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Nucleotide Polymorphism in Chloroplast DNA of *Nicotiana debneyi*

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Summary. EcoRI restriction endonuclease analysis of chloroplast DNA isolated from several distinct populations of *Nicotiana debneyi* has revealed a naturally occurring polymorphism. The chloroplast DNA of seven of the nine populations analysed possessed an additional EcoRI site. The origin of the additional restriction endonuclease fragments was confirmed by hybridisation of [^{32} P]-cRNA to fractionated EcoRI restricted chloroplast-DNA fragments adsorbed to nitrocellulose filters. Reciprocal F_1 hybrids between plants carrying the variant chloroplast-DNA's confirmed maternal inheritance of chloroplast-DNA.

Key words: Nucleotide polymorphism – Chloroplast DNA – *Nicotiana debneyi*

Introduction

Within species, plant populations are known to be polymorphic for a large number of nuclear-coded gene functions, many of which probably result from nucleotide substitution. Because nuclear and chloroplast genomes cooperate in a number of important functions (Bogorad 1975; Kung 1977) it follows that chloroplasts, and their genomes, should also display intraspecific variability. However, the evidence, based largely on the analysis of chloroplast-coded functions such as that for the large subunit of ribulose bisphosphate (RuBP) carboxylase (Chen et al. 1977; Kung 1977), indicate that chloroplast DNA (cp-DNA) appears to be highly conserved. More recently, restriction endonuclease analysis of cp-DNA (Atchison et al. 1976; Vedel et al. 1976; Frankel et al. 1979) and peptide mapping of the large subunit of RuBP carboxylase (Holder 1978; von Wettstein et al. 1978) have demonstrated greater variability in cp-DNA between closely related species than previously expected.

Intraspecific variability in mitochondrial DNA has been demonstrated in animals (Potter et al. 1975; Buzzo et al. 1978) and in maize (Levings and Pring 1976; Pring and Levings 1978), and in wheat it has been suggested that mitochondrial DNA is heterogeneous (Quetier and Vedel 1977). There has been no reported case (apart from deleterious albinos which result from mutation pressure alone) of persistent intraspecific variation in the cp-DNA of plants.

This paper describes a naturally occurring EcoRI restriction endonuclease site polymorphism in cp-DNA of an Australian species of tobacco, *Nicotiana debneyi*. The polymorphism resides in an additional EcoRI restriction site in some populations. Reciprocal F_1's between plants carrying the different cp-DNA types provide direct evidence for the maternal inheritance of cp-DNA.

Material and Methods

Origin of N. debneyi Populations

N. debneyi occurs in a 300-400 km wide coastal strip of eastern Australia from Cape York Peninsula (lat. 15°S) to Sydney (lat. 34°S), and on Lord Howe Island and in New Caledonia (Good-

Table 1. Origin of *N. debneyi* populations, and cp-DNA type as revealed by EcoR1 restriction endonuclease analysis

Population	Collection site and date		cp-DNA type
TS233	Biloela, Qld	1956	D2
TS232	Mt. Isa, Qld	1956	D2
TS6	Cape York, Qld	1953	D1
TS98	Mt. Etna, Qld	1953	D1
TS287	Carnarvon Range, Qld	1956	D1
ZY173	Cunningham's Gap, Qld	1973	D1
RCE10109	Royal National Park, N.S.W.	1978	D1
AN1288	New Caledonia	1959	D1
AN1382	New Caledonia	1963	D1

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speed 1954; Burbidge 1960). The species usually grows on sandy sites near the sea, but it has been collected amongst regrowth in disturbed forests and also in alluvial soils. This species is distinguished from other Australian species by the staminal filaments which are free from the corolla and by a haploid chromosome number of 24.

Of the nine populations studied (Table 1) TS232 was probably a fortuitous introduction since the species range does not extend as far west at Mt. Isa. It has not been possible to determine the precise collection sites of the two New Caledonia populations.

The chromosome number of at least four plants from each population was determined from anther squashes, previously fixed in glacial acetic acid: alcohol (1:3) and stained with aceto-orcein.

Cp-DNA Isolation and Restriction Endonuclease Analysis

Chloroplast DNA was isolated from bulked young plants, subjected to EcoRI restriction endonuclease digestion and fractionated on agarose gels according to Frankel et al. (1979).

Isolation of EcoRI cp-DNA Fragments

DNA fragments from EcoRI digests were fractionated in low melting point agarose (Sea-Plaque Agarose, Marine Colloids, Maine, USA) and extracted from the melted agarose by a method which employs hexadecyl trimethyl ammonium bromide (Langridge et al. 1979).

Preparation of [^{32}P]-cRNA

[α-^{32}P]-labelled ribonucleotide 5^1-triphosphates, prepared by a modification (Dennis, pers. comm.) of Symons (1974), were used to synthesise [^{32}P]-labelled RNA complementary to EcoRI fragments of cp-DNA essentially as described by Appels et al.(1978).

Filter Membrane Hybridisation

EcoRI restriction endonuclease fragments of cp-DNA, previously fractionated in agarose gels, were transferred to nitrocellulose strips (Southern 1975), dried, and incubated with [^{32}P]-cRNA in 3 X SSC containing 50% formamide and 0.1% SDS for 3 h at 45°C (Dennis, pers. comm.). Unbound cRNA was removed with pancreatic RNase, the filters exhaustively washed in 2 X SSC containing 0.1% SDS and dried. Hybridisation was detected by autoradiography.

Phosphoglucomutase Isozyme Analysis

The isozyme techniques used were modified (Brown et al. 1978) from Brewer and Sing (1970). Leaf extracts were fractionated in horizontal starch gels (histidine gel buffer, pH 8.0; sodium citrate electrode buffer, pH 8.0) and assayed for phosphoglucomutase (E.C.2.7.5.1) isozymes.

Results

EcoRI Restriction Site Polymorphism

The different EcoRI restriction patterns were initially seen in a comparison of cp-DNA isolated from plants of the TS287 and TS233 populations. The TS287 pattern is designated D1 and that of TS233, D2. A comparison of tracks A and D in Figure 1 shows that the difference resides in the absence of the 4.95 Mdalton fragment along with the appearance of a 2.60 and a 2.38 Mdalton frag-

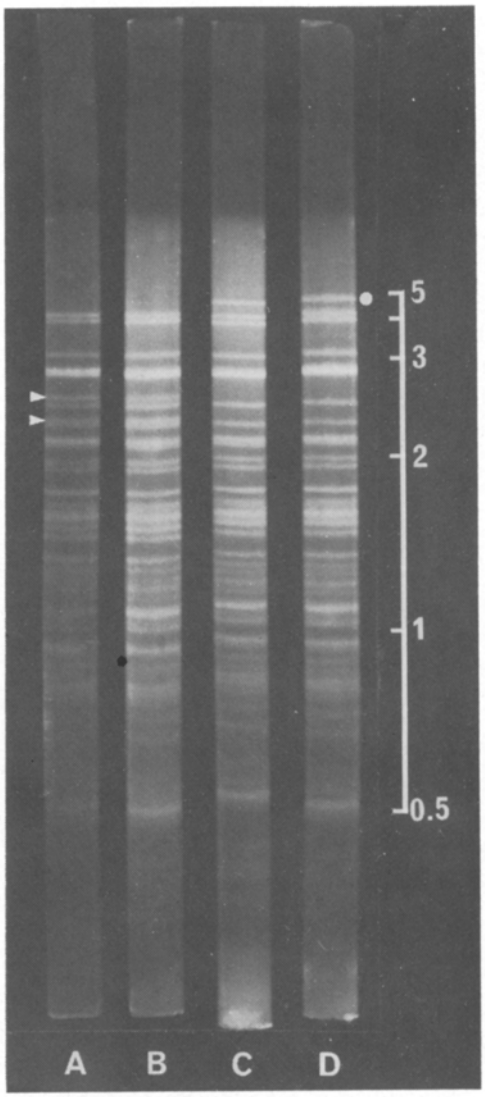

Fig. 1. Electrophoretic fractionation of EcoRI restriction endonuclease digests of chloroplast DNA isolated from D1 (TS287 – track A) and D2 (TS233 – track D) type populations and from the reciprocal F_1 hybrids (TS287♀ X TS233♂ – track B; TS233♀ X TS287♂ – track C). ▷ = 2.60 and 2.38 Mdalton fragments of TS287 (cp-DNA type D1): • = 4.95 Mdalton fragment of TS233 (cp-DNA type D2). Molecular weight scale in Mdaltons

ment in the D1 pattern, relative to D2. The sum of the molecular weights of these two additional fragments is essentially equal to that of the missing larger fragment. No other differences were apparent. Excess EcoRl and additional incubation time failed to degrade the 4.95 Mdalton fragment of the D2 pattern.

The EcoRl restriction endonuclease patterns of the cp-DNA from other populations conformed to either the D1 or D2 patterns. The only other population that had the D2 pattern was TS232; the remaining seven populations had the D1 pattern (Table 1). No other differences could be discerned.

All populations had a haploid chromosome number of 24 and in each population the staminal filaments were free from the corolla, thus confirming that these populations belong to the same species.

Morphological Differences Between Populations

The greenness of the rosette leaves of D2 type populations was more intense than that of D1 type populations. The basal rosette of D2 populations was also more compact than that of D1 types. The height of the main stem at flowering was greater in D1 (900-1000 mm) than in D2 (450-550 mm) populations. Anthocyanin pigmentation was also more noticeable in the D1 type populations. Of distinctive note was a pink-purple patch on the dorsal surface of the corolla lobes of plants from D1 populations, whereas the corolla lobes of D2 plants were completely white. It is assumed that these differences are controlled by nuclear-coded genes.

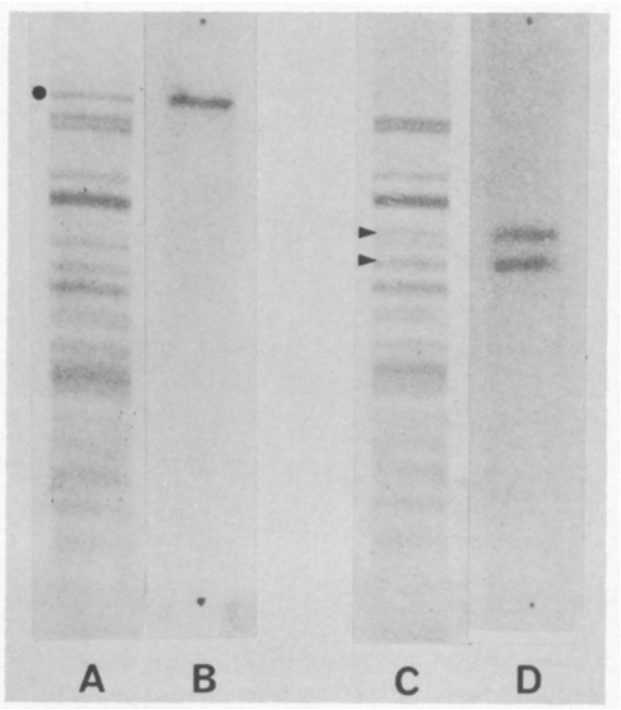

Fig. 2. Homology between [^{32}P]-RNA complementary to the 4.95 Mdalton fragment of TS233 and EcoRl fragmentation patterns of TS233 and TS287. The ethidium bromide stained gels of the fractionated EcoRl digests of TS233 (track A) and TS287 (track C) were photographed prior to transfer to nitrocellulose filters, enlarged to the same magnification as the autoradiographs and aligned by an independent scale. Autoradiographs resulted from the hybridisation of [^{32}P]-RNA, complementary to the TS233 4.95 Mdalton fragment, to each of the EcoRl digests of TS233 (track B) and TS287 (track D). • = 4.95 Mdalton DNA fragment of TS233; ▸ = 2.60 and 2.38 Mdalton DNA fragments of TS287

[^{32}P]-*cRNA Hybridisation*

The hybridisation of [^{32}P]-RNA, complementary to the 4.95 Mdalton fragment isolated from the EcoRl digests of TS233 (D2), with EcoRl digests of cp-DNA from D1 and D2 are shown in Figure 2. As expected, the D2 4.95-cRNA specifically hybridises to the 4.95 Mdalton fragment of the fractionated digest of TS233. There was no hybridisation to any other fragment even after prolonged exposure of the X-ray film. When hybridised to the fractionated EcoRl digest of TS287 (D1), the D2 4.95-cRNA hybridised to both the 2.60 and 2.38 Mdalton fragments. This demonstrates that the nucleotide sequence of these two D1 fragments is homologous with that of the 4.95 Mdalton fragment of TS233 (D2).

Inheritance of cp-DNA Polymorphism

Reciprocal F_1 hybrids between TS287 and TS 233 were made, and the cp-DNA of the F_1 plants subjected to EcoRl analysis. Tracks B and C of Figure 1 show the restriction fragmentation patterns where the female parent was TS287 or TS233, respectively. In each case the pattern in the F_1 was identical to that of the female parent.

The F_1's were shown to be genuine nuclear hybrids by analysis of the nuclear-coded phosphoglucomutase enzyme. The two parents were first screened by starch gel electrophoresis for several different enzymes to find isozyme variants which distinguished TS287 from TS233. For phosphoglucomutase, TS287 (D1) carried a fast allele and TS233 (D2) a more slowly migrating variant (Fig. 3). Each of the F_1's carried both the fast and slow migrating variants (Fig. 3), thus confirming their respective hybrid genotypes.

The hybrid nature of both F_1's was further confirmed by the anthocyanin pigmentation on the corolla lobes which was intermediate in intensity relative to the non-pigmented TS233 and the pink purple colour of TS287.

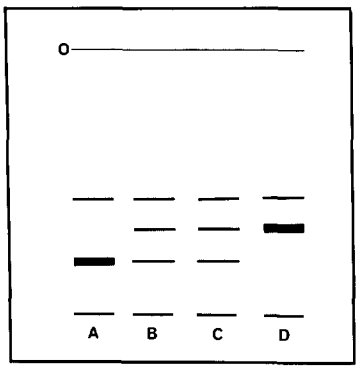

Fig. 3. Diagrammatic representation of starch gel electrophoresis of isozyme variants of phosphoglucomutase in leaf extracts of *N. debneyi*. A = TS287 (cp-DNA type D1); B = F_1 (TS287♀ × TS233♂); C = F_1 (TS233♀ × TS287♂); D = TS233 (cp-DNA type D2); O = origin

Discussion

A polymorphism in cp-DNA of *N. debneyi* was revealed by EcoRI restriction endonuclease analysis. An additional EcoRI site occurs in some populations (DI type) resulting in the cleavage of the largest fragment (4.95 Mdaltons) into two smaller fragments (2.60 and 2.38 Mdaltons). Homology between each of the two smaller fragments and the 4.95 Mdalton fragment was demonstrated by hybridisation of the smaller fragments to [^{32}P]-cRNA prepared from isolated DNA of the larger fragment. Of the nine populations analysed, seven had cp-DNA with the additional EcoRI site. While no attempt was made to determine intrapopulation variability, there was no discernible heterogeneity in the fragmentation patterns of the cp-DNA isolated in bulk from up to 15 plants for each population.

No function can be ascribed to the polymorphism but several morphological characters distinguished the D1 and D2 populations. D2 populations had darker green leaves and a more compact growth habit than D1 populations, which in turn had more anthocyanin pigmentation in the corolla lobes than D2.

The cp-DNA polymorphism could be due to the fixation of a nucleotide substitution which either generated a new EcoRI recognition sequence or alternatively, abolished a pre-existing one. Seven of the nine populations screened had the additional site in the cp-DNA suggesting that this is the more common variant. This favours the tentative conclusion that the D2 type variant arose as a result of a mutational event which abolished a pre-existing EcoRI recognition site.

The polymorphism could also be due to methylation of one of the bases in one EcoRI site of the D2 type populations, which would preclude the cleavage of the 4.95 Mdalton fragment. While possible, it is unlikely that modification by base methylation would occur in just one specific EcoRI site. Moreover, cp-DNA has no discernible methylated bases (Whitfeld and Spencer 1968; Kung 1977). From the analysis of the F_1's it also follows that cp-DNA methylation would have to be chloroplast coded.

A third possibility could be interspecific hybridisation followed by recurrent natural backcrossing with *N. debneyi*. *N. debneyi* is coincident with part of the distribution of both *N. megalosiphon* and *N. suaveolens*. However, these two species, which have identical EcoRI fragmentation patterns (Frankel et al. 1979), differ significantly in fragmentation patterns from both the D1 and D2 type patterns of *N. debneyi*. The restriction pattern of D2 is more like that of *N. megalosiphon* and *N. suaveolens* than is D1, but even so there are three discernible differences.

Assuming that the cp-DNA polymorphism resulted from nucleotide substitution, it can be argued that just one mutational event had occurred in the entire chloroplast genome and that it became apparent through serendipity. It is more likely however, that differences revealed by restriction endonuclease analysis reflect the average rate of nucleotide substitution in the genome. Consequently restriction endonuclease analysis provides a method of sampling the nucleotide sequence, from which sequence divergence can be estimated (Upholt 1977). This assumes that the occurrence of restriction sites is a function of the nucleotide proportions. In tobacco the molecular weight of cp-DNA, estimated from *Sal*, *Bam* and *Pst* endonuclease restriction patterns (Frankel et al. 1979) is 97 Mdaltons (156.7 kilobase pairs). Based on a random distribution of nucleotides and a GC content of 40.6% for tobacco cp-DNA (Whitfeld and Spencer 1968), 50 EcoRI sites (GAATTC/CTTAAG) would be expected (Upholt 1977) in the chloroplast genome. The actual number of discernible fragments in the original prints of Figure 1 was 41, and from densitometer traces of negatives, the molar proportions of 11 of these was approximately two-fold, and of 1 fragment, three-fold. The total number of 53 discernible fragments is in close agreement with expectation. Thus it appears that EcoRI sites are randomly distributed within the tobacco chloroplast genome.

These 53 RI sites represent the potential for change at only 318 nucleotide pairs, which represents only 0.2% of the entire cp-DNA genome. In the *N. debneyi* polymorphism therefore, the deletion of a pre-existing EcoRI site could reflect an average of 500 nucleotide substitutions which distinguish the chloroplast genome of D2 from that of D1 types. This suggests that cp-DNA of tobacco may be a great deal more variable than previously deduced from the analysis of variation in the chloroplast-coded large subunit of Fraction I protein (Chen et al. 1977). However, the large subunit gene may not be truly representative of the chloroplast genome because the require-

ment for biological activity of RuBP carboxylase may constrain the extent of genetic variability which can be tolerated.

Substantial evidence from the analysis of the progeny of intraspecific hybrids of tobacco indicates that the chloroplast genome is maternally inherited (Kung 1977; Frankel et al. 1979). The EcoRI analysis of reciprocal F_1 hybrids between representatives of D1 and D2 type populations confirm maternal inheritance of cp-DNA at the nucleotide level and extend it to provide definitive proof of maternal inheritance within a species.

Differences in organellar genomes, as determined by restriction endonuclease analysis, appear to be associated with male-sterility in wheat (Quetier and Vedel 1977), maize (Levings and Pring 1976; Pring and Levings 1978) and tobacco (Belliard et al. 1978; Frankel et al. 1979). Such association were used to impute either mitochondria or chloroplasts as the cytoplasmic determinants of male-sterility. Both D1 and D2 populations, which differ in cp-DNA, and the F_1's between them, are fully male-fertile. Thus differences in the nucleotide sequence of organelles do occur without concomitant male-sterility. Consequently, previous studies which imply a causal relationship between organelle DNA differences and male sterility must be treated with circumspection.

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