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Study of the divergence of moderately repetitive sequences in *Nicotiana* species and in protoclones of *Nicotiana plumbaginifolia* Viviani

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Summary. Molecular DNA markers can be very useful to assess the amount of genetic variation and are thus important for taxonomic studies. Two moderately repetitive sequences were isolated from *N. plumbaginifolia* leaf DNA and used to screen various *Nicotiana* species. A huge variability was detected among species belonging to the same subgenus or the same section, which could be utilized for a molecular taxonomy of the genus *Nicotiana*. Although variation at the DNA level between somaclonal lines was reported, we did not find evidence for variability of both repetitive sequences in established callus culture obtained from protoplasts of *Nicotiana plumbaginifolia*.

Key words: Repetitive DNA – *Nicotiana plumbaginifolia* – Genomic divergence – Somaclonal variation

Introduction

Although the use of molecular DNA and RNA markers is widespread in animals, especially *Drosophila* (Riede et al. 1983; Martin et al. 1983), they are not often used in plants. Repetitive sequences are of particular interest when used to detect variation by representing multiband patterns on hybridizations to genomic DNA on Southern blots. As most plants have a larger fraction of their genome under the form of repetitive sequences than animals, these markers could be very valuable in plant taxonomy and plant breeding. The amount of heterogeneity in a range of DNA markers can be used to determine the evolutionary divergence between related species. The genus *Nicotiana* has a well established hierarchical taxonomic division (Smith 1972) based on biometrical, cytogenetical and biochemical (isozymes and alkaloids) data, and is therefore a good object of study. The results obtained by the use of DNA markers can be compared to, and complement, the above-mentioned data.

Somaclonal variation is the variation found between plants derived from any form of cell culture (Larkin and Scowcroft 1981). It seems to be closely linked with the in vitro phase and can be considered an unavoidable burden (if the cloning of cell lines is the desired goal) or a novel source of genetic variability. Rearrangements of repetitive DNA and mobilization of transposable elements are among a series of possible origins for such a variation, as suggested by the high rate of transposition in tissue culture lines and laboratory stocks of *Drosophila* (Junakovic et al. 1984) and the variability found in mitochondrial DNA in tissue cultured cells of maize (McNay et al. 1984). The use of moderately repetitive sequences as probes may represent a way to assay such rearrangements. Alterations on DNA level between different protoclone lines from *Solanum tuberosum* hybridized with random DNA clones were reported by Landsmann and Uhrig (1985). The same markers used to screen evolutionary divergence can be used to detect somaclonal variation, especially those which show extensive evolutionary instability.

Materials and methods

Plants and calli

All *Nicotiana, Lycopersicon* and *Petunia species* were grown in greenhouse conditions. Seeds from the different *Nicotiana* species were obtained from the Laboratorium voor Systematiek, University of Nijmegen (Netherlands). Protoplasts were isolated from the haploid *N. plumbaginifolia* line P_1P (Negru-

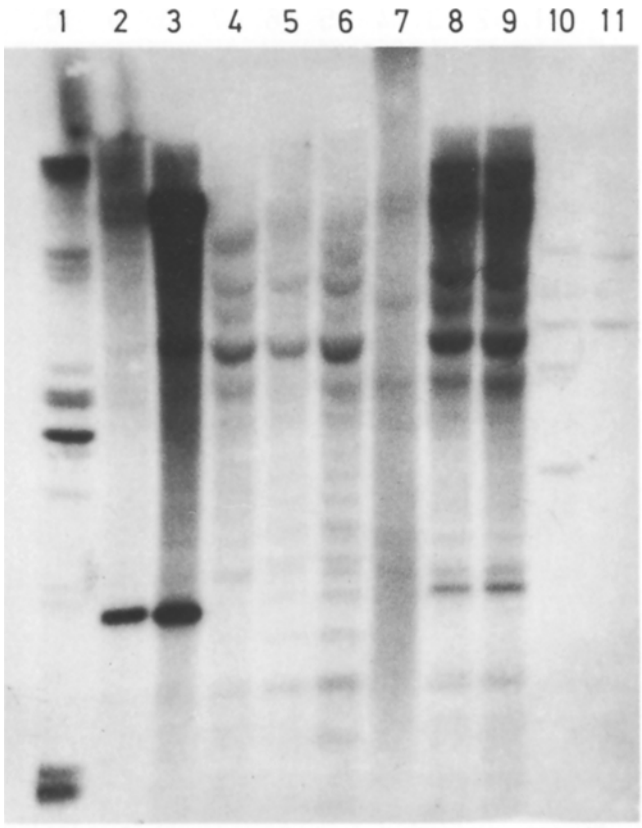

Fig. 1. Southern blot hybridization with p5Np1 (1.0 kb sequence) on different *Nicotiana* species digested with EcoRI. *Lane 1* PstI, *2 N. plumbaginifolia* (callus), *3 N. plumbaginifolia* (leaf), *4 N. sylvestris, 5 N. tabacum, 6 N. rustica, 7 N. longiflora, 8 N. alata, 9 N. alata, 10 Lycopersicon esculentum, 11 Petunia hybrida*

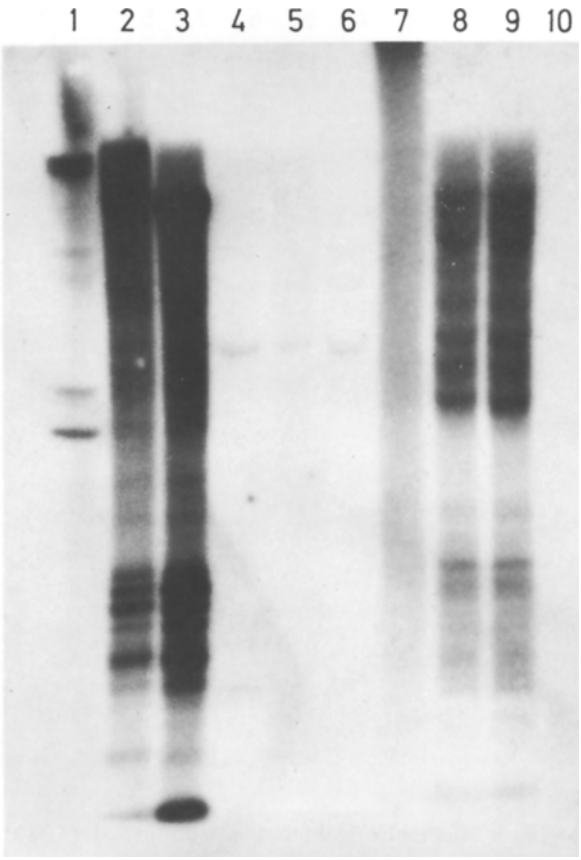

Fig. 2. Southern blot hybridization with p5Np2 (0.4 kb sequence) on different *Nicotiana* species digested with EcoRI. *Lane 1* PstI, *2 N. plumbaginifolia* (callus), *3 N. plumbaginifolia* (leaf), *4 N. sylvestris, 5 N. tabacum, 6 N. rustica, 7 N. longiflora, 8 N. alata, 9 N. alata, 10 Lycopersicon esculentum, 11 Petunia hybrida*

tiu and Mousseau 1980) and the calli were subcultured on R_4M medium (2 month intervals) for more than 30 months.

Plant DNA cloning

Plant and callus DNA was prepared according to Dellaporta et al. (1983). Restriction enzyme digestions were performed in the buffers suggested by the suppliers (Boehringer and Pharmacia) for 2 h at 37 °C. For callus DNA the addition of spermidine (20 m*M*) proved indispensible to obtain complete digestion. Plant DNA was cloned randomly into the EcoRI site of pBR325 (Bolivar 1978) using equimolar 'sticky ends' ratio plant/pBR325. Selection for inserts was done using crystalviolet 6 µg/ml (Proctor and Rownd 1982) and carbeniciline (100 µg/ml). Plasmids were isolated according to Birnboim and Doly (1979). Restriction digests were run on 0.8% agarose TEB gels (Maniatis 1982).

To provide molecular markers a high excess of lambda digested with PstI DNA was used on the gels, resulting in a background banding pattern on the Southern blots (lane *1* of Figs. 1–4).

DNA hybridization

Plant and plasmid DNA was labeled with P^{32} by nick translation using DNA Polymerase I (Rigby et al. 1977) to a specific activity of about 5.10^7 cpm/µg. For Southern blot hybridization (Southern 1975) nylon membranes were used (PALL Biodyne) with 10–20 µg plant DNA per lane. The nylon filters were exposed to short-wavelength UV light (transilluminator) for 5 min and baked for 2 h at 80 °C. DNA was hybridized at 50 °C with 31.4% formamide for 24–72 h (Cannon et al. 1984). The membranes were washed under stringent conditions: 5 min in 2×SSC, 0.1% SDS at room temperature and 2×1 h in 0.1×SSC, 0.1% SDS at 50 °C and exposed to Fuji RX X-ray film with an intensifying screen at –70 °C for 1–7 days.

Results

Random *N. plumbaginifolia* DNA clones were used because repetitive sequences are an important part of plant genomes (e.g. 70% in *N. tabacum;* Zimmerman and Goldberg 1977). A first selection for moderately repetitive sequences was made by hybridizing total plant DNA to Southern blots of EcoRI digested clones.

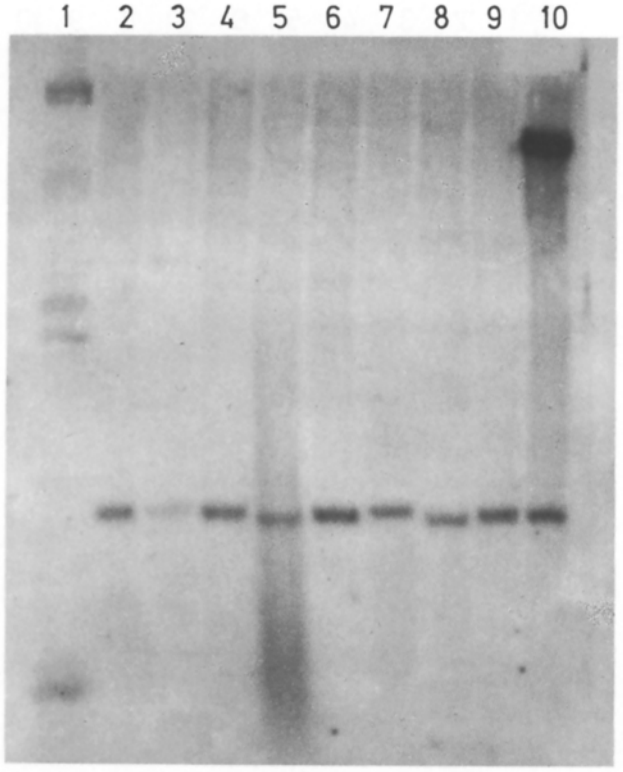

Fig. 3. Southern blot hybridization with p5Np1 (1.0 kb sequence) on *N. plumbaginifolia* protoclone lines digested with EcoRI. *Lane 1* Pst1, *2* cNp 57, *3* cNp 65, *4* cNp 82, *5* cNp 150, *6* cNp 203, *7* cNp 217, *8* cNp 231, *9* cNp 273, *10* leaf (wild type)

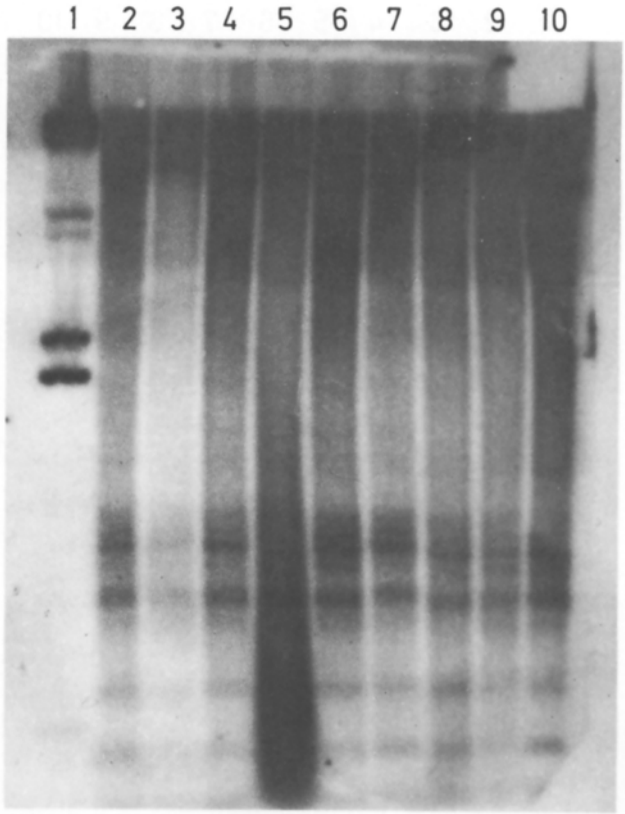

Fig. 4. Southern blot hybridization with p5Np2 (0.4 kb sequence) on *N. plumbaginifolia* protoclone lines digested with EcoRI. *Lane 1* Pst1, *2* cNp 57, *3* cNp 65, *4* cNp 82, *5* cNp 150, *6* cNp 203, *7* cNp 217, *8* cNp 231, *9* cNp 273, *10* leaf (wild type)

The clones showing a strong signal supposedly contained highly repetitive sequences. One of these, with a 2.4 kb insert and a copy number of about 2,000, was described previously (Speeckaert and Jacobs 1984). A number of clones with a low signal, but still significantly higher than the background, were hybridized to Southern blots of *N. plumbaginifolia* DNA. From these sequences two were chosen which showed a multiband pattern. Clones p5Np1 and p5Np2 contained 1.0 and 0.4 kb inserts, respectively.

Clone p5Np1 hybridized to a pattern of at least 16 bands on Southern blot with *N. plumbaginifolia* DNA (Fig. 1), ranging from 0.6–12.0 kb. The 3 most prominent bands, of respectively 1.0 kb (the sequence itself), 7.0 and 8.0 kb were absent in all other *Nicotiana* species investigated except in *N. alata,* where an 8.0 kb band was visible. The 3.0 kb and the 3 bands of about 2.6 kb were found in all *Nicotiana* species but not in *Lycopersicon esculentum* or *Petunia hybrida.*

The second clone, p5Np2, hybridized to a large number of bands ranging from 0.4–12.0 kb. Twenty bands were clearly determined (Fig. 2). The variability between the species was even greater when compared to the 1.0 kb fragment. In *N. alata* the sequence was represented but with a clearly different pattern. In *N. sylvestris, N. rustica* and *N. tabacum* there were only small traces of hybridization.

We also looked for variability between callus lines derived from single cell protoplast cultures, defined as protoclones by Shepard et al. (1980). Our two considered clones were hybridized to DNA obtained from a range of established callus lines from *N. plumbaginifolia.* They were subcultured for different periods: 3 years for cNp numbers < 200 and 18 months for cNp numbers > 200. Although the callus lines showed the expected phenotypical heterogeneity (ranging from friable yellow to hard dark green calli), no variability of DNA banding patterns was found (Figs. 3 and 4). When compared to leaf DNA, however, the relative intensities of the hybridization signals of the large (12.0 kb) and small (1.0 kb) bands were completely different. We cannot entirely exclude the possibility of a different effect of the extraction and digestion procedures in callus tissue when compared to leaves.

Discussion

Moderately repetitive sequences were selected and used to screen for variability at two levels: an evolutionary level and a tissue culture level. Clones p5Np1 and p5Np2 with 1.0 kb and 0.4 kb *Nicotiana plumbaginifolia* sequences are considered here. The two sequences showed a totally different hybridization pattern even between closely related species, both quantitatively (reiteration frequency) as well as for the presence (or absence) of bands on Southern blots. Sequences are absent or occur in a very low frequency in more distantely related species of the *Solanaceae* family (*Lycopersicon esculentum* or *Petunia hybrida*). In the same genus, in most species the sequence are less represented (fewer bands with a lower intensity) than in *N. plumbaginifolia.* The resemblance in hybridization pattern and relative abundancy in three species is remarkable: *N. rustica, N. sylvestris* and *N. tabacum* are taxonomically classified in three separate subgenera (although *N. tabacum* is supposed to be a hybrid between *N. sylvestris* and *N. tomentosiformis*). The banding patterns of *N. plumbaginifolia* and *N. sylvestris* are completely different for both sequences, although they both belong to the *Alatae* section in the *Petunioides* subgenus (Table 1). These data show the utility of molecular markers in taxonomy, even though they are rarely used in this discipline. Of course many more sequences will have to be considered to get an idea of a 'molecular' taxonomy of the genus *Nicotiana.* The right molecular markers could also provide convincing proof for the origin of the second ancestor of *N. tabacum* besides *N. sylvestris* (*N. tomentosiformis* or *N. otophora;* Gray et al. 1974).

Several models have been developed recently in which a substantial fraction of the moderately repetitive sequences in plants are mobile (Freeling 1984). According to the concept of 'genomic fluidity' (Walbot and Cullis 1985) the genome is considered to be in a constant state of flux. Somaclonal variation might be one of the appearances of this genetic flexibility. In vitro culture of maize seems to increase the mobility of some transposable elements (Peshke et al. 1986). Detection of somaclonal variation on potato protoclones through the use of random DNA markers (and a rDNA probe) has been reported (Landsman and Uhrig 1985), but the only variation was due to small relative intensity differences between bands and was difficult to interpret.

In our protoclone lines and sequences we did not find evidence for clear divergence, but some differences in relative hybridization between leaves and all callus tissues were nevertheless observed. This could imply some form of under-replication of some fragments in callus tissue. Although many more sequences need to be screened, our current data do not favour the existence of highly mobile repetitive sequences. The use of molecular DNA markers will certainly become a valuable technique in such disciplines as taxonomy and plant breeding.

Table 1. Classification of the considered *Nicotiana* species (Smith 1968)

Sub-genus	Section	Species	Authority	Chromo-some no.
Rustica	*Rusticae*	*rustica*	Linneaus	48
Tabacum	*Genuinae*	*tabacum*	Linneaus	48
Petunioides	*Alatae*	*sylvestris*	Spegazzini and Comes	24
		longiflora	Cavanilles	20
		alata	Link and Otto	18
		plumbaginifolia	Viviani	20

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