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Investigation of chloroplast DNA heteroplasmy in *Medicago sativa* L. using cultured tissue

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Summary. *Medicago sativa* L. cv Regen S is heteroplasmic for chloroplast DNA (cpDNA). Previous analyses of regenerated plants have shown a predominance of one of the cpDNAs which we have designated type A (the other we have designated type B). Studies of the replication of the two cpDNAs in tissue culture were carried out using leaflet explants with defined cpDNA types and a distinguishing probe. The explants obtained showed a bias toward type A cpDNA during tissue culture. The data suggest that chloroplasts with different DNAs in a common nuclear background can multiply at different rates.

Key words: Chloroplast DNA – Heteroplasmy – *Medicago sativa* – Tissue culture – Preferential replication

Introduction

Medicago sativa L. cv Regen S is heteroplasmic for chloroplast DNA (Rose et al. 1986; Lee et al. 1988; Masoud et al. 1990). The cpDNA of *M. sativa* lacks the inverted repeat typical of most angiosperms (Palmer et al. 1987) and has been shown to be biparentally inherited (Smith et al. 1986; Corriveau et al. 1988; Masoud et al. 1990).

Chloroplast heteroplasmy is unusual as in most angiosperms it is a transient state with plastids sorting out during shoot growth and development. However, in *M. sativa* the two cpDNAs are maintained in the population (Rose et al. 1986; Johnson and Palmer 1989). In *M. sativa* L. cv Regen S we have denoted the two types of cpDNAs as type A and type B and they can be distinguished by the absence or presence of an *Xba*I restriction site respectively. The restriction site difference has been located in a 6.2-kb *Pst*I fragment of the chloroplast genome (Johnson and Palmer 1989). This has been confirmed by isolation and cloning of two *Eco*RI fragments which have a restriction site difference (Fitter and Rose, unpublished data).

The initial evidence for the presence of these two DNAs came from tissue culture and plant regeneration studies (Rose et al. 1986) and raised questions about the relative ability of the type A and type B cpDNAs to be transmitted from the explant to the regenerated plants, as most regenerated plants had type A cpDNA. This evidence was based on ethidium bromide staining of *Xba*I digests of chloroplast DNA separated on agarose gels. In the present study, hybridisation with a distinguishing probe has been used to examine the replication of the two types of cpDNA in tissue culture. Data reported in this paper show a preferential replication of type A cpDNA during tissue culture.

Materials and methods

Plant material

Cuttings from long-established plants were grown in the glasshouse (temperature 14–30 °C, daylength 13–14 h, and light intensity 55–2000 μmol m^{-2} s^{-1}) with each plant identified on the basis of cpDNA type. Plants were cut back regularly to encourage regrowth for fresh culture material. Leaf explants were taken from actively growing stems and placed into culture.

Culture protocols

Leaf tissue was sterilised for 5 min in a 33% saturated solution of calcium hypochlorite to which a few drops of 7X detergent

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had been added, followed by four rinses with sterile distilled water.

Tissue culture was conducted using a sequence of media for callusing (C1), embryo initiation (C2), embryo development (C3), and embryo germination (P40). C media contains KWM major salts (Kao and Wetter 1977) but with NH_4NO_3 at 600 mg l^{-1}, B5 minor salts and vitamins (Gamborg et al. 1968), MS casamino acids (Murashige and Skoog 1962), FeNaEDTA (after Dalton et al. 1983), 3% sucrose, pH 5.8, and 0.8% agar. In addition to the basal medium, C1 medium contains 5 μM NAA, 1 μM 2,4-D, 1 μM BAP; C2 contains 22.5 μM 2,4-D, 1.5 μM kinetin; and C3 contains 30 mM proline. P40 medium (Thomas et al. 1990) contains normal KWM major salts, lacks myo-inositol, and contains casamino acids at 1/4 MS levels (250 mg l^{-1}).

Tissue was incubated on C1 medium in the dark at 27 °C for 14–16 days then subcultured to C2 medium, incubated in the dark for a further 10 days then placed under fluorescent lights (15–30 μmol m^{-2} s^{-1}). Embryo development was monitored and embryos/embryogenic calli were subcultured to C3 medium when embryos were clearly formed, and at the cotyledonary stage. Advanced embryos were subsequently transferred to P40 medium for germination. The time from initiation of culture to analysis of a regenerated plantlet was 120–136 days by which time two or three trifoliate leaves had fully formed.

DNA extraction

Total DNA was extracted from leaf explants, calli, and regenerated plantlets using a modification of the procedure of Mettler (1987). Tissue was frozen with liquid nitrogen and ground with a mortar and pestle and 0.1 g of alumina powder. The ground tissue was incubated in 2–3 volumes of buffer (1% sarkosyl, 400 mM NaCl, 40 mM Na_2EDTA pH 8.0, 100 mM Tris pH 8.0, 5%-β-mercaptoethanol) at 50 °C for 60 mins then extracted with phenol (saturated with 0.5 M NaCl, 100 mM Tris pH 8.0). The DNA was recovered by ethanol precipitation with 1/10 vol of 3.0 M NaOAc, then reprecipitated with ethanol and 1/2 vol of 7.5 M NH_4OAc. The DNA was dried under vacuum and dissolved in T1/2E buffer (10 mM Tris-Cl pH 8.0, 0.5 mM EDTA pH 8.0).

DNA restriction, electrophoresis and Southern hybridisation

DNA was digested with *Xba*I in REact 2 buffer (BRL, Gaithersburg, Maryland USA) and run on 0.85% agarose gels (SEB buffer of 0.04 M trizma base, 0.005 M NaOAc, 0.001 M $Na_2EDTA \cdot 2H_2O$ buffered to pH 7.8 with CH_3COOH) at 60 V for 14–16 h. Gels were stained with ethidium bromide (0.5 μg ml^{-1}), rinsed in distilled water and photographed with Polaroid type 55 Land film under UV light. DNA was transferred to Zeta-Probe membranes (Bio-Rad, Richmond, California, USA) by alkaline transfer (Bio-Rad protocol after Reed and Mann 1985) for 12–15 h. The 6.2-kb *Pst*I fragment of *M. sativa* L. cpDNA cloned into a pUC19 plasmid vector was used as a probe to distinguish between types A, B, and AB cpDNA in *Xba*I-digested DNA. Probe DNA was labelled with ^{32}P dNTP by multiprime labelling (Amersham, Buckinghamshire, England) and unincorporated nucleotides were removed using a Sephadex G-50 spun column (Maniatis et al. 1982). Hybridisation was carried out using the Bio-Rad standard protocol for hybridisation (1 mM Na_2EDTA, 0.5 M $NaHPO_4$ pH 7.2, 7% SDS) and washing (two washes in 1 mM Na_2EDTA, 40 mM $NaHPO_4$ pH 7.2, 5% SDS at 65 °C for 30 min, followed by two washes in 1 mM Na_2EDTA, 40 mM $NaHPO_4$ pH 7.2 1% SDS at 65 °C for 30 min). Kodak XAR-5 film was exposed to the membranes in cassettes between intensifying screens, for 0.5–16 h at room temperature.

Results

In order to carry out studies on the cpDNA in cultured tissue it was necessary to obtain leaflets with defined cpDNA types. Southern hybridisation analyses revealed plants that were predominantly type A, B, or AB (Fig. 1).

In preliminary experiments type AB cpDNA leaf explants were taken from actively growing stems of plants and placed into tissue culture and the shoots were then allowed to grow for a further 7 days after which DNA was extracted from two trifoliate leaves. The leaf immediately below the explant source was combined with the one above the explant source which had grown during the 7 days. The explant tissue was cultured for 37 days and then the calli were extracted for total DNA. *Xba*I digestion of these total DNA samples was carried out and the samples electrophoresed, followed by Southern hybridisation using the 6.2-kb *Pst*I alfalfa fragment probe. As shown in Fig. 2 there was a preferential amplification of type A cpDNA during culture.

In further experimentation a modified procedure (Fig. 3) was adopted in which the explant source was

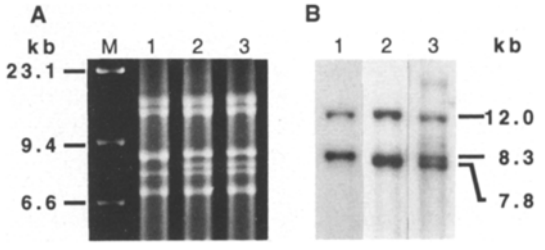

Fig. 1A, B. Representative banding patterns for type A, B, and AB cpDNAs, from *M. sativa* L. cv Regen S plants, digested with *Xba*I. **A** Ethidium bromide staining of purified cpDNA, and **B** using the 6.2-kb *Pst*I fragment of *M. sativa* cpDNA as a probe for Southern hybridisation. *Lanes 1*, type A cpDNA; *lanes 2*, type B cpDNA; *lanes 3*, type AB cpDNA; *lane M*, Lambda marker, *Hin*dIII fragments

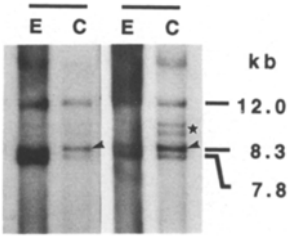

Fig. 2. Evidence for preferential replication of type A cpDNA (*arrowed*) in tissue culture from explant to callus (*lane E*, total DNA from explant; *lane C*, total DNA from callus after 37 days of culture; –, non-specific binding which was largely eliminated in later experiments: see also in Masoud et al. 1990)

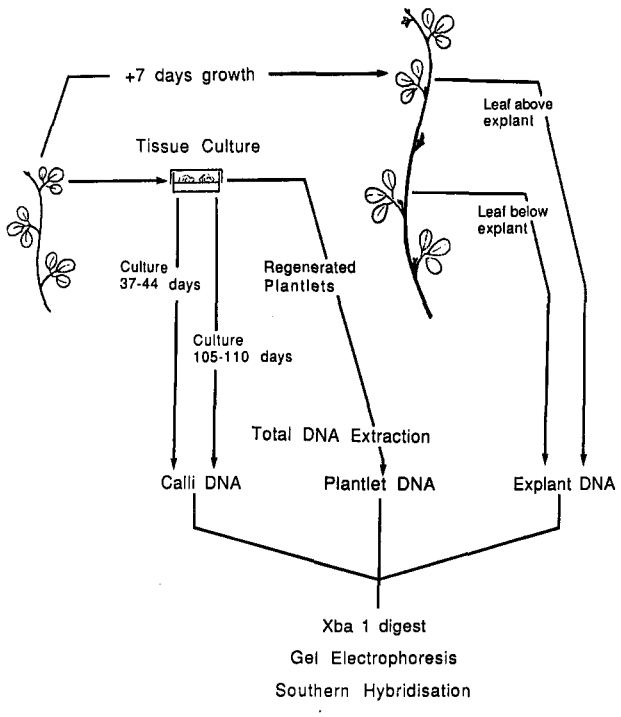

Fig. 3. Experimental protocol for investigation of *M. sativa* L. cpDNA from explant, through tissue culture to callus, and regenerated plantlets

extracted for total DNA as before, except that this time the leaf below the explant and the newly emerged leaf that had grown in the 7 days from initiation of culture were extracted separately. This latter protocol provided more precise information on the cpDNA type in the starting tissue.

A sample of callus tissue was extracted for total DNA at 37–44 days and then another sample extracted at a 105–110 days to determine the longer term effects of culture. In addition to the events prior to and during culture a sample of regenerated plants was also examined. Whole embryo/plantlets were sacrificed and extracted for total DNA. The whole plantlet was used so that there could be no error due to loss of one cpDNA type as a result of sorting out during growth of the regenerated plantlet. The sample would then be representative of the cpDNA type that the plantlet had at regeneration.

At 44 and 107 days after initiation of culture, explants from type A cpDNA leaves (RS1.1/4) were found to be type A in the callus phase (Fig. 4A, B, Table 1).

Type AB leaves were found to be type AB cpDNA in the callus tissue but with a bias toward type A (Fig. 4D, E). In 8/9 cases where the explant was type AB, regardless of the proportion of A or B initially,

Table 1. cpDNA type revealed by Southern hybridisation with the 6.2-kb *Pst*I fragement of *M. sativa* to total DNA extracts from explant source tissue, callus tissue after 44 days or 107 days, or regenerated plantlets (underlined character indicates the predominant cpDNA type; addition of # indicates a strong predominance of the underlined type of cpDNA; *indicates faint images on autoradiographs)

Plant no.	cpDNA type in tissue							
	Explant tissue		Callus tissue		Regenerated plants			
	Above	Below	44 days	107 days	1	2	3	4
RS1.1/4	A	A	A	A	A	–	–	–
	A	A	A	A	A	–	–	–
	A	A	A	A	A	A	–	–
RS1.2/3	A<u>B</u>	A<u>B</u>#	<u>A</u>B	<u>A</u>B*	–	–	–	–
	*	<u>A</u>B	<u>A</u>B	<u>A</u>B*	–	–	–	–
RS1.8/3	A<u>B</u>	A<u>B</u>	<u>A</u>B	<u>A</u>B	A<u>B</u>	–	–	–
	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B*	–	–	–	–
	<u>A</u>B*	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B	–	–	–
	<u>A</u>B*	*	<u>A</u>B	<u>A</u>B*	<u>A</u>B	<u>A</u>B	–	–
	B	B	<u>A</u>B	<u>A</u>B	<u>A</u>B#	<u>A</u>B#	–	–
RS1.8/4	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B	–	–	–
	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B	<u>A</u>B
	B	B	<u>A</u>B	<u>A</u>B	<u>A</u>B#	–	–	–
RS1.8/5	<u>A</u>B	<u>A</u>B	<u>A</u>B*	<u>A</u>B#	<u>A</u>B	–	–	–
	*	*	*	*	<u>A</u>B	<u>A</u>B#	<u>A</u>B#	–
RS1.2/4	B	B	AB	<u>A</u>B	–	–	–	–
	B	B	<u>A</u>B	<u>A</u>B	–	–	–	–
	B	B	AB	<u>A</u>B	<u>A</u>B	–	–	–

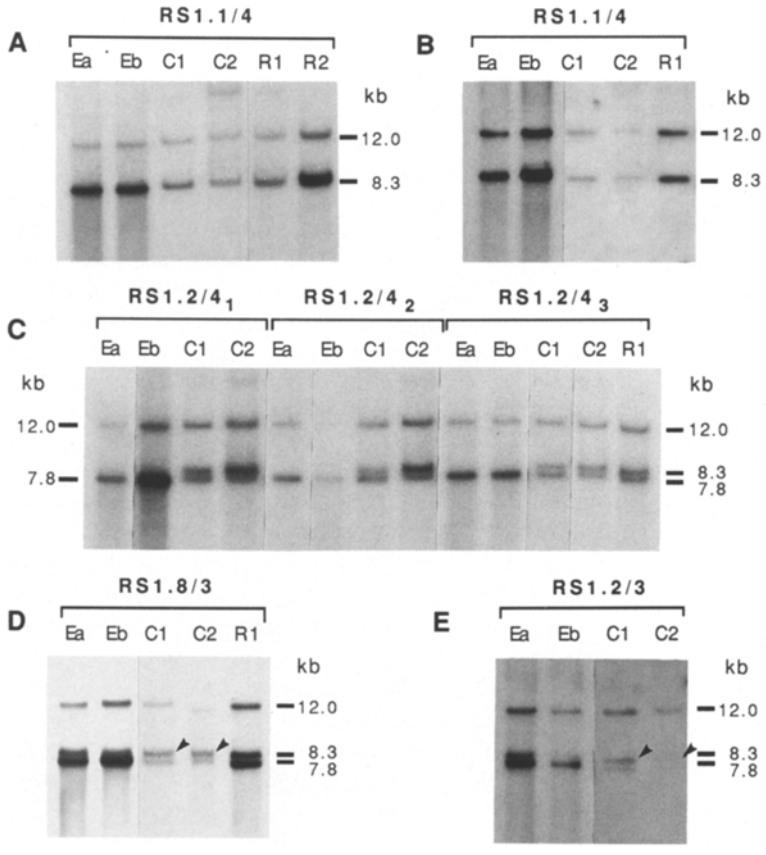

Fig. 4A–E. Southern hybridisation patterns for total DNA extracts from: explant sources (*Ea*, leaf above explant; *Eb*, leaf below explant), callus tissue after 44 days (*C1*) and 107 days (*C2*), or regenerated plants (*R1*, *R2*) as revealed by hybridisation with the 6.2-kb *Pst*I fragment of *M. sativa* cpDNA. Typical patterns are shown for cultures initiated from: type A cpDNA explants (**A, B**), type B cpDNA explants (**C**), and type AB cpDNA explants (**D, E**) showing preferential replication of the type A cpDNA band in callus tissue (➧)

the callus had predominantly type A cpDNA at both 44 days and 107 days (Table 1). In the case of the plant RS1.8/3, although the explants from two different stems showed one with a predominance of type A and the other of type B, the callus was found to have a greater amount of type A in both cases. Where the RS1.8/3 and RS1.8/4 explants were identified as type B in the later experiments (due to sorting out within the plant) then type A cpDNA was detected in callus DNA at 44 days and at 107 days. Changes in type B explants in culture are examined further below.

RS1.2/4 explants, identified as type B, gave callus tissue with type A cpDNA present at 44 days and as the major band at 107 days (Fig 4C). Callus grown from type B leaves (RS1.2/4, RS1.8/3, RS1.8/4) was found to have a type A band in the callus tissue DNA revealing that type A cpDNA molecules may exist in putative type B leaves but at a level below the detection limits of the analysis methods used (Table 1). Clearly, however, plant RS1.2 did have shoots with type A cpDNA and sorting out may not have been complete when cuttings were taken. A similar situation occurred in plant RS1.8 (see RS1.8/3 and RS1.8/4).

Plants regenerated from type A explants were found to have type A cpDNA in all cases (4/4). All regenerated plantlets from type B or AB explants were found to be heteroplasmic. In plants regenerated from type AB explants the relative amounts of each type of cpDNA followed a trend of having a predominance of type A cpDNA, although in one case a regenerated plant (RS1.8/3 line 1 Table 1) had a predominance of type B cpDNA. Of 17 regenerated plants derived from AB or B explants there were 16 which had a predominance of type A in their mixed (AB) cpDNA and of these, five plants (two of RS1.8/3, one of RS1.8/4, and two of RS1.8/5) showed a strong predominance (#, see Table 1) of type A cpDNA in their DNA.

Discussion

The results obtained provide good evidence for the preferential replication of the type A cpDNA molecule during tissue culture. Though such a situation has not previously been shown in tissue culture for cpDNA, similar data have been obtained for mitochondrial DNA. In cultured cells of *Brassica campestris* rapid structural alterations in mtDNA are suggested to result from a preferential replication of minor forms of the mt genome (Shirzadegan et al. 1991), while in

Chenopodium album L. substoichiometric molecules of DNA occur in different copy number in plants and cell cultures (Dorfel et al. 1989). In somatic hybrids of *Graminae* amplification of specific mtDNA sequences is commonly found (Ozias-Akins et al. 1988), and in wheat a differential replication rate of subgenomic molecules of mtDNA is associated with in-vitro culture (Hartmann et al. 1989).

What is also clear from the data is that chloroplasts with different cpDNAs in a common nuclear background can have different rates of cpDNA replication. This situation is similar to that which is well documented for *Oenothera* (Chiu et al. 1988). The basis for this is unclear given the nuclear control of chloroplast division (Rose et al. 1990); however, differential replication of *Oenothera* plastids has been suggested to be controlled by individual chloroplast genomes (Chiu and Sears 1992).

The results presented here could provide a partial explanation for those of Rose et al. (1986) where regenerated plants contained mainly type A cpDNA from starting material containing both type A and B cpDNA molecules. In addition, however, no type B regenerated plants have as yet been found from our explant cultures. Type B tissue shows a distinct recalcitrance to produce embryos, while type A and type AB tissue have shown quite high regeneration efficiencies, with type A tissue producing greater numbers of embryos than type AB tissue.

There is a trend in the data toward type B becoming the major type of cpDNA present in the intact plant (e.g., Table 1; plant 1.2); however, the tissue-culture data have shown that despite type A not being detectable in leaf samples the band was found in callus tissue after 44 days and 107 days. This may simply be the result of too few type A cpDNA molecules being present in the explant to enable detection by Southern hybridisation techniques. When a plant apparently does not contain any type B cpDNA (i.e., it is type A) then it remains true to type regardless of time or growth status. Plants identified as type A initially were also of type A after 2 years.

In heteroplasmic plants the proportion of each type of cpDNA may differ, and it seems unusual that plants simply do not consistently sort out to either type A or B. In chlorophyll-deficient mutants of *M. sativa* sorting of plastids has resulted in pure sectors of tissue occurring during development (Smith et al. 1986; Lee et al. 1988). Sorting of mutant and normal plastids has been correlated with cpDNA restriction patterns (Lee et al. 1988); however, since we have no morphological marker for either of the cpDNAs, we have not been able to observe such a correlation in our work. Conversely, the persistence of mixed plastids in shoots re-grown following top growth removal has also been reported (Masoud et al. 1990).

Why the heteroplasmic condition is maintained in the population is unclear at this time. The chloroplast genome of *M. sativa* has been shown to be bi-parentally inherited. This factor would tend to maintain both types of cpDNA in a population provided the correct combination of gametes occurred at fertilization. Additionally, *Medicago* is an outbreeding species and this would also tend to favour the maintenance of heterogeneity.

Schumann and Hancock (1989) have suggested that paternal plastids may be preferentially replicated in zygotes of *M. sativa*. The in-vitro system emphasises the in-planta situation and it is possible that regulation of cpDNA replication in-vitro may be similarly controlled in a preferential fashion. It is only one or a few cells that initiate an embryo in-vitro (Haccius 1978). In the germinated embryo and developing plant a new set of controls may come into play that could give rise to the observed cpDNA types in regenerated plants which show differences from the callus.

A resolution of the reasons for heteroplasmy of cpDNA in *M. sativa* and the of nature of the changes in the genome during tissue culture will be of benefit not only for an understanding of the biology of the chloroplast genome but should also prove useful in terms of the manipulation of the chloroplast genome where a culture phase is involved and the amplification of a new cpDNA type is required.

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