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Heteroplasmy of the chloroplast genome of *Medicago sativa* L. cv 'Regen S' confirmed by sequence analysis

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Abstract The heteroplasmy of chloroplast DNA (cpDNA) observed in *Medicago sativa* L., which involves the presence (type B) or absence (type A) of an *Xba*I restriction site, was examined using closed fragments covering the variable *Xba*I site from type-A and type-B cpDNA. The 6.2-kb *Pst*I fragment of DNA from type-A cpDNA (−*Xba*I) and from type-B cpDNA (+*Xba*I) was cloned into pUC19 plasmids. *Eco*RI fragments bearing the variable *Xba*I site from the type-A and type-B 6.2-kb *Pst*I fragments were subcloned into pUC19. DNA sequences of both types of the 696-bp *Eco*RI fragments were determined and computer-assisted analysis of the sequence data carried out. Type-A cpDNA was found to differ from type-B cpDNA by 1 base, a G to T conversion, which results in a non-recognition site for *Xba*I in the type-A cpDNA. The sequence difference was in a non-coding region. Cloning and sequencing of the fragments verified the individual identity of the type-A and type-B cpDNA.

Key words Heteroplasmy · Chloroplast DNA · *Medicago sativa* L. · Sequence analysis

Introduction

Plant regeneration studies from isolated protoplasts first revealed heteroplasmy in *M. sativa* L. (Rose et al. 1986), and in inheritance studies both normal and mutant plastids have been observed in cells of individual plants (Lee et al. 1988). Other investigations established single plant heteroplasmy of chloroplast DNA (cpDNA) in a number of *Medicago* species (Johnson and Palmer 1989; Schumann et al. 1989; Masoud et al. 1990). In tissue culture studies of *M. sativa* L. cv 'Regen S', heteroplasmy was observed in explants, callus tissue and regenerated plants (Fitter and Rose 1993).

The polymorphism detected by Rose et al. (1986) involves the presence or absence of an *Xba*I restriction site that has been located on the *Pst*I map of the chloroplast genome (Palmer et al. 1987) in the 6.2-kb fragment (Johnson and Palmer 1989). The 6.2-kb fragment can be used as a distinguishing probe to differentiate between type-A and type-B cpDNA (our denotion) in Southern hybridisation analyses of *Xba*I digests of cpDNA or total DNA following electrophoresis and transfer to nylon membranes (Fitter and Rose 1993). It was first used in inheritance studies of cpDNA by Masoud et al. (1990).

Mapping studies using nine restriction enzymes (Johnson and Palmer 1989) suggested that the *Xba*I difference was most likely due to a small difference in the cpDNA sequence, since comparisons of the restriction fragments revealed little or no difference in the genome size. Despite this observation the possibility of a small insertion, deletion or inversion affecting the *Xba*I recognition site could not be ruled out. Insertions and deletions have been observed in the chloroplast genome of *M. sativa* in the intergenic region between *psbA* and *trnH* (Aldrich et al. 1988). Furthermore, the presence of small repeat sequences giving rise to recombinational events was possible since dispersed repeats have been reported from the chloroplast genomes of clover (Palmer 1985) and *Trifolium subterraneum* (Palmer et al. 1987), two other legumes.

Chloroplast genomes of higher plants that contain an inverted repeat exist as two equimolar populations that differ in the relative orientation of the large single-copy region (Palmer 1983). This situation is dependent on

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reciprocal recombination at the ends of the inverted repeat region (Palmer 1983). Despite evidence of recombination between two forms of cyanelle DNA in *Cyanophora paradoxa* (Bohnert and Loffelhardt 1982) direct evidence from cpDNAs in higher plants has been lacking in investigations involving sexual crosses (Chiu and Sears 1985) or cultured tissues (Fluhr et al. 1984), and there is only limited evidence from somatic hybrid analyses (Medgyesy et al. 1985; Thanh and Medgyesy 1989). In those species that lack the inverted repeat, recombination as described by Palmer (1983) may not be possible. While *M. sativa* chloroplast genomes lack an inverted repeat (Palmer et al. 1987), the presence of smaller repeats in analagous positions could still allow recombination to occur. The chloroplast genome of black pine, *Pinus thunbergii*, for example, lacks the ribosomal RNA genes but retains a 495-bp fragment of the inverted repeat region that contains the $tRNA^{Ile}$ gene and a portion of the *psbA* gene (Tsudzuki et al. 1992).

In this work the basis of the differences between the type-A and type-B cpDNAs was examined in detail using cloned fragments covering the variable *Xba*I site from type-A and type-B cpDNA.

Materials and methods

DNA extractions

Purified cpDNA was extracted from isolated chloroplasts, from approximately 10 g of leaves, harvested from individual plants held for 24 h in the dark, after the method of Kemble (1987). Plasmid DNA was isolated by a mini-prep method according to a modification of the Birnboim and Doly (1979) method. DNA for sequencing was extracted according to a modification of the lysozyme method of Sambrook et al. (1989).

Preparation and transformation of competent *Escherichia coli* (DH5α)

Transformation competent *E. coli* DH5α cells (BRL Laboratories Life Technologies) were prepared by the calcium chloride method (Seidman 1987). Recombinant plasmids transformed into competent cells, using standard transformation techniques, were selected by blue/white selection selection on LB media containing 100 μg ml^{-1} carbenicillin, and the substrates 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-1-thio-β-D-galactoside (IPTG).

Cloning of the 6.2-kb *Pst*I cpDNA fragments

Chloroplast DNA, extracted from previously defined type-A and type-B cpDNA plants was digested with *Pst*I and mixed with *Pst*I-digested, dephosphorylated pUC19 and then ligated with a T_4 DNA ligase (BRL).

Cloning of the *Eco*RI fragments of type-A and type-B cpDNA

The *Eco*RI fragments of a type-A and a type-B cpDNA plant were cloned from the 6.2-kb *Pst*I clones by digesting the 6.2-kb *Pst*I clone mini-prep DNA with *Pst*I and then *Eco*RI so that the only fragment which had an *Eco*RI site on both ends was the desired fragment. Ligation was carried out with *Eco*RI-digested, phosphatased pUC19 DNA.

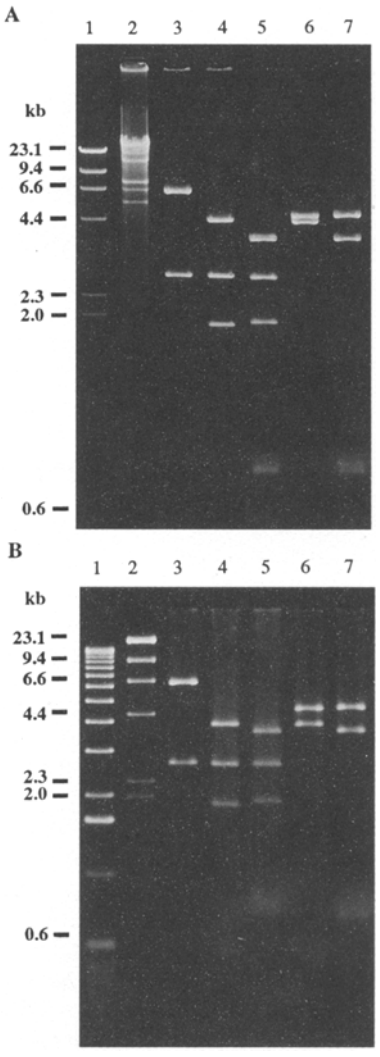

Fig. 1 A,B Restriction fragments from digests of selected pUC19 clones bearing type-A (**A**) or type-B (**B**) 6.2-kb *Pst*I inserts of cpDNA from *M. sativa* L. cv 'Regen S'. **A** *Lane 1* $^{\lambda}$*Hin*dIII fragment marker, *lane 2* cpDNA digested with *Pst*I. **B** *Lane 1* 1-kb ladder, *lane 2* $^{\lambda}$*Hin*dIII fragment marker. **A** and **B** Clone DNA digested with. *Pst*I (*lane 3*). *Pst*I/*Xba*I (*lane 4*). *Pst*I/*Eco*RI (*lane 5*). *Xba*I (*lane 6*). *Eco*RI (*lane 7*)

Verification of the type-A and type-B cpDNA 6.2-kb *Pst*I fragments and 696-bp *Eco*RI fragments

Restriction digestion and gel electrophoresis were used to verify the identity of fragments inserted and to map the inserts.

DNA sequencing

Purified plasmid/insert DNA was denatured in 0.2 *N* NaOH, and then the DNA was passed through a Sephadex G-50 (fine) column (G-50/TE/0.05% Azide).

DNA sequencing was carried out using a USB Sequenase Version 2.0. T7 DNA polymerase kit (United States Biochemical). Conditions for the sequencing reactions were modified according to the manufacturers instructions (USB sequencing manual, Handbook 6th edn.) to achieve a maximum readable sequence. Sequencing was carried out in both directions with M13/pUC primers for forward sequence and

reverse sequence. Oligonucleotide primers were also prepared as internal sequencing primers for the forward and reverse directions to bridge gaps and achieve a consensus sequence for the fragments in each direction.

Results

*Pst*I clones

Clones containing the 6.2-kb *Pst*I chloroplast DNA fragment from type-A cpDNA or type-B cpDNA plants were analysed by restriction digestion and agarose gel electrophoresis. Type-A cpDNA digested with *Pst*I was run alongside the type-A 6.2-kb *Pst*I clone to verify the fragment identity (Fig. 1A). The 6.2-kb *Pst*I fragment of cpDNA (P8) is evident (lane 2, Fig. 1) and digestion of the type-A clone DNA with *Pst*I produced a single insert band in an equivalent position (lane 3). Type-B clones also produced a single insert fragment of 6.2 kb (Fig. 1B) upon digestion with *Pst*I, however digestion with *Xba*I revealed an extra fragment (lanes 4 and 6) of approximately 500 bp. with a corresponding reduction in the size of the other fragments in the same lanes so that the total plasmid/insert length was the same as for type-A clones. Digestion with *Pst*I/*Xba*I, *Pst*I/*Eco*RI, *Xba*I and *Eco*RI (lanes 4–7, respectively) allowed mapping of the insert fragments for the type-A and type-B clones (Fig. 3).

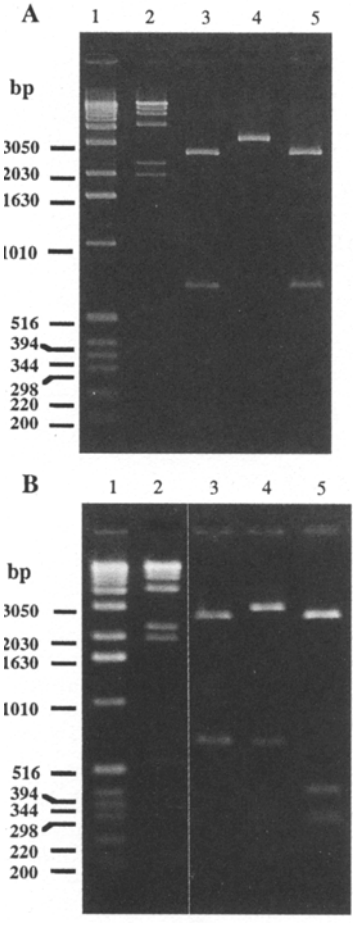

Fig. 2 A,B Restriction fragments from digests of selected pUC19 clones bearing type-A (**A**) or type-B (**B**) 696-bp *Eco*RI inserts of cpDNA from *M. sativa* L. cv 'Regen S'. **A** and **B** *Lane 1* 1-kb ladder, *lane 2* '*Hin*dIII fragment marker, *lane 3 Eco*RI digest, *lane 4 Xba*I digest, *lane 5 Xba*I/*Eco*RI digest

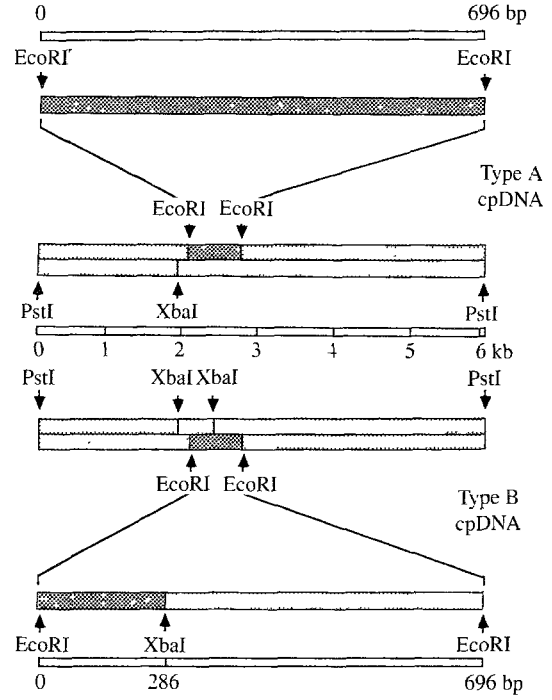

Fig. 3 Map details for 6.2-kb *Pst*I fragments of cpDNA from type-A and type-B cpDNA from *M. sativa* L. cv 'Regen S'. Each cpDNA type *Pst*I fragment contains a 696-bp *Eco*RI fragment. The type-A fragment lacks an *Xba*I site that is present in the type-B fragment

*Eco*RI clones

Subcloning of the *Eco*RI fragments produced clones of type-A and type-B cpDNA *Eco*RI fragments suitable for sequencing. Restriction analysis confirmed the identity of the clone inserts (Fig. 2A, B). *Eco*RI digestion of clone DNA yielded a single, approximately 700-bp insert fragment in addition to the plasmid band (2.7 kb) in both type-A and -B clones. Digestion with *Xba*I, however, linearised the plasmid/insert to yield a band of approximately 3.2 kb in type-A clones while producing two bands, one of approximately 550 bp and one of approximately 2.9 kb in type-B clones. Double digests with *Eco*RI and *Xba*I produced fragments approximately 700 bp and 2.7 kb in length in type-A clones, while in type-B clones fragments of approximately 400 bp, 300 bp and 2.7 kb were produced.

Mapping data (Fig. 3) confirmed one *Xba*I site in the type-A cpDNA 6.2-kb *Pst*I fragment and two *Xba*I sites in the type-B cpDNA 6.2-kb *Pst*I fragment. The variable *Xba*I site is about 500 bp distant from the common *Xba*I site and contained within a 696-bp *Eco*RI fragment that excludes the common site.

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1                                                  30                                                  60
GAATTCACCA  TACTCTATTT  ATATACTATG  TTTTTTTTAT  AATATAAATA  TACTATTACT
GAATTCACCA  TACTCTATTT  ATATACTATG  TTTTTTTTAT  AATATAAATA  TACTATTACT

61                                                 90                                                  120
TTCGTATTCA  ATACATAAGC  AACGGCGGAT  AGCGGGAATC  GAACCCGCGT  CTTCTCCTTG
TTCGTATTCA  ATACATAAGC  AACGGCGGAT  AGCGGGAATC  GAACCCGCGT  CTTCTCCTTG

121                                                150                                                 180
GCAAGGAGAA  ATTTTACCAT  TCAACTATAT  CGCATTTTTG  TTTTTATTGT  ACTTTATACT
GCAAGGAGAA  ATTTTACCAT  TCAACTATAT  CGCATTTTTG  TTTTTATTGT  ACTTTATACT

181                                                210                                                 240
TTACCATTTT  ATTTGTGCAG  AACTATGTCT  GATATCTATT  AGGAATAATT  AAAAAAAGTA
TTACCATTTT  ATTTGTGCAG  AACTATGTCT  GATATCTATT  AGGAATAATT  AAAAAAAGTA

241                                                270                                                 300
GAAATCTATA  GATCTATAGT  TAGACTAGAA  TATATAACTA  TTAGATTCTA  TAATCTATAT
GAAATCTATA  GATCTATAGT  TAGACTAGAA  TATATAACTA  TTAGATTCTA  GAATCTATAT

301                                                330                                                 360
AGATTCTATT  TAGATATCTA  TCTATATTAT  AGATAGAATC  TATATACTAT  ATCTCTTTCT
AGATTCTATT  TAGATATCTA  TCTATATTAT  AGATAGAATC  TATATACTAT  ATCTCTTTCT

361                                                390                                                 420
ATTCTAAATA  GACCTTATTT  GTATATAAGA  ATATAGATCT  ATATATTAAT  TAAATTCATT
ATTCTAAATA  GACCTTATTT  GTATATAAGA  ATATAGATCT  ATATATTAAT  TAAATTCATT

421                                                450                                                 480
AAAAAAATAG  AATTACAGAT  TTCTAAAATA  TAAAAAATAA  ATTCGAATAT  ATTCCCCTAG
AAAAAAATAG  AATTACAGAT  TTCTAAAATA  TAAAAAATAA  ATTCGAATAT  ATTCCCCTAG

481                                                510                                                 540
ATACTAGTCC  CATATTTTTC  TATTTTCTTA  TCTTGTTTTT  AGTTTTACTA  TATATATCAT
ATACTAGTCC  CATATTTTTC  TATTTTCTTA  TCTTGTTTTT  AGTTTTACTA  TATATATCAT

541                                                570                                                 600
ATTATATATA  TAATATATAG  TATAAGATAT  AAGCTTTTTC  CAATAGAAGA  AGTTTGACCC
ATTATATATA  TAATATATAG  TATAAGATAT  AAGCTTTTTC  CAATAGAAGA  AGTTTGACCC

601                                                630                                                 660
CTCCCATTAA  TAATAATAAT  GTTTTAGTTT  TCTTTATTTG  TGGGGTCTTA  TCTATTCCAT
CTCCCATTAA  TAATAATAAT  GTTTTAGTTT  TCTTTATTTG  TGGGGTCTTA  TCTATTCCAT

661                                                690  696
TTTCATTTTA  ATGTGCCTCA  CAACCCGAAA  GAATTC
TTTCATTTTA  ATGTGCCTCA  CAACCCGAAA  GAATTC
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Fig. 4 Complete sequences of type-A (*upper*) and type-B (*lower*) cpDNA *Eco*RI fragments from *Medicago sativa* L. cv 'Regen S' The variable *Xba*I site is shown *underlined* in the type-B cpDNA sequence from base 287 to 292 Base 291 differs between the two sequences and is within the *underlined* sequence. Sequences used for oligonucleotide primer preparation are *underlined* in the type-A fragment sequence (forward primer–base 196–220, reverse primer–base 474–499)

Sequence data for the type-A and type-B *Eco*RI cpDNA fragments

The complete sequence for the type-A and -B cpDNA *Eco*RI fragments (Fig. 4) revealed that both of the fragments are 696 bp in length and differ from each other by a single base pair at a position 291 bp from the insertion point of the fragment. In type-A cpDNA the sequence from base 287 reads 5'..TCTATA..3', and in type-B cpDNA it reads 5'..TCTAGA..3' (note asterisk Fig. 5A). The sequence 5'..TCTAGA..3' is the recognition sequence for *Xba*I. In type-A cpDNA then the substitution of a T for a G results in a non-recognition site for the *Xba*I enzyme. The sequence data was confirmed by the overlap of sequence, and use of the oligo primers confirmed that the opposite strand sequence was in the region of the variable *Xba*I site (Fig. 5B).

Knowing the sequence and having primers on either side of the *Xba*I site (Fig. 4), we were able to use the polymerase chain reaction(PCR) to detect type-A and type-B cpDNAs. Amplified fragments of type B contain an *Xba*I site, whereas those of type A do not contain the *Xba*I site. The digestion of amplified fragments with *Xba*I distinguishes the two cpDNAs. As shown in Fig. 6, after PCR of mini-prep DNA from clones verified for type, type-A cpDNA gives a single band of 304 bp, whereas type B gives two bands, one of 212 bp and the other 92 bp.

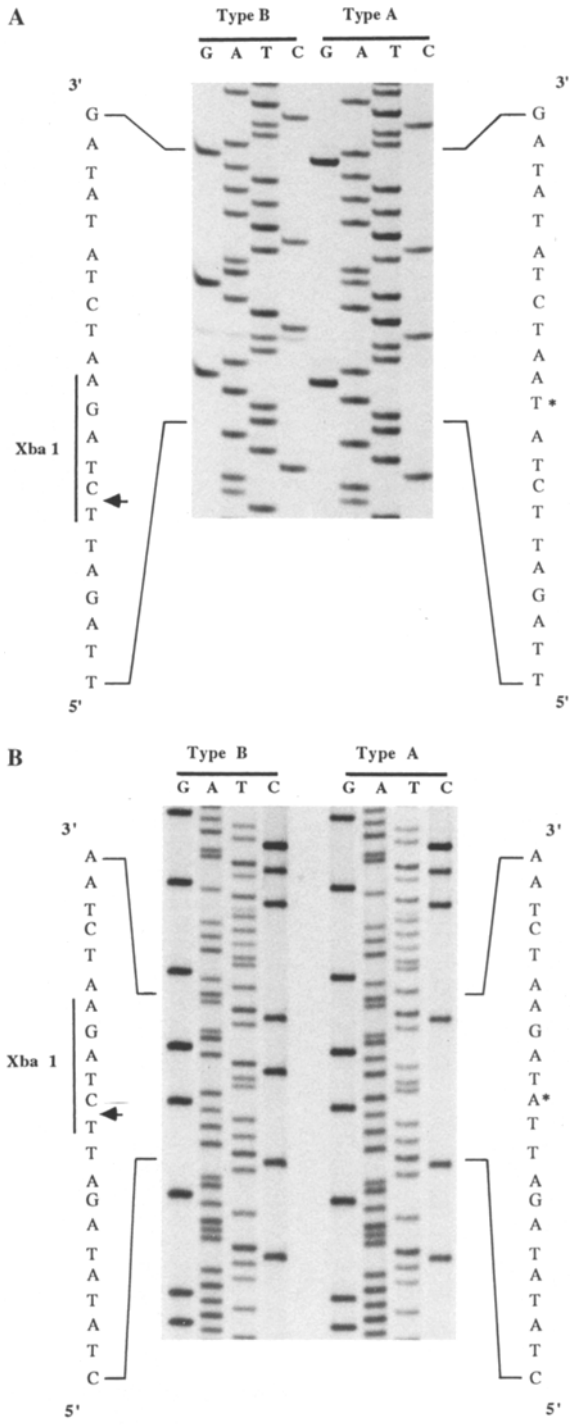

Fig. 5A,B **A** Portion of sequencing gel autoradiograph showing nucleotide sequence of forward strand of 696-bp *Eco*RI type-A and -B cpDNA fragments from *M sativa* L. cv 'Regen S' from base 281 to 302. The *Xba*I site, indicated by a bar is present in type-B cpDNA, and the variant base which results in a non-recognition site in type-A cpDNA is marked with an *asterisk*. **B** Portion of sequencing gel autoradiograph showing nucleotide sequence of inverse strand of 696-bp *Eco*RI type-A and -B cpDNA fragments from *M. sativa* L cv 'Regen S' from base 394 (302 fwd) to 415 (281 fwd) The *Xba*I site, indicated by a bar is present in type-B cpDNA, and the variant base which results in a non-recognition site in type-A cpDNA is marked with an *asterisk*. Sequence shown is complementary to that shown in **A**

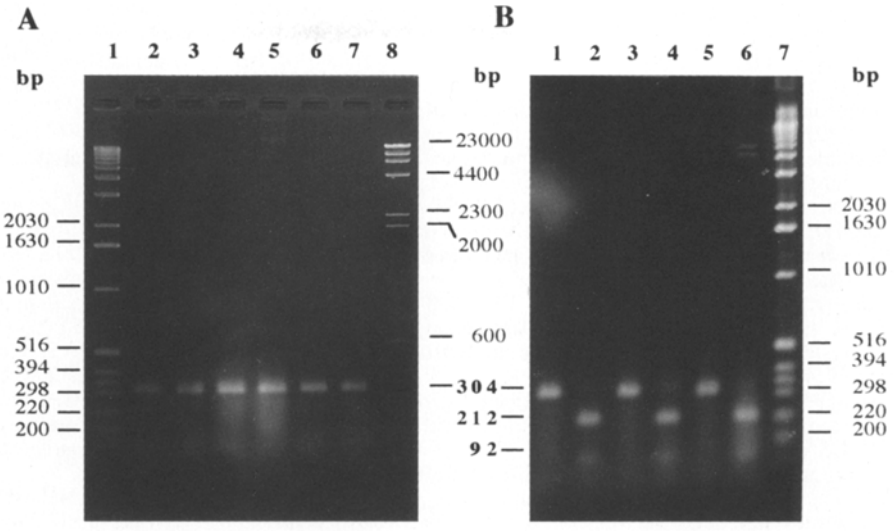

Fig. 6 A,B **A** Fragments of 304 bp from PCR amplification of 5 ηg DNA from mini-preps of selected pUC19 clones bearing type-A (*lanes 2, 3, 4*) or type-B (*lanes 5, 6, 7*) cpDNA fragment inserts. *Lane 1* 1-kb ladder, *lanes 2, 3* 696-bp *Eco*RI clone, *lane 4* 6.2-kb PstI clone, *lane 5* 6 2-kb *Pst*I clone, *lanes 6, 7* 696-bp *Eco*RI clones, *lane 8* '*Hin*dIII fragment marker **B** Fragments from digestion of PCR-amplified fragments of DNA with *Xba*I. *Lanes 1, 2* from 6.2-kb *Pst*I clones of type A and type B, respectively, *lanes 3, 5* from 696-bp *Eco*RI clones of type-A cpDNA, *lanes 4, 6* from 696-bp *Eco*RI clones of type-B cpDNA, *lane 7* 1-kb ladder

Discussion

The individual identity of the type-A and type-B cpDNA molecules from *M. sativa* L. cv 'Regen S' was confirmed by cloning of the 6.2-kb *Pst*I fragments from shoots with cpDNA having unique restriction profiles (Fitter and Rose 1993). Previous reports of heteroplasmy of cpDNA in *M. sativa* (Rose et al. 1986; Lee et al. 1988; Johnson and Palmer 1989; Schumann and Hancock; Masoud et al. 1990; Fitter and Rose 1993) were based on restriction fragment length polymorphism (RFLP) data. Heteroplasmy in gymnosperms was detected using distinguishing DNA markers specific to parental plants (Govindaraju et al. 1988; White 1990; Sutton et al. 1991), and in *Chlamydomonas* it was observed in *rbcL* mutant studies (Spreitzer and Chastain 1987; Zhang and Spreitzer 1990). The results of this study confirms two chloroplast genomes in *M. sativa* L. cv 'Regen S' by cloning and sequencing of the individual *Eco*RI restriction fragments from each of the two cpDNA types, A and B, containing the variable *Xba*I site. Consistent with the data from earlier work (Fitter and Rose 1993), one of the genomes (type B) contains an extra *Xba*I site in the *Eco*RI fragment that is located in the 6.2-kb *Pst*I fragment of cpDNA (Fig. 3). No other differences were found in the comparisons between the two sequences.

Sequence data revealed the basis of the difference between the type-A and type-B cpDNA to be a single base pair substitution, G to T, which results in a non-recognition site for the *Xba*I restriction enzyme in the type-A cpDNA. This data also confirms that the RFLP is not due to methylation of the cpDNA. In amyloplasts of cultured *Acer pseudoplatanus* L. cells methylation has been associated with differential transcription of plastid DNA (Ngernprasirtsiri et al. 1988).

The single base pair difference, of a T in type-A cpDNA for a G in type B, located at base 291 of the *Eco*RI fragment is most likely the result of a point mutation. The base pair difference is in a non-coding region of the chloroplast genome between the $tRNA^{Gly}_{GCC}$ and *psaB* genes. This provides no ready explanation for the preferential replication of type-A cpDNA in tissue culture (Fitter and Rose 1993). A better understanding of cpDNA biology in *M. sativa* may help provide an explanation, and the ability to use the PCR to detect type-A and type-B cpDNAs (Figs. 6A and 6B) will aid future investigations. The cross-pollination and bi-parental inheritance of plastids (with a paternal bias) characteristic of *M. sativa* (Zhu et al. 1993) would facilitate heteroplasmy. However, given that type-A and type-B cpDNA can show differences in replication (Fitter and Rose 1993), it would be expected that sorting out would still eventually occur. In *Chlamydomonas* heteroplasmy has been related to a balanced polymorphism of organellar alleles (Yu and Spreitzer, 1992). However, there is no evidence on this point in *M. sativa*. A complete explanation for heteroplasmy in *M. sativa* may depend on a knowledge of the complete sequence of the type-A and type-B cpDNAs.

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