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Genome Modifications in Protoplast-Derived Tobacco Plants: Phenotypic Evaluation and RFLP Analysis

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Abstract. Genomic instability of protoplast-derived tobacco plants was studied by means of phenotypic evaluation, karyological analysis, and Southern blot experiments. Of the total number of 91 regenerants belonging to 35 different protoclones 57 plants displayed various morphological and/or functional aberrations, some of them being inherited into the progeny. A karyological study of 20 randomly chosen plants revealed 15 tetraploid and 5 diploid chromosome sets. A Southern blot hybridization analysis of three regenerants displayed some DNA polymorphism (RFLP) and thus confirmed that in such plants alterations in the genome structure could be found and that genotypes of protoplast-derived plants frequently differ from the parental genotype.

Additional index words: Genome instability – Protoplast-derived plants – Somaclonal variation – RFLP analysis – *Nicotiana tabacum*

It has been well established that eukaryotic genomes display some extent of structural and functional instability. A high degree of genome plasticity could be observed in higher plants perhaps reflecting their high ability of adaptation to a changing environment (Walbot and Cullis 1985). A good example of this variability are flax genotrophs obtained by plant cultivation under suboptimal conditions, which differ both in phenotypes and genomic structure (Cullis and Cleary 1986). Another manifestation of genome plasticity in plants is that which results in a widespread phenomenon called somaclonal variation (Larkin and Scowcroft 1981). Due to the capacity of cultured cells to regenerate whole plants a large number of species can now be cloned using *in vitro* techniques. Among these regenerants a remarkably high proportion of non true-to-type plants are often found. This variation can be of epigenetic (modulation of gene expression including DNA methylation) or genetic (changes in DNA structure) origin and both types are frequently inherited into the plant progeny (Phillips *et al.* 1990). A broad spectrum of true genetic changes have so far been described in this respect, including polyploidy, aneuploidy, gross chromosomal aberrations as well as changes detectable at the DNA level altering the primary structure or the copy numbers of some DNA sequences.

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In this work we have studied genome plasticity in tobacco plants regenerated from leaf mesophyll protoplasts. To monitor these changes phenotypic evaluations, karyological analyses, and Southern blot hybridization experiments were performed.

MATERIAL AND METHODS

Plant Material

The plant material used was *Nicotiana tabacum* L. cv. Vielblättriger. To exclude natural genetic variation among the individual plants, only one typical plant (futher designated as T3) was selected and used for all the experiments.

Protoplast Isolation and Regeneration

Leaf mesophyll protoplasts were prepared in an enzyme solution consisting of 2 % Cellulolysin and 1 % Macerozyme (CALBIOCHEM) dissolved in K3 medium with 0.4 M sucrose. Three-week-old cultures of microcolonies were diluted and mixed with the melted agarose K3 medium to reach a low plating density. The microcalli were subcultured on a modified MS medium containing 2 mg/l NAA and 0.2 mg/l BAP. The resulting calli were transferred on the same agar media but with the opposite ratio of NAA to BAP (0.2 mg/l NAA and 2 mg/l BAP) to induce shoot formation. After rooting and a short period of hydroponic culture the regenerants were grown up in a greenhouse under the same conditions as the donor control T3 plant.

Cytological Analysis

Cytological squash preparations were made from root tips of some regenerated plants. Roots were fixed in Carnoy mixture, hydrolysed in 1N HCl for 10 min at 60 °C, and stained with Schiff reagent.

DNA Isolation

Total DNAs from fresh tobacco leaves were isolated using a modified procedure of Murray and Thompson (1980).

DNA Probes and Southern Hybridization

Four different nuclear repeated DNA sequences were used to monitor genomic changes in protoplast-derived plants. (i) HRS60.1 – a 184 bp member of the HRS60 family of tandem DNA repeats (Koukalová *et al.* 1989), (ii) a 2.4 kbp DNA repeat designated as R8.1 (Kuhrová *et al.* 1991), (iii) a 5.5 kbp DNA repeat designated as R8.3 (Kuhrová *et al.* 1991). In some experiments two parts of tomato rDNA operon (1.7 kbp and 2.5 kbp coding 18S and 25S rRNA, respectively) were also used (Kiss *et al.* 1988). Restriction endonuclease digestion, gel electrophoresis, DNA/DNA hybridization, and ^{32}P-labelling of

DNA probes were performed as described by Koukalová *et al.* (1989). To test the completeness of endonuclease digestion, each blot was hybridized with a cloned fragment of the tobacco chloroplast DNA (Sugiura *et al.* 1986). This method uses the chloroplast DNA present in the samples of total DNA as an inner standard.

RESULTS

Morphology of Regenerants and Their Progenies

Ninty-one tobacco regenerants from 35 different callus clones of protoplast origin have been isolated. Up to 7 plants were regenerated from the individual calli, grown up, and futher analysed. Of the whole population of regenerants only 34 plants (*i.e.* 37.4 %) totally confirmed the original T3 phenotype, the rest of plants displaying one or more morphological and/or functional aberrations. The most frequent findings included stem splitting (31.9 %), leaf deformations (29.7 %) irregular branching of leaf veins (8.8 %), flower malformations including reproductive parts (11.0 %), and leaf curling (2.2 %) (Fig. 1a–c).

The R_0 plants were self-pollinated and the seeds tested for germination and hereditability of their phenotypic aberrations. In general the germination was significantly decreased (ranging from 31.0 to 89.0 % in the individual progenies) in comparison with the T3 seeds (98.4 %). The progeny of 14 randomly selected regenerants was checked for the inheritance of the induced phenotypic changes. In all of them some aberrant phenotypes appeared without any relation to the phenotypes of the respective R_0 plants.

Changes in Ploidy Level

A karyological analysis of 20 randomly chosen plants showed that only 5 of them retained the original diploid number of chromosomes ($2n = 48$), the others being tetraploids (Fig. 1d). There was no correlation between the increased chromosome number and the type of a specific phenotype change.

Southern Blot Analysis of DNA from R_0 Plants

Three of the regenerants which had shown some morphological aberrations, were chosen for further analysis. The plant DNAs were cleaved by restriction endonucleases and Southern hybridizations were carried out with the R8.1 and *HRS*60.1 probes or with the 18S and 25S rDNA probes.

Of the four digests made by different enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Pst*I) only several combinations of the enzymes and probes resulted in hybridization patterns with discrete bands (Fig.2). In Fig. 2a differences can be seen in hybridization patterns of DNA from the standard plant T3 (lane 1) and from

three regenerated plants (lanes 2,3,4) hybridized with the probe R8.1 and digested with *Bam*HI or *Eco*RI. The probe *HRS*60.1 provided a ladder of hybridization bands which corresponded to multiples of the 184bp basic unit of the *HRS*60 family of tandem DNA repeats, both with *Bam*HI (Fig. 2c) and with *Eco*RI (Fig. 2d) digested DNAs. In the case of the *Bam*HI digests there were considerable differences in the strength of hybridization signals, if the control T3 DNA and the DNAs of R_0 plants have been compared. Mutations in the *Bam*HI sites in the *HRS*60 DNA of some R_0 plants seem to be the plausible explanation.

In the case of *Eco*RI digests the ladder of hybridization bands was not so prominent; the *Eco*RI recognition sites in the *HRS*60 repeats are much less frequent than the *Bam*HI sites (Koukalová *et al.* 1989).Nevertheless, each DNA sample tested displayed a different hybridization pattern, suggesting that mutations in some *Eco*RI sites had occured. Two probes of the rDNA operon applied in a mixture to these plant DNAs also revealed differences in hybridization patterns in both the *Bam*HI (Fig. 2e) and the *Eco*RI (Fig. 2f) digested DNAs, revealing some changes in the rDNA in the R_0 regenerants.

DISCUSSION

During *in vitro* culture and plant regeneration a broad spectrum of genetic and epigenetic variation evolves (Karp and Bright 1985). Recent molecular analyses of regenerants confirmed changes both in natural DNA sequences (nuclear – Dennis *et al.* 1987, organelle – Li *et al.* 1988) and in transgenes (Peerbolte *et al.* 1987).

In this work many various changes at the level of both phenotype and genotype were found. The Southern analyses also showed some changes in the restriction patterns of DNAs isolated from the R_0 plants probed with the DNA repeats. New positions of DNA restriction fragments were not frequent, but some probes documented local DNA amplifications or deletions. These results support the quantitative hybridization data showing that in the population of plant regenerants gross and abundant DNA changes can be detected (Vyskot *et al.* 1991). They seem to be a widespread phenomenon since recent data have shown that tissue culture-derived rice plants also display increased levels of DNA polymorphism and it was demonstrated in different functional genomic domains (Brown *et al.* 1990).

The occurrence and high frequency of morphological and karyological changes in protoplast-derived plants are not surprising and correspond to many other date (*e.g.* Prat 1983). In this connection it is interesting to mention that an evidence for both their induction during cell culture (Lörz and Scowcroft 1983) and the transmission of pre-existing heterogeneity from original tissue sources (Magnien *et al.* 1982) was demonstrated. No correlation could be found between the character of DNA alterations observed and the phenotypes of

regenerated plants. Similar results were obtained recently in rice (Müller *et al.* 1990). The relations between genotype and phenotype, in the case of genome restructurations, may be rather complicated. Except for the possible effects of unstable epimutations due to the changes in DNA metylation status (Holliday 1987, Phillips *et al.* 1990) and to the position effects (Weisling *et al.* 1988), the changes in chromatin folding could also be involved: abundance and/or distribution of DNA repeats may be expected to change the "chromatin code" (Vogt 1990) and subsequently also the expression of phenotype or it may be a result or gross chromatin reorganizations.

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Figs. 1 and 2 at the end of the issue.