Theor Appl Genet (1987) 74:65–70

Nuclear DNA amplification in cultured cells of *Oryza sativa* L.

K. L. Zheng [1, *], S. Castiglione [1], M. G. Biasini [1], A. Biroli [1], C. Morandi [1] and F. Sala [2]

[1] Department of Genetics and Microbiology, University of Pavia, Via S. Epifanio, I-27100 Pavia, Italy
[2] Institute of Botany, University of Parma, Via Farini, Parma, Italy

Received November 6, 1986; Accepted December 24, 1986
Communicated by F. Salamini

Summary. Highly repeated nuclear DNA sequences from suspension cultured cells of *Oryza sativa* L. cv. 'Roncarolo' have been cloned in pBR322. Ten clones with specific digestion patterns have been randomly selected. Nine sequences appear to be organized in a clustered tandem array while one is interpersed in the rice genome. The clones have been used to gather information on: (a) their modulation in cultured cells as compared to whole plant and (b) their distribution in different rice cultivars belonging to the *Japonica* or *Indica* subspecies of *Oryza sativa* L. Hybridization with nuclear DNA isolated either from suspension or from seedlings of the 'Roncarolo' cultivar revealed extensive quantitative variations, with most cloned sequences showing amplification (up to 75-fold) in cultured cells. Hybridization with nuclear DNA isolated from seedlings or suspension cultured cells from different cultivars belonging to the *Japonica* or to the *Indica* subspecies of *O. sativa* have shown that (a) amplification also occurs in a similar pattern in the case of DNA from the other tested suspension cultured cell types but not in the case of DNA from seedlings; (b) in some cases the tested sequences show minor but significant variations in different rice accessions.

Key words: DNA amplification – Cultured cells – Dot hybridization – *Oryza sativa* L.

Introduction

A large proportion of the plant nuclear genome is made up of repeated DNA sequences (Flavell 1982; Evans et al. 1983). These sequences, with few exceptions, are not transcribed and play a yet unknown function.

The electrophoretic analysis of restriction endonuclease fragments obtained from the nuclear DNA of distantly related plant species, as well as cross-hybridization experiments, have shown a large degree of species-specificity of repeated sequences (Sals et al. 1985).

Species-specificity of repeated sequences has also been demonstrated in closely related species (Ranjekar et al. 1978; Evans et al. 1983; Pental and Barnes 1985; Metzlaff et al 1986). In the case of rice, qualitative differences were found when two repeated sequences isolated from *Oryza sativa* were used as hybridization probes for the DNA from different species of the same genus (Pental and Barnes 1985).

De Paepe et al. (1982) found heritable quantitative and qualitative changes in the nuclear DNA of homozygote diploid plants obtained by consecutive cycles of pollen culture of *Nicotiana sylvestris*: repeated sequences increased with additional haploid cycles.

However, too scanty data are available on the modulation of repeated sequences in the plant kingdom: the study of highly preserved repeated sequences may shed light on their functional significance while species-specific sequences might be used as markers to study phylogenetic relationships. We also need more data on the modulation of repeated sequences in specific plant species during the differentiation or dedifferentiation (induction of callus growth) processes.

We have thus decided to isolate and characterize a number of highly repeated sequences from rice and to verify both their modulation when passing from in vivo (leaves) to in vitro (suspension cultured cells) and their presence in different rice cultivars belonging to the *Japonica* and *Indica* subspecies of *Oryza sativa* L.

Materials and methods

Materials

Cellulase "Onozuka" RS was from Kinkly Yakult MFG Co. LTD, Japan; Pectinase was from Serva; Proteinase K, Ribo-

* On leave from China National Rice Research Institute, Hangzhou, China

nuclease A and T1 and the restriction endonucleases were from Boehringer; α-^{32}P-dCTP and the "Multiprime Labelling System" (RPN. 1601) were from Amersham.

Cell cultures and seedling growth

The following rice (*Oryza sativa* L.) cultivars were used: 'Roncarolo', 'Arborio', 'Balilla' (all belonging to the subspecies *Japonica*) 'Dourado precoce' and 'IR50' (both of the subspecies *Indica*). All seeds were kindly supplied by Ente Nazionale Risi, Mortara, Pavia, Italy.

Suspension cultures of the rice cvs. 'Roncarolo' and 'Dourado precoce' were established and serially subcultured in the R2 culture medium as described (Sala et al. 1985). At the moment of use cell cultures were 8 years old in the case of 'Roncarolo' and 3 months old in the case of 'Dourado precoce'.

Seeds were sterilized (Sala et al. 1979) and grown on ½ MS medium.

Preparation of nuclear DNA from protoplasts

Leaf protoplasts were prepared from 14 day-old etiolated seedlings by cutting them into longitudinal strips and then digesting these with 2% cellulase RS, 0.5% driselase and 0.5% macerozyme in 0.6 M mannitol and salts (pH 5.8) essentially as described by Lai and Liu (1978). Protoplasts from suspension cultured cells were isolated as described previously (Sala et al. 1985).

Nuclei were isolated essentially as described by Kirk et al. (1970) by resuspending freshly prepared protoplasts in a buffer containing 0.05 M Tris-HCl (pH 7.6 at 0 °C), 0.1 M EDTA, 0.5 M sucrose and 1% Triton-X-100.

Nuclear preparations were lysed essentially as described by Blin and Stafford (1976) by resuspending the nuclear pellet in SSC buffer (150 mM NaCl, 15 mM Na-citrate, pH 7.0) containing 10 mM EDTA, 0.5% Na dodecyl sulphate and 100 µg/ml proteinase-K and incubating at 42 °C for 4 h. DNA was extracted and purified from RNA and proteins by standard procedures (Kirk et al. 1970). From 20 to 40 µg of high molecular weight nuclear DNA was usually obtained from 50×10^6 protoplasts.

Extraction and purification of cloned sequences

Rapid isolation of plasmid DNA was performed with the alkaline lysis method as described by Maniatis et al. (1982). The procedure yields DNA virtually free from protein and RNA.

Quantification of DNA

This was routinely performed by spotting serial dilutions of DNA samples onto a plastic wrap, mixing the samples with a solution of ethidium bromide (2 µg/ml) and photographing the spots under a UV illuminator, as described by Maniatis et al. (1982). Quantification was performed by comparing the intensity of fluorescence in the samples with that of standard DNA solution.

Restriction endonuclease digestion and agarose gel electrophoresis

Restriction digestions were usually performed at 37 °C for 2–3 h with 2.5 units of enzyme per µg DNA. Digestions were carried out in the buffer recommended by the supplier. After digestion the proteins were eliminated by phenol extraction. Horizontal agarose gels were run in TBE (89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA) with agarose concentrations varying between 0.6 and 0.9% (W/V).

Electrophoresis was at 70 V for 3–4 h or at 30 V overnight. The gel was stained with ethidium bromide (0.5 µg/ml).

Labelling of DNA

Nick translated DNA was used for colony hybridization. It was prepared essentially as described by Maniatis et al. (1982), using DNA polymerase I and α-^{32}P-dCTP. On average $1–2 \times 10^8$ d.p.m. were incorporated per µg of DNA.

In all other cases the "Multiprime Labelling System" was used, following the procedure described by the manufacturer. On average, 2×10^9 d.p.m. of α-^{32}P-dCTP were incorporated per µg of DNA.

Colony and dot-blot hybridization

For colony hybridization, *E. coli* colonies were lysed and fixed on nitrocellulose filters following the procedure of Grunstein and Hogness (1975). Hybridization with radioactive nuclear DNA was carried out overnight at 42 °C, in 50% formamide, containing 4×SSC, 0.1 M Na phosphate, 0.5% SDS, 4×Denhardt's solution, 25 µg/ml herring sperm DNA.

Dot-blot hybridization was performed essentially as described by Kafatos et al. (1979) and modified by Gasser et al. (1982) by blotting 0.4 µg nuclear DNA on nitrocellulose filters (Schleicher and Schuell, BA85) placed in a microsample filtration manifold (Minifold) from Schleicher and Schuell, USA.

Pre-hybridization and hybridization with the labelled pladmid DNA was performed essentially as described by Maniatis et al. (1982).

Autoradiographs were obtained by exposure to a Kodak film (X-Omat SO-282), dots were then cut and radioactivity counted in a Packard scintillation mixture using a Packard Tri-Carb (C2425) liquid scintillation spectrometer.

Southern blotting

DNA fragments in agarose gels were partially depurinated by two 15-min treatments with 0.25 M HCl and transferred to nitrocellulose filters essentially as described by Southern (1975). The filters were then hybridized with radioactive DNA probes.

Results

Cloning of rice highly repeated sequences

DNA was isolated from nuclei of cultured rice cells (cv. 'Roncarolo') digested with BamHI, ligated to BamHI-digested pBR322 and used to transform *E. coli*.

One hundred and ninety-two bacterial clones containing plasmids with an insert have been randomly selected. Out of these, 13 clones harboring plasmids with highly repeated sequences have been identified by in situ colony hybridization with ^{32}P-labelled rice DNA (Fig. 1).

Size of the inserts and restrictions patterns

The 13 plasmids carrying the identified highly repeated sequences were digested with BamH1 (Fig. 2) and BglII, ClaI and EcoRI (Fig. 3A, B and C) and electro-

phoresed on an agarose gel. Most of the inserts are of 4–5 Kbp; a few of them (pD5 and pD11) are of 2–2.5 Kbp.

The results also show that pD11, pE2 and pE4 have an insert of a similar size (Fig. 2) and share the same restriction sites (Fig. 3). Thus it was assumed that they are identical. The same situation was found for pE7 and pE10.

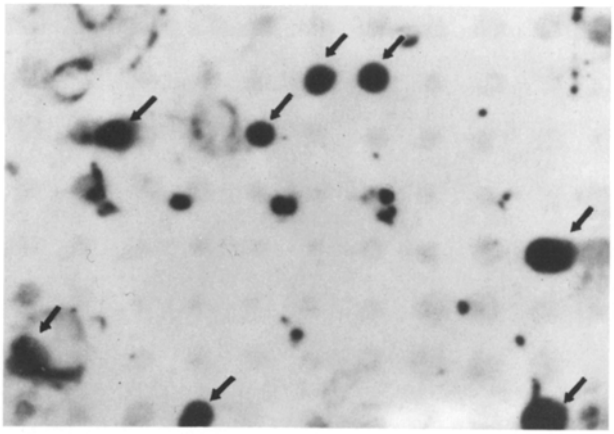

Fig. 1. Colony hybridization of 96 *E. coli* colonies containing BamHI sequences of rice nuclear DNA inserted in pBR322. The colonies were probed with rice ^{32}P-labelled nuclear DNA. Arrows indicate clones that have been selected for the insertion of highly repeated plant DNA sequences, as indicated by the strong radioactive signal in the autoradiography

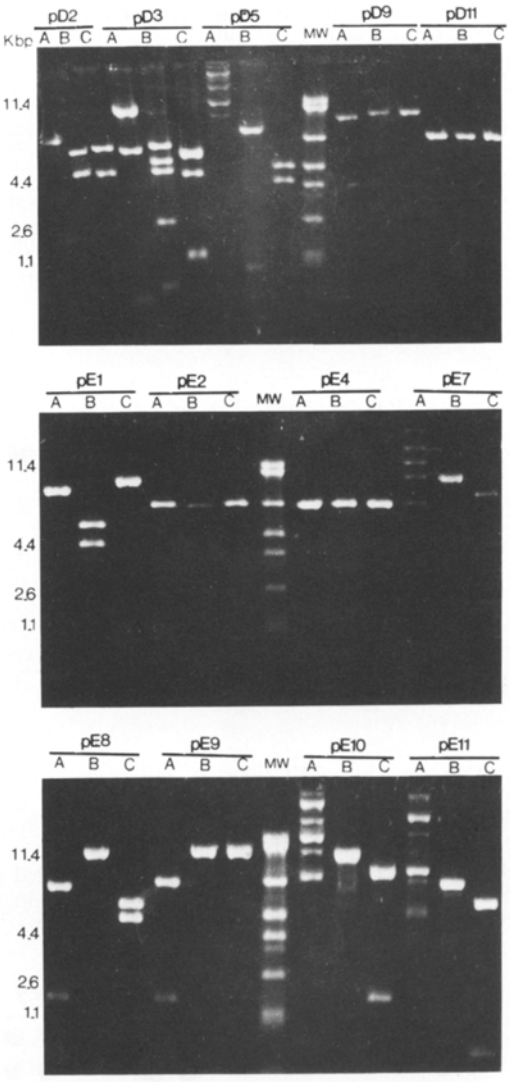

Fig. 3. Electrophoresis in 0.7% agarose of rice highly repeated sequences after digestion with BglIII (A), ClaI (B) and EcoRI (C). Experimental details were as Fig. 2

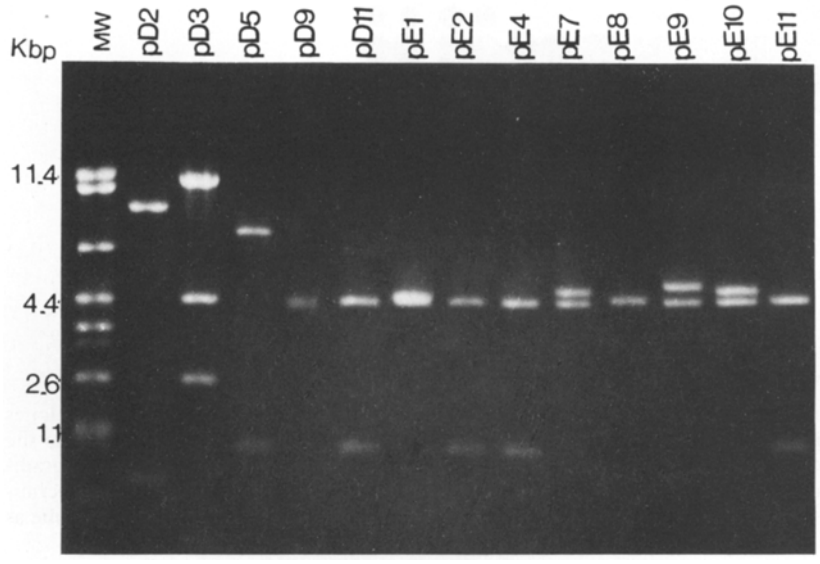

Fig. 2. Electrophoresis in 0.7% agarose gel of rice highly repeated sequences after digestion with BamHI. 0.5 μg of digested DNA was used for each lane. Molecular weight markers were produced by ClaI digestion of λ phage DNA

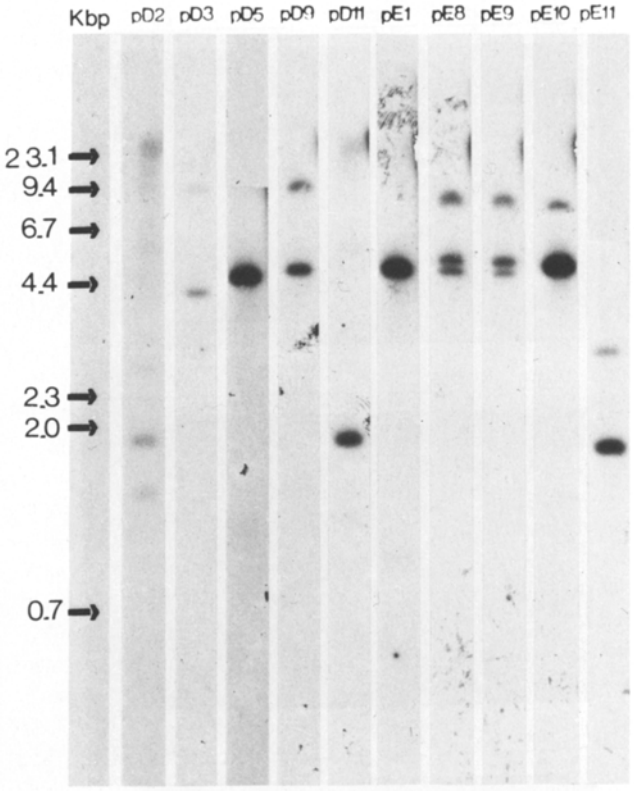

Fig. 4. Southern blots of nuclear DNA of *O. sativa* cv. 'Roncarolo' after digestion with BamHI and electrophoresis on a 0.7% agarose gel. Blots were hybridized with the ^{32}P-labelled plasmid DNAs indicated in the figures. Molecular weight markers were produced by HindIII digestion of λ phage DNA

Subsequent experiments were thus restricted to the 10 plasmids: pD2, pD3, pD5, pD9, pD11, pE1, pE8, pE9, pE10, pE11.

The sequence cloned in pD3 is a ribosomal DNA fragment, as assessed by Southern blot hybridization with a cloned flax rDNA sequence (not shown).

Distribution in the nuclear genome

Following restriction endonuclease digestion and agarose gel electrophoresis of nuclear DNA, repeated DNA will predominantly give a single strong band if present in tandem array while it will appear as a smear if interspersed in the genome.

To gather information on the distribution of the cloned, highly repeated sequences in the genome of the cv. 'Roncarolo', the 10 ^{32}P-labelled plasmids were hybridized to Southern blots of BamHI-degested 'Roncarolo' nuclear DNA electrophoresed on an agarose gel. As Fig. 4 shows, the rice sequence cloned in pD2 is interspersed in the genome, while the others appear to be essentially organized as clustered tandem sequences.

Distribution of the cloned DNA sequences in the DNA obtained from different rice sources

The cloned DNA sequences were isolated from suspension cultured 'Roncarolo' cells. It was of interest to verify if qualitative or quantitative variations occur: (a) during the dedifferentiation processes from in vivo

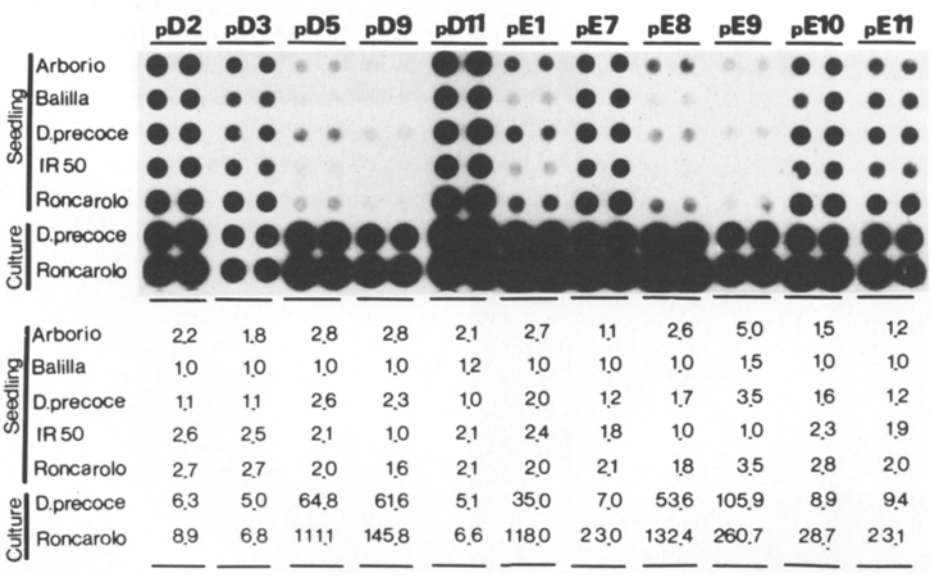

Fig. 5. Dot hybridization of nuclear DNAs from leaves or cultured cells of different *O. sativa* genotypes with 11 ^{32}P-labelled clones of DNA repeated sequences isolated from cultured cells of the cv. 'Roncarolo'. 0.4 µg of each DNA preparation was bound to the nitrocellulose filter and hybridized with labelled DNA (5×10^7 d.p.m. of a probe at $1.5–3.0\times10^9$ d.p.m. µg^{-1}). After autoradiography, spots were utilized for radioactivity count. Dots are in replicas, while quantitative data are the average of the two determinations. Data reported in the table are the ratios among the counts, taking the lowest value obtained with each radioactive probe as 1.0 (which was 1,825, 625, 178, 106, 5,284, 342, 1,207, 233, 60, 812, 709 from pD2 to pE11, in that order)

(plant) to in vitro (cell culture) conditions or (b) among rice cultivars of different origin.

To this purpose, nuclear DNA was extracted from suspension cultured cells of cv. 'Roncarolo' and 'D. Precoce' and from leaves of 'Roncarolo', 'D. Precoce', 'Arborio', 'Balilla' and 'IR50', and blotted in equal quantity (0.4 μg/spot) into nitrocellulose strips. The two identical sequences in pE7 and pE10 were both included in the experiment.

The DNA on the blots was then hybridized with ^{32}P-labelled plasmid DNA probes. The results as seen in Fig. 5 show no qualitative differences in the tested rice cultivars, either in the differentiated or in the undifferentiated state. However, remarkable quantitative differences are observable. The most striking are those found within the same cultivar ('Roncarolo' and 'D. precoce') when comparing leaves and cultured cells: all tested sequences are amplified in the undifferentiated cells, with amplification ranging from a factor of about 3 (pD3, pD11) to a factor of about 100 (pD9, pE1, pE8, pE9). This does not seem to be directly related to the period of time the cells were kept in culture (8 years in the case of 'Roncarolo') since a recently established cell culture (3 months in the case of 'D. precoce') also shows similar behaviour.

At a lower degree quantitative variations are also to be observed when comparing seedlings from different rice cultivars. Cv. 'Balilla' shows the lowest copy number for most tested repeated sequences while the others show amplification up to 5 times from the lowest tested values.

Discussion

Ten highly repeated DNA sequences have been cloned from suspension cultures of the rice cultivar 'Roncarolo'. The clones are different, at least on a basis of size of insert and restriction enzyme pattern. However, as cross-hybridization experiments between clones have not been performed we cannot be sure that some of them are not somehow related. Indeed, some clones hybridized to bands of similar size in the plant digest.

Of the ten isolated sequences, nine were found to be rice specific while one (pD3), a ribosomal DNA fragment, was common to all tested species (*Daucus carota, Lycopersicon esculentum* and *Nicotiana plumbaginifolia*), as assessed by in situ colony hybridization (to be published).

The cloned sequences were used as probes to assess their distribution in different rice cultivars as well as their modulation during tissue dedifferentiation.

The results reported in this paper have shown that the sequences are present in all tested rice cultivars, both in leaves and in cultured cells. However, in the case of leaves of the five tested cultivars, significant quantitative differences were found, with some repeated sequences present at a multiplicity of 3–5 times as compared, in each case, to the lowest value. The pattern of distribution of the tested probes in the different cultivars can thus be proposed as a tool for the recognition of different genotypes.

From an evolutionary point of view, no clear correlation can be drawn between the results reported in Fig. 5 and the classification of the assayed genotypes within the *Japonica* or *Indica* subgroup. In fact, the differences noticed within the same subspecies ('Arborio', 'Balilla', and 'Roncarolo' of the *Japonica* group and 'D. precoce' and 'IR50' of the *Indica* group) are as marked as those among subspecies. 'Balilla' shows the lowest amplification values for all tested sequences, while some sequences are amplified up to five times in the other two closely related Italian cultivars ('Arborio' and 'Roncarolo') as well as in 'D. precoce' (from Brazil) and 'IR50' (from the Philippines).

On the other hand, striking quantitative variations were observed when comparing the distribution of the selected repeated sequences in the nuclear DNA of leaves and cultured cells. All selected sequences had undergone amplification (from 1 to 74 times) when cells were cultured in the undifferentiated state.

The amplification does not depend on the age of cultured cells since it is evident in both long (8 years for 'Roncarolo') and newly (3 months for 'D. precoce') established cultures. Furthermore, the extent of amplification does not depend on the subspecies group, since 'Roncarolo' (Japonica) and 'D. precoce' (*Indica*) show a similar amplification pattern.

All tested sequences turned out to be amplified when going from the in vivo to the in vitro cell condition; this poses a number of questions. What is the reason and functional significance for this extensive phenomenon? Is there a modulation of these sequences during the differentiation an dedifferentiation cycles? Are the quantitative changes related to somaclonal variation in regenerated plants? What is the relation between these amplification phenomena and chromosome number modifications in cultured cells? As for the latter question we have observed a tendency of our cultured rice cells to retain their typical chromosome number ($2n = 24$) even after years in culture. In the case of cultured 'Roncarolo' cells, from which the cloned DNA sequences were obtained, chromosome number was 21 ± 3 with a mode of 19.

Recently, Oono et al. (1986) gave preliminary results showing variations in the copy number of two rice repeated DNA sequences, one being amplified and the other reduced in copy number in the dedifferentiation process. Significant molecular diversity has also been observed in the mitochondrial DNA of suspension cultured cells of *Vicia faba* as compared to plant mitochondrial DNA (Negruk et al. 1986),

thus suggesting that the whole plant genome may be affected during cell dedifferentiation.

The observed increase in copy number of rice repeated sequences after induction of dedifferentiation cannot be ascribed to changes in ploidy level. In fact, polyploidization would not affect results obtained when hybridization of repeated sequences is performed on identical nuclear DNA quantities. Furthermore, aneuploidy for a few chromosomes would not be sufficient to account for amplification of sequences up to the observed 74 times, even in view of the observed relatively constant chromosome number of rice culture cells.

Thus, how has the nuclear genome been modified in the dedifferentiated cell? The most likely possibility is amplification of the tested repeated sequences, either as chromosomal or extrachromosomal entities. If so, further analysis may reveal increase in the DNA content per nucleus and, perhaps, the appearance of satellite DNA (D'Amato 1978).

An understanding of the molecular bases of the observed modulations of repeated sequences in the different cultural conditions is a prerequisite for the elucidation of their functional significance. To this purpose it would be of interest to follow the behaviour of the amplified sequences during the differentiation of plants from cultured cells. However, an experimental difficulty is represented by the impossibility of verifying the genomic constitution of the few initial cells from which the plants differentiate. A possible material for these studies could be the synchronous embryogenesis system developed in carrot (Giuliano et al. 1983).

Acknowledgements. This investigation was supported by funds of C.N.R., Italy, special grant IPRA, sub-project 1, of the "Biotechnology Action Programme" (n. 0084I) of the Commission of the European Communities, Contract BAP no. 0084-I and of the Ministero della Pubblica Istruzione.

References

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Blin N, Stafford DW (1976) A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res 3:2303–2308

Cohen SN, Chang ACY, Hsu L (1972) Non chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci USA 69:2110–2114

D'Amato F (1978) Chromosome number variation in cultured cells and regenerated plants. In: Thorpe TA (ed) Frontiers of plant tissue culture. University of Calgary Offset Printing Service, Calgary, pp 287–295

De Paepe R, Prat D, Huguet T (1982) Heritable nuclear DNA changes in double haploid plants obtained by pollen culture of *Nicotiana sylvestris*. Plant Sci Lett 28:11–28

Evans IJ, James AM, Barnes SR (1983) Organization and evolution of repeated DNA sequences in closely related plant genomes. J Mol Biol 170:803–826

Flavell RB (1982) Sequence amplification, deletion and rearrangement: major sources of speciation during species divergence. In: Dover GA, Flavell RB (eds) Genome evolution. Academic Press, London, pp 301–325

Gasser CS, Simonsen CC, Schilling JW, Schimke RT (1982) Expression of abbreviated mouse dihydrofolate reductase genes in cultured hamster cells. Proc Natl Acad Sci USA 79:6522–6526

Giuliano G, Rosillini D, Terzi M (1983) A new method for the purification of the different stages of carrot embryoids. Plant Cell Rep 2:216–218

Grunstein M, Hogness (1975) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc Natl Acad Sci USA 72:3961–3965

Kafatos FC, Jones CW, Efstratiadis A (1979) Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res 7:1541–1552

Kirk JTO, Rees H, Evans G (1970) Base composition of nuclear DNA within the genus *Allium*. Heredity 25: 507–512

Lai KL, Liu LF (1978) Studies on the rice protoplasts. Ultrastructural changes during enzymatic isolation. J Agri Assoc of China, N S 102:11–23

Maniatis T, Fritsch EF, Sambrood J (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

Metzlaff M, Troebner W, Baldauf F, Schlegel R, Cullum J (1986) Wheat specific repetitive DNA sequences-construction and characterization of four different genomic clones. Theor Appl Genet 72:207–210

Negruk VI, Eisner GI, Redichkina TD, Dumanskaya NN, Cherny DI, Alexadrov AA, Shemyakin MF, Butenko RG (1986) Diversity of *Vicia faba* circular mtDNA in wholo plants and suspension cultures. Theor Appl Genet 72: 541–547

Nielsen E, Rollo F, Parisi B, Cella R, Sala F (1979) Genetic markers in cultured plant cells: differential sensitivity to amethopterin, azetidine-2-carboxylic acid and hydroxyurea. Plant Sci Lett 15:113–125

Oono K, Kikuchi S, Takaiwa (1986) DNA amplification and diminution in rice callus culture. In: Somers DA, Gengenbach BG, Biesboer DD, Backett WP, Green CE (eds) Abstracts of 6th Int Congr Plant Tissue Cell Culture. University of Minnesota, Minneapolis Minn, (USA), p 287

Pental D, Barnes SR (1985) Interrelationship of cultivated rices *Oryza sativa* and *O. glaberrima* with wild *O. perennis* complex. Theor Appl Genet 70:185–191

Ranjekar PK, Pallotta D, Lafontaine JG (1978) Analysis of plant genomes. 5. Comparative study of molecular properties of DNAs of seven *Allium* species. Biochem Genet 16:957–971

Sala C, Biasini MG, Morandi C, Nielsen E, Parisi B, Sala F (1985) Selection and nuclear DNA analysis of cell hybrids between *Daucus carota* and *Oryza sativa*. J Plant Physiol 118:409–419

Sala F, Cella R, Rollo F (1979) Freeze-preservation of rice cells grown in suspension culture. Physiol Plant 45: 170–176

Saul MW, Potrykus I (1984) Species-specific repetitive DNA used to identify interspecific somatic hybrids. Plant Cell Reps 3:65–67

Southern E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517