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DNA variations in regenerated plants of pea (*Pisum sativum* L.)

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Summary. The aim of this study was to determine whether DNA variations could be detected in regenerated pea plants. Two different genotypes were analyzed by cytogenetic and molecular techniques: the "Dolce Provenza" cultivar and the "5075" experimental line. "Dolce Provenza" regenerated plants showed a reduction in DNA content, particularly at the level of unique sequences and ribosomal genes. Moreover, regeneration was associated with an increase in DNA methylation of both internal and external cytosines of the CCG sequence. On the other hand, the DNA content of the "5075" line remained stable after regeneration. DNA reduction was found only in "5075" plants regenerated from callus cultures maintained for long incubation periods (about a year). The DNA variations observed are discussed both in relation to the genotype source and the role of tissue-culture stress.

Key words: In-vitro culture – Nuclear DNA variation – *Pisum sativum* – Tissue-culture stress – Underrepresentation – DNA methylation

Introduction

Tissue-culture techniques are commonly used in many plant species, and may be useful either for applied purposes or for studying plant genetics and physiology. It is known that, in many cases, a consistent proportion of the regenerants differ from the parental type, a phenomenon called "somaclonal variation" (Larkin and Scowcroft 1981). Somaclonal variation is thought to derive either from the release of genetic diversity pre-existing in the explant or else from variability originating during cell de-differentiation or callus maintainance in vitro (D'Amato 1985; 1990). The mutational events might depend on the plant species, the genotypes involved, the type of explant, and the culture media and conditions (Meins 1983; Karp and Bright 1985); they may involve genomic, chromosomal and gene mutations (D'Amato 1985; Brown and Lorz 1986), the activation of transposable elements (Peschke et al. 1987; Planckaert and Walbot 1989), or changes in methylation pattern (Brown 1989).

While karyological changes in response to tissue culture have been amply documented (D'Amato 1985), more cryptic gene alterations, such as amplification or loss of DNA and re-arrangements of nuclear DNA, are now being shown to be affected by tissue-culture stress.

Many of these genome alterations are known to occur during either de-differentiation or callus formation. Amplification of highly repetitive sequences was found in de-differentiating cells of *Nicotiana glauca* (Durante et al. 1983); more recently, differential amplification of five genes was described in *Oxalis* callus cultures (Escandon et al. 1989). As far as regenerated plants are concerned, amplification of repetitive sequences was shown in double haploids of *Nicotiana sylvestris* (DePaepe et al. 1982) and *N. tabacum* (Dhillon et al. 1983). Chromatin diminution (including unique DNA sequences) was described by Deumling and Clermont (1989) in both calli and regenerated plants of *Scilla siberica*. Variations in ribosomal DNA amounts have been reported in regenerated plants of *Solanum tuberosum* (Landsmann and Uhrig 1985), flax (Cullis and Cleary 1986) and *Triticale* (Brettell et al. 1986). Variability (either amplification or diminution) in the quantity of cloned repetitive sequences was found during callus culture and plant regeneration in flax (Cullis and Cleary 1986) and rice (Kikuchi et al. 1987). Finally, DNA rearrangements and methyla-

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tion pattern changes were observed in rice regenerants by Muller et al. (1990).

In the present study we report the results of cytophotometric and molecular analyses aimed at assessing whether similar cryptic alterations occur in regenerated plants of two *Pisum sativum* genotypes; moreover, the effect of tissue-culture stress has been studied by analyzing plantlets regenerated after different periods of callus culture.

Materials and methods

In vitro culture

In vitro culture of vegetative pea shoots was performed according to Natali and Cavallini (1987) on two *P. sativum* genotypes: the cv. "Dolce Provenza" and the experimental line "5075". Pea seeds were surface-sterilized with 2% sodium hypochlorite for 20 min and then rinsed twice with sterile distilled water. Seeds were germinated on hormone-free MS (Murashige and Skoog 1962) medium solidified with 0.8% agar.

When shoots were 3–4 days old, vegetative apices were excised and mechanically macerated under sterile conditions with a scalpel in a drop of hormone-free MS medium and using a dissection microscope. The explant was composed of the apical dome plus three to four leaf primordia. The cell masses were placed on solid MS medium supplemented with 3% sucrose, 0.5 mg/l 6-benzylaminopurine and 0.2 mg/l naphthalene-acetic acid (pH 5.7) to promote callus formation. The explants were kept in a temperature-controlled room (25°±1 °C) under cool white fluorescent light of 2,000 lux, using a light and dark cycle of 16/8 h.

After 20–30 days of culture, regenerated shoots, which developed on the callus surface, were excised and cultured on the same medium to achieve plant development. Calli from "5075" line meristems were maintained in culture by subculturing monthly on the same regeneration medium for at least 1 year.

Cytophotometric analyses

For cytophotometric analyses, vegetative shoots, portions of callus after 20 days of culture, and adventitious shoot apices in two different developmental stages (1–2 cm and 4–5 cm long), were collected. Samples were fixed in ethanol-acetic acid 3:1 (v/v), treated with a 4% solution of pectinase (Sigma) for 15–30 min at 38 °C, and then squashed under a cover-slip in a drop of 45% acetic acid. After removal of the cover-slips, the slides were simultaneously hydrolyzed in N HCl at 60 °C for 7 min and then stained in 0.5% fuchsin-Schiff for 1 h at room temperature. This hydrolysis time ensures optimal staining in this species (Cavallini and Natali 1991). After three 10-min washes in SO_2 water, the slides were dehydrated and mounted in DPX (BDH).

Preparations used to collect data which were to be directly compared were processed simultaneously whenever possible. When there were too many such preparations, squashes made with the root tips of a single plantlet of *Vicia faba* were concurrently stained for each group of slides and were used as standards. Absorptions measured in *V. faba* (4C = 53.31 pg, Bennett and Smith 1976) were also used to convert relative Feulgen units into picograms of DNA. The amount of DNA per nucleus was measured by a Barr and Stroud integrating microdensitometer, type GN5, at a wavelength of 550 nm.

With the same instrument, and at the same wavelength, the Feulgen/DNA absorptions of chromatin fractions with differing condensation were determined by measurements on one and the same shoot tip interphase nucleus, after selecting different thresholds of optical density in the instrument (see Cavallini et al. 1981; Cavallini and Natali 1991); where optical density is greater than the preselected limit the instrument does not read those parts of the nucleus, regarding them as a clear field.

DNA extraction

DNA was extracted from in vivo- or in vitro-regenerated vegetative apices (about 15 µg of tissue) or from whole shoots (from 1 to 5 g of tissue). The tissue was homogenized in liquid nitrogen and lysed for 30 min at 60 °C in 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 2% N-laurylsarcosine, 100 mM diethyldithiocarbamic acid. The extract was treated with Proteinase K (0.1 mg/ml), then, after a phenol extraction, submitted to a CsTFA (Pharmacia) gradient. DNA prepared by this method was essentially RNA-free and could be cut by restriction enzymes, as checked by gel-electrophoresis of DNA fragments obtained by digestion with different restriction enzymes, using λ-DNA as an internal marker. DNA concentration was estimated spectrophotometrically. Average DNA fragment length (Lw) was determined after densitometric scanning according to Singer et al. (1979).

Probes

Nuclear DNA from leaves was fractionated according to the Cot values of Murray et al. (1978). Highly repetitive (HR: $0.01 < Cot < 2$), medium repetitive (MR: $2 < Cot < 200$) and unique (U:$Cot > 1000$) sequences were separated by hydroxylapatite as previously described (Durante et al. 1989) and used as probes; other probes were: (1) plasmid pBG35: a *Bam*HI 18+25 S rDNA repeat of flax cloned into the vector pAT 153 (Goldsbrough and Cullis 1981); (2) plasmid pBG 13: a *Bam*HI 5 S rDNA repeat of flax cloned into the vector pAT 153 (Goldsbrough et al. 1981); (3) plasmid VIP 143: an *Eco*RI/*Hin*dIII chalcone synthase (CHS) cDNA of petunia cloned into pTZ 19 (Reif et al. 1985).

DNA probes were labeled with α-32P-dCTP using a Random Primer Kit (Amersham).

Slot blot and hybridization

For each hybridization a 1 µg sample of DNA was denatured by heating at 37 °C for 10 min in 0.5 M NaOH and neutralized by the addition of an equal volume of 2 M ammonium acetate; scalar dilutions from 0.5 µg to 0.03125 µg were loaded onto nylon filters (Hybond-N Amersham) using a commercial slot-blotting apparatus (Minifold II, Schleicher and Schuell).

Hybridization was performed according to Maniatis et al. (1982). After hybridization filters were washed sequentially in 2x, 1x, 0.3x SSC containing 0.05% SDS at 65 °C. Then the filters were exposed to Amersham Hyperfilms with two intensifying screens at −70 °C. Autoradiographs were scanned in a Vernon type PH1 densitometer and the tracings were used for quantitative determinations.

All the experiments were repeated at least six times. The differences between different DNA preparations were within the range of variation between identically loaded slots. As a control all filters were re-hybridized with a single-copy gene (plasmid VIP 143).

Results

Cytophotometric analyses

Table 1 lists the mean 4C nuclear DNA content as determined by cytophotometric measurement of the Feulgen

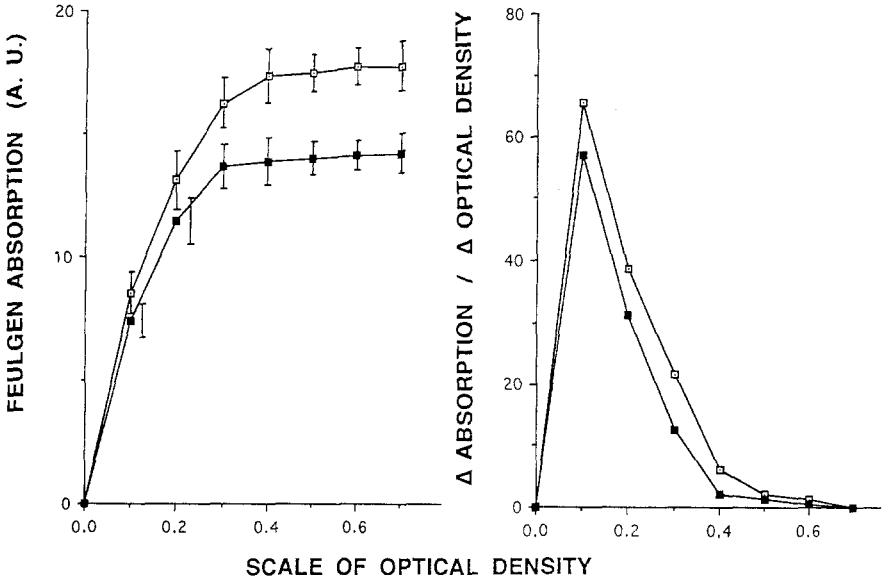

Fig. 1. Feulgen absorptions (mean ±SE, arbitrary units) and first derivative curves (right) at different thresholds of optical density of DNA presynthetic (G_1) nuclei in vegetative apices of plants of *P. sativum* cv. "Dolce Provenza", (□—□) in vivo and (■—■) regenerated in vitro. Twenty nuclei for each of three plantlets per sample were measured. Fiduciary limits at $P = 0.01$

Table 1. Feulgen absorption of early prophases in vegetative apices of normal and regenerated diploid plants (in two subsequent developmental stages) of pea "Dolce Provenza" and "5075" cultivars. Feulgen absorption corresponding to 4C in 20-day old callus (calculated after microdensitometric analysis of interphase nuclei) and early prophase Feulgen absorption in regenerated plants progenies are also reported

Cv.	Early prophase absorption in vivo[a]	Absorption corresponding to 4C in the callus	Early prophase absorption in regenerated vegetative apices (R_o)		Early prophase absorption in vegetative apices of R_1 plants
			First stage	Second stage	
"Dolce Provenza"	31.9±0.9	27.0	27.3±0.5	26.9±0.8	29.8±0.6
"5075"	27.6±1.0	26.4	27.5±1.0	27.8±0.7	27.4±0.8

[a] Mean ±SE (arbitrary units)

absorption of early-prophase nuclei at different stages of in-vitro culture of pea meristems; namely, explants, calli, 1–2 cm- and 4–5 cm-long regenerated diploid plantlets. As chromosomal mosaicism has been described in some regenerated plants from pea meristems (Natali and Cavallini 1987), cytophotometric analyses were performed only on strictly diploid plants (confirmed by chromosome counts of metaphase plates).

It is observed (Table 1) that: (1) while the two genotypes show different 4C DNA contents in vivo (4.92 vs 4.25 pg per haploid nucleus) a 15.6% reduction of mean 4C DNA content takes place during callus formation in the cultivar "Dolce Provenza"; (2) regenerated plantlets of this cultivar maintained the reduced 4C DNA content at both the stages analyzed; (3) when analogous stages were analyzed no variation in nuclear DNA content was found in the "5075" experimental line, either in the callus or in regenerated plants; however, when plantlets regenerated after 12–13 monthly callus subcultures were analyzed, a 6.6% reduction of 4C DNA content was also observed (note, long-term cultures are no available for cv. "Dolce Provenza").

To clarify whether particular chromatin fractions are involved in the nuclear DNA content reduction in "Dolce Provenza" regenerated plantlets, optical density curves of interphase ($2C = G_1$) nuclei of both regenerated and normal plantlets were measured (Fig. 1). Significant differences do not appear at high thresholds of optical density, while first derivative optical density curves (0.1–0.3 thresholds) (Fig. 1 B) show that the nuclei of regenerated plants have a lower content of less-condensed chromatin.

Molecular analyses

To identify the sequences or portions of the genome involved in the variations, slot-blot DNA hybridization analysis was performed using sequences of different degrees of repetitiveness and with specific genes. For both genotypes studied, DNA was extracted from two tissue types of 4–5 cm long shoots: vegetative apices (that consist only on meristem cells) and whole shoots (mostly composed of differentiated cells). In Fig. 2 a representative series of slots is shown together with the correspond-

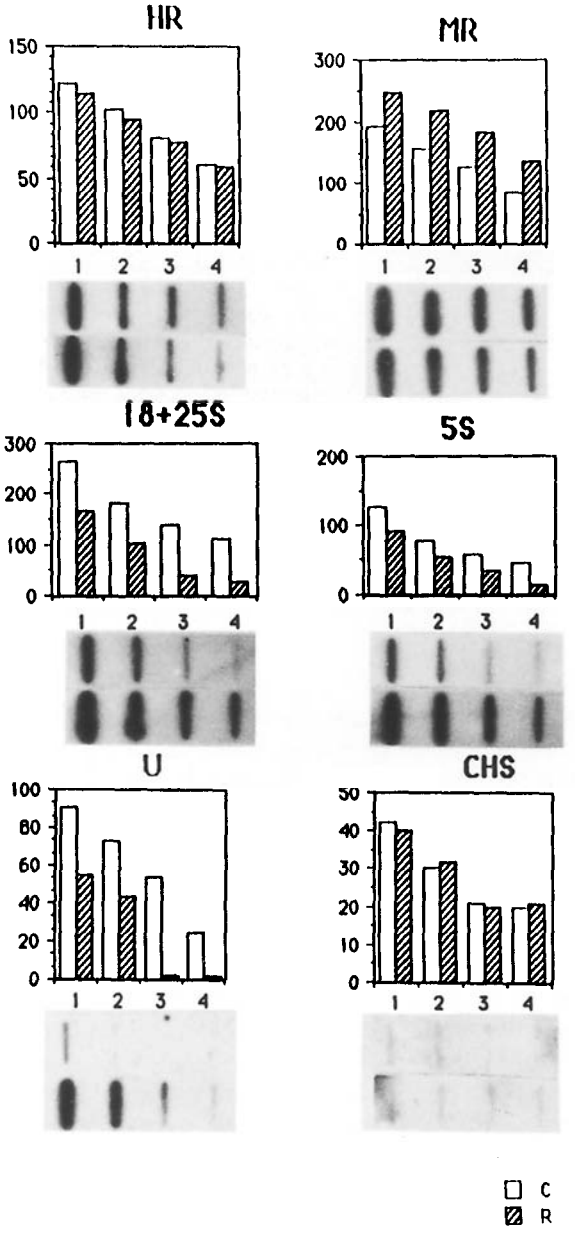

Fig. 2. A representative series of slot-blots ("Dolce Provenza" DNA from vegetative apices) is shown together with the histogram obtained by the densitometric scanning of the autoradiograms. On the top of each graph is shown the sequence used to probe the slot (*C*, control plants; *R*, regenerated plants)

Table 2. Average DNA fragment length values (Lw) after restriction enzyme digestion of genomic DNA from normal (C) and regenerated (R) plants. The standard deviation was always below 1%

Digest	C (Lw)	R (Lw)
*Hpa*II	4,936 bp	5,124 bp
*Msp*I	4,458 bp	5,035 bp
*Sma*I	5,194 bp	5,366 bp
*Xma*I	4,605 bp	5,199 bp

ing densitometrically obtained value compiled in histogram form.

As far as "Dolce Provenza" regenerated plants are concerned, meristematic cells showed a reduction both of unique sequences and of genes for 18+25S and 5S rDNA relative to normal plants. HR, MR and CHS remained invariant (Fig. 3A). On the other hand, with the exception of unique sequences and 5S rDNA, shoot tissues showed a different degree of amplification for all the other sequences used as probes (Fig. 3B).

The slot-blot analysis of DNA from the "5075" line confirmed the cytogenetic analysis. No variation in the copy numbers of the sequences used as probes was detected in meristematic cells of regenerated plants (Fig. 3C). DNA from whole shoots was somewhat amplified at the level of 18+25S rDNA (Fig. 3D).

A higher degree of genetic instability, relative to in-vivo plants, was evidenced in regenerated plants of the "5075" line obtained from callus cultures maintained for longer incubation periods (1 year) than in those from shorter incubation periods (20–30 days). With the exception of the CHS gene (Fig. 4), regenerated apices showed a significant reduction of all the sequences used as probes.

An analysis with restriction enzymes sensitive to DNA methylation was performed on regenerated plants of both the genotypes. Relative to normal plants, DNA from regenerated shoots of "Dolce Provenza" showed an increase of DNA methylation at the level of both the internal and the external cytosines of the CCG sequence, as evidenced by DNA digestion with the isoschizomers *Msp*I and *Hpa*II or *Sma*I and *Xma*I (Table 2). By contrast, no differences in the degree of DNA methylation were revealed in the "5075" line and no differences were found using other enzymes sensitive to cytosine methylation at different DNA sequences in "Dolce Provenza".

Discussion

Recent results show a wide range of DNA modifications in response to tissue culture in the absence of gross chromosomal changes (see Introduction), although the molecular basis of such somaclonal variation remains as-yet unknown. The aim of our experiments was to study DNA variation in regenerated plants of pea with different genotypes after different periods of maintenance in culture before regeneration.

Cytogenetic analyses showed a significant reduction of nuclear DNA content in regenerated plants of "Dolce Provenza" whereas the DNA content remained stable after regeneration in the "5075" line. Molecular analysis of meristematic cells revealed a reduction of specific subfamilies of MR sequences; namely, the 18+25S and 5S ribosomal genes. Contrary to expectation, HR sequences

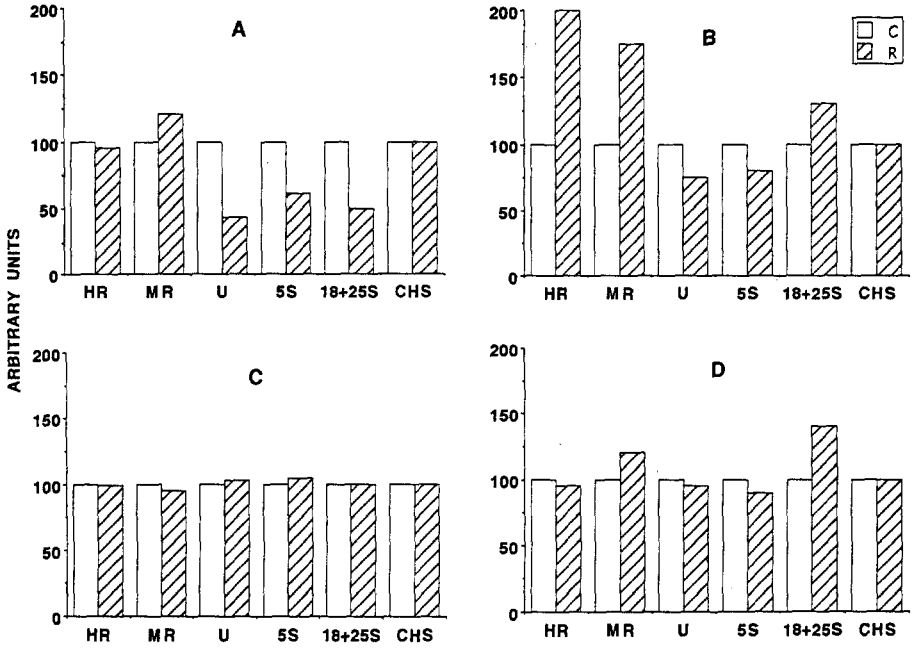

Fig. 3A–D. The densitometric values (each repeated six times) of the slot-blot experiments were averaged and normalized (control = 100) for each probe. Thus the figures show the degree of variation of the different DNA sequences (*C*, control plants; *R*, regenerated plants). The DNAs were extracted respectively from vegetative apices (**A**) and shoots (**B**) of cv. "Dolce Provenza" and from vegetative apices (**C**) and shoots (**D**) of "5075" line plants. Fiduciary limits at $P = 0.01$ never exceeding $\pm 5\%$ (data not-shown)

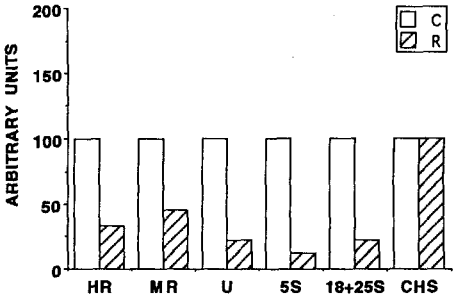

Fig. 4. Normalization of the densitometric values of slot-blot experiments (see the legend of Fig. 3). The DNAs were extracted from vegetative apices of normal plants (*C*) and plants regenerated from cells of long-term cultures (*R*) of the "5075" cell line. Fiduciary limits as in Fig. 3

remained invariant, while a marked reduction of kinetically unique sequences has been found. These data were confirmed by cytophotometric analysis, which showed nuclei with a lower content of less condensed chromatin in regenerated plants. The origin of this reduction may depend on the nuclear fragmentation known to occur during the first phase of callus induction (Natali and Cavallini 1987).

On the other hand, differentiated cells regained their normal DNA content and selectively amplified different sequences or portions of the genome. A similar phenomenon of selective loss and regain of chromatin was described by Deumling and Clermont (1989) in *Scilla siberica*. Here, regenerated plants showed a drastic reduction of satellite DNA and of certain protein-coding genes, whereas the same sequences increased to 30–40% of their normal proportion after a year of growth in the garden. A possible explanation of this phenomenon of euchromatin loss is that it may involve structural genes which are redundant in the plant genome, namely small gene-families, without complete loss of genetic information. Indeed, in our experiments, regenerated pea plants only rarely show phenotypic variation, indicating that DNA reduction does not affect the plant phenotype.

A well known epigenetic process of rapid modification of gene expression, which could be involved in response to stress, is the modification of the degree of endogenous DNA methylation (Durante et al. 1989; Brown 1989). We analyzed DNA methylation patterns of the two genotypes studied by restriction with specific isoschizomers. The DNA of "Dolce Provenza" was hypermethylated at the level of both of the cytosines of the CCG sequence. This extended hypermethylation of the genome in regenerated plants could be a rapid mechanism for silencing potential lethal genes during stress conditions (Muller et al. 1990). In the "5075" line we did not detect any modification in the DNA methylation pattern by restriction enzyme analysis. However, confirmation of these results will depend on more sensitive approaches, such as nucleotide HPLC analysis. This will be undertaken soon.

The results presented here clearly suggest that DNA variations related to culture and regeneration stress are dependent, at least in part, on the genotype: thus, in terms of the experiments we have performed, the "5075" line is more stable than the "Dolce Provenza" cultivar.

However, DNA content variability was found in "5075" plants regenerated from calli maintained in culture for a long incubation period (even if they showed good regeneration capacity). This result confirms the hy-

pothesis that the genetic constancy of regenerants is also a function of the length of time such cultures are maintained as a callus (Meins 1983; Muller et al. 1990).

That DNA reduction in regenerated plants largely involves unique sequences seems of particular interest. It is conceivable that these are non-coding sequences, since regenerated plants show a normal phenotype; in this sense, the sequences involved would belong to the so-called "conformational DNA" (Nagl 1990) that determines general nuclear structure and function. Single-copy, non-coding sequences, which often make up a significant fraction of plant genomes, might predominantly be the decaying relicts of former repeats (Smyth 1991). Further studies are in progress to establish the nature of the sequences involved in the variation.

We are currently focusing on DNA modifications during callus culture which seem to be of importance in determining the genotype and phenotype of the regenerants. We are also testing whether the DNA modifications observed are epigenetic (transient) or whether they are maintained in the sexual progeny of regenerated plants.

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