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Heteroplasmy of chloroplast DNA in *Medicago*

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Abstract

Two chloroplast DNA (cpDNA) regions exhibiting a high frequency of intra- or inter-species variation were identified in 12 accessions of the genus *Medicago*. Restriction maps of both regions were prepared for alfalfa, and the probable nature of the events causing the DNA differences was identified. Specific DNA fragments were then cloned for use in identification of variants in each region. Two each of *M. sativa* ssp. *varia* and ssp. *caerulea* and one of six *M. sativa* ssp. *sativa* single plants examined possessed cpDNA heterogeneity as identified by screening extracts for fragments generated by the presence and absence of a specific *Xba* I restriction site. Three plants of *M. sativa* ssp. *sativa*, two of each of sspp. *varia* and *caerulea*, and three *M. scutellata* were also examined for single-plant cpDNA heterogeneity at a hypervariable region where differences resulted from small insertion-deletion events. A single *M. scutellata* plant with mixed cpDNAs was identified. Sorting out was seen when one ssp. *sativa* plant with mixed plastid types identifiable by the *Xba* I restriction site difference was vegetatively propagated. This indicated that the initial stock plant was heteroplastidic. Controlled crosses will be required in order to test whether heteroplasmy results from chloroplast transmission in the pollen and to examine the dynamics of sorting out. However, heteroplasmy is apparently not a rare situation in *Medicago*.

Introduction

Cells of flowering plants possess many copies of their chloroplast genome [1, 3, 4], in contrast to only one or a few copies, depending on ploidy level, of their nuclear genome. Chloroplast genome numbers are estimated to range from 1 900 to 50 000 copies per cell, with multiplicity occurring in both plastid number and DNA copies per plastid [1]. It is generally observed, however, that single plants exhibit homogeneity of chloroplast DNA (cpDNA), presumably as the result of a relatively rapid "sorting out" of plastids by vegetative segregation [3, 4]. Examples of this plastid sorting out have been clearly established in studies with variegated plants [4, 9, 30] and with somatic hybrid plants regenerated following protoplast fusion [8, 23].

There are a few exceptions, however, to the above generalizations. For example, Moon *et al.* [16] claimed the existence of a persistent cpDNA heterogeneity in single rice plants. Lax *et al.* [10] have described a plastome mutant of cotton that exhibits a slow rate of sorting out, resulting in a persistence of mixed cells possessing both wild type and mutant plastids. Heteroplasmy is possible in several species

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of conifers that exhibit paternal plastid inheritance [33, 34]. In contrast to the apparent rarity of heteroplasmy in cpDNA, numerous cases of heteroplasmic mitochondrial DNAs have been reported in animals (reviewed in [31]).

We became interested in the possibility of locating heteroplastidic plants in alfalfa, *Medicago sativa* L., after a report from our laboratory by Rose *et al.* [24] documenting the recovery of one or the other of two types of cpDNA in 23 plants regenerated from protoplasts (protoclones). Results were most easily explained if the original, seed-derived protoplast donor plants possessed both types of cpDNA, but with sorting out having occurred in individual shoots, probably prior to protoplast isolation.

Medicago would seem to be a logical plant genus within which to look for at least occasional single-plant cpDNA heterogeneity. Studies by Smith *et al.* [30], using two chlorophyll-deficiency mutants in alfalfa, provided developmental and genetic evidence suggesting that both were plastid-encoded mutations and that there was a high frequency of transmission of these mutant plastids through the pollen. There is also cytological evidence for biparental inheritance of plastids in alfalfa [6]. An earlier report of biparental plastid transmission exists for *M. truncatula* [12]. The biparental transmission of cpDNA in *Medicago* should provide a mechanism for re-establishing cpDNA heterogeneity.

CpDNAs of the genus *Medicago* are also characterized by the lack of the large inverted repeat ([20], Johnson and Palmer, unpublished) that is found in most other land plant cpDNAs. The absence of this repeat correlates with an increased frequency of plastid DNA evolutionary rearrangement [22], and nucleotide substitution is significantly reduced in the inverted repeat of cpDNAs relative to single copy regions [36]. Thus studies involving a genus lacking this repeat structure might provide a greater number of mutants with cpDNAs readily distinguishable by restriction patterns with which to work.

In this paper, the identification and characterization of several cpDNA regions with a high frequency of intraspecific and interspecific variability in the genus *Medicago* is described. Cloned fragments from these regions are then used to demonstrate single-plant cpDNA heterogeneity and heteroplasmy.

Materials and methods

Medicago accessions obtained from the USDA Regional Plant Introduction Station, Ames, Iowa, were *M. lupulina* L. (PI289310), *M. orbicularis* (L.) Bart. (PI385011), *M. sativa* L. ssp. *sativa* (PI26590), *M. sativa* L. ssp. *falcata* (PI234815), *M. sativa* L. ssp. *varia* (PI238145), *M. sativa* L. ssp. *caerulea* (PI206453), and *M. cancellata* Marschall von Bieberstein (PI315458). *M. carstiensis* Wulf. (TM993), *M. arborea* L. (UAG1497), and *M. papillosa* Boiss. ssp. *papillosa* (UAG98), initially designated *M. dzhawakhetica* [29], were provided by T. McCoy, University of Arizona, Tucson. E. L. Sorensen, Kansas State University, provided seed for *M. sativa* L. ssp. *falcata* cv. Anik, as did I. D. Kaehne, Dept. of Agriculture and Fisheries, Adelaide, South Australia, for *M. scutellata* cv. Robinson. Several Regen S alfalfa [2] clones and protoclones, including RS-K1, RS-K2, and K2-66S, were utilized, and have been maintained by propagation of shoot cuttings since the initial studies of their cpDNA by Rose *et al.* [24].

Chloroplast DNAs from bulked plant samples were extracted from plastids banded in sucrose gradients and then pelleted by centrifugation [18, 20]. Following plastid lysis, cpDNAs were banded twice in CsCl gradients, dialyzed, and stored at 4 °C [18, 20]. The sucrose gradient step was typically omitted from the procedure for single-plant extracts.

Restriction endonuclease digestion, 0.7 or 1.0% agarose slab gel electrophoresis, ethidium bromide staining and gel photography, blotting to Zetabind (AMF CUNO, Meriden, CT), nick translation, filter hybridization, and autoradiography were done after Palmer [18, 20]. Probes used included a cloned set of eight alfalfa [20] and four mung bean [20, 21] fragments spanning the entire chloroplast genome.

Several smaller subclones were prepared from the aforementioned alfalfa probes by digestion, electrophoresis, and excision of the desired subfragment from an agarose gel, followed by electroelution, phenol and diethyl ether extraction, and ethanol precipitation [18]. Fragments were next ligated into the appropriately cut pIC20H vector [15], which was then transformed into competent cells of *Escherichia coli* strain JM83. Recombinant white colonies were then selected on ampicillin/X gal plates,

and inserts characterized from plasmid DNA minipreps [5].

Results

The first cpDNA polymorphism selected for study involved the *Xba* I region suggested by Rose *et al.* [24] to reveal heteroplasmy in certain Regen S alfalfa donor clones. A preliminary comparison of *Xba* I fragmentation patterns of the cpDNAs of bulk extracts of single accessions of *M. sativa* ssp. *sativa*, ssp. *varia*, and ssp. *caerulea* revealed a reduced ethidium bromide staining in gels of cpDNAs of these three subspecies for the same 7.8 kb fragment observed variable by Rose *et al.* [24] relative to other singlet bands such as the one at 7.5 kb (Fig. 1A). Increased intensity of staining also occurred within a triplet of bands of 8.3 to 8.5 kb. This suggested heterogeneity in these subspecies, prompting further investigation and ultimately single plant analyses.

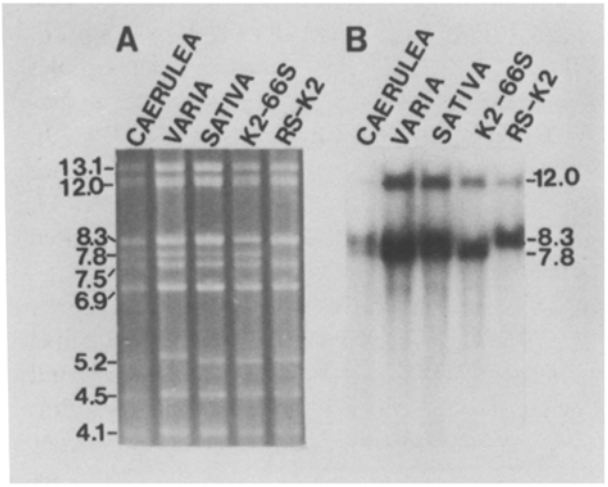

Fig. 1. Evidence of cpDNA polymorphism in *Medicago sativa* populations following *Xba* I digestion. From left to right, ssp. *caerulea*, ssp. *varia*, ssp. *sativa* PI26590, ssp. *sativa* K2-66S, and ssp. *sativa* RS-K2. (A) Ethidium bromide-stained gels showing possible heterogeneity as a consequence of variable staining of a 7.8 kb fragment relative to other singlet bands such as the one at 7.5 kb for the first three accessions. Increased staining intensity in a triplet of bands at 8.3 to 8.5 kb suggested involvement of one of these fragments in this heterogeneity. (B) Autoradiogram of blot from this same gel following hybridization with the alfalfa 6.2 kb cpDNA fragment of Palmer *et al.* [20]. Fragments of both 8.3 and 7.8 kb from sspp. *caerulea, varia*, and *sativa* PI26590 hydridized to this probe, thus revealing cpDNA heterogeneity within each population.

The variable fragment was first located on the alfalfa cpDNA restriction map of Palmer *et al.* [20] by hybridization with cloned fragments that cover the alfalfa chloroplast genome. These include eight alfalfa *Pst* I fragments and four mung bean fragments that span the single uncloned alfalfa *Pst* I fragment of 34 kb. These clones were hybridized with blots containing *Xba* I digests of cpDNAs of protoclone K2-66S possessing the fragment [24] and a recently located RS-K2 plant lacking the fragment (Fig. 1A, B). The 6.2 kb alfalfa probe clearly showed the location of the difference, hybridizing to an 8.3 kb *Xba* I fragment with the RS-K2 extract and the 0.5 (not shown) and 7.8 kb fragments with the K2-66S extract. Without the *Xba* I site under study, the slightly larger 8.3 kb fragment comigrated with several other fragments of 8.3 to 8.5 kb. Further mapping studies indicated that the *Xba* I difference under study was due to a single restriction site change, presumably the result of a single base pair substitution, because no differences were observed with any of the other nine enzymes whose sites were mapped (Fig. 2A). However, the possibility of a small insertion, deletion or inversion altering or removing the site involved, yet causing an imperceptible change in the size of the 0.8 kb *Eco* RI fragment, the smallest fragment studied containing the site, cannot be eliminated.

In order to simplify the interpretation of autoradiograms from single plant extracts, a smaller probe was prepared by subcloning a 3.2 kb *Xba* I-*Sac* I fragment from the cloned *Pst* I 6.2 kb fragment of Palmer *et al.* [20]. As predicted from the map shown in Fig. 2A, this subclone hybridized to a 3.2 kb *Xba* I-*Sac* I fragment when the *Xba* I site was missing and to 2.7 kb and 0.5 kb fragments when the site was present. It likewise hybridized to the expected 8.3, and 7.8 and 0.5 kb fragments, respectively, when *Sac* I was omitted from the digestions. When blots of bulk extracts of cpDNAs of sspp. *sativa, varia* and *caerulea* were hybridized with either the 6.2 kb (Fig. 1B) or the 3.2 kb probes, all three showed evidence of heterogeneity. In the case of *varia*, most hybridization was to the 7.8 kb fragment, but it was clear when single plants were examined that heter-

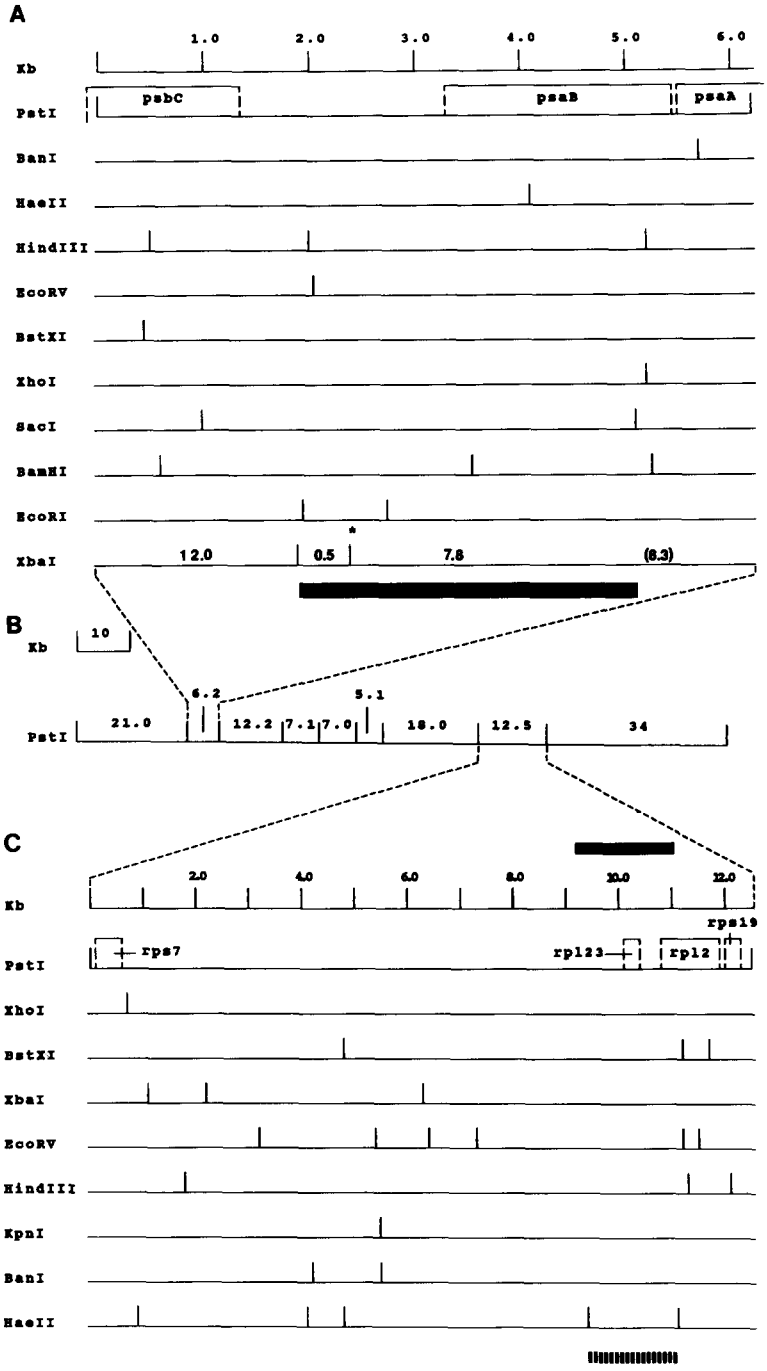

Fig. 2. (A) Restriction map of the 6.2 kb *Pst* I fragment of alfalfa cpDNA for 10 additional enzymes. The fragment contains no *Bst* EII or *Kpn* I sites. Shown in bold numbers are the sizes in kb of the *Xba* I fragments. The *Xba* I site marked by an asterisk (*) is responsible for the polymorphism under study. When present, 0.5 and 7.8 kb fragments occur. When absent, an 8.3 kb fragment (in parentheses) is seen instead. The solid bar indicates the extent of the 3.2 kb *Xba* I-*Sac* I fragment used as a hybridization probe in Fig. 3. Approximate gene positions are from the unpublished mapping data of J. Palmer. (B) Linearized map of alfalfa cpDNA *Pst* I fragments after Palmer *et al.* [20]. (C) Restriction map of the 12.5 kb *Pst* I fragment of alfalfa cpDNA for eight additional enzymes. The fragment contains no *Bst* EII or *Sac* I sites. The vertically striped bar below the *Hae* II map delineates the hypervariable region of interest. The solid bar above the map indicates the approximate location of the 1.9 kb *Bam* HI fragment used as a hybridization probe in Fig. 5. Approximate gene positions are from the unpublished mapping data of J. Palmer.

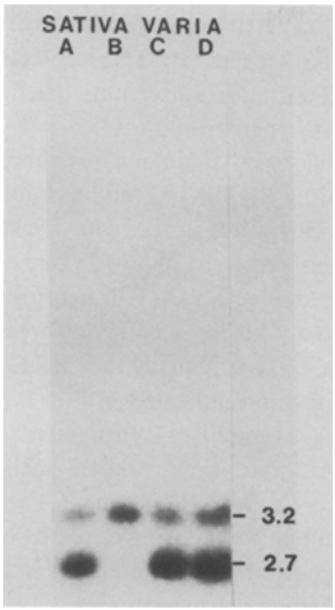

Fig. 3. Evidence of single plant heterogeneity of cpDNA in *Medicago*, as shown in an autoradiogram of a gel blot containing cpDNA double-digested with *Xba* I and *Sac* I. Hybridization was with a nick-translated, cloned 3.2 kb *Xba* I-*Sac* I fragment (Fig. 2) derived from the alfalfa 6.2 kb *Pst* I fragment of Palmer *et al.* [20]. Estimated fragment sizes in kb are shown on the right. The region of the autoradiogram shown is from the origin to about 2.4 kb. Lanes A and B contained digests from single *M. sativa* ssp. *sativa* plants, lanes C and D digests from single *M. sativa* ssp. *varia* plants.

ogeneity existed within the accession (Fig. 3).

A small number of single plant DNAs were then double digested with *Xba* I and *Sac* I and the resulting fragments separated by electrophoresis. Filter blots containing the restriction fragments were then hybridized with the 3.2 kb probe. One of two ssp. *sativa* plants and each of two ssp. *varia* plants provided clear evidence of heterogeneity at this *Xba* I site (Fig. 3), as did each of two plants of ssp. *caerulea* (data not shown). Note that digestion was complete, as evidenced by both the total lack of hybridization of the 3.2 kb probe to any fragment larger than 3.2 or 2.7 kb (Fig. 2) and by the hybridization of other clones to only those *Xba* I fragments expected in a complete digest. In addition, this heterogeneity was reproducible in several different digestions of the same DNA, digests that were otherwise complete by the criteria just mentioned. Therefore we feel that this heterogeneity is not an artifact of partial digestion, but is real, resulting from single plants containing two populations of cpDNA molecules, one with and one without the *Xba* I site marked by an asterisk in Fig. 2A. A later extraction of four different ssp. *sativa* plants revealed one possessing a 3.2 kb fragment and three with a 2.7 kb fragment, but no heterogeneity.

Plant A of ssp. *sativa* was propagated by rooting of two shoot tip cuttings, and the resultant plants were tested for cpDNA heterogeneity. Hybridization experiments revealed that both plants contained detectable amounts of only the 2.7 kb fragment, the predominant one in the original plant A, documenting the eventual occurrence of sorting out [3, 4, 9, 30].

The second variable region of alfalfa cpDNA that we selected for study was initially identified during efforts to determine phylogenetic relationships within the genus *Medicago* through the use of cpDNA

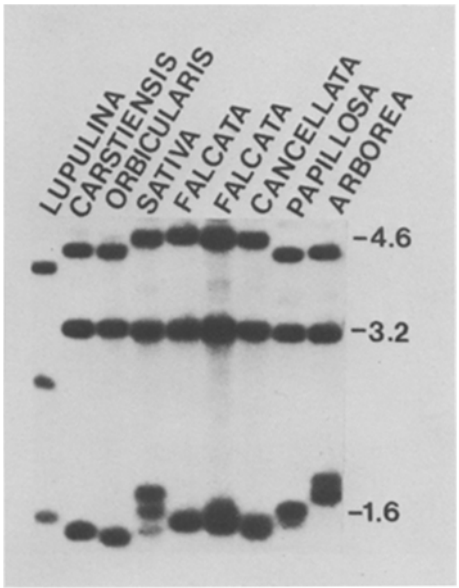

Fig. 4. Hypervariable (HV) region in cpDNA from nine *Medicago* accessions, as seen in a portion of an autoradiogram of a gel blot containing *Hae* II-digested cpDNA. Hybridization was with a cloned, nick-translated 12.5 kb *Pst* I fragment from alfalfa cpDNA [20]. Estimated fragment sizes in kb are shown on the right. Accessions from left to right are *M. lupulina, M. carstiensis, M. orbicularis, M. sativa* ssp. *sativa, M. sativa* ssp. *falcata, M. sativa* ssp. *falcata* cv. Anik, *M. cancellata, M. papillosa*, and *M. arborea*. The HV region contains fragments of approximately 1.45 to 1.8 kb among the accessions shown.

restriction mapping (L. B. Johnson and J. D. Palmer, unpublished). Figure 4 shows the hybridization of a 12.5 kb alfalfa clone (Fig. 2B) to *Hae* II digests of cpDNAs from a group of *Medicago* accessions. Two regions of fragment size polymorphism are obvious. A region of interspecific variation only is located within a group of fragments of 4.0 to 4.6 kb and was not studied further. The second region, encompassing a nearly continuous range of size forms from 1.45 to 1.8 kb, exhibited both interspecific and intraspecific variation (Fig. 4). The cpDNAs in this figure were prepared from approximately 30 to 100 pooled plants, and it was clear that not all fragments were equally represented within those mixed populations, e.g. ssp. *sativa*. A similar pattern of fragment size variation as revealed by *Hae* II digestion (Fig. 4) was also documented with several other enzymes (*Ava* I, *Bam* HI, *Cla* I, *Eco* RV) that produce relatively small fragments in this region of the genome (data not shown; but see Fig. 5B). This result clearly implicates length variation (insertions/deletions) as the cause of this hypervariation.

A 1.9 kb *Bam* HI fragment was subcloned from the parent 12.5 kb *Pst* I clone to produce a more specific hybridization probe. The 1.9 kb fragment has not been precisely mapped, but with alfalfa cpDNA digested with *Hae* II it hybridizes strongly to the 1.5 to 1.7 kb fragments, and weakly only to the adjacent 4.6 kb fragment. This places it in the approximate position indicated in Fig. 2C.

Blots of *Hae* II and *Eco* RV fragments from single plant DNAs were then probed with the nick-translated 1.9 kb *Bam* HI fragment. Figure 5 shows hybridization patterns for three plants of *M. sativa* ssp. *sativa* (two of which were used in Fig. 3), two *sativa* ssp. *varia* (both used in Fig. 3), two *sativa* ssp. *caerulea*, and three *M. scutellata*. None of the *sativa* subspecies shown exhibited single-plant heterogeneity for these fragments under study. However, cpDNA of one *M. scutellata* (plant J) is clearly heterogeneous in this region, possessing roughly equal amounts of the two *Hae* II fragments (1.45 and 1.55 kb) and two *Eco* RV fragments (3.7 and 3.8 kb). This heterogeneity was demonstrable with both the 1.9 and 12.5 kb probes. The cpDNA possessing the larger of these two fragments must have been relatively rare in our *M. scutellata* population, in that the fragment was not observed in blots from bulk extracted cpDNA (data not shown).

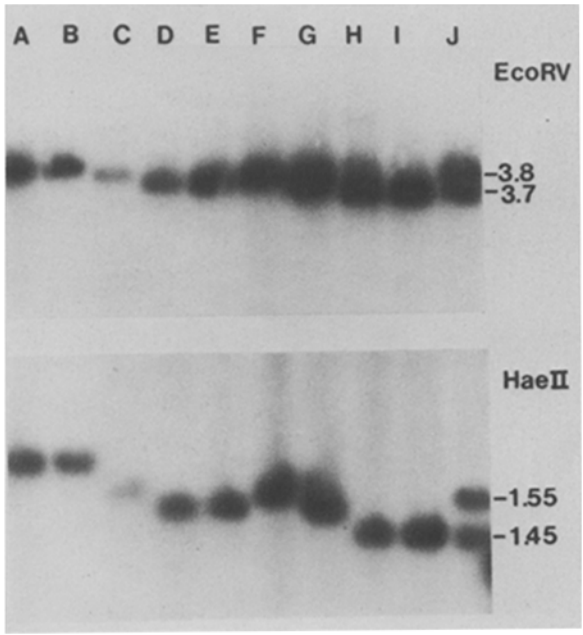

Fig. 5. Evidence of single plant heterogeneity of cpDNA in *Medicago*, as shown in autoradiograms of gel blots containing single-plant cpDNAs digested with *Eco* RV (top) or *Hae* II (bottom). Hybridization was with a nick-translated, cloned 1.9 kb *Bam* HI fragment derived from the alfalfa 12.5 kb *Pst* I fragment of Palmer *et al.* [20]. Estimated fragment sizes in kb are shown on the right. Single plant digests for both enzymes include *M. sativa* ssp. *sativa* (lanes A–C), *M. sativa* ssp. *varia* (lanes D and E), *M. sativa* ssp. *caerulea* (lanes F and G) and *M. scutellata* (lanes H–J). Single plant heterogeneity is seen in lane J.

Discussion

Our results clearly establish that single plants in the genus *Medicago* can possess more than one type of plastid DNA. This heterogeneity was of two types. In the first type, the two DNAs were distinguishable using *Xba* I, and differed by a single restriction site, possibly resulting from a single base pair change. Heteroplasmic plants possessing both the *Xba* I forms (Figs. 1, 3) were identifiable in three different subspecies of *sativa*, and plants with only one form or the other were also found. Sorting out was demonstrated, as was apparently also observed by Rose *et al.* [24]. Thus heteroplasmy is clearly in-

volved. Its high frequency of occurrence strongly suggests its perpetuation by pollen transmission, although if some mechanism existed to maintain heteroplasmy at levels below our detection limit, these two *Xba* I variants might conceivably crop up repeatedly in different plants.

The second type of cpDNA heterogeneity observed was the result of frequent insertion or deletion events that have occurred both within and between species (Fig. 4). Nine individual plants of three subspecies of *M. sativa* were all monomorphic at the size-variable locus, even though size heterogeneity was present in the single population studied. We detected single-plant heteroplasmy at this locus in only one *M. scutellata* plant (Fig. 5). Reasons for the low frequency of single-plant heteroplasmy in the *sativa* subspecies relative to that of the *Xba* I variants are unclear, although it may be a reflection of chance, given the relatively small sample number.

There is little likelihood that either nuclear or mitochondrial DNAs contribute to the polymorphisms under question, even though the presence of cpDNA in both nuclear [26, 35] and mitochondrial [7, 13, 14, 16, 26, 28, 32, 35] genomes of plants is well documented. Nuclear DNA can be ruled out in the *M. sativa* ssp. *sativa* plant A that initially possessed both forms of *Xba* I-variant molecules, since somatic sorting out occurred as a result of vegetative propagation. In addition, several of our mixed plants (e.g. *M. scutellata* plant J) contained nearly equimolar amounts of the two DNAs. This is a most unlikely occurrence if one fragment came from either the nuclear or mitochondrial genomes, considering the high copy number of cpDNA molecules in leaves [1] and the several-fold enrichment of cpDNA in our extraction procedure. It is also clear, considering the hypervariable, length-polymorphic region, that most plants contained only one of the many different fragments, which always clearly mapped to the chloroplast genome.

The nature of the DNA sequences responsible for the frequent size variation in the hypervariable region of our *Medicago* accessions is unknown. One possible explanation for this variation is that the region contains a short tandem repeat element that undergoes copy number changes via unequal crossover to produce the observed diversity. There is precedent for this phenomenon with other cpDNAs. A tandem repeat was sequenced in *Euglena* that produces a high degree of size polymorphism within that region [25]. Pea also possesses a size variable region hypothesized by Palmer *et al.* [19] to result from a short tandem repeat. In addition, as cited by Solignac *et al.* [31], numerous studies have documented that heteroplasmy in animal mtDNA results from variable number tandem repeats. Sequencing of this hypervariable region in cpDNAs from several of our *M. sativa* ssp. *sativa* plants, where the variation occurs within a single populations of plants, could do much to reveal mechanisms involved.

Our results were obtained with a random sample of plants, most selected from populations containing cpDNA heterogeneity. From our small population it is difficult to assess how frequently single-plant cpDNA heteroplasmy exists in *Medicago*. We assume that the phenomenon stems in large part from the reported biparental transmission of plastids [6, 30], which occurs at high frequency in alfalfa [30], and should at least initially manifest as heteroplasmy at the single-cell level. Controlled crosses between plants of differing cpDNA types will be required before the frequency of this phenomenon can ultimately be assessed.

It is noteworthy that alfalfa is a perennial and largely self-sterile outcrosser, while *M. scutellata* is a self-pollinated annual [11], yet both exhibited single-plant cpDNA heterogeneity in our study. *M. truncatula*, another annual [11], like alfalfa [6, 30] has also been reported to exhibit biparental plastid inheritance [12]. Based on these observations it is interesting to speculate that either heteroplasmy or the preservation of plastid variation may confer some evolutionary advantage in *Medicago*. Alternatively, pollen plastid transmission is of trivial importance in alfalfa and presumably other perennial *Medicago* species, and was merely retained during the postulated evolutionary divergence of the annuals from the perennials. Should *M. scutellata* not possess plastid biparental inheritance, the heteroplastidic plant observed may simply possess a recent mutation that ultimately would sort out, and as such may be a reflection of the extreme hypervariability of this particular region of the genome.

The alfalfa cpDNA heterogeneity that we ob-

served appears different from that in rice in that we have observed sorting out. In rice, single plants were reported by Moon *et al.* [16] to maintain a relatively constant rate of two cpDNA types, although evidence for both forms of the putative rice cpDNA being of true chloroplast origin is not entirely convincing [17]. Lax *et al.* [10] studied sorting out in variegated cotton containing a plastome mutant, and suggested that the high frequency of plants heterozygous for cpDNA was the result of a slow sorting out, possibly facilitated by plastid fusions that produced within-plastid DNA heterogeneity. We could not measure sorting out rate, both because our cpDNA mutants did not result in chlorophyll-deficient plants and because it is unlikely that all plants assayed were even fertilized by pollen possessing a different cpDNA type.

Acknowledgements

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