

Distribution of telomere DNA in mitotic and polytene nuclei of the anther tapetum of a tetraploid hybrid bean, *Phaseolus vulgaris* x *P. acutifolius*

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ABSTRACT

The distribution of telomeres was analysed by *in situ* hybridization with biotin labelled synthetic oligomers in root tips and anther tapetum nuclei of the amphidiploid *Phaseolus vulgaris* x *P. acutifolius*. Most metaphase telomeres were labelled with probe, although with varying signal strength. Diploid interphase nuclei presented some signals near the nuclear envelope and some scattered throughout the nucleus. Differentiated interphase cells showed more condensed chromocentres with signals at both termini, suggesting more restricted chromosome domains. Polytene cells, on the other hand, presented a proportionally smaller number of signals which were preferentially located near or at the chromocentres. No association between telomeres and the nuclear envelope was observed. Some signals were exceptionally large, indicating fusion or differential amplification. Although the general structure of diploid and polytene nuclei seems to be conserved, some molecular aspects of the nuclear organization may be altered.

INTRODUCTION

After sequencing the telomere repeat of *Arabidopsis* (Richards and Ausubel, 1988), cytological analysis by *in situ* hybridization demonstrated the presence of this sequence at chromosomal extremities of plant species from many different families (Cox *et al.*, 1993). Molecular studies in several organisms have drawn attention to the instability of the repeat number of this sequence in nuclei and chromosomes (Biessmann and Mason, 1994), and particularly in plants it seems to be the most variable sequence of the genome (Broun *et al.*, 1992). The instability of telomere DNA is particularly high in very specialized nuclei, such as the macronuclei of ciliated protozoa (Blackburn, 1991) and in some cells with finite doubling capacity (Harley *et al.*, 1990).

Polytene cells are commonly found in many specialized tissues in plants (Nagl, 1981). Like animal polytene cells, they show increased metabolic activity and are unable to divide mitotically. In contrast to Diptera polytenics, which are interphase structures, plant polytene chromosomes have a condensation-decondensation cycle which makes them visible at prophase as chromatid-bundles and at interphase as large chromocentres (Guerra and Carnevalheira, 1994; Carnevalheira and Guerra, 1994). In fact, they are rarely clearly observed, mainly because the sister-chromatids are not synapsed, as in Diptera, and the terminal regions of each chromosome are generally very diffuse.

The best analysed plant polytene chromosomes are those from embryo suspensor tissue of *Phaseolus*, where they are more amplified and more easily individualised. After *in situ* hybridization with the human telomere probe, Nagl (1991) observed that the embryo suspensor polytenics of *Phaseolus* presents a

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group of dots or compact bands at the telomeres. However, in the polytene nuclei of the anther tapetum, where the chromosomal termini are far more dispersed, telomere sequences have not been demonstrated.

The amphidiploid *Phaseolus vulgaris* x *P. acutifolius* characterized by the presence of large chromocentres and diffuse chromosome ends, is a hybrid extensively studied genetically and cytologically as a tool in the transfer of drought, heat, pest and disease resistance from *P. acutifolius* to *P. vulgaris* (Thomas *et al.*, 1983).

MATERIAL AND METHODS

Plant material

Seeds of the amphidiploid *Phaseolus vulgaris* x *P. acutifolius* cv. 18016/2 were supplied by the Agriculture and Livestock Research Enterprise of the State of Pernambuco (IPA) and were grown in pots in the Department of Genetics of the Universidade Federal de Pernambuco. The plants were partially sterile, each developing only a few pods and about eight seeds per plant.

Slide preparation

Mitotic metaphases were obtained from primary root tips of seedlings germinated in Petri dishes. They were pretreated with 0.002 M 8-hydroxyquinoline at 18°C for 6 h before fixation in ethanol-acetic acid (3:1) overnight at room temperature and storage at -20°C for some months. For analysis of tapetal polytene cells, young flower buds were sliced longitudinally and fixed and stored in the same way as the root tips.

For slide preparation, the root tips were washed twice in distilled water (10 min each), digested with a 2% cellulase-10% pectinase solution at 37°C for 20 min and squashed on a clean slide in a drop of 45% acetic acid. Anthers of different sizes were also washed and macerated in the same enzyme solution at 37°C for 8 min and gently squashed in a drop of 45% acetic acid. After freezing and coverslip removal, slides were desiccated at -20°C for one to seven days before processing.

In situ hybridization

Oligomer primers T1 (5'-TTTAGGG-3')₅ and T2 (5'-AAATCCC-3')₅ were synthesized, amplified and kindly supplied by Anthony Cox (Jodrell Laboratory,

see Cox *et al.*, 1993). The *in situ* hybridization procedure was as described by Parokony *et al.* (1992) except that the hybridization mixture contained 2 x SSC, 50% formamide, 10% dextran sulphate, 450 µg ml⁻¹ autoclaved salmon sperm DNA and biotinylated probe to a final concentration of 5 µg ml⁻¹.

Hybridized biotinylated DNA was detected with fluoresceinated avidin (Vector Laboratories). Total DNA was counterstained with 1 µg ml⁻¹ propidium iodide in water and 2 µg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) in McIlvaine's buffer. Photographs were taken with Kodak Ektachrome 800/1600 colour transparency film, processed at 800 ASA.

RESULTS

The karyotype of the tetraploid hybrid *P. vulgaris* x *P. acutifolius* consists of 2n = 44 two-armed, small and similar-sized chromosomes (Figure 1a). After *in situ* hybridization, almost all chromosomes of root tip metaphase cells were labelled at both terminal regions as one or two dots of bright FITC fluorescence (Figure 1b). Only a few chromosomes were labelled at a single terminal region and none of them was unlabelled.

The absolute size of signals was small (under 0.4 µm), giving the impression of limited variation. However, they presented continuous variation in signal strength, ranging from apparent absence of labelling to a signal length as large as 1/4 of the whole chromosome arm. This difference in size of the hybridized area was observed even between telomeres of the same chromosome. However, since it was not possible to characterize individual chromosomes due to the karyotype uniformity, we could not verify whether it was an artefactual or a structural variation. No other chromosomal segment was clearly labelled, although in some cells the proximal region of a few chromosomes showed a yellowish colour (Figure 1c), suggesting diffuse hybridization with the probe in this region.

Interphase diploid nuclei of root tip and anther cells presented an areticate structure with some conspicuous chromocentres (Figure 1a,d). The FITC signals were scattered throughout the whole nuclear area, although most of them were associated with chromocentres (Figure 1b,e). Very commonly, signals were located at the limits of the nucleus, attached to the nuclear envelope.

In differentiated and more condensed interphase nuclei, FITC fluorescence was observed on either side of most of the individual chromocentres, although chromocentres without labelling and isolated signals

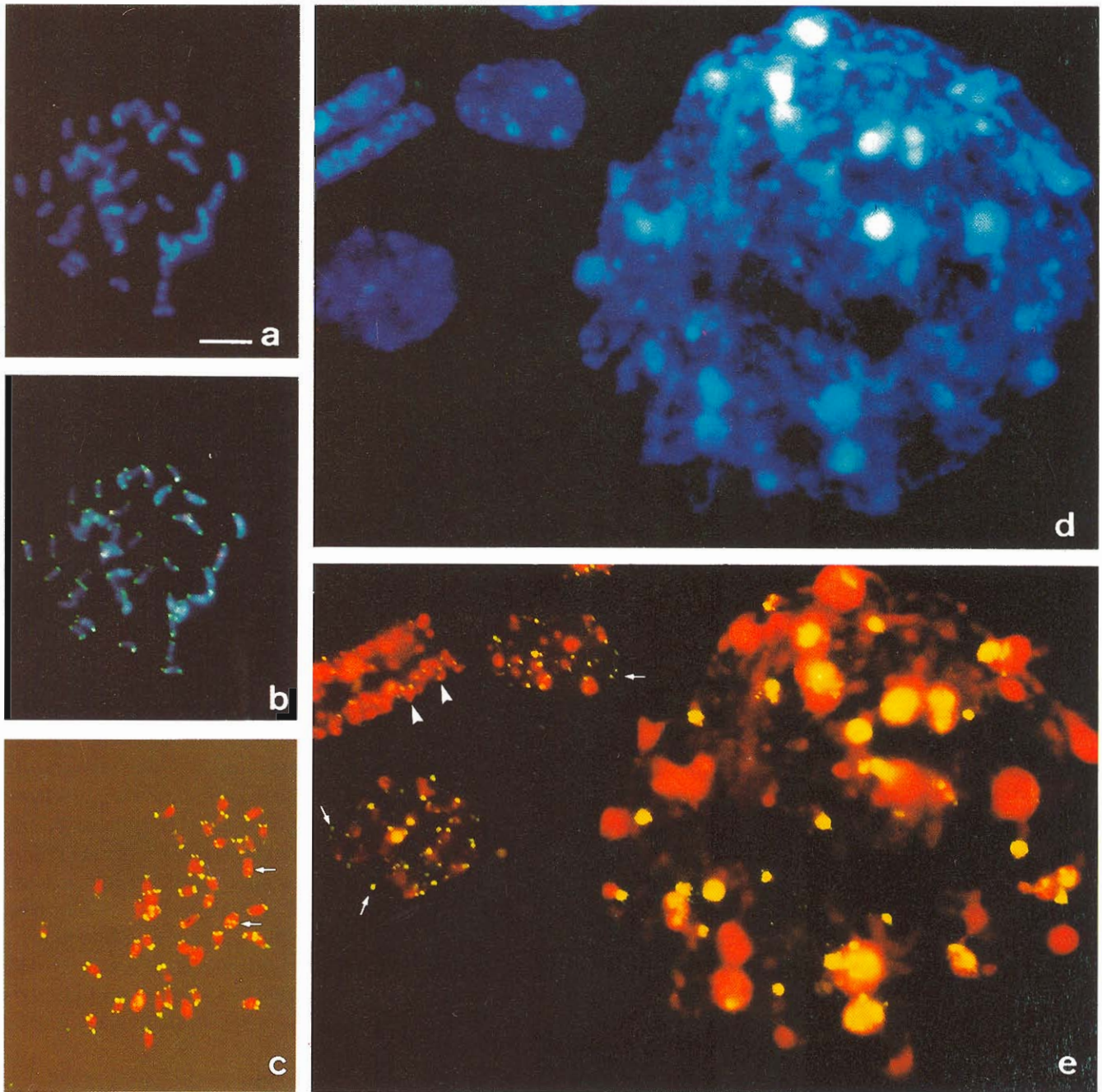


Figure 1 - Telomere DNA signal distribution in root tip metaphases and anther tapetum cells. a, Metaphase chromosomes stained with DAPI showing the symmetry of the chromosome complement. b, The same metaphase as in a recorded by double exposure of the photographic film showing FITC signal distribution counterstained with propidium iodide (weekly) and with DAPI. Note the presence of the label at almost all telomeric ends. c, Metaphase stained with propidium iodide showing less intensive FITC signals at some proximal regions (arrows). d, DAPI stained diploid nuclei (left) and one polytene nucleus (right) of the tapetum. Note the polarization of the polytene nucleus with many parallel chromatid bundles concentrated at the centromere pole (upper half). e, Same nuclei as in d, showing the distribution of *in situ* hybridized probe. Observe the difference in signal size variation between diploid and polytene nuclei, the presence of labels associated with the nuclear periphery only in diploid cells (arrows) and signals at both sides of each chromatid aggregate in differentiated nuclei (arrowheads). Bar represents 5 μ m.

were also found (Figure 1e). In some cases, chromocentres with four or more FITC spots were observed, indicating fusion of individual chromocentres. This association of signals with chromocentres was less evident in meristematic cells. In both cell types, the labels seemed to be of the same size and number as

those observed in metaphase chromosomes. Some larger labelled areas were also observed, suggesting occasional fusion of individual signals.

In the polytene nuclei many chromocentres were particularly large and sometimes associated with partially condensed chromosome segments. The cen-

tromere-telomere polarization often observed in non-pretreated meristematic cells, was only occasionally found in polytene nuclei (Figure 1d). After *in situ* hybridization, the FITC fluorescent areas observed were always larger than those of diploid nuclei. They were distributed randomly in the nuclear area and association with the nuclear boundary was never observed (Figure 1e).

In all polytene cells the signals were far less numerous and more variable in size than those of diploid nuclei, probably due to telomere clustering or fusion. In some cases dozens of FITC dots were clustered together, forming a huge chromocentre (Figure 1e). Most of the signals were associated with chromocentres, but a relation of 2 signals to 1 chromocentre was only rarely found. More commonly only one signal was located at the chromocentre or beside it. The large nucleolar area, observed with DAPI staining as an unstained space in the nucleus, was always free of signals.

DISCUSSION

The synthetic oligomers (5'-TTTAGGG-3')₅ and (5'-AAATCCC-3')₅ hybridized intensely with telomeric regions of all mitotic chromosomes of the amphiploid *Phaseolus vulgaris* × *P. acutifolius*, as has been demonstrated in some other unrelated angiosperm species (Cox *et al.*, 1993). The signal strength variation observed indicates a wide range of telomeric sequence copy number per chromosome end. The labelled area seemed to vary from no labelling to as much as 1/4 of the chromosome arm length. The size of the blocks may be overestimated due to the brightness of fluorochrome labelling. Similar variation in strength of signal has been reported in many other plant species (Schwarzacher and Heslop-Harrison, 1991; Wang *et al.*, 1991; Werner *et al.*, 1992). The apparent lack of labelling in some terminal regions may best be interpreted as due to the amount of hybridized probe below the detection threshold of the technique (Schwarzacher and Heslop-Harrison, 1991) rather than to lack of telomere sequences. A high intraspecific variation in plants has been quantified by molecular methods (Richards and Ausubel, 1988; Ganal *et al.*, 1991; Broun *et al.*, 1992; Burr *et al.*, 1992), but not by *in situ* hybridization, where this variation seems to be hindered by technical constraints.

Additionally, diffuse labelling was observed in the proximal region of some chromosomes. Since the overall background level was very low, we suppose that those labels indicate the ectopic location of a small number of telomeric repeats or very similar sequences. Non-telomeric sites of the telomeric sequences have

been demonstrated widely in vertebrate chromosomes (Meyne *et al.*, 1990) and in some invertebrate species (Pelliccia *et al.*, 1994). In plants, on the other hand, ectopic location of telomeric repeats have been reported as a weak signal in only a few chromosomes of a few species (Ganal *et al.*, 1991). Because the ectopic sites are preferentially located in the centromeric or pericentromeric region, they may be associated with fusion of telocentric chromosomes, but, in general, its functional or evolutionary significance remains completely unknown.

Although the telomere sequence distribution has some similarity with the C-band pattern described by Schweizer (1976) for *P. vulgaris* and *P. coccineus*, it was clearly different. In root tip cells, the heterochromatin was distributed at telomeric regions, variable in length, in one or both arms of all chromosomes. Additionally, large C-bands were found in the NORs and in all centromere regions, suggesting that the heterochromatin is only partially co-localized with telomere probe sites and that they may represent two unrelated sequences. In some other species, one or more telomere associated satellite DNAs have been observed which also hybridize with non-telomeric bands (Lapitan, 1992; Kenton *et al.*, 1993).

In polytene nuclei labellings of different size were observed, most of them associated with chromocentres. The size of individual FITC signals was enhanced, suggesting that the telomere was duplicated during the endoreplication cycles, in spite of the fact that after DAPI or Giemsa staining the terminal regions were less dense and less stainable than the proximal ones. A terminal band or group of dots, like those observed in the embryo suspensor polytene chromosomes of *Phaseolus vulgaris* and *P. coccineus*, after C-banding (Schweizer, 1976) or after *in situ* hybridization with a human telomere probe (Nagl, 1991), was not found.

The nuclear structure of diploid and polytene cells, after conventional or DAPI staining, seemed to be very similar (Carvalho and Guerra, 1994). However, the *in situ* hybridization with telomere DNA showed that at least some aspects of the basic molecular organization in diploid cells were not completely conserved in polytene nuclei of the anther tapetum.

The most evident change was the loss of telomere signals associated with the nuclear envelope. This association is widely documented in many plant and animal cell types (Comings, 1980; Fussell, 1983) and seems to be lost only in some special chromosome types, such as pigeon lampbrush chromosomes (Solovei and Macgregor, 1995) and Diptera polytenic ones (Agar and Sedat, 1983). In plant polytene nuclei, however, this

association has never been reported. The telomere-nuclear envelope association is often related to centromere-telomere polarization (Hilliker and Appels, 1989; Rawlins *et al.*, 1991; Werner *et al.*, 1992). However, in tapetal polytene cells of *Phaseolus*, neither a telomere-nuclear envelope association nor a preferential distribution of FITC signals was found, even in the polarized nuclei. Our preliminary unpublished observations in tapetal cells of *Vigna unguiculata* with this same telomere probe also showed a similar distribution.

In polytene nuclei of *Drosophila*, Agar and Sedat (1983) observed that the chromosomes retained the same centromere-telomere orientation observed in mitotic cells, despite the absence of this telomere repeat in Diptera (Biessmann and Mason, 1994). Nevertheless, the ends of *Drosophila* polytene chromosomes were not attached to the nuclear membrane and the spatial organization seemed to be guaranteed by several intercalary heterochromatin loci in contact with the nuclear envelope (Agar and Sedat, 1983; Saumweber, 1987). In spite of the absence of telomere-nuclear envelope association in the anther tapetum nuclei, large chromocentres were observed at the nuclear periphery of tapetal cells in some *Phaseolus* species (Carvalho and Guerra, 1994).

The small number and variable size of FITC signals in tapetal polytene nuclei may be interpreted as a consequence of widespread telomere fusion. Similar results have often been observed in heterochromatic regions of interphase nuclei in many species (see, e.g. Greilhuber and Speta, 1978; Saumweber, 1987). However, to explain the very large signals we must consider the possibility of intense chromosome end displacement, which has not been observed in somatic cells of different tissues (Fussell, 1983). Moreover, fusions alone cannot explain the disappearance of the signals associated with the nuclear envelope.

An alternative explanation is that during the endoreplication cycle the association with the nuclear envelope has been disrupted and the telomeres have not been amplified equally. Differential amplification of repetitive sequences in polytene nuclei of *Phaseolus* was first claimed by Lima de Faria *et al.* (1975), later confirmed by Forino *et al.* (1979) and Cionini *et al.* (1982). More recently, we found indication of differential replication of rDNA sites in polytene tapetal cells of *Vigna unguiculata* (Guerra *et al.*, in press). On the other hand, unstable telomere replication has been documented widely (Blackburn, 1991) and may also be involved in the rapid replication cycles of polytene nuclei.

A further change in nuclear organization has been observed in some different diploid cell types of the

anther tapetum. In nuclei of differentiated cells, a telomere was found on either side of most of the chromocentres, suggesting a well defined and restricted nuclear volume for each chromosome. In meristematic cells such an organization was not common, indicating that the expansion of some chromosome domains may change during the differentiation process. Rearrangement of telomere signals has also been observed in nuclei from different forms of *Trypanosoma brucei* (Chung *et al.*, 1990). The implications of such changes in the nuclear architecture for chromosome activity have been thoroughly discussed by Bennett (1984).

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RESUMO

A distribuição de telômeros em núcleos de pontas de raiz e do tapete de antera do anfidiplóide *Phaseolus coccineus* x *P. acutifolius* foi analisada por hibridização *in situ* com oligômeros sintéticos marcados com biotina. Na maioria das metáfases os telômeros foram marcados, embora com sinais de intensidades diferentes. Nos núcleos interfásicos diplóides alguns sinais apareceram localizados próximos ao envelope nuclear e outros se encontraram dispersos na cromatina. Interfases mais diferenciadas mostraram cromocentros mais condensados e com sinais em ambos os terminais, sugerindo domínios cromossômicos mais restritos. Por outro lado, células politênicas apresentaram sinais menos numerosos localizados nos cromocentros ou próximos a estes. Em nenhum caso foi observada associação de telômeros com o envelope nuclear. Alguns sinais se mostraram excepcionalmente grandes, indicando a ocorrência de fusões ou amplificação diferencial. Embora a estrutura nuclear pareça estar conservada em células diplóides e politênicas, os resultados sugerem que alguns aspectos da organização nuclear podem estar alterados.

REFERENCES

- Agar, D.A. and Sedat, J.W. (1983). Three-dimensional architecture of a polytene nucleus. *Nature* 302: 676-681.
 Bennett, M.D. (1984). Nuclear architecture and its manipulation. In: *Gene Manipulation in Plant Improvement*

- (Gustafson, J.P., ed.). Plenum Publishing Corporation, New York, pp. 469-502.
- Biessmann, H. and Mason, J.M.** (1994). Telomeric repeat sequences. *Chromosoma* 103: 154-161.
- Blackburn, E.H.** (1991). Structure and function of telomeres. *Nature* 350: 569-573.
- Broun, P., Ganal, M.W. and Tanksley, S.D.** (1992). Telomeric arrays display high levels of heritable polymorphism among closely related plant varieties. *Proc. Natl. Acad. Sci. USA* 89: 1354-1357.
- Burr, B., Burr, F.A., Matz, E.C. and Romero-Severson, J.** (1992). Pinning down loose ends: mapping telomeres and factors affecting their length. *Plant Cell* 4: 953-960.
- Carvalho, G. and Guerra, M.** (1994). The polytene chromosomes of anther tapetum of some *Phaseolus* species. *Caryologia* 59: 211-217.
- Chung, H.M.M., Shea, C., Fields, S., Taub, R.N. and Van der Ploeg, L.H.T.** (1990). Architectural organization in the interphase nucleus of the protozoa *Trypanosoma brucei*: location of telomeres and mini-chromosomes. *EMBO J.* 9: 2611-2619.
- Cionini, P.G., Cavallini, A., Corsi, R. and Fogli, M.** (1982). Comparison of homologous polytene chromosomes on *Phaseolus coccineus* embryo suspensor cells: Morphological, autoradiographic and cytophotometric analysis. *Chromosoma* 86: 383-396.
- Comings, D.E.** (1980). Arrangement of chromatin in the nucleus. *Hum. Genet.* 53: 131-143.
- Cox, A.V., Bennett, S.T., Parokony, A.S., Kenton, A., Callimassia, M.A. and Bennett, M.D.** (1993). Comparison of plant telomere locations using a PCR-generated synthetic probe. *Ann. Bot.* 72: 239-247.
- Forino, L.M.C., Tagliasacchi, A.M. and Avanzi, S.** (1979). Different structure of polytene chromosomes of *Phaseolus coccineus* suspensors during early embryogenesis. *Protoplasts* 101: 231-246.
- Fussell, C.P.** (1983). Telomere arrangement of differentiated interphase cells of *Allium cepa*: a function of chromosome arm lengths at anaphase-telophase. *Can. J. Genet. Cytol.* 25: 478-486.
- Ganal, M.W., Lapitan, N.L.V. and Tanksley, S.D.** (1991). Macrostructure of the tomato telomeres. *Plant Cell* 3: 87-94.
- Greilhuber, J. and Speta, F.** (1978). Quantitative analyses of C-banded karyotypes, and systematics in the cultivated species of the *Scilla siberica* group (Liliaceae). *Pl. Syst. Evol.* 129: 63-109.
- Guerra, M. and Carvalho, G.** (1994). Occurrence of polytene chromosomes in the anther tapetum of *Vigna unguiculata* (L.) Walp. *J. Hered.* 85: 43-46.
- Guerra, M., Kenton, A. and Bennett, M.D.** rDNA sites in mitotic and polytene chromosomes of *Vigna unguiculata* (L.) Walp. and *Phaseolus coccineus* L. revealed by *in situ* hybridization (in press).
- Harley, C.B., Futcher, A.B. and Greider, C.W.** (1990). Telomeres shorten during ageing of human fibroblasts. *Nature* 345: 458-460.
- Hilliker, A.J. and Appels, R.** (1989). The arrangement of interphase chromosomes: structural and functional aspects. *Exp. Cell Res.* 185: 297-318.
- Kenton, A., Parokony, A.S., Gleba, Y.Y. and Bennett, M.D.** (1993). Characterization of the *Nicotiana tabacum* L. genome by molecular cytogenetics. *Mol. Gen. Genet.* 240: 159-169.
- Lapitan, N.L.V.** (1992). Organization and evolution of higher plants nuclear genomes. *Genome* 35: 171-181.
- Lima de Faria, A., Pero, R., Avanzi, S., Durante, M., Stahle, U., D'Amato, F. and Granström, H.** (1975). Relation between ribosomal RNA genes and the DNA satellites of *Phaseolus coccineus*. *Hereditas* 79: 5-20.
- Meyne, J., Baker, R.J., Hobart, H.H., Hsu, T.C., Ryder, O.A., Ward, O.G., Wiley, J.E., Wuster-Hill, D.H., Yates, T.L. and Moyzes, R.K.** (1990). Distribution of non-telomeric sites of the (TTAGGG)_n telomeric sequence in vertebrate chromosomes. *Chromosoma* 99: 3-10.
- Nagl, W.** (1981). Polytene chromosomes of plants. *Int. Rev. Cytol.* 73: 21-53.
- Nagl, W.** (1991). Two human DNA sequences (aromatase, telomere) detected in *Phaseolus* (Fabaceae) by respectively blot and *in situ* hybridization. *Polish Bot. Stud.* 2: 159-164.
- Parokony, A.S., Kenton, A.Y., Meredith, L., Owens, S.J. and Bennett, M.D.** (1992). Genomic divergence of allopatric sibling species investigated by molecular cytogenetics of their F₁ hybrids. *Plant J.* 2: 695-704.
- Pelliccia, F., Volpi, E.V., Lanza, V., Gaddini, L., Baldini, A. and Rocchi, A.** (1994). Telomeric sequences of *Asellus aquaticus* (Crust. Isop.). *Heredity* 72: 78-80.
- Rawlins, D.J., Highett, M.I. and Shaw, P.J.** (1991). Localization of telomeres in plant interphase nuclei by *in situ* hybridization and 3D confocal microscopy. *Chromosoma* 100: 424-431.
- Richards, E.J. and Ausubel, F.M.** (1988). Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell* 53: 127-136.
- Saumweber, H.** (1987). Arrangement of chromosomes in interphase cell nuclei. In: *Results and Problems in Cell Differentiation*. Vol. 14. *Structure and Function of Eukaryotic Chromosomes* (Hennig, W., ed.). Springer-Verlag, Berlin, pp. 223-234.
- Schwarzacher, T. and Heslop-Harrison, J.S.** (1991). *In situ* hybridization to plant telomeres using synthetic oligomers. *Genome* 34: 317-323.
- Schweizer, D.** (1976). Giemsa and fluorochrome banding of polytene chromosomes in *Phaseolus vulgaris* and *P. coccineus*. In: *Current Chromosome Research* (Jones, K. and Bradham, P., eds.). Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 51-56.
- Solovei, I. and Macgregor, H.** (1995). Star-like complexes of chromosomes in the diplotene oocytes of pigeon. *Chromosome Research* 3 (Suppl. 1): 62 (Abstract).
- Thomas, C.V., Manshardt, R.M. and Waines, J.G.** (1983). Teparies as a source of useful traits for improving common beans. *Desert Plants* 5: 43-48.
- Wang, S., Lapitan, N.L.V. and Tsuchiya T.** (1991). Characterization of telomeres in *Hordeum vulgare* chromosomes by *in situ* hybridization I. Normal diploid barley. *Jpn. J. Genet.* 66: 313-316.
- Werner, J.A., Kota, R.S. and Gill, B.K.** (1992). Distribution of telomere repeats and their role in the healing of broken chromosome ends in wheat. *Genome* 35: 844-848.