Studies on Backcross Progeny of *N. rustica* × *N. tabacum* Interspecific Hybrids II. Genomic DNA analysis

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Studies on the mammoth gene and its segregation in the *rustica-tabacum* interspecific hybrid progenies have shown that the introgression of the mammoth gene into *N. rustica* genome has given rise to new expressions in flower induction and flower morphogenesis (Smith 1950, 1973). This may be due to the differential response of the mammoth gene to a different genetic background. The inheritance and expression of the mammoth gene was more complex in *N. rustica* than in *N. tabacum*. The altered flowering responses in the *rustica-tabacum* hybrid progeny fell into two main categories. Our previous studies revealed that 3% of the backcross progeny of the hybrids remained non flowering for an indefinite period of time (Non flowering plants, NF) and 9% of the hybrids showed a flower reversion phenomenon (Flower revertants, FR). The majority of the hybrid segregants (88%) were normal (N) plants, similar to the recurring parent *N. rustica* (see related paper in this volume).

Segregation in the backcross hybrid progeny for FR and NF plants was heterogeneous and did not conform to known Mendelian ratios. Cytological studies both at mitosis and meiosis indicated a general cytological instability in the hybrid progeny segregating for FR and NF characters. Cytological analysis of the normal (N) plants revealed regular mitosis with 48 chromosomes. It was important to investigate whether the cytological disorders were associated with changes in the molecular composition of nuclear DNA. The molecular composition of the genomic DNA of several eukaryotic species is well established. But very little information is available on the pattern of molecular changes associated with the reorganization of genomes after interspecific hybridization. A comparison of the composition of the genomic DNA of the hybrid progenies (FR and NF) and their parental species was therefore undertaken and the present paper describes the results of these studies.

Materials and methods

Plant material

The backcross hybrid progeny, (*N. rustica* × *N. tabacum*) × *N. rustica* [line 34753 (7925-4) from the BC₄F₆ generation], segregating for the mammoth gene and the parental species *N. tabacum* cv. Maryland Mammoth and *N. rustica* were used in this investigation. The seeds were provided by Prof. Harold H. Smith (USA) and were grown in a glasshouse at 25°C.

Extraction and purification of genomic DNA

Genomic DNA was extracted from leaf material collected from *rustica-tabacum* backcross hybrid segregants (FR and NF plants) and their parental species *N. rustica* and *N. tabacum*. Leaf material collected from healthy plants was quickly frozen for 10–15 min in liquid nitrogen, ground to a fine powder and suspended in extraction buffer (0.1 M NaCl, 20 mM EDTA, 50 mM Tris (pH 8.5) and 1 M NaClO₄) to which diethylpyrocarbonate at a final concentration of

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1% (W/V) and 0.1 M sodium diethyl dithiocarbamate were added just before use. The suspension was centrifuged at 4°C for 10 min at 2000 rpm in an MSE high spin-21 centrifuge and the supernatant containing the cytoplasmic organelles was discarded. The nuclear pellet was resuspended in cold lysis buffer (0.1 M NaCl, 20 mM EDTA, 50 mM Tris (pH 8.5) and 1% Sarkosyl) and ground with a pestle to help lysis. The suspension was recentrifuged and the supernatant was repeatedly extracted with an equal volume of chloroform-octanol (99:1) until no protein precipitate was visible at the interface. The genomic DNA was further purified from contaminating polysaccharides, RNA and protein by CsCl density gradient centrifugation at 40,000 rpm (130,000 g) for 48 hr at 20°C.

Thermal denaturation analysis of DNA

Thermal denaturation of DNA was carried out in an automated SP1800 spectrophotometer having electronically heated cell blocks. Purified genomic DNA samples were dialysed extensively against $0.1 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M NaCl}$, $0.015 \text{ M trisodium citrate}$, pH 7.0) in a final concentration of $20 \mu\text{g}/\mu\text{l}$ (an optical density of 0.4–0.5). In order to achieve equal ionic concentrations, the different samples to be compared were dialysed simultaneously. Initial absorbance of the DNA samples were recorded at 25°C. The temperature of the cell block was quickly raised to 50°C and the absorbance of the DNA sample was recorded at 260 nm after stabilising for 5 min. The temperature was then gradually increased at the rate of $0.25^\circ\text{C}/\text{min}$ and the increase in the optical density of DNA (hyperchromicity) was automatically recorded. The absorbance measurements, which were corrected for solvent expansion using the table of Mandel and Marmur (1968), were plotted as a ratio A_t/A_{25} (absorbance at temperature 't' divided by the initial absorbance at 25°C) against temperature. From the slope of the melting profile, T_m values (the temperature corresponding to half the final increase in the relative absorbance) of DNA were calculated and extrapolated to $1 \times \text{SSC}$. The average G + C contents were estimated using the equation of Kemp and Sutton (1976). The average base compositional heterogeneity of the DNA samples were estimated according to Mandel and Marmur (1968).

Analytical density gradient analysis of DNA

Analytical isopycnic centrifugation of genomic DNA was carried out in an MSE Centrican-75 analytical ultracentrifuge. Purified genomic DNA samples (1.5 to $2 \mu\text{g}$) were centrifuged to equilibrium in neutral CsCl solution (density 1.71 g/ml) in 0.5 ml analytical cells. *Micrococcus lysodeikticus* DNA ($0.25 \mu\text{g}$) of known buoyant density (1.731 g/ml) was added to each sample as an internal standard. The DNA samples and the blank solutions were centrifuged at 40,000 rpm for 24 hr at 20°C. At equilibrium, the density gradients were optically scanned at $254 \mu\text{m}$ and plotted. The mean buoyant density of the DNA sample was calculated with reference to the buoyant density of the marker DNA (Mandel *et al.* 1968). The buoyant density, the G + C contents and the mole fraction of 5-methyl deoxycytidine contents of the DNA samples were calculated using the modified equations of Schildkraut *et al.*, (1962) as given by Kemp and Sutton (1976).

Results

Purified genomic DNA of FR, NF hybrids and the parental species *N. tabacum* and *N. rustica* were compared to find out the differences in the overall base composition by thermal denaturation analysis and analytical neutral CsCl density gradient analysis. The normal (N) segregants were similar to recurring parent *N. rustica* in external morphology and in karyotype arrangement. Although chromosomal aberrations were detected at meiosis the molecular

Fig. 1. DNA melting profiles of *Nicotiana* genomic DNA. (a) *N. tabacum* cv. Mammoth. (b) Flower revertant (FR) plant. (c) Non flowering (NF) plant. (d) *N. rustica*.

composition of the genomic DNA of normal (N) plants was similar to that of *N. rustica*.

Melting profile analysis

The melting profiles of the genomic DNA of *N. tabacum*, *N. rustica*, FR and NF gave information about base pair ratio and also about the heterogeneity in the dispersion of the base pairs within the DNA (Figs. 1a, b, c, d). The hyperchromicity, T_m values and G+C contents of different samples derived from DNA melting profiles are given in Table 1. The average G+C content of the four genomic DNA samples estimated from the melting profiles range narrowly from 49% (*N. rustica* and FR) to 51.5% in *N. tabacum*. Although FR and *N. rustica* had similar T_m values and G+C contents, they differed in base compositional heterogeneity.

Fig. 2. Analytical buoyant density gradient profiles of *Nicotiana* genomic DNA in neutral CsCl. (A) *N. tabacum* cv. Mammoth. (B) *N. rustica*. (C) Flower revertant (FR) plant. (D) Non flowering (NF) plant. Arrowheads denote the presence of G+C rich satellite DNA sequences. Purified DNA of *Micrococcus lysodeikticus* with a known buoyant density of 1.731 g/ml was used as the marker.

The base compositional heterogeneity, which is a measure of base pair distribution of GC and AT base pairs, varied between the parental species and between the two hybrid segregants. There were no detectable differences in DNA composition between plants within FR and NF phenotypes.

Analytical density gradient analysis

The differences in the buoyant density gradient profiles between *N. tabacum*, *N. rustica*, FR and NF plants are shown in Fig. 2. The asymmetric profiles suggest the presence of satellite DNA with an altered base pair composition. The average buoyant densities and the G+C contents are given in Table 2. The buoyant densities varied between 1.685 g/ml to 1.689 g/ml. The G+C content estimated from the buoyant densities ranged from 32.1% to 36.2%.

A comparison between data in Table 1 and Table 2 shows that the G+C contents estimated from the buoyant densities are considerably lower than the values estimated from the melting profiles. These differences in the G+C contents could result from a reduction in buoyant densities due to the presence of unusual base pairs or due to the methylation of deoxycytidine residues in the DNA. It is already established that while the T_m is unaffected by

Table 1. Melting analysis of nuclear DNA

Material	T _m (°C) in 0.1 × SSC	Hyperchromicity	Base compositional heterogeneity	T _m (°C) extrapolated to 1 × SSC	% G + C content
<i>N. tabacum</i>	73.6	0.250	15.47	90.1	51.5
Flower revertant	72.6	0.342	14.35	89.1	49.0
Non flowering	73.0	0.290	16.93	89.5	50.0
<i>N. rustica</i>	72.6	0.314	17.86	89.1	49.0

Table 2. Analytical density gradient analysis of nuclear DNA

Material	Density (g/ml)	% G + C content	Estimated* 5-methyl deoxycytidine	Estimated Density (g/ml)	Estimated % G + C Content
<i>N. tabacum</i>	1.685	32.1	0.296	1.704	51.5
Flower revertant	1.685	32.1	0.258	1.702	49.5
Non flowering	1.689	36.2	0.211	1.703	50.5
<i>N. rustica</i>	1.686	33.2	0.242	1.702	49.5

* Expressed as a proportion of the total deoxycytidine residues in mole fraction.

the amount of 5-methyl deoxycytidine in DNA, the buoyant density is lowered by $0.004 \text{ gm} \cdot \text{cm}^{-3}$ for every 0.0625 mole fraction of 5-methyl deoxycytidine (Kemp and Sutton 1976). The mole fractions of 5-methyl deoxycytidine calculated from the buoyant densities varied from 0.296 moles in *N. tabacum* to 0.211 moles in NF plants (Table 2). *N. rustica* and FR segregants were found to have intermediate values of 0.242 and 0.258 moles respectively. Though *N. tabacum* and FR DNA have shown the same buoyant density values of 1.685 g/ml, they differed in the extent of methylation of cytosine residues.

Using known values of methylated deoxycytidine residues of the four DNA samples, it was possible to correct the buoyant densities. The estimated buoyant densities (Table 2) of the parental species and that of the *rustica-tabacum* segregants ranged from 1.702 to 1.704 g/ml. The G + C contents calculated from the estimated buoyant densities (Table 2) did not differ from the values calculated from the T_m values (Table 1). The results confirm the differences in DNA composition between FR and NF plants as observed from the DNA melting experiments.

Variation in satellite DNA sequences

Distinct satellite DNA fractions could be detected in the genomic DNA profiles of FR, NF and *N. rustica* (Fig. 2). The density gradient profile of *N. tabacum*, although asymmetrical, could not be resolved into a well defined satellite fraction. The amounts of satellite DNA sequences in FR, NF and *N. rustica* were estimated as a proportion of the total genomic DNA using a digitizer. The satellite DNA sequences in *N. rustica* made up about 10.9% of the total genome size. The amount of satellite DNA sequences was 9.3% of the total genome size in FR and 8.5% in NF plants. Like the thermal denaturation profiles, the analytical density gradients did not reveal differences between plants within FR and NF genotypes.

Discussion

Molecular studies on the composition of genomic DNA

The extensive cytological abnormalities in the hybrids could lead to changes in the composition of genomic DNA. The DNA melting analysis has revealed considerable differences in base pair dispersion within and between the four DNA samples (*N. tabacum*, FR, NF

and *N. rustica*). The differences in G+C content between the two parental amphidiploid species could be expected from the divergence in phylogeny and extensive genome differentiation between the two species. The observed differences in the G+C content and base compositional heterogeneity between FR and NF, suggest significant DNA sequence rearrangements in the segregating progenies. The differences in the buoyant densities of the nuclear DNA of FR and NF plants, as revealed by analytical density gradient analysis of the genomic DNA, supports the above conclusion.

The variation in the degree of cytosine methylation in the hybrid segregants could result from the unequal segregation of chromosomes at mitosis and meiosis, or from the changes in methylation modification subsequent to interspecific hybridization. Such variation in the degree of methylation of cytosine residues in the nuclear DNA is reported to have an important role in gene regulation and expression (Razin and Riggs 1980).

Unlike the genomic DNA of *N. tabacum*, the DNA of FR, NF and *N. rustica* revealed distinct G+C rich satellite DNA sequences in their analytical density gradient profiles. As the *rustica-tabacum* hybrid was repeatedly backcrossed to *N. rustica*, the G+C rich satellite DNA fraction, clearly seen in the hybrids, must have been transferred from the *rustica* genome. The variation in the amount of satellite DNA observed between FR and NF hybrid plants could also be due to differential segregation of chromosomes frequently seen in the hybrid plants.

Species that are stable under natural selection display stability in genome organization. Interspecific hybridization, on the one hand, confers a genetic advantage to the nascent hybrids, but on the other, leads to an imbalance in genome organization. It has already been shown that, in the evolution of amphidiploid species in *Nicotiana*, genome reorganization has occurred subsequent to interspecific hybridization. This was often associated with changes in genome size due to deletion or accretion of mainly non-coding nuclear DNA sequences (Narayan 1987, 1988). *Nicotiana* amphidiploid species *N. arentsii* which is relatively recent in origin, on an evolutionary time scale, shows large scale cytological abnormalities in the present day populations. The variation in the buoyant densities, in the amount of satellite DNA sequences and in the 5-methyl deoxycytidine content of the total DNA could result from the chromosomal instability both at mitosis and meiosis.

Summary

The mammoth gene of *Nicotiana tabacum* has been transferred to *N. rustica* genome through interspecific hybridization followed by repeated backcrossing to *N. rustica*. Our previous studies have revealed that the expression and segregation of the mammoth gene was more complex in the *N. rustica* genetic background than in *N. tabacum*. Plants with abnormal floral expressions such as non flowering (NF) and flower revertants (FR) with altered responses to environmental factors such as photoperiod and thermoperiod frequently appeared in the backcross progeny of *rustica-tabacum* hybrids. The segregation of plants for FR and NF characters among the progeny was highly heterogeneous and chromosome instabilities were found both at mitosis and meiosis.

Characterization of the genomic DNA of *rustica-tabacum* hybrid segregants (FR and NF) and the parental species (*N. tabacum* and *N. rustica*) was carried out in the present study using thermal denaturation and analytical density gradient analyses. Differences were observed in the DNA composition among the hybrid plants segregating for abnormal flowering expressions, FR and NF, as well as the parental species. Variation was observed in the mole fraction of 5-methyl deoxycytidine in the genomic DNA of the hybrid progenies and the parental species. Variation in the amount of satellite DNA fraction was also found between the hybrid plants, FR and NF and the recurrent parental species *N. rustica*. The differences in the buoyant densities, satellite

DNA fraction, 5-methyl deoxycytidine contents between FR, NF and *N. rustica* suggests reorganization of genomes of the hybrid progenies following interspecific hybridization. The variation could result in part from the abnormal segregation of chromosomes and chromosome aberrations frequently found in the backcross progeny of *rustica-tabacum* hybrids at mitosis and meiosis.

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