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DNA variation in tissue-culture-derived rice plants

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Summary. Regenerants of rice were examined by RFLP analysis to determine the occurrence and extent of somaclonal variation. DNA polymorphisms were observed both among plants regenerated from different callus cultures as well as among sibling plants derived from a single callus. Regardless of the basal medium, a higher degree of genetic instability was found among plants regenerated from callus cultures maintained for longer incubation periods (67 days) than among those from shorter incubation periods (28 days). Detailed analysis showed that in several regenerants, there was a close correlation among those plants exhibiting DNA rearrangements and those with apparent methylation changes. Such alterations were observed with both structural and housekeeping genes.

Key words: Rice – Tissue culture – Somaclonal variation – RFLP – Methylation

Introduction

Regeneration of plants via tissue culture should result in the production of clones that are phenotypically and genetically identical to the material from which they were originally derived. In many cases however, a proportion of the regenerated plants exhibits deviations from the parental type, a phenomenon termed somaclonal variation (Larkin and Scowcroft 1981). One factor of apparent importance in the genetic constancy of the regenerants is the length of time such cultures are maintained as callus (Meins 1983). McCoy et al. (1982) found that the frequency of chromosomal abnormality in oat plants regenerated from callus cultures rose from 49% after 4 months in culture, to 88% after 20 months. RFLP analysis has shown that both callus cultures and regenerated plants of *Zea mays* L. exhibit high levels of DNA polymorphism (Brown 1989).

In an attempt to determine whether similar variation could be found in other cereals and whether such alterations could be related to specific aspects of the tissue culture process, molecular analysis of tissue-culture-derived *Oryza sativa* L. plants was undertaken. Indica rice lines can be established in culture and plants can be regenerated from callus cultures, but the resulting progeny often exhibit high levels of mutation from the parental form (Oono 1985).

During our establishment of a tissue culture system for Indica-type rice lines (Hartke and Lörz 1989), comparisons were made between two callus induction media and culture periods, as well as between two regeneration media. The application of RFLP analysis to rice plants from cultures derived from different callus induction periods should enable us to determine whether there is a direct correlation between length of callus culture and changes in DNA stability. Secondly, we hoped to distinguish whether any DNA changes could be ascribed to rearrangements rather than to the effect of methylation-induced variation.

Materials and methods

Rice varieties, including the line IR 40, were kindly provided by Dr. G. Khush, IRRI, The Phillipines. Embryogenic callus was initiated from immature embryos (10–12 days after pollination) on modified CC callus induction medium (Potrykus et al. 1979), supplemented with 2 mg/l 2,4-D. The callus was maintained for

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28 days (later termed short-phase callus induction) or 67 days (long-phase callus induction) on callus induction medium, before embryogenic parts were transferred to CC medium with 1 mg/l IAA and 0.05 mg/l zeatin riboside (CCIZ), or to CC regeneration medium without hormones. Small plantlets (ca. 2 cm) were subcultured on one-half MS medium to improve rooting, and well-developed plants were then transferred to the greenhouse. Regenerants and seed-derived control plants were cultivated in a hydroponic culture solution (Levatit, Bayer) in the greenhouse.

Methods for the isolation, purification, and Southern analysis of genomic DNA were as described in Brown (1989). The probes used were the 3-kb actin gene of soybean (Dr. D. Shah, Athens/GA, courtesy of Dr. B. Baker, Albany/CA), the ATP/ADP translocator gene (courtesy of Prof. C. Leaver, Edinburgh), the maize-derived waxy gene (courtesy of Dr. Z. Schwarz-Sommer, Köln), the cytosolic glyceraldehyde-3-phosphate-dehydrogenase gene (courtesy of Dr. W. Martin, Köln), and the ribosomal gene (courtesy of Prof. K. Yakura, Kanazawa).

Results

Tissue culture

For the molecular analysis of somaclonal variation in Indica-type rice lines, regenerants from two different experiments were chosen. First, plantlets were regenerated from a single, immature-embryo-derived callus via somatic embryogenesis, after a callus induction phase of 28 days on medium containing 2,4-D, followed by subculture on regeneration medium (CCIZ). In the second experiment, regenerants were obtained from two different calli after 67 days on callus induction medium and were subsequently regenerated on CC-based medium without any hormones. The frequency of plants showing signs of phenotypic variation such as dwarfism or albinism was less than 1% (data not shown).

Conditions for RFLP analysis

The first requirement for a successful RFLP analysis of the regenerated plants was to define a suitable restriction enzyme and gene marker system. This had to be sufficient to distinguish between different rice cultivars as well as to expose any variation within them after regeneration. As rice is an inbred species with outcrossing being the exception, the parental control line is not expected to exhibit heterozygosity. Both 4- and 6-bp recognition restriction enzymes were tested. With most of the probes tested, we found that a stronger signal and larger fragment size was obtained with 6-bp enzymes such as HindIII. Of the characterized sequences used for probing, the 3-kb HindIII fragment of the soybean actin gene produced the best results, with a number of strongly hybridizing fragments and an ability to clearly differentiate between different rice lines (Fig. 1 A). Probes such as the maize ATP/ADP translocator gene also gave strong signals, although the ability to differentiate between different lines was reduced (Fig. 1 B). Extensive probing of genomic DNA

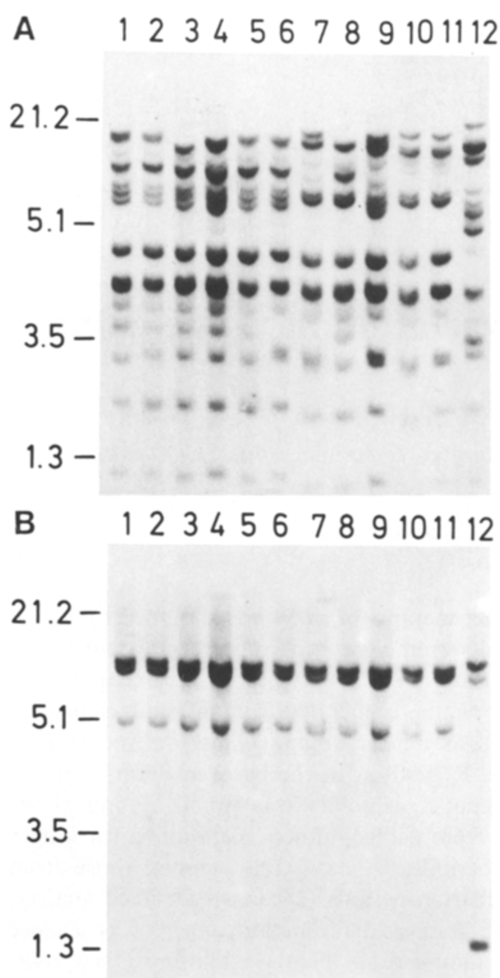

Fig. 1 A, B. RFLP analysis of different rice lines: genomic DNA digested with restriction enzyme HindIII and probed with a 3-kb actin gene fragment (**A**), and the same filter probed with a 1.2-kb ATP/ADP translocator gene fragment (**B**). *Tracks 1–9: Oryza sativa* L. (ssp. Indica); *1* IR 54, *2* IR 64, *3* IR 28150, *4* IR 28288, *5* IR 31802, *6* IR 31868, *7* IR 32429, *8* IR 35366, *9* IR 39385. *Tracks 10–11: Oryza sativa* L. (ssp. Japonica); *10* Minehikari, *11* Yamabiko. *Track 12 Oryza longistaminata*

from large numbers of control, seed-grown IR40 plants with the different restriction enzyme/gene combinations, including the HindIII/actin gene, revealed no obvious heterogeneity between seed-grown control plants (data not shown).

Analysis of regenerants

Preliminary experiments demonstrated that RFLP analysis of plants regenerated from different calli exhibit DNA polymorphisms regardless of whether the plants had a normal or deviant phenotype (data not shown). To determine whether such polymorphism could also be detected between sibling plants derived from the same callus and whether the length of culture as undifferentiated

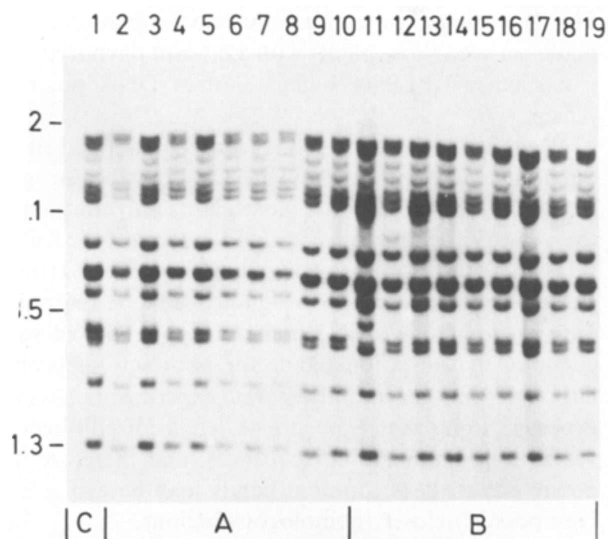

Fig. 2. RFLP analysis of regenerated IR 40 rice plants. *A* – plants from short callus induction phase (28 days); **B** – plants from long callus induction phase; *C* control plant DNA. Genomic DNA digested with restriction enzyme HindIII and probed with 3-kb actin gene fragment

callus affected DNA constancy in the subsequently regenerated plants, genomic DNA from plants regenerated from calli maintained on 2,4-D supplemented medium for two different time periods was undertaken. The plants tested were a random assortment of phenotypically normal and abnormal plants of the line IR 40. The results demonstrate that not only can DNA polymorphisms occur between plants derived from the same callus, but that the longer cultures are kept as callus, the greater the number of regenerants that subsequently show DNA polymorphisms (Fig. 2).

Comparative analysis of the amount of DNA polymorphism reveals that plants derived from short-term cultures (28 days) (Fig. 2A) only infrequently show polymorphisms. Conversely, long-term callus culture (67 days) (Fig. 2B) results in a large number of RFLP (tracks 11, 12, 13, and 17), as demonstrated here for the actin gene. The overall percentage of plants exhibiting such variation was low: approximately 19% of the regenerated IR 40 plants examined showed RFLP variations, with plants regenerated after 28 or 67 days in culture showing 6.3% or 23% RFLP changes, respectively. Thus, doubling the culture period (from 28 to 67 days) resulted in a fourfold increase in the level of variation detectable in one gene.

Figure 3 demonstrates that, in plants regenerated after longer callus culture periods, the RFLP pattern was considerably different from the polymorphism seen in the plants regenerated after short culture periods. Figure 3A represents the RFLP analysis of the actin gene in a number of phenotypically normal and abnormal sibling

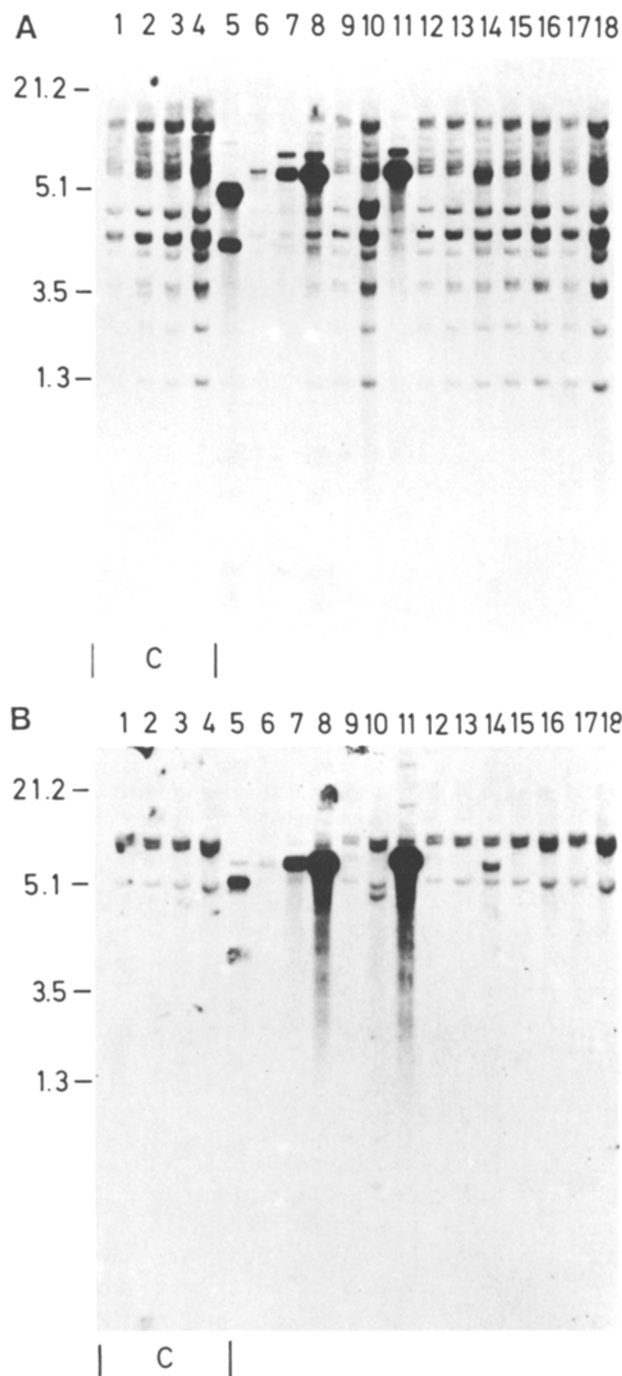

Fig. 3A, B. RFLP analysis of regenerated IR 40 rice plants derived from a single callus. Genomic DNA digested with restriction enzyme HindIII and probed with a 3-kb actin gene fragment (**A**), and the same filter probed with a 1.2-kb ATP/ADP translocator gene fragment (**B**). *C* – Control plant DNA

IR 40 plants, all derived from the same callus. The results clearly demonstrate that both genetically normal and abnormal plants can be derived from a single callus. The degree of polymorphism ranged from the apparent loss of one restriction site (track 14) to plants where there had obviously been significant DNA alterations (tracks 5 and

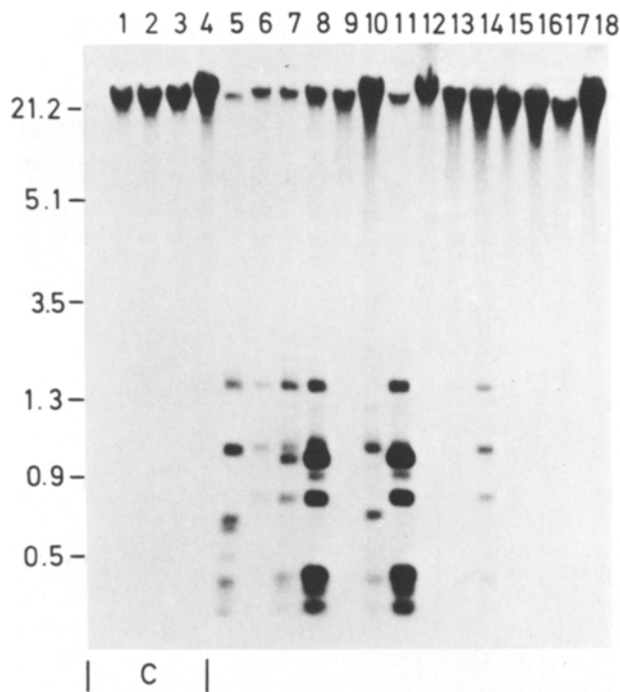

Fig. 4. RFLP analysis of regenerated IR 40 rice plants derived from a single callus. The same genomic DNA samples as in Fig. 3 were now digested with restriction enzyme DpnI and probed with a 3-kb actin gene fragment. *C* – Control plant DNA

11). The results also confirm that no correlation can be made between those plants with a deviant phenotype and a particular RFLP or higher level of DNA polymorphism.

Removal of the actin probe from the filter and subsequent probing with the ATP/ADP translocator gene (Fig. 3 B) showed that only those plants polymorphic for the actin gene are correspondingly polymorphic for the ATP/ADP translocator gene, which suggests that these plants may have undergone significant DNA rearrangements throughout their genome. The presence of some common hybridization bands for both sets of probes, although found in several separate experiments, was unexpected. Long-term exposure of dehybridized filters revealed no contaminating sequences, and therefore it is possible that these common bands may be artifacts or even possibly closely homologous regions.

One possible explanation for these results, since HindIII is a methylation-sensitive enzyme, is that restriction sites are lost when the 5′ adenosine of the recognition sequence is methylated, i.e., in the presence of 6-methyladenine (6-mAd). This base is generally regarded exclusively as a constituent of prokaryotic DNA. To determine whether the results were a reflection of direct DNA polymorphism or the presence of the minor base 6-mAd, identical digestions to those used in Fig. 3 were made using the

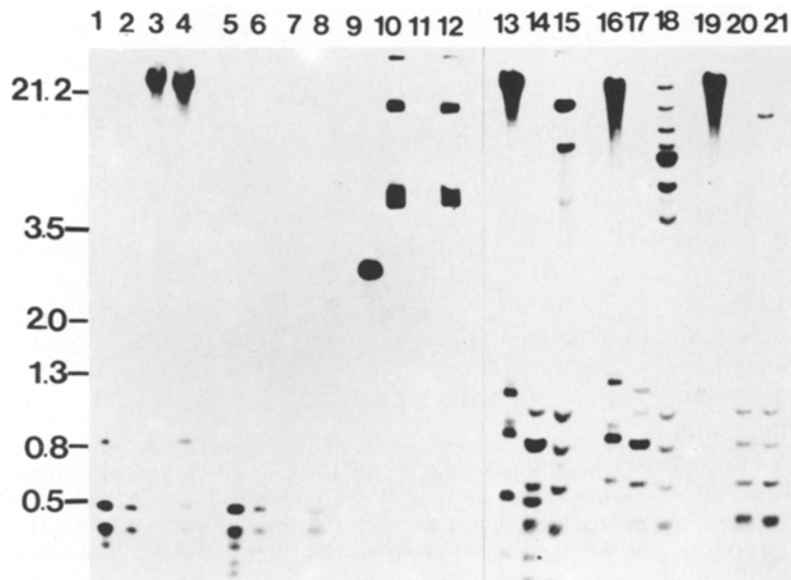

Fig. 5. RFLP analysis of plasmid and genomic DNA from control (*tracks 1–12*) and three regenerated rice plants (*tracks 13–21*). The 3-kb actin gene fragment was digested with DpnI (*track 1*), Sau3A (*track 5*), or MboI (*track 9*). The actin-gene-containing plasmid DNA was similarly digested with DpnI (*track 2*), Sau3A (*track 6*), or MboI (*track 10*). Genomic DNA from control plants was digested together with plasmid DNA, with DpnI (*track 4*), Sau3A (*track 8*), or MboI (*track 12*); genomic DNA from control plants was digested without plasmid DNA, with DpnI (*track 3*), Sau3A (*track 7*) or MboI (*track 11*). *Tracks 13–21* show genomic DNA from three different regenerants digested with DpnI (*13, 16, 19*), Sau3A, (*14, 17, 20*) or MboI (*15, 18, 21*)

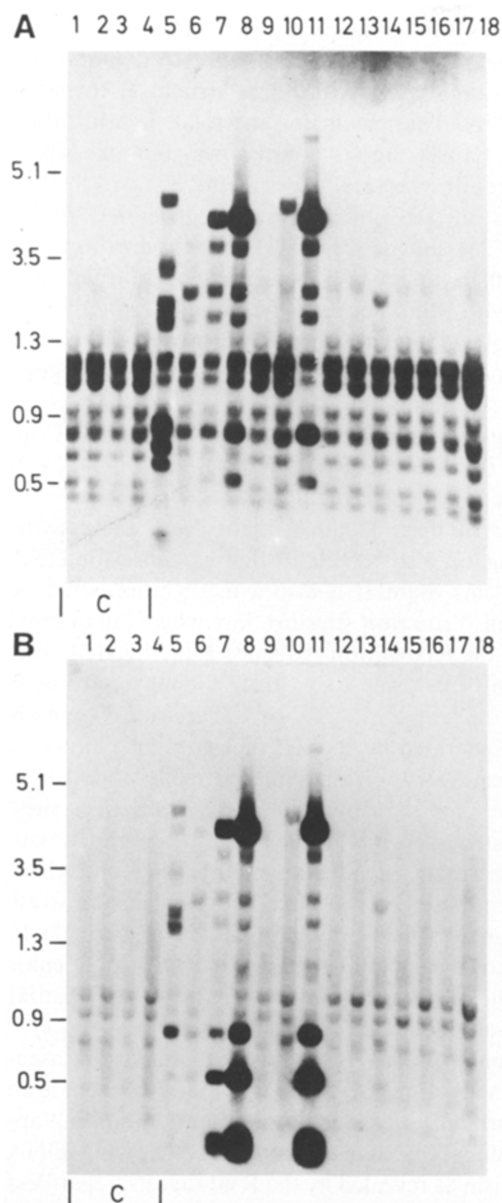

Fig. 7 A, B. RFLP analysis of regenerated IR 40 rice plants derived from a single callus. The same genomic DNA samples as in Figs. 3, 4, and 6 were now digested with restriction enzyme RsaI and probed with a 3-kb actin gene fragment (**A**), and the same filter was probed with a 1.2-kb ATP/ADP translocator gene fragment (**B**). *C* – Control plant DNA

generated rice plants. Arguments as to the causes of somaclonal variation have revolved around whether this is due to genetic heterogeneity of the explant or whether it is a reflection of changes induced by tissue culture. The results here, which show that there can be significant genetic differences even between plants regenerated from a single callus, would tend to substantiate the experiments of Edallo et al. (1981) and Fukui (1983), who suggested that callus cultures contain genetically different sectors. The dissimilarity of some of the RFLP patterns obtained from sibling plants in these experiments would tend to confirm that callus should be regarded as a genetic mosaic rather than as genetically homogeneous.

Significantly lower levels of DNA polymorphism could be detected in plants regenerated from callus maintained for short periods, compared to the obvious and sometimes extensive variation found in some regenerants from longer-maintained cultures. Although comparisons were made from only two culture periods and two regeneration media, this was the only dissimilarity between the two sets of plants. Hence, the differences in DNA stability shown by RFLP analysis between plants regenerated from these two periods would suggest that length of the callus growth phase may be one of the major factors in the induction of molecular and somaclonal variation. Whether this effect is due to the maintenance of a proliferative callus phase or to the associated long exposure to such potential mutagens as 2,4-D is not clear, and is the subject of further investigation. Regeneration of maize plants resulted, in many cases, in extremely high levels of phenotypic variation (Brown 1989). Although no direct correlation was made between subsequent molecular analysis and this disturbance of phenotype, it would be logical to assume that high levels of genetic variation would be reflected in a large percentage of plants showing genetic changes. Among rice regenerants, the level of phenotypic variation was considerably lower than that found in maize, regardless of whether the plants were regenerated from long- or short-term cultures. Nevertheless, plants with a normal phenotype could still reveal an altered DNA restriction pattern. Consequently, the difference in level of DNA polymorphism between maize and rice as a result of tissue culture is very small compared to the large differences in phenotypic change.

RFLPs can arise by two mechanisms: (1) direct point or structural mutations, or (2) a change in methylation pattern. In this work we demonstrate that a change in methylation pattern may explain some of the genetic variation seen among regenerated plants. Furthermore, these results indicate clearly an elevation of 5-methylcytosine as well as the possible presence of the minor base 6-methyladenine, in a percentage of tissue-culture-derived plants.

The latter result is totally unexpected, as there is little evidence for the presence of 6-methyladenine in eukaryotic DNA. Despite an extensive search of the literature, we have been able to find only few references for the presence of this base in higher plant DNA (Dunn and Smith 1958; Vanyushin et al. 1971). The fact that both control plants and regenerants were grown under the same conditions would tend to limit the possibility of a bacterial contamination affecting only regenerants. Restriction of DNA by the enzyme DpnI is dependent entirely on the presence of 6-mAd. Therefore, the presence

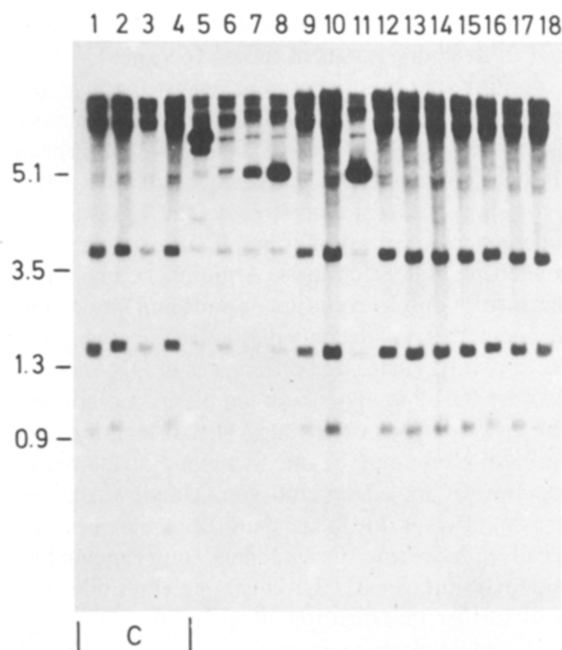

Fig. 6. RFLP analysis of regenerated IR 40 rice plants derived from a single callus. The same genomic DNA samples as in Figs. 3 and 4 were now digested with restricition enzyme BamHI and probed with a 3-kb actin gene fragment. *C* – Control plant DNA

restriction enzyme DpnI. This enzyme is useful in that digestion will only occur in the presence of 6-mAd. Analysis of this digest with the actin gene (Fig. 4) reveals that there is no digestion of control plant DNA, confirming the low level of this base in eukaryotic DNA and the fact that only those plants that show polymorphisms in Fig. 3 do, in fact, show a number of low-molecular-weight bands upon DpnI digestion. The possibility that this apparent restrictability is an artifact due to bacterial contamination can be readily discounted.

To confirm whether tissue culture had resulted in an increase in the amount of 6-mAd, further analysis using the DpnI isoschizomers Sau3A and MboI was undertaken (Fig. 5). To ensure that the results were not an experimental artifact due to incomplete DNA digestions, the actin gene containing plasmid was introduced into the dam+ *E. coli* strain MC 1061 and subjected to digestion by these enzymes, as both an isolated fragment (tracks 1, 5, and 9), and as a plasmid (tracks 2, 6, and 10). Control plant DNA was also correspondingly digested both with (tracks 4, 8, and 12) and without (tracks 3, 7, and 11) plasmid DNA. The results show that all three control DNAs react to digestion in an expected way, with control plant DNA showing resistance to digestion with DpnI. Although the DNA is susceptible to restriction with both MboI and Sau3A, the similarity in hybridization patterns confirms that there is no evidence for any methylated adenine residues. In the actin fragment and plasmid DNA, growth in a dam+ *E. coli* host resulted in the adenine bases becoming methylated. The three bands present in the MboI-digested plasmid DNA do not represent restriction sites but the three structural forms of plasmid DNA. The reason for the weak hybridization signal with Sau3A digests is unknown, but this was a consistent result in several experiments.

Analysis of DNA from three regenerated plants (tracks 13–21) shows differences in hybridization pattern, particularly in the low-molecular-weight fragments. Digestion with MboI suggests that a number of adenine bases are not methylated. However, comparative analysis of these bands with those from Sau3A and DpnI digests for two of the plants (tracks 12–15 and 16–18) would indicate that adenine methylation has occurred. Conversely, the third regenerated plant (tracks 19–21) shows no evidence for adenine methylation.

Analysis of these identical plants with the enzyme BamHI (Fig. 6), also reveals further evidence for DNA polymorphisms. BamHI is also a methylation-sensitive enzyme, and restriction sites are lost when the internal cytosine of the recognition sequence is methylated. This methylation of cytosine to produce 5-methylcytosine is widespread in eukaryotic DNA. Therefore, polymorphism demonstrated by BamHI digestion may either be due to direct DNA changes or indirectly to cytosine methylation and subsequent loss of restriction sites. There is a strong correlation between those plants with apparent increases in adenine methylation and those plants which, based on the BamHI digests, demonstrate alterations in cytosine methylation. Two plants (tracks 10 and 14), however, revealed changes only in adenine methylation, and their restriction patterns on BamHI digestion were identical to controls.

Digestion of plant DNA with the methylation-insensitive enzyme RsaI (Fig. 7) demonstrates that there is an absolute correlation between those plants exhibiting apparent methylation changes and those showing DNA polymorphism as revealed by the RsaI digests, regardless of whether the probe used is the complete actin gene (Fig. 7A) or the ATP/ADP translocator gene (Fig. 7B).

Discussion

The aim of these experiments was to determine whether, as a consequence of tissue culture of rice, any resulting DNA rearrangements could be detected by RFLP analysis even between sibling plants produced from the same callus; secondly, whether extended culture periods as undifferentiated calli affected the subsequent genetic constancy of the regenerated plants; and thirdly, whether the new observed RFLPs were due to actual DNA sequence alterations or to changes in the methylation patterns.

The results demonstrate that tissue culture can be responsible for significant DNA alterations in some re-

of hybridization bands cannot be regarded as a polymorphism based on changes in recognition sequence. The fact that in the control plants there was no digestion would strongly support the theory that this base is not present normally in eukaryotic DNA, while the evidence of DNA digestion from tissue culture-derived plants would indicate that, in a number of these plants, this base may be present.

Further analysis with methylation isoschizomers and a range of different controls also indicates that this base is present. Confirmation of this result will be dependent, however, on more direct evidence such as HPLC analysis. This will presently be undertaken.

Analysis of regenerants with such pronounced methylation changes, by nonmethylation-sensitive enzymes such as RsaI, reveals that there is a direct correlation between those plants with methylation changes and those with DNA alterations. Work with *E. coli* (Coulondre et al. 1978) and the human genome (Barker et al. 1984), suggests that there was a correlation between mutational „hotspots" and methylated sequences. It is conceivable, therefore, that the results here may be a reflection of one of two opposing hyptheses: namely, that tissue culture stress results in an increase in DNA methylation levels which, as potential mutation hotspots, result in the DNA polymorphisms detected here or, conversely, that tissue culture stress results in direct DNA rearrangements in sequences that are then subsequently transcriptionally inactivated in an attempt to silence potentially lethal genes.

In conclusion, therefore, it is clear that tissue culture and, in particular, the length of time during which the callus phase is maintained, is an important factor in the degree of DNA variation subsequently found in regenerated plants. There has been considerable discussion as to whether variation in regenerated plants is due to tissue culture production of, and selection for, mutant types, or whether it is a reflection of genetic heterozygosity in the starting material (Lörz and Scowcroft 1983). The results here clearly suggest that such variation found in regenerants is predominantly due to the stress of tissue culture.

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