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Cytoplasmic DNA variation in a potato protoclonal population

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Summary. Mitochondrial DNA variation was detected in potato plants (protoclones) regenerated from leaf mesophyll protoplasts. Two forms of variation were evident; (1) DNA sequence alterations within the high molecular weight mitochondrial chromosome and (2) the appearance of an additional low molecular weight mitochondrial DNA species. Variation in chloroplast DNA was not detected. The data suggests that protocloning can introduce molecular diversity into mitochondrial genomes and thereby assist in overcoming the cytoplasmic genetic uniformity prevalent in most major crops.

Key words: Potato – Protoclones – Mitochondrial DNA – Chloroplast DNA – *Solanum tuberosum*

Introduction

In plant species, genetic information embodied within individual cells is distributed across nuclear, mitochondrial, and chloroplastic genomes. Mitochondrial and chloroplast specific DNAs reside in the cytoplasm and, in most crops, are only inherited from the female parent. Consequently, mixed or recombined cytoplasmic genomes do not arise from sexual crosses which produce diversity in the nuclear genome. This limitation leads to genetic uniformity within the heritable cytoplasmic elements.

It is becoming clear that genetic diversity is desirable in crop plants for cytoplasmic as well as nuclear genes. For example, while the molecular bases of heterosis are unclear, it is recognized that interactions between nuclear and cytoplasmic genetic elements are most certainly involved.

The most thoroughly documented case of cytoplasmic gene function is found in the Southern corn leaf blight disease where Texas (T) male sterile maize cytoplasm confers susceptibility to toxins liberated by the fungal pathogen *Helminthosporium maydis* race T. Evidence from several sources has implicated mitochondrial membranes and genes encoded in mitochondrial DNA (mtDNA) as major components of this susceptibility. By 1970, 85% of all hybrid corn in the USA carried the T cytoplasm and this vulnerability resulted in one of the most severe disease epidemics of modern times. From this, Ullstrup (1972) concluded that, "... never again should a major cultivated species be molded into such uniformity that it is so universally vulnerable to attack by a pathogen, insect or environmental stress. Diversity must be maintained in both the genetic and cytoplasmic constitution of all important crop species".

Given the fact that extranuclear DNA can influence plant phenotype, work reported here was performed to determine whether plants regenerated from mesophyll protoplasts of potato (protoclones) exhibited variation in mtDNA and chloroplast DNA (cpDNA). It is well known that potato protoclones exhibit a wide array of phenotypic alterations even when the parental chromosome number is retained (Shepard et al. 1980) and it is possible that alterations in cytoplasmic genetic elements may contribute some of the diversity.

Materials and methods

Protoplasts of the tetraploid (2n = 4x = 48) potato (*Solanum tuberosum* L. spp. *tuberosum*) cultivar 'Russet Burbank' were prepared and cultured as previously described (Shepard 1980a). Two protoclonal populations were regenerated from potato protoplasts. One set, termed unselected protoclones,

represents all plants regenerated by standard methods (Shepard 1980b) in one sequence of experiments. The second population, designated as the 'vigor series', was cultured in the same manner as the first through the stage where primordial shoots were initiated on Medium-D. Calli were then transferred to Medium-T (Shepard 1982) for shoot elongation and rooting rather than to the Medium-E composition used for unselected protoclones. The rate at which shoots develop differs considerably between protoplast-derived calli on Medium-T, but to a far lesser extent on Medium-E. Shoots which developed the most rapidly during a 10 day culture period on Medium-T, i.e. attained a length greater than 4.3 cm, were selected for the vigor series and the remainder were discarded. The overall frequency of selection was approximately one "vigor protoclone" per 40 total protoclones. In all cases, plants were grown to maturity and tubers collected.

MtDNA was extracted from 1.5 g to 7.5 g of protoclone leaves according to the methods of Kemble et al. (1980). CpDNA was isolated from the same amounts of tissue by a modification of the method of Palmer (1982). The two cesium chloride gradient centrifugations were replaced by three phenol-chloroform extractions. In our hands, the cesium chloride gradients and the phenol-chloroform steps resulted in cpDNA of equivalent purity. However, not only did the phenol-chloroform extractions allow the cpDNA to be recovered in higher concentration but it also greatly increased the rapidity of the method. After ethanol precipitation, the DNAs were digested to completion with six different restriction enzymes and electrophoresed on agarose gels (Kemble et al. 1980).

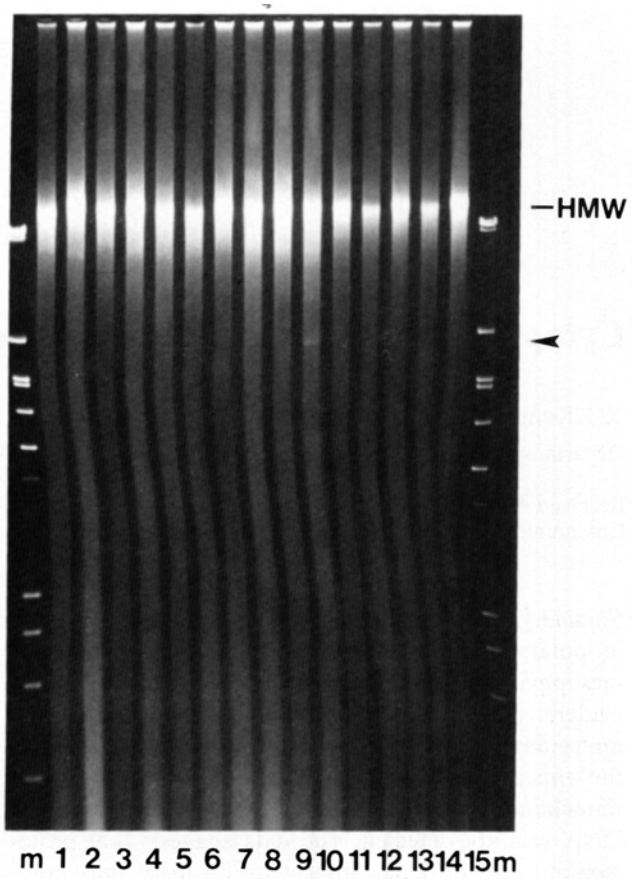

Fig. 1. Electrophoresis on 1% agarose gel of mtDNAs, not subjected to restriction enzyme digestion, from *lane 1,* parental 'Russet Burbank'; *lanes 2 to 15,* 14 vigor clones. *Lanes m* contain size marker fragments produced by independent digestions of lambda DNA with Eco RI and Hae III. *HMW* refers to the position of the high molecular weight mitochondrial genome. The arrow indicates the position of an additional mtDNA species in *lane 10*

Results

Analyses of mtDNA and cpDNA were performed on 26 protoclones selected on the basis of vegetative vigor on Medium-T, on 21 unselected clones and on parental 'Russet Burbank' plants. Plants used were raised from tubers collected from first generation protoclones. Chromosome counts of root tip cells revealed that unselected protoclones having 47, 48, 49, 72, 93 and 96 chromosomes were represented in the study. Chromosome counts were not performed on the vigor clones.

Electrophoresis of 'Russet Burbank' mtDNA not subjected to restriction enzyme digestion indicated that the DNA was present as a single highly fluorescent diffuse band which is characteristic of all plant mtDNAs and corresponds to the high molecular weight mitochondrial chromosome (Fig. 1, lane 1). No additional mtDNA molecules were evident in any preparations from 'Russet Burbank' or from 45 of the 47 clones (Fig. 1). However, one vigor clone and one unselected clone contained a nucleic acid component in addition to the high molecular weight mitochondrial chromosome (Fig. 1, lane 10). The size of this additional band was estimated at 7.15 kb by comparison with the mobility of linear DNA size markers on gels. It was the same size in each of the two clones and was resistant to RNase A but suceptible to DNase I.

Restriction enzyme analyses using Bam HI, Xho I, Hin dIII, Pst I, Eco RI and Bgl II indicated that one of the 26 vigor clones exhibited a variant high molecular weight mitochondrial chromosome (Fig. 2, lane 7). As compared to 'Russet Burbank' and all other protoclones in the vigor class, this clone lacked one Xho I fragment of 18.6 kb but possessed additional fragments of 13.7 and 9.4 kb. Fragments less than approximately 1 kb were not retained on the gels shown in Figs. 1, 2 and 4.

High molecular weight mtDNA variation was detected in four of the 21 unselected clones studied (Fig. 3). Restriction enzyme digestions indicated that in one of the variant clones, Bam HI fragments of 4.7 kb and 2.8 kb were absent (Fig. 3, lane 10). Another variant clone also lacked a fragment of 4.7 kb but possessed an additional fragment of 6.8 kb (lane 7) whereas in two variant clones a fragment of 14.5 kb was absent (lanes 4 and 14). Fragments smaller than ap-

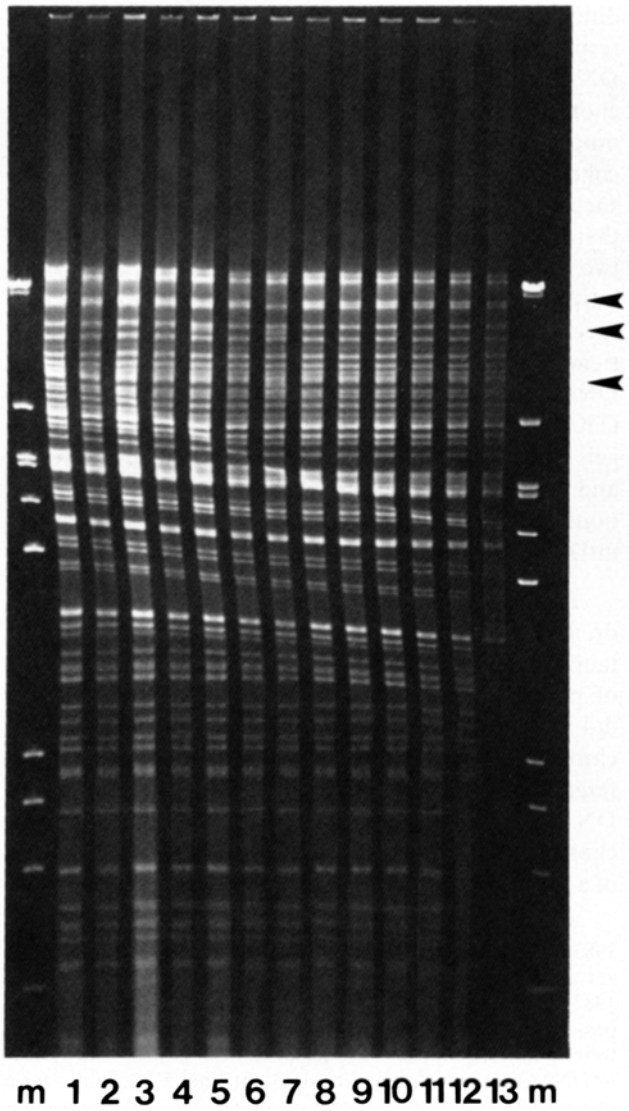

Fig. 2. Electrophoresis on 1% agarose gel of Xho I fragmented mtDNA from *lane 1*, parental 'Russet Burbank'; *lanes 2 to 13*, 12 vigor clones. *Lanes m* contain size markers as in Fig. 1. The additional and missing fragments of the sample in *lane 7* are indicated by *arrows*

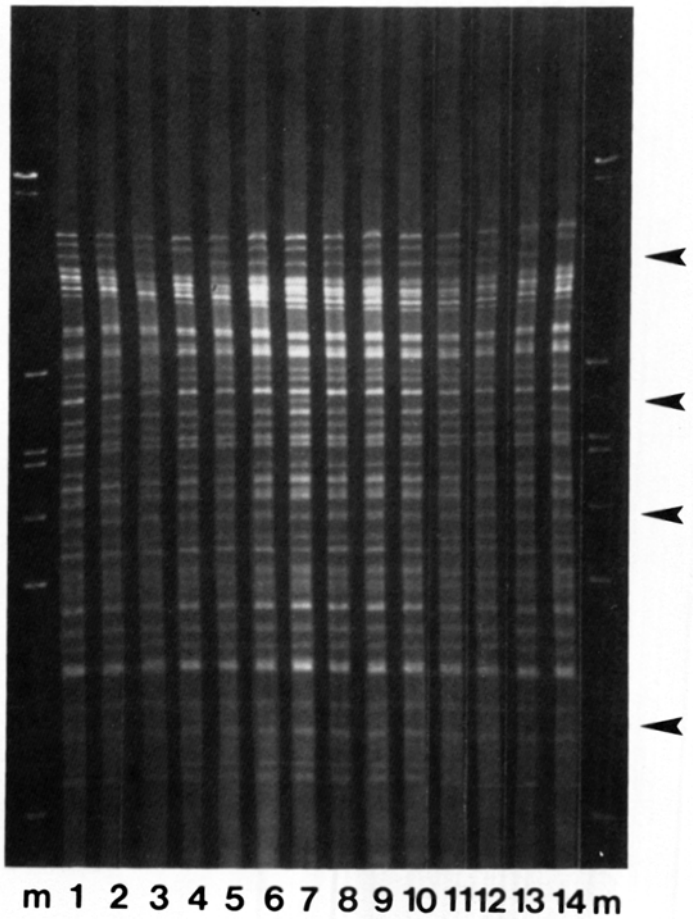

Fig. 3. Electrophoresis on 0.7% agarose gel of Bam HI fragmented mtDNA from *lane 1*, parental 'Russet Burbank'; *lanes 2 to 14*, 13 unselected clones. *Lanes m* contain size markers as in Fig. 1. The additional or missing fragments of samples in *lanes 4, 7, 10* and *14* are indicated by *arrows*

proximately 2 kb were not retained on the gel shown in Fig. 3. Of the four unselected protoclones that exhibited mtDNA variation, three were tetraploid (48 chromosomes) while the remaining one possessed 47 chromosomes.

No mtDNA variation was detected in 'Russet Burbank' plants which had been propagated by conventional techniques.

Summation of restriction fragment sizes, taking into account the differences in stoichiometry between fragments, gave an estimate of approximately 400 kb for the complexity of the 'Russet Burbank' mitochondrial chromosome.

CpDNA variation was not detected in any of the vigor or unselected clones with the six restriction enzymes employed. Figure 4 shows a representative gel containing Bam HI fragmented cpDNA from 'Russet Burbank', two vigor clones and two unselected clones.

Discussion

MtDNA variation was detected in seven of the 47 protoclones analysed (equivalent to 14.9%). This variation was expressed as additional low molecular weight mtDNA species in two of the clones (4.3%) and as sequence alterations of the high molecular weight mitochondrial chromosome in five of the clones (10.6%). This evidence establishes that mitochondrial genome variation occurs in protoclones suggesting that there is potential for introducing mtDNA diversity into potato germplasm. Similar diversity in mtDNA is not detected

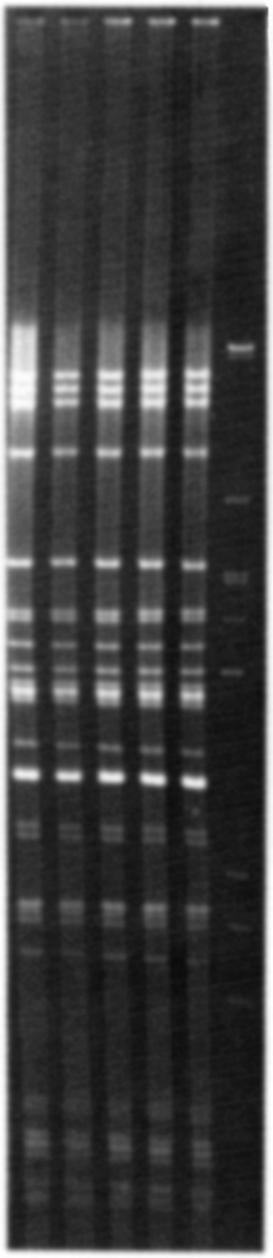

Fig. 4. Electrophoresis on 1% agarose gel of Bam HI fragmented cpDNA from *lane 1,* parental 'Russet Burbank'; *lanes 2 and 3,* two vigor clones; *lanes 4 and 5,* two unselected clones. *Lane m* contains size markers as in Fig. 1

in progeny obtained by conventional propagation. If other restriction enzymes or more precise approaches, such as DNA sequencing, had been employed to detect DNA variation it is likely that even greater mtDNA diversity would have been detected in the protoclonal population.

On electrophoretic gels, mtDNAs isolated from one vigor clone and one unselected clone displayed an additional discrete DNA species with a mobility corresponding to that of a linear molecule of 7.15 kb. This DNA co-purified with the high molecular weight mitochondrial chromosome extracted from DNase I treated mitochondria. Since we are not aware of any DNA-containing potato pathogen, viz. a DNA virus, which is specifically associated with mitochondria, we conclude that it is a naturally occurring mtDNA species in these two protoclones. This is the first report of extra mtDNAs in the genus *Solanum.* Because this 7.15 kb DNA species appeared as a single band on gels, our experience with other mitochondrial plasmids and virus-like DNAs suggests that it may be linear in conformation. Circular mtDNAs normally appear as three bands on gels corresponding to the supercoiled, open-circular and linear conformations. Unfortunately, tissue limitations prevented further characterization of this 7.15 kb mtDNA.

Restriction enzyme fragmentation of the mitochondrial chromosomes from the two 7.15 kb DNA-containing protoclones produced patterns identical to those of parental 'Russet Burbank'. Thus, the 7.15 kb DNA did not arise by precise excision from the mitochondrial chromosome because the loss of an equivalent size fragment was not detected in the high molecular weight DNA. If the 7.15 kb DNA did evolve from the mitochondrial chromosome, it could represent duplication of a specific region.

Maize (Kemble and Bedbrook 1980; Kemble et al. 1980, 1983; Kemble and Thompson 1982; Pring et al. 1977; Weissinger et al. 1982), sorghum (Dixon and Leaver 1982; Pring et al. 1982) and *Brassica* (Palmer et al. 1983) have been reported to possess linear mtDNAs in addition to the high molecular weight mitochondrial chromosome. In maize, the linear mtDNAs are covalently attached, at the 5′ termini, to a protein which may play a role in replication of the molecules (Kemble and Thompson 1982). Small circular mtDNAs have been found in maize (Kemble and Bedbrook 1980; Kemble et al. 1980, 1983), sugar beet (Powling 1981) and *Vicia faba* (Boutry and Briquet 1982; Negruk et al. 1982; Nikiforova et al. 1983). Thus, while parental 'Russet Burbank' mtDNA possessed only the high molecular weight mitochondrial chromosome, two protoclones possessed an additional mtDNA species akin to those present in the aforementioned crops. Since the range of phenotypic variation among potato protoclones is broad, it is not possible to ascribe any obvious phenotypic alteration to the presence of this 7.15 kb species.

The variation displayed in the mitochondrial chromosomes of the protoclones probably results from substantial DNA sequence rearrangements (e.g., deletions and additions) and cannot be explained by simple point mutations. Both unselected clones and those selected on the basis of rapid growth showed variation. Two clones displayed identical mitochondrial chromosomal variation and another two clones exhibited some degree of similarity in variation. This suggests that there may be one or only a few region(s) of the mito-

chondrial chromosome which is (are) particularly susceptible to sequence reorganizations.

A similar type of mtDNA variation has been found in plants regenerated from cultured immature embryos of both T and N (normal, male fertile) cytoplasm maize. The maize regenerants exhibited mtDNA variation when unselected and also when subcultured under a *H. maydis* race T toxin selection pressure (Gengenbach et al. 1981; Kemble et al. 1982; Kemble and Pring 1982; Umbeck and Gengenbach 1983). It has recently been reported that mtDNA variation did not occur in five streptomycin resistant and four lincomycin resistant protoclones of *Nicotiana plumbaginifolia* (Nagy et al. 1983). However, mtDNA variation may nonetheless be detected in *Nicotiana* if a larger protoclonal population is analysed using a greater number of restriction enzymes.

There are reports of mtDNA recombination occurring in somatic hybrids produced by protoplast fusions (reviewed by Evans 1983). The results reported here suggest that caution should be shown in interpreting mtDNA data obtained from these plants. In such cases, the mtDNA rearrangements observed could be caused by variation in one of the parental genomes and be completely independent of the protoplast fusion process.

There was no correlation between chromosome number and the degree of mtDNA diversity among unselected potato protoclones. Variation appeared in three of the 12 tetraploid unselected clones and one of the four aneuploid clones. Diversity was not detected in the hexaploid or octaploid protoclones analysed but this may have been due to the limited number analysed.

Allowing for differences in fragment stoichiometry, summation of the restriction enzyme fragments indicate that the 'Russet Burbank' mitochondrial chromosome has an approximate complexity of 400 kb. This value is larger than previous estimates of 91 kb and 136 kb obtained from *S. tuberosum* var. 'Bintje' callus tissue by electron microscopic measurements and restriction fragment analysis, respectively (Quetier and Vedel 1977). Evidence from other systems, however, suggests that 400 kb may also be an underestimate. The size of the maize mitochondrial chromosome has, for example, been estimated at 200 kb to 500 kb by restriction enzyme analysis, whereas a more accurate estimate of 1,200 kb has recently been obtained by cosmid mapping (Lonsdale et al. 1983).

Our inability to detect cpDNA variation is in accord with previous evidence which suggests that chloroplast genomes, e.g. of potato, which contain two inverted repeat regions, are highly stable and resistant to sequence rearrangements (Fluhr and Edelman 1981; Palmer and Thompson 1982). Interestingly cpDNA restriction fragment patterns of 'Russet Burbank' and those of a putative plastome mutant (designated protoclone no. 116) could not be differentiated with any of the ten enzymes utilized.

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