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RFLP analysis of *Zea mays* callus cultures and their regenerated plants

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Summary. Tissue culture of the *Zea mays* inbred line A188 resulted in the regeneration of plants having a high level of phenotypic variation compared to seed-grown control plants. To determine how such variation was induced and whether this could be related to specific in vitro culture methods, callus cultures were established and maintained on different, commonly used culture media. Plants were regenerated and the genomic DNA of callus cultures and regenerants analysed for RFLP differences. The results show that regardless of the gene probe used, callus formation resulted in significant deviations from the DNA pattern normally found in seed-grown control plants. Alterations in gene copy number also occurred. As differentiation and organogenesis began, the level of DNA variation fell, and most of the regenerated plants showed a genetic similarity to the controls; those with RFLP differences were the somaclonal variants.

Key words: Callus cultures – RFLP – *Zea mays* – *Oryza sativa* – Regeneration – Somaclonal variation

Introduction

During an investigation into the high level of phenotypic variation found in tissue culture-derived maize plants, Brown (1989) discovered that many of these plants, regardless of phenotype, contained significant molecular deviations from seed-grown controls. These alterations included gene methylation changes, DNA re-arrangements and alterations in copy number. Subsequently, RFLP analysis of callus cultures and regenerated plants has been undertaken to determine which aspects of the tissue culture process may be responsible for these alterations, in the hope that if the changes can be related to particular culture conditions, subsequent modifications in the culture systems may reduce or eliminate the variations that occur in regenerated plants.

The source of genetic variability in tissue culture-derived plants is the subject of speculation: is it a reflection of variation already present in the explant or is it due to the tissue culture process itself (for review, Morrish et al. 1987). Swedlund and Vasil (1985) examined chromosome numbers in the immature inflorescences of *Pennisetum americanum* that were used as explants for tissue culture. While 95.8% of the explants were predominantly diploid, some were found to be tetraploid (2.5%) and others aneuoploid (1.2%). Of the resultant embryogenic calli 92% were diploid after 1 month in culture; this fell to 76% after 6 months. The level of tetraploidy was 4%. The proportion of aneuploid cells rose from 10% after 1 month culture to 14% after 6 months. After analysing maize callus cultures, Edallo et al. (1981) proposed that any single callus should be regarded as an assembly of genetically different cells. The effect of culture conditions on the karyological status of callus cultures has been extensively reviewed (D'Amato 1985; Karp and Bright 1985).

The effect of a modification of the culture conditions on the ability of cereal explants to produce callus-developing somatic embryos has been described in barley by Lührs and Lörz (1987). The initiation and development of somatic embryos usually requires the presence of an auxin, often 2,4-D, in the culture medium, although subsequent shoot development requires a decreased quantity

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or even the omission of such hormones (Green and Phillips 1975). As there is some evidence that growth regulators, especially 2,4-D, may have an effect on chromosomal stability (Hangyel Tarczy et al. 1986), there remains the possibility that the main effect of such compounds is to select for a specific genotype of cells present in the explant. Such factors may be related to the findings of Müller et al. (1990), who demonstrated that extending the time of callus culture from 28 to 67 days resulted in the proportion of tissue culture-derived rice plants with an abnormal RFLP pattern for the actin gene probe rising from 6.3% to 23%. Evidence for a correlation between tissue culture-induced stress and transposition has been shown for the Ac element in maize cultures (Peschke et al. 1987).

The purpose of the work described here was to determine whether the molecular deviations found in some regenerated *Zea mays* plants can be related to variation already present in the explant or to any particular aspect of the tissue culture process.

Materials and methods

Tissue culture and plant regeneration

Donor plants of the inbred line A188 were grown in a greenhouse with additional lighting (the photoperiod was 16 h light/8 h dark) and self-pollinated by hand. Immature embryos were collected 10–12 days post-pollination when they had reached a length of 1.0–1.5 mm (from base to tip of the scutellum). Kernels were removed from the dehusked ears, surface sterilized in 70% ethanol (v/v) followed by sodium hypochlorite (1.5% active Cl) for 15 min and rinsed three times with sterile distilled water. The embryos were aseptically dissected out from the caryopses and placed on agar-solidified culture medium with the embryo axis in contact with the medium.

For callus induction and callus proliferation the basal media used were MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968) and CC (Potrykus et al. 1979). The MS medium was supplemented with sucrose (30 g/l), coconut water (50 ml/l) and 2.0 mg/ml 2,4-dichlorophenoxy-acetic acid (2,4-D). Gamborg's B5 medium was supplemented with sucrose (30 g/l), proline (0.7 g/l) and 1.0 mg/l 2,4-D. To the basal formula of the CC medium, sucrose (20 g/l), mannitol (36.4 g/l), coconut water (100 ml/l) and 2,4-D in concentrations of either 1.0, 2.0, 4.0 or 8.0 mg/l were added.

To induce plant regeneration MS medium devoid of any hormone but supplemented with sucrose (100 g/l) and coconut water (100 ml/l) was used. Developing shoots were transferred to half-strength MS medium containing 10 g/l sucrose. The pH of all media was adjusted to 5.8 before autoclaving, and media were solidified with 0.8% Difco Bacto agar.

For callus development and proliferation all cultures were incubated in darkness at 25 °C and transferred to fresh medium every 4 weeks. Plant regeneration was achieved by transferring embryogenic callus sections or groups of somatic embryos to the regeneration medium and then incubating these at 25 °C in 16 h light and 8 h dark. Plantlets 1–2 cm long were transferred to 20-cm high glass containers with half-strength MS medium for further development. After the plantlets had established an adequate root system, they (height 10–15 cm) were potted in a mixture of soil and sand (3:1) and kept in the greenhouse under a transparent plastic foil for acclimatization for approximately 2 weeks. Thereafter the plantlets were transplanted into soil and grown to maturity.

Table 1. Culture media used for *Zea mays* callus cultures

Medium[a] (basal formula)	2,4-D (mg/l)	Sucrose (g/l)	Mannitol (g/l)	L-Proline (g/l)	Coconut milk (%)
B5	1	30	–	0.7	–
MS	2	30	–	–	5
CC	1, 2, 4, 8	20	36.4	–	10

[a] B5 medium: Gamborg et al. 1968; MS medium: Murashige and Skoog 1962; CC medium: Potrykus et al. 1979

Isolation and analysis of DNA

DNA isolation from all of the plant material was according to the modified CTAB procedure as described by Taylor and Powell (1985). The DNA was then centrifuged overnight on Caesium chloride/ethidium bromide gradients and subsequently washed with *n*-butanol before being extensively dialysised against TRIS/EDTA buffer (10 m*M* TRIS/1 m*M* EDTA) pH 7.6. Samples were precipitated by the addition of 50% 7.5 *M* ammonium acetate and two volumes of 95% ethanol, before being re-dissolved in 200 µl TRIS/EDTA buffer. The concentration and purity of the DNA were checked by spectrophotometer readings at 260 nm and 280 nm. Samples with an OD260/OD280 ratio of more than 1.8 were washed with phenol:chloroform:isoamyl alcohol (50:49:1) and rechecked.

DNA samples for analysis were digested overnight with restriction enzymes according to the manufacturer's specifications. To ensure that full digestion had occurred, a second aliquot of enzyme was added after the overnight digestion, and the reaction was allowed to proceed for a further 5 h. Phage lambda DNA was used as a control to confirm that full digestion had occurred. DNA restriction fragments were separated by electrophoresis on 1% agarose gels and transferred to Hybond TM (Amersham) using the manufacturer's protocol. The filters were pre-hybridised for 5 h at 45 °C in prehybridisation buffer consisting of 50% formamide, 5× Denharts solution (1× Denhart's solution contains 0.02% Ficoll, 0.02% BSA, 0.02% PVP), 5× SSPE (1× SSPE contains 0.8 *M* NaCl, 10 *M* $Na_2HPO_4 \cdot 2H_2O$, 1 m*M* EDTA, pH 7.0), 0.1% SDS and 100 µg/ml denatured herring sperm DNA.

Characterised, cloned sequences were isolated by restriction digestion of recombinant plasmids and separation on (0.8%) agarose gels. The desired fragment was removed, and the DNA was isolated by electrophoretic dialysis. The DNA was then run on a second agarose gel to ensure that the sequence was not contaminated by vector DNA. The DNA was isolated as previously described, washed with phenol:chloroform:isoamyl alcohol (50:49:1), ethanol precipitated and then re-dissolved in 100 µl TRIS/EDTA buffer. As a final check of purity, a small sample of the insert DNA was used as a probe against digested vector DNA in a Southern analysis to ensure that there was no cross-hybridisation.

For Southern analysis (Southern 1975) the cloned gene sequences were labelled with alpha ^{32}P-dCTP by nick-translation (Rigby et al. 1977) to a high specific activity ($0.5-2.0 \times 10^9$) cpm/µg) and then hybridised to the filters overnight at 42 °C. The filters were then rinsed in 2 × SSC buffer, and stringently washed: 15 min in 2 × SSC buffer containing 0.5 SDS, two times 15 min in 2 × SSC, 10 min in 1 × SSC, 5 min in 0.5 × SSC and

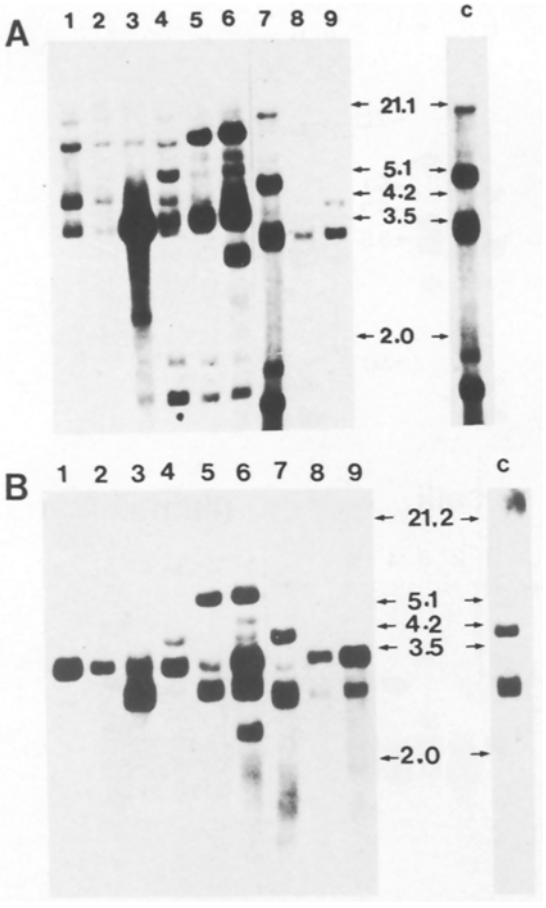

Fig. 1 A, B. Comparative analysis of DNA changes in independent calli after 5 months in culture on modified B5 medium; note wide-ranging changes in DNA organisation, including obvious alterations in hybridisation strength. Genomic DNA was digested with HindIII and probed with (**A**) the 4.5-kb BamHI fragment of the sucrose synthase gene and (**B**) the 1.2-kb PstI fragment of the ATP/ADP Translocator gene. The control (*C*) was pooled genomic DNA from 40 seed-grown seedlings

finally 5 min in 0.1 × SSC. The washing temperature depended on the homology of the probe used, but was usually 65° or 70 °C. The washed filters were then placed against Kodak XAR-5 X-ray film at −70 °C for 24–48 h using Dr. Goos casettes and intensifying screens. Slot-blot assays were carried out according to the protocol of Rivin et al. (1986).

Results

RFLP analysis of callus cultures and regenerated plants

Analysis of the sucrose synthase gene in a number of independent calli grown on the same medium (modified B5 medium) (Fig. 1 A) shows that for most of these calli, there is a widespread alteration in the gene. Repetition of this analysis with other gene sequences such as the ATP/ADP Translocator gene (Fig. 1 B) revealed a similar level of changes. There are also significant differences in hybridisation strength, and therefore presumably in copy number of the gene, between each callus with evidence

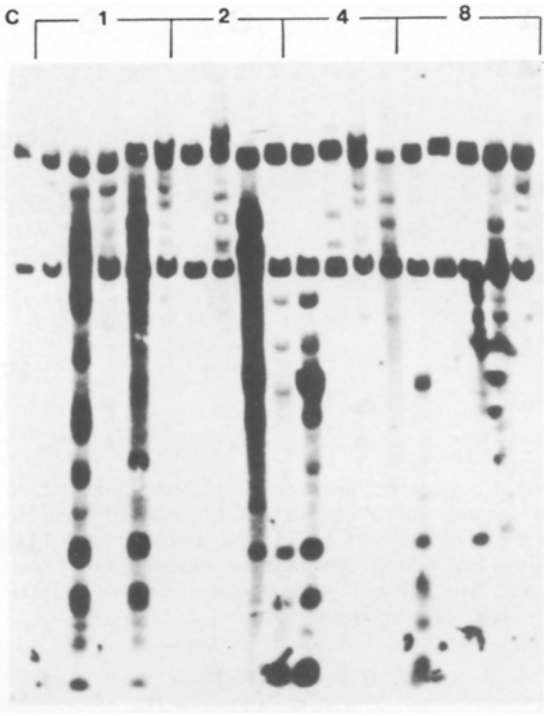

Fig. 2. Comparative analysis of increasing levels of the synthetic auxin 2,4-D on the sucrose synthase gene in independent callus cultures grown on CC medium supplemented with 1, 2, 4 and 8 mg/l 2,4-D. Genomic DNA was restricted with HindIII and probed with the 4.5-kb BamHI sucrose synthase fragment. The control (*C*) was pooled genomic DNA from 40 seed-grown seedlings

for both amplification (Fig. 1 A, tracks 3 and 6) and de-amplification (Fig. 1 A, track 2).

Analysis of the sucrose synthase gene from calli grown on the same basal medium but differing in the concentration of 2,4-D, (Fig. 2) revealed that the level of DNA variation was independent of the concentration of 2,4-D in the medium. Removal of this hybridisation signal and re-probing with sequences such as the ATP/ADP Translocator gene or the actin gene (results not shown) confirmed that the differences are independent of the sequence examined.

Comparison of independent calli grown on different cereal tissue culture media (Fig. 3) or on a single medium such as MS supplemented with growth hormones prior to regeneration (Fig. 4) suggested that the DNA polymorphisms are independent of the media used. Comparative analysis of 11 independent calli (Fig. 4) that derived originally from the same single embryogenic callus suggested that callus may be a genetic mosaic; this was revealed by the similarity of hybridisation patterns between calli 3, 5 and 9. Further evidence for changes in gene copy number could also be seen. This apparent change in copy number was specific for each gene/callus combination. Subsequent quantification of copy number changes by slot blot assays (Rivin et al. 1986) revealed changes of up

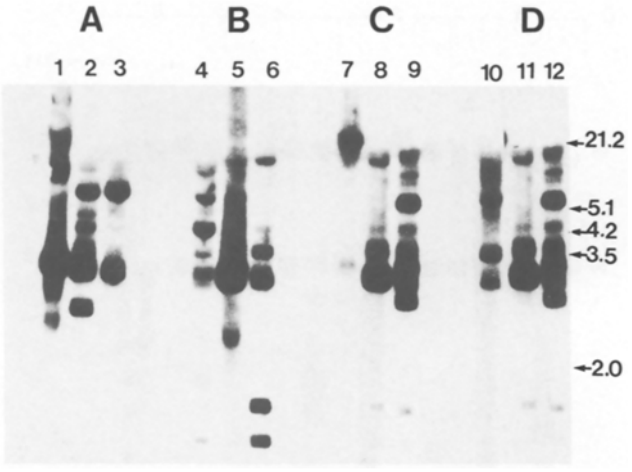

Fig. 3A–D. Comparative analysis of 12 independent calli grown on four different culture media. **A** Gamborg B5 medium; **B** Potrykus CC medium with 2 mg/l 2,4-D and **C** 8 mg/l 2,4-D; **D** Murashige and Skoogu MS medium. Genomic DNA was digested with HindIII and probed with the 4.5-kb BamHI sucrose synthase gene fragment

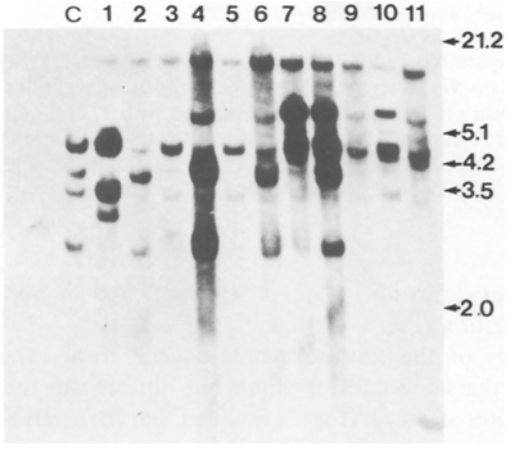

Fig. 4. Comparative analysis of 11 different calli derived from one single embryogenic callus. Genomic DNA was digested with HindIII. The probe was the 247-bp PstI fragment of the waxy gene. The control (*C*) was pooled genomic DNA from 40 seed-grown seedlings

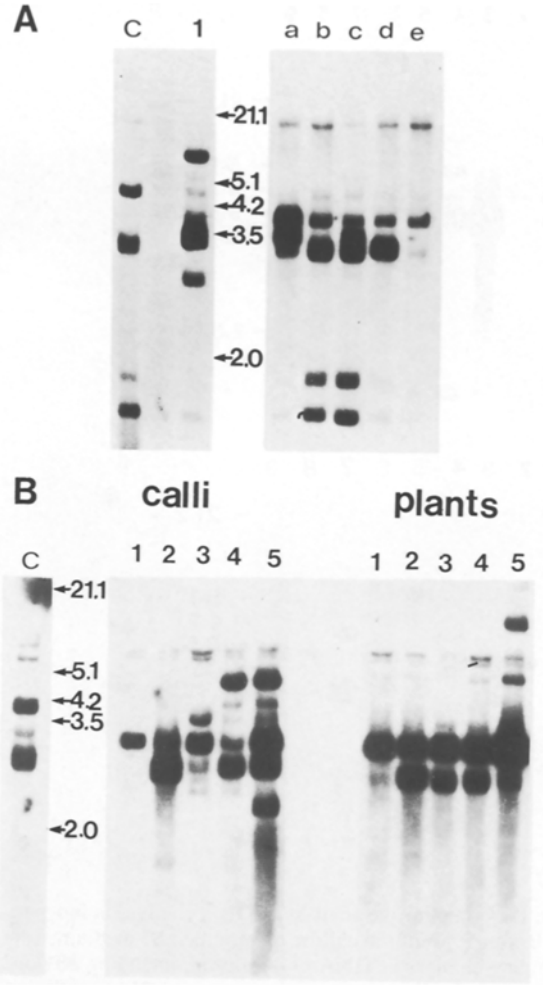

Fig. 5A and B. **A** RFLP changes in five plants (*lanes a–e*), all regenerated from the same callus (*lane 1*). Genomic DNA was restricted with HindIII and probed with the 4.5-kb BamHI fragment of the sucrose synthase gene. **B** demonstrates RFLP changes in five independent calli and their respective regenerants. Genomic DNA was digested with HindIII and probed with the 1.2-kb PstI fragment of the ATP/ADP Translocator gene. The control (*C*) was pooled genomic DNA from 40 maize seedlings

to 65 fold in some sequences, such as that of the actin gene (results not shown).

Figure 5A shows the changes in the sucrose synthase gene in five plants all derived from the same embryogenic callus, whilst Fig. 5B shows changes in the ATP/ADP Translocator gene in five plantlets derived from five different calli. Although it is obvious that significant DNA differences exist between these direct regenerants and controls, it is evident that the DNA variation was considerably reduced with respect to that found in callus.

To determine whether such alterations were specific to this particular maize line (A188), we repeated the analysis of cultures with different maize lines (results not shown) and three different Indica rice cultivars ('IR40', 'IR45' and 'IR54'). In only one line of rice callus ('IR54') (Fig. 6) was there evidence of a significant alteration in the examined gene, although there were obvious stoichiometric differences between the DNA from callus and that from seed-grown control plants.

Analysis of RFLP polymorphisms in a number of genes including the sucrose synthase gene (Fig. 7A) and the ATP/ADP Translocator gene (Fig. 7B) in direct regenerants from maize callus showed that DNA polymorphisms ocurred between regenerants. Although the situation is made more complex by the naturally occurring polymorphism found in control maize lines, variation levels between the regenerants here and those recorded levels of gene polymorphism for seed grown controls

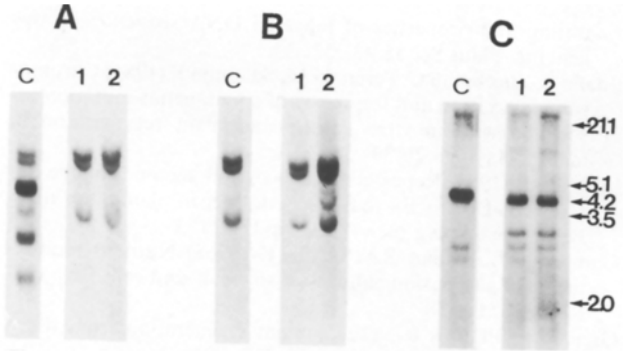

Fig. 6A–C. RFLP analysis of rice callus. Genomic DNA was restricted with the enzyme RsaI and probed with the 4.5-kb BamHI sucrose synthase gene fragment. **A** is rice cv 'IR45'; **B** is rice cv 'IR40'; **C** is rice cv 'IR54'. *Lane 1* is callus grown on CC medium (Potrykus et al. 1979); *Lane 2* is callus grown on L50 medium (Hartke and Lörz 1989); Control (*C*) was genomic DNA from seed-grown rice plants

(Johns et al. 1983) revealed a significantly increased polymorphism rate.

Discussion

The question posed at the beginning of this study was whether somaclonal variation is a reflection of changes induced by in vitro culture and whether these changes can be related to particular aspects of in vitro culture systems. It is obvious that the level of DNA polymorphism in calli, regardless of the gene examined, is so great as to preclude the possibility that this is a reflection of explant heterogeneity alone. The conclusion must be that tissue culture itself is responsible for a greater part of this variation. The results also demonstrate that differences in culture medium cannot be responsible for this variation, neither in callus cultures nor in the direct regenerants. Consequently, two questions must be raised: firstly, how it is possible for the type of genetic variation found here to occur, and secondly, how, in view of the significant changes found during callus growth, is it possible for the majority of regenerated plants to "revert" to the parental genotype?

Whilst accepted mechanisms for inducing variation in genes such as translocations, inversions and duplications, etc. may be responsible for a proportion of the changes shown here, it is unlikely that they could account for such a degree of change within such a short time. Flavell (1985) concluded that in contrast to conserved coding sequences, repetitive DNA rapidly evolves and has a high turnover. Studies by Hake and Walbot (1980) have shown that in *Zea mays*, 20% of the genome is highly repetitive DNA and 40% is middle repetitive DNA. Interspersed within this middle repetitive DNA can be found up to one-third of the unique sequences. Presumably, therefore, if tissue culture is responsible for producing a "genetic stress", the possibility is that this

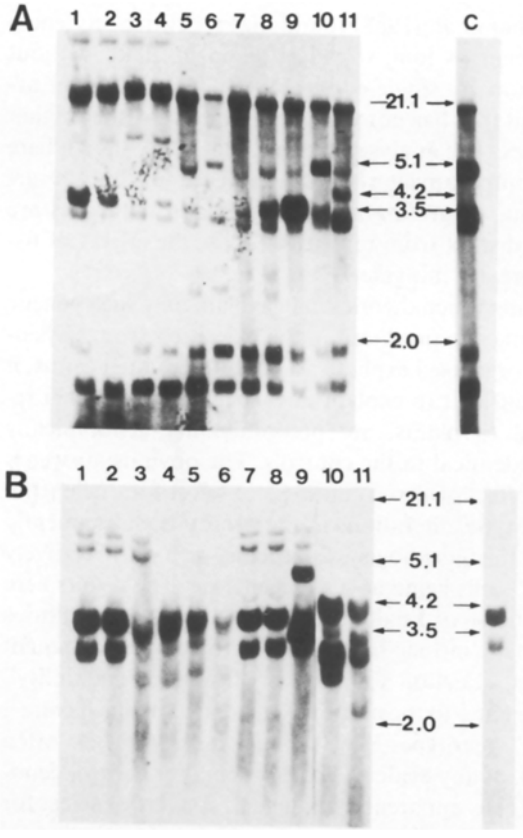

Fig. 7A and B. RFLP analysis of regenerated maize plants of the inbred line A188. Genomic DNA was digested with HindIII and probed with the 4.5-kb BamHI sucrose synthase gene fragment (**A**). The hybridisation was removed from the filter and re-hybridised with the 1.2-kb PstI fragments of the ATP/ADP Translocator gene (**B**). The control (*C*) was pooled genomic DNA from 40 maize seedlings

will affect predominantly repetitive sequences and concomitantly a large proportion of this single-copy DNA. Lapitan et al. (1988) were able to show by C-banding analysis of tissue culture-derived wheat × rye hybrids, amplification of 480-bp repeated sequences in two rye chromosomes. Similarly, Zheng et al. (1987) cloned highly repetitive sequences from suspension cultures of *Oryza sativa* and used a random selection to determine quantitative changes in suspension-cultured cells compared to controls. Most cloned sequences showed an amplification of up to 75 fold in cultured cells compared to seedlings. Caboche and Lark (1981) isolated nuclei from suspension cultures of soybean and demonstrated that repetitive sequences are preferentially replicated from such cultured cells. They hypothesise that the replication of single-copy and repetitive DNA may be controlled differently, the DNA produced under in vitro conditions apparently being "biased" towards replication of repetitive sequences.

A possible strengthening of this theory are the results demonstrating the comparative stability of rice callus

DNA. Dhar et al. (1988) found that repetitive sequences in rice occur as long stretches of up to 20 kb without interruption by single-copy DNA. This is a novel arrangement that has not yet been reported in other higher eukaryotes. The analysis of rice callus DNA shown here reveals comparatively very little evidence for DNA re-arrangement, suggesting that when unique sequences are spatially distant from repetitive DNA, the effects of tissue culture are mitigated.

Although such theories may explain how such genetic changes may be produced in the transition from physiologically organised explants to de-differentiated callus, it is more difficult to explain how such cultures, when regenerated to plants, are predominantly genotypically close or identical to the controls. The obvious answer is that the effect of tissue culture is to select for one particular genotype, alterations in the genotype, subsequently affecting the morphogenesis process, with only relatively "normal" cells being able to regenerate. The results here confirm those of Edallo et al. (1981) that callus cultures are a genetic mosaic of cells. Therefore, as development continues, selection for genetic "fitness or suitability" occurs, leading to most of the regenerated plants having a "normal" genotype. How though, in view of the often total lack of any evidence for a parental or control genotype, the cells apparently manage to convert or select for such a form remains an open question.

The results reported in this paper answer the questions that were originally set as to the source of variation and the relevance of different aspects of tissue culture to the induction of this variation. However, the results have also raised more questions, such as the relevance of changes in repetitive DNA to alterations in coding sequences and also the ability of cells under culture to undergo a rapid genetic change as well as their ability to select, under regeneration pressure, for a more normal genotype. These questions are presently under investigations.

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