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The Nucleolus Organizers of Diploid Wheats Revealed by in Situ Hybridization

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Summary. Labelled RNA, transcribed in vitro from wheat ribosomal DNA cloned in a bacterial plasmid, has been hybridised to metaphase chromosomes of five diploid wheats. Autoradiography of the chromosomes has provided unequivocal evidence that these genotypes possess two pairs of nucleolus organizer chromosomes. The diploid wheat accessions used possess widely differing numbers of ribosomal RNA genes.

Key words: Nucleolus organizers – In situ hybridisation – Diploid wheats – Ribosomal RNA genes

Introduction

The classical cytogenetical characters used to describe chromosomes are morphological, e.g. chromosome size, arm ratio, number and position of secondary constrictions and the presence and location of conspicuous heterochromatic knobs. With the emergence of molecular biological techniques, however, it is now possible to determine the location of specific DNA sequences using the technique of in situ hybridization (Pardue and Gall 1975). This technique, therefore, enables many unresolved problems of classical cytogenetics to be examined further.

One such problem is the number of nucleolus organizers in diploid wheats (2n = 14). Earliest reports suggested that there were two pairs of nucleolus organizer chromosomes on the basis that nucleolus organizers are correlated with secondary constrictions visible in mitotic metaphase chromosomes (Pathak 1940; Camara 1943). Some subsequent investigations, however, have provided evidence for only one pair of nucleolus organizer chromosomes (Riley et al. 1958; Upadhya and Swaminathan 1963; Coucoli and Scorda 1966; Patil and Deodicar 1967). Recently, on the basis of the number of nucleoli formed in telophase and early interphase nuclei, Anastassova-Kristeva et al. (1978) have supported the original suggestion of two pairs of nucleolus organizer chromosomes in *Triticum monococcum*.

A nucleolus organizer is the site of clusters of the genes for the 18, 5.8 and 25S ribosomal RNA molecules. It can therefore be detected autoradiographically by the in situ hybridization of labelled ribosomal RNA to metaphase chromosomes (Pardue and Gall 1975). In this investigation we have employed in situ hybridization, using a probe prepared from wheat ribosomal RNA genes cloned in a bacterial plasmid (Gerlach and Bedbrook 1979), to demonstrate the existence of two pairs of chromosomes carrying major blocks of ribosomal RNA genes in five diploid wheats.

Materials and Methods

Stocks

The following diploid wheat stocks (2n = 2x = 14) from the collection at the Plant Breeding Institute, Trumpington, Cambridge were studied (one accession of each): *Triticum monococcum; T. thaoudar; T. aegilopoides; T. urartu; T. sinskajae*; The hexaploid wheat *T. aestivum* var. 'Chinese Spring' (2n = 6x = 42) was also studied.

In Situ Hybridization

Root tip mitotic metaphase nuclei were accumulated in seedlings using colchicine (Gerlach 1977) or 1-bromonapthalene pre-treatments. In a number of experiments it has been found that the method of pre-treatment does not affect the amenability of the subsequent cytological preparations to the in situ hybridization technique. The technique used for in situ hybridization and preparation of autoradiographs is described in detail elsewhere (Appels et al. 1979; Gerlach and Peacock 1980). The nucleic acid probe used for in situ hybridization was tritium labelled RNA (specific activity = 3×10^7 cpm/μg) transcribed by *E. coli* RNA polymerase from DNA plasmid pTA71 as described by Gerlach and Bedbrook (1979). This chimaeric plasmid consists of a single

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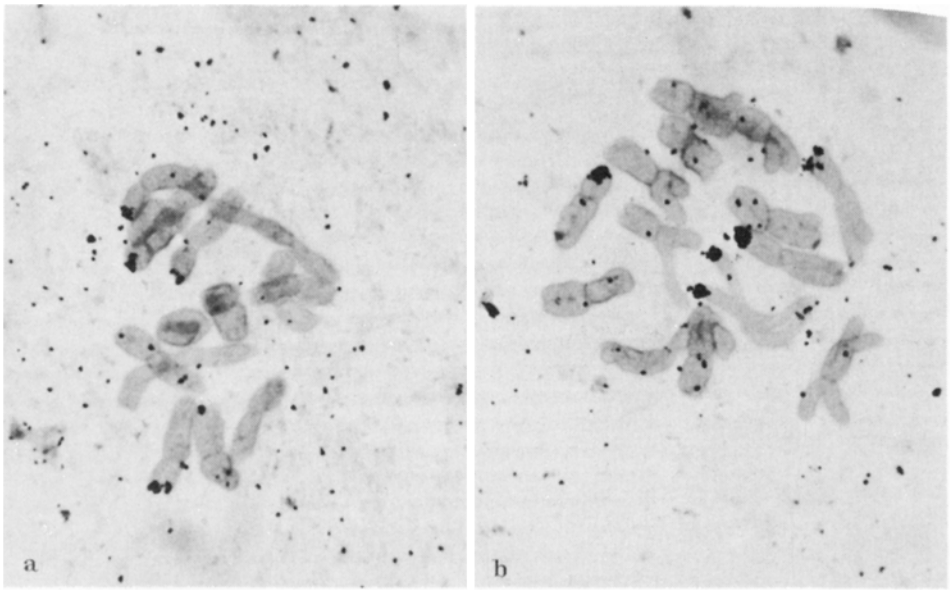

Fig. 1a and b. In situ hybridization of RNA complementary to nucleolus organizer DNA in mitotic metaphase chromosomes of (a) *T. urartu* and (b) *T. aegilopoides*

wheat ribosomal RNA gene repeating unit (i.e., 18S, 5.8S, 25S rRNA genes + associated spacer DNAs) in the vector plasmid pAC184 (Gerlach and Bedbrook 1979).

Nucleic Acid-purification and Hybridizations on Nitrocellulose Filters

The purification of the ribosomal RNA and DNAs was carried out as described previously (Flavell and Smith 1974). The DNA was loaded on to millipore nitrocellulose filters (about 10 μg/filter) and all the filters incubated in the same vial with labelled RNA in 6 × SSC at 70°C for 3 hrs, also as described previously (Flavell and Smith 1974). The radioactivity bound to each filter was then counted and the amount of DNA on each filter determined by acid hydrolysis. All results were corrected for any radioactivity bound to filters lacking DNA. 5′ ^{32}P-labelled ribosomal RNA and ^{32}P-labelled RNA transcribed upon the pTA71 template were prepared in vitro as described by Gerlach and Bedbrook (1979).

Results and Discussion

Radioactive RNA copies of a plasmid containing a wheat ribosomal DNA unit repeat were hybridized to metaphase chromosomes of five diploid wheats and also of hexaploid wheat. In all the lines tested two pairs of ribosomal RNA gene clusters were detected autoradiographically. The results for *T. urartu* and *T. aegilopoides* are illustrated as examples in Figure 1a and b. This shows unequivocally that diploid wheats have at least two pairs of nucleolus organizer chromosomes. The autoradiographic exposure time required to display the ribosomal RNA gene clusters to a similar extent was much longer for the diploid wheats (53 days) than for hexaploid wheat (12 days) even though all the hybridization conditions were the same.

Four explanations could account for this difference. (1) The diploid wheats studied could have considerably fewer genes than the hexaploid wheat studied. (2) It is possible that a large fraction of the ribosomal RNA genes are dispersed in the diploid wheat chromosomes but not in hexaploid wheat. The dispersed genes would not have been detected because only clustered genes are recognised by in situ hybridization under the conditions used in this study. (3) If the spacer DNAs between the rRNA genes were very different in the diploid wheats from those in hexaploid wheat, then the amount of labelled RNA able to hybridize to the nucleolus organizer regions in the diploid wheats might be considerably less. This is because the labelled RNA probe contained sequences homologous to the spacer DNAs in hexaploid wheat. (4) Ribosomal RNA genes in the diploid wheat metaphase chromosomes may be less accessible to the labelled ribosomal RNA probe due to differences in chromatin conformation.

To investigate some of these possibilities, labelled probe RNAs were hybridized to purified DNAs immobilized on nitrocellulose filters and the amount of probe bound per μg of DNA determined. DNAs were purified from accessions of *T. monococcum, T. urartu. T. thaoudar, T. aegilopoides* and *T. aestivum*, var. 'Chinese Spring'. Two sorts of RNA probe were used. The first was ribosomal RNA purified from wheat and labelled in vitro at 5′ ends with ^{32}P γlabelled ATP using the enzyme polynucleotide kinase. This RNA was used in concentrations which are sufficient to saturate wheat rDNA (Flavell and

Table 1. Hybridization of RNA to *Triticum* DNAs immobilized on nitrocellulose filters

	Experiment A	Experiment B
	Ribosomal RNA cpm/μg × genome size (pg)[a]	RNA complementary to pTA 71 cpm/μg × genome size (pg)[a]
T. monococcum	333 ± 13	417 ± 15
T. urartu	447 ± 36	495 ± 24
T. thaoudar	1429 ± 57	1214 ± 25
T. aegilopoides	540 ± 27	583 ± 14
T. aestivum	1153 ± 63	1101 ± 58

[a] 1C genome sizes were taken from Bennett and Smith (1976). The hybridization value for each genotype is the mean of five replicates ± the standard deviation of the mean

Smith 1974). The second was RNA synthesized in vitro by *E. coli* RNA polymerase using the recombinant plasmid pTA 71 as template i.e. the RNA used for the in situ hybridizations. This RNA, in contrast to the first, should contain transcripts of the hexaploid wheat spacer DNAs. It was used in non-saturating concentrations. The RNA hybridized to each kind of DNA is given in Table 1 as cpm/μg DNA × genome size in pg. These values provide relative estimates of the number of rRNA genes in each genotype. Within each experiment the values for each genotype have the same quantitative relationships to one another. This indicates that the RNAs homologous to the spacer DNAs hybridize to diploid and hexaploid wheats to a similar extent. The hybridization values also indicate that the *T. monococcum, T. urartu* and *T. aegilopoides* accessions have fewer than half the number of rRNA genes than *T. aestivum* var. 'Chinese Spring' which has about 9,500 (Flavell and O'Dell 1976, 1979). The *T. thaoudar* accession, however, has more rRNA genes than *T. aestivum*, var. 'Chinese Spring'.

These results eliminate explanation (3) for the difference in autoradiographic exposure time required to display the nucleolus organizers in the diploid and hexaploid wheats examined. Longer exposure times would be expected for the diploids with fewer than half the rRNA genes in the hexaploid wheat (explanation (1)) but not for *T. thaoudar*, assuming that the plants used for the extraction of DNA for the filter hybridizations had the same number of rRNA genes as the plant used for the in situ hybridizations. Because of the *T. thaoudar* anomaly and the exceptionally long exposure time for the diploids, even accounting for the lower number of rRNA genes, the possibility of explanation (4) remains, i.e. a smaller proportion of the rRNA genes may be accessible to the labelled RNA probe in diploid wheats. Szabo et al. (1977) have shown that only approximately 5 to 10 per cent of the chromosomal DNA sequences complementary to 5S RNA in *Drosophila melanogaster* salivary gland chromosomes are available for hybridization in situ, which indicates the hybridization is strongly influenced by the chromatin structure. From data currently available it is not possible to determine whether some of the rRNA genes are dispersed in diploid chromosomes (explanation (2)).

Although the experiments reported here unequivocally establish the presence of two pairs of nucleolus organizer chromosomes in diploid wheats, they do not identify the chromosomes involved. The major blocks of rRNA genes in hexaploid wheat are on chromosomes 1A, 1B, 5D and 6B with considerable variation between varieties in the number of rRNA genes at each site (Flavell and O'Dell 1976, 1979). The variety 'Chinese Spring' used in this work has most of its rRNA genes clustered on chromosomes 1B and 6B (Flavell and O'Dell 1976; Appels et al. 1980). Chromosomes 1A and 5D carry very few rRNA genes.

Because a diploid wheat constitutes the A genome of hexaploid wheat, it is reasonable to presume that chromosome 1A of the diploid wheats carries one nucleolus organizer. Whether chromosome 5A or chromosome 6A carries the second remains unknown. There have been no confirmed reports that chromosome 5A or chromosome 6A in hexaploid wheats carries a nucleolus organizer, but chromosome $5C^u$ of *Aegilops umbellulata* is known to have a secondary constriction. The absence of a second nucleolus organizer in the A genome of the hexaploid wheats so far examined suggests that the rRNA genes may have been lost from the second A genome site in these varieties.

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