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Genome Modifications in Protoplast-Derived Tobacco Plants: Contents of Repetitive DNA Sequences

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Abstract. Plasticity of the tobacco genome was studied by testing the DNAs of protoplast-derived regenerants with three different repetitive DNA sequences by the method of quantitative DNA/DNA hybridizations. A large population of 91 regenerants belonging to 35 different protoclones was analysed and a high degree of heterogeneity in the contents of the different DNA repeats was detected. The contents of middle repetitive sequences of two types were more stable or changed in the same direction, while the highly repetitive sequence varied independently and displayed a significant reduction in comparison with the two other sequences. Comparing the variation within the subpopulations of plants of the same clonal origin and the variation among the protoclones led to a conclusion that the pre-existing DNA variability in the starting plant material and/or the *in vitro* stress during the very early stages of protoclone regeneration played a decisive role in the formation of modified genomes in regenerants.

Additional index words: Genome instability – Protoplast-derived plants – Quantitative DNA/DNA Hybridization – Repetitive DNA sequences – *Nicotiana tabacum*

Plant genomes are generally large and a great part of the DNA is repeated. The role of repetitive sequences, if any, is essentially unknown. Despite of this, one can speculate that homology between multiple copies of repetitive DNA sequences could promote recombination events. These sequences are easily prone to change in structure and copy number which can influence the genome as an integrated system. The most likely causes of such genome changes are unequal crossing over, gene conversion, slippage DNA synthesis, DNA amplification, transposition, and retrotransposition (for a review see, *e.g.* Flavell 1985). It is generally assumed that the changes in copy number of DNA sequences and complexity of genome are a stochastic process which can be induced by various kinds of stress. Conditions of *in vitro* culture are a good example of such a stress resulting in a great genome variation in both cell cultures and regenerated plants (somaclonal variation, Evans and Sharp 1986).

The first evidence for the somaclonal variation at the molecular level was a rDNA deficiency in protoplast-derived potato plants (Landsmann and Uhrig

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1985). Since that time a variation in the copy number of various nuclear repeats was frequently detected in tissue culture regenerants of triticale (Brettell *et al.* 1986), rice (Zheng *et al.* 1987), *in vitro* derived wheat × rye hybrids (Lapitan *et al.* 1988) or in flax calli (Cullis and Cleary 1986). A number of models have been suggested explaining the mechanisms of their amplification or reduction. Quite recently an evidence was also given for an extrachromosomal circular DNA molecule harbouring tandemly repeated units amplified in cell cultures of rice (Cuzzoni *et al.* 1990).

The aim of this work was to study the changes in the content of repetitive DNA sequences in tobacco plants regenerated from protoplasts. The variation in copy numbers of three different middle or highly repetitive sequences were analysed and the causes of this variation were discussed.

MATERIAL AND METHODS

Plant Material

The plant material used was *Nicotiana tabacum* L. cv. Vielblättriger. The protoplast isolation, culture, and plant regeneration were described by Vyskot *et al.* (1991).

Content of Specific DNA Sequences in Genomic DNAs

Total DNAs from tobacco leaves were isolated using a modified procedure of Murray and Thompson (1980). The method of Labarca and Paigen (1980) was used to estimate DNA concentrations. To measure contents of repetitive DNA sequences in plant DNA samples a technique of quantitative filter-bound hybridization called "slot-blotting" was used (Rivin 1986). Samples of denatured DNA from the individual plants were applied onto nitrocellulose using a Schleicher and Schuell Minifold II slot-blot apparatus. After hybridization with ^{32}P-labelled probes (DNA repeats) the autoradiograms were scanned on a laser scanning densitometer.

Three different nuclear tobacco repeated DNA sequences isolated in this laboratory were used to monitor genomic changes in protoplast-derived plants - HRS60.1 (a 184bp member of the HRS60 family of tandem DNA repeats, Koukalová *et al.* 1989), R8.1 (a 2.4kbp middle repetitive DNA sequence, Kuhrová *et al.* 1991), and R8.3 (a 5.5kbp middle repetitive sequence, Kuhrová *et al.* 1991). The number of copies per tobacco haploid genome are 1.6×10^5, 1.0×10^3, and 1.6×10^3 for HRS60, R8.1, and R8.3, respectively.

RESULTS AND DISCUSSION

Samples of DNA were isolated from the R_0 plants and the contents of the individual repeated sequences were estimated using the slot-blot DNA/DNA hybridizations. We evaluated relative amounts of the R8.1, R8.3, and HRS60

sequences in the R_0 plants compared with the control T3 plant and the ratios of R8.1 /R8.3, R8.1/ HRS60, and R8.3/HRS60 instead of direct evaluation of copy numbers of individual repetitive sequences. The reference values of the T3 plant represent average values for mesophyll cell populations. Only the protoclones which yielded three or more plants were used for statistical analysis of the results.

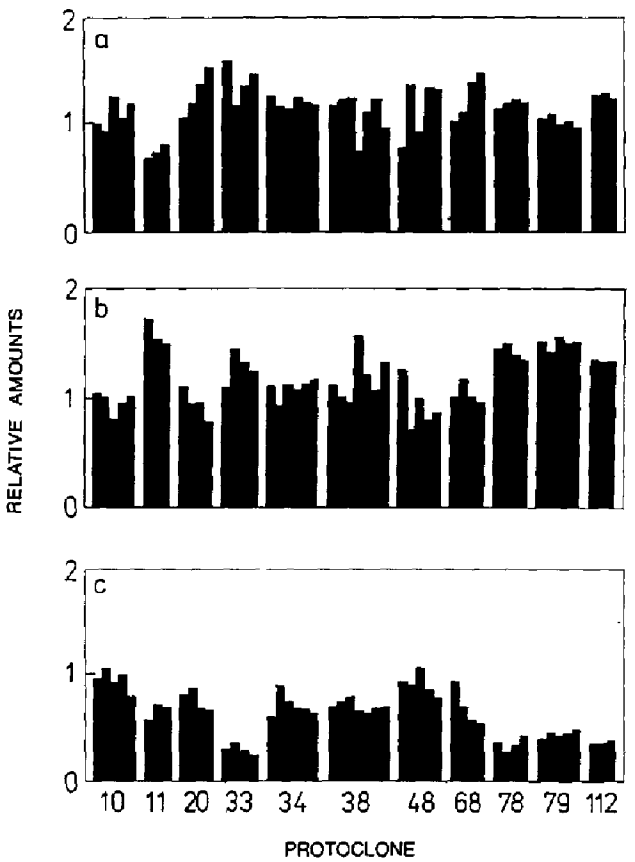

Fig. 1. Distribution of the relative amounts of the repetitive DNA sequences in the protoplast-derived plants as detected by slot hybridizations. Three different DNA probes were used – R8.1 (a), R8.3 (b), and HRS60.1 (c). All the values are expressed as multiples of the amounts present in the original T3 plant. The symbols representing the plants originated from the same protoclone are joined together.

The slot-hybridization data showed that the relative contents of two middle repetitive sequences R8.1 and R8.3 were increased in most cases, while the abundance of the highly repetitive sequence was generally lower (Fig. 1). The ratio of R8.1/R8.3 was constant in most protoclones and was close to 1, while the other two ratios were more variable among the individual protoclones and

their values were higher than 1 (Fig. 2). These data imply that the contents of sequences R8.1 and R8.3 are constant or changed (*i.e.*, amplified or reduced) in the same direction, while the HRS60 content varies more often and independently on the previous ones.

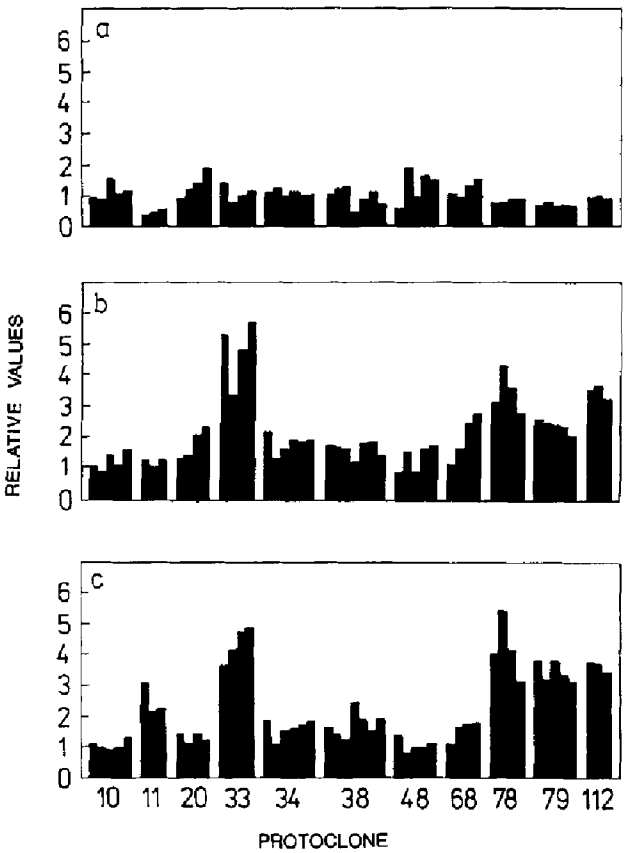

Fig. 2. Demonstration of mutual ratios of the repetitive DNA contents in the clones of R_0 plants: (a) R8.1/R8.3, (b) R8.1/HRS60, (c) R8.3/HRS60. The columns representing the plants of the same protoclone origin are joined together.

The interclonal and intraclonal differences in the contents of repeated sequences were evaluated by the *F*-test (Tab. 1). The results show that the variation in the contents of the repeats is significantly higher between the protoclones than within the protoclones. It means that most of the genetic variation observed is the result of events which occured during the first steps of the *in vitro* culture or reflect the variation already present in the parental mesophyll cells. This conclusion was also suppported by comparing the different clones using the multiple *t*-test (data not mentioned). A statistically very

TABLE 1.

The contents of the three repetitive sequences R8.1, R8.3 and HRS60 in the DNA of R_0 plants belonging to 11 protoclones. The contents are related to the standard plant T3 where all three values are given as 1 and the sum of all of them to three. The table shows the averages for each protoclone and the corresponding standard errors, the results of F tests for the null hypothesis (that the relative contents of the three repetitive sequences within the clone are unchanged). The last line shows the F values for comparisons of intraclonal and interclonal variance in the whole set of data for each of the sequences and the corresponding P values.

No. of clone (number of plants)	repetitive sequence			*F*-test
	R8.1	R8.3	HRS60	
No. 10 (5)	1.085 ± 0.13	0.98 ± 0.086	0.94 ± 0.091	$F_{(2,12)} = 2.23$ $P > 0.1$
No. 11 (3)	0.75 ± 0.052	1.61 ± 0.10	0.65 ± 0.060	$F_{(2,5)} = 98.9$ $P << 0.0005$
No. 20 (4)	1.29 ± 0.19	0.965 ± 0.12	0.75 ± 0.090	$F_{(2,9)} = 11.9$ $P < 0.005$
No. 33 (4)	1.40 ± 0.15	1.30 ± 0.13	0.30 ± 0.035	$F_{(2,9)} = 77.3$ $P << 0.0005$
No. 34 (6)	1.20 ± 0.050	1.10 ± 0.073	0.70 ± 0.10	$F_{(2,15)} = 61.6$ $P << 0.0005$
No. 38 (7)	1.03 ± 0.17	1.20 ± 0.20	0.69 ± 0.046	$F_{(2,18)} = 18.8$ $P << 0.0005$
No. 48 (5)	1.15 ± 0.25	0.945 ± 0.20	0.91 ± 0.093	$F_{(2,12)} = 1.94$ $P > 0.10$
No. 68 (4)	1.26 ± 0.19	1.06 ± 0.080	0.69 ± 0.15	$F_{(2,9)} = 11.05$ $P < 0.05$
No. 78 (4)	1.20 ± 0.031	1.45 ± 0.058	0.355 ± 0.054	$F_{(2,9)} = 407.7$ $P << 0.0005$
No. 79 (5)	1.03 ± 0.045	1.52 ± 0.046	0.44 ± 0.030	$F_{(2,12)} = 703.0$ $P << 0.0005$
No. 112 (3)	1.275 ± 0.025	1.35 ± 0.009	0.37 ± 0.015	$F_{(2,6)} = 3119.0$ $P << 0.0005$
F – tests	$F_{(10,39)} = 3.99$ $P = 0.001$	$F_{(10,39)} = 12.1$ $P << 0.0001$	$F_{(10,39)} = 25.4$ $P << 0.0001$	

significant decline in the content of the HRS60 sequence compared with the R8.1 and R8.3 in most of the clones was proved again as well as the same or similar relative abundances of the R8.1 and R8.3 sequences.

It has already been shown that quantitative DNA changes also take place in plant tissues *in vivo* and they can influence morphogenetic capacity of these tissues *in vitro* (Altamura *et al.* 1987). It is worth to stress that the protoclones yielded rather homogenous populations of plants. This indicates that the R_0 genomes remained stable during the later stages of callus culture and plant morphogenesis. The ratios of the content of the three repetitive sequences indicate that the variability was of different extent for each repeat and that the changes in the sequences R8.1 and R8.3 were coupled (*i.e.* constant or changed in the same direction), but that the highly repetitive sequence HRS60 varied independently and most of all. This sequence also displayed a very significant reduction compared with R8.1 and R8.3.

The DNA probes used in this work to monitor genome changes in protoplast-derived tobacco plants correspond to both the middle and highly repetitive DNA sequences. *In situ* hybridization analyses showed that their homologous genomic sequences were localized on a majority of tobacco chromosomes thus covering a significant part of the *N. tabacum* genome and represent suitable probes for monitoring overall structural genomic changes (Bezděk *et al.*, 1991, Kuhrová *et al.*, 1991). The widespread genomic heterogeneity reported in this paper is in a good correlation with the data published by Brown *et al.* (1990) where DNA polymorphism for many gene probes representing different functional domains were detected.

REFERENCES

Altamura, M. M., Bassi, P., Cavallini, A., Cionini, G., Cremonini, R., Monacelli, B., Pasqua, G., Sassoli, O., Tran Thanh Van, K., Cionini, P. G.: Nuclear DNA changes during plant development and the morphogenetic response *in vitro* of *Nicotiana tabacum* tissues. – Plant Sci. **53**: 73–79, 1987.

Bezděk, M., Koukalová, B., Brzobohatý, B., Vyskot, B.: 5-azacytidine induced hypomethylation of tobacco tandem DNA repeats in tissue cultures. – Planta **184**: 487–490, 1991.

Brettell, R. I. S., Pallotta, M. A., Gustafson, J. P., Appels, R.: Variation at the *Nor* loci in triticale derived from tissue culture. – Theor. appl. Genet. **71**: 637–643, 1986.

Brown, P. T. H., Kyozuka, J., Sukekiyo, Y., Kimura, Y., Shimamoto, K., Lörz, H.: Molecular changes in protoplast-derived rice plants. – Mol. gen. Genet. **222**: 324–328, 1990.

Cullis, C. A., Cleary, W.: DNA variation in flax tissue culture. – Can. J. Genet. Cytol. **28**: 247–251, 1986.

Cuzzoni, E., Ferretti, L., Giordani, C., Castiglione, S., Sala, F.: A repeated chromosomal DNA sequence is amplified as a circular extrachromosomal molecule in rice (*Oryza sativa* L.). – Mol. gen. Genet. **222**: 58–64, 1990.

Evans, D. A., Sharp, W. R.: Applications of somaclonal variation. – Bio/Technology **4**: 528–532, 1986.

Flavell, R. B.: Repeated sequences and genome change. – In: Hohn, B., Dennis, E. S. (ed.): Genetic Flux in Plants. Pp. 139–152. Springer Verlag, Wien 1985.

Koukalová, B., Reich, J., Matyášek, R., Kuhrová, V., Bezděk, M.: A *Bam*HI family of highly repeated DNA sequences of *Nicotiana tabacum*. – Theor. appl. Genet. **78**: 77–80, 1989.

Kuhrová, V., Bezděk, M., Vyskot, B., Koukalová, B., Fajkus, J.: Isolation and characterization of two middle repetitive DNA sequences of nuclear tobacco genome. – Theor. appl. Genet. **81**: 740–744, 1991.

Labarca, C., Paigen, K.: Simple, rapid, and sensitive DNA assay procedure. – Anal. Biochem. **102**: 75–86, 1980.

Landsmann, J., Uhrig, H.: Somaclonal variation in *Solanum tuberosum* detected at the molecular level. – Theor. appl. Genet. **71**: 500–505, 1985.

Lapitan, N. L. V., Sears, R. G., Gill, B. S.: Amplification of repeated DNA sequences in wheat × rye hybrids regenerated from tissue culture. – Theor. appl. Genet. **75**: 381–388, 1988.

Murray, M. G., Thompson, W. F.: Rapid isolation of high molecular weight plant DNA. – Nucl. Acids Res. **8**: 4321, 1983.

Rivin, C.: Analysing genome variation in plants. – Methods Enzymol. **118**: 75–86, 1986.

Vyskot, B., Fajkus, J., Kuglík, P., Koukalová, B., Kuhrová, V.: Genome modifications in protoplast-derived tobacco plants: phenotypic evaluation and RFLP analysis. – Biol. Plant. **33**: 455–460, 1991.

Zheng, K. L., Castiglione, S., Biasini, M. G., Biroli, A., Morandi, C., Sala, F.: Nuclear DNA amplification in cultured cells of *Oryza sativa* L. – Theor. appl. Genet. **74**: 65–70, 1987.

Evangelopoulos, E. A., Changeux, J. P., Packer, L., Sotiroudis, T. G., Wirtz, K. W. A. (ed.): Receptors, Membrane Transport and Signal Transduction. – NATO ASI Series, Series H: Cell Biology, Vol. 29. Springer-Verlag, Berlin–Heidelberg 1989, 387 pp. Hardcover DM 168,–.

The most of cell processes is induced and controlled by the external stimuli – i.e. by the signals. These signals – generally of either chemical or physical nature – are recognized by the receptors and transduced to the efficient structures inside the cell.

This publication offers a comprehensive account of the fields of membrane receptore (special interest is paid to receptors for neurotransmitters in Part II: "Membrane receptors and neurotransmitters"). The general principles of signal transduction pathways are presented in Part I: "G-proteins, adenylate cyclase and protein phosphorylation". The conversion of hormonal signal into appropriate intracellular metabolic signals, detection, integration and amplification of the external signals and the role of G-proteins in these processes are discussed here in context of specific cellular processes. In Part III: "Membrane transport and bioenergetics", transport processes mainly in various bacteria are discussed in relation to the signal transduction and to the growth.

This book covers recent development in membrane receptor research and signal transduction and membrane transport mechanisms in the context of current knowledge of the structure and function of membranes. It should be very useful to researchers and students interested in the fields of mechanism of action of hormones, signal transduction, membrane transport and generally in cell growth and development.

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