffelhardt, 1982) and the chloroplast of *Phaseolus vulgaris* (Palmer, 1983). Such recombination processes could promote extensive genomic rearrangement in plant mitochondria and could account in large part for the complexity and non-stoichiometry of restriction profiles, the existence of a heterogeneous population of linear molecules and the presence of a heterogeneous population of circular molecules containing discrete size classes.

As a working hypothesis, therefore, we envisage the plant mitochondrial genome as a dynamic entity in which intragenomic rearrangement via site-specific recombination is frequent. Certainly, there is evidence that extensive rearrangements have occurred during the evolution of mtDNA in the genus *Zea* (Sederoff *et al.*, 1981; Borck and Walbot, 1982). Spontaneous rearrangements in the mtDNA of whole plants (Levings *et al.*, 1980) and of cultured cells (Gengenbach *et al.*, 1980; Dale *et al.*, 1981) have been documented, as have extensive mtDNA rearrangements in somatic hybrids of *Nicotiana* species (Belliard *et al.*, 1979; Nagy *et al.*, 1981). These latter observations, in particular, strongly imply the presence of an active recombination system in plant mitochondria.

If repeated sequences in plant mtDNA do promote intragenomic rearrangement through site-specific recombination, then differences in the number and/or distribution of such sequences could contribute to the very divergent restriction patterns of mtDNAs from even closely-related plants. For example, one might expect to see an increase in the complexity of the mtDNA restriction pattern as the number of distinct repeat classes increases, since this would allow additional possibilities for intragenomic recombination. This could account for the pronounced variability in the size and restriction patterns of mtDNA even within the same family of plants (e.g., Ward *et al.*, 1981). The apparent absence of heterogeneity in sequences flanking rRNA genes in maize mtDNA (Stern *et al.*, 1982; Iams and Sinclair, 1982) suggests that recombination sites are not associated with mitochondrial rRNA genes in maize, as they seem to be in wheat. Nevertheless, repeated sequences do occur in maize mtDNA (Lonsdale *et al.*, 1981), and recently such sequences have been implicated in site-specific recombination events of the type outlined in Figure 6a, both in maize (Lonsdale *et al.*, 1983) and in *Brassica* (Palmer and Shields, 1983) mtDNA. Reciprocal recombination between repeated sequences therefore appears to be a common feature of plant mtDNAs. It should be pointed out, however, that each repeat class cannot be highly reiterated, since in general plant mtDNA

renatures as if it contained primarily a single kinetic component (Kolodner and Tewari, 1972; Wong and Wildman, 1972; Vedel and Quetier, 1974; Ward *et al.*, 1981; but cf., Wells and Birnstiel, 1969).

A dynamic model of the plant mitochondrial genome must be able to accommodate the highly reproducible character of restriction patterns, which are qualitatively identical for specific sub-populations (e.g., linear or circular) of a given plant mtDNA (Sparks and Dale, 1980). This constancy requires that any recombination be highly specific, occurring only at a limited number of sites. On the other hand, perturbations in the frequency of recombination between specific sites could lead to changes in the steady-state level of particular alternative forms, which might then be reflected in a quantitative change in the stoichiometry of certain restriction fragments (see, e.g., Borck and Walbot, 1982), rather than a qualitative change in the restriction profile itself.

There is evidence that 26S rRNA genes, also, are repeated in the wheat mitochondrial genome (Bonen and Gray, 1980; D.Falconet and F.Quetier, unpublished results). Ribosomal RNA genes should therefore prove to be particularly useful markers in further studies of site-specific recombination in wheat mtDNA.

Materials and methods

Recombinant plasmids containing wheat mitochondrial 18S and 5S rRNA genes

A wheat mtDNA library has been constructed by cloning *Sal*I fragments into pBR322 (B.Lejeune and F.Quetier, unpublished data). Standard procedures (Maniatis *et al.*, 1982) were used and details will be presented elsewhere. Ampicillin-resistant tetracycline-sensitive (Ap^RTc^S) transformants of *Escherichia coli* HB101 were selected and screened for mitochondrial rRNA genes according to the procedure of Grunstein and Hogness (1975), as modified by Howell (G.Ledoigt, personal communication). Total rRNA from wheat mitochondria, 5′ end-labelled *in vitro* with ^{32}P (Bonen and Gray, 1980), was used as a probe. Positive clones were further characterized by the small scale plasmid isolation procedure of Birnboim and Doly (1979), combined with Southern (1975) hybridization using purified wheat mitochondrial 26S, 18S and 5S [^{32}P]rRNA probes (Bonen and Gray, 1980). For large scale isolation of the four recombinant plasmids (pTam-S21, pTam-S19, pTam-S5,6a, pTam-S5,6b) used in the present study, the 'cleared lysate' method of Clewell and Helinski (1969) was followed.

Mapping strategy

Restriction sites were mapped by standard techniques (Maniatis *et al.*, 1982) based on analysis of single, double and triple digests generated by six-base cutters. This was coupled with Southern (1975) hybridization experiments to identify fragments containing 18S and 5S coding sequences. For more detailed mapping, certain fragments were isolated by preparative electrophoresis in low melting point agarose gels and digested further with four-base cutters. In a number of cases, isolated fragments were end-labelled and restriction sites mapped by partial endonuclease digestion (Smith and Birnstiel, 1976). Restriction digests were resolved by electrophoresis in 2% agarose gels, and λ DNA cleaved with *Eco*RI + *Hind*III was included as a mol. wt. marker. Fragment sizes were determined from a plot of log (log mol. wt.) *versus* mobility. With this empirical method, correlation coefficients were usually >0.995 for fragments up to 5 kbp. Fragment sizes larger than this were determined by difference from the estimated size of each *Sal*I insert.

Acknowledgements

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