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J. Lima-Brito[1*], A. Carvalho[1], C. Matos[1], Pat Heslop-Harrison[2] and H. Guedes-Pinto[1]

1 Department of Genetics and Biotechnology, CGB/ICETA, University of Tras-os-Montes and Alto Douro, 5000-911 Vila Real, Portugal., [2] Department of Biology, University of Leicester, Leicester LE1 7RH, United Kingdom
To whom editorial correspondence should be addressed, e-mail: jbrito@utad.pt

rRNA GENE EXPRESSION AND LOCATION IN TRITICALE ASSAYED BY SILVER STAINING AND *IN SITU* HYBRIDISATION TECHNIQUES

ABSTRACT

In durum wheat × rye hybrids and the derived amphiploid triticale, AABBRR, the expression of the 1R rRNA genes is largely suppressed. Alloauto-octoploid triticales, AABBRRRR, allows the evaluation if rye NOR inactivation can be overcome by the increase of rye genome number.

In the present work, we used silver staining and *n situ* hybridisation techniques in order to study the nucleolar activity and to localize the rRNA genes in hexaploid and alloauto-octoploid triticales.

The use of rye genomic DNA as probe allowed the identification of the rye chromosomes present in both hexaploid and octoploid triticales (14 and 28, respectively). The simultaneous use of the ribosomal sequence enables the localisation of 18S-25S rDNA on the satellite chromosomes of both triticales. On hexaploid triticale we detected six rDNA sites (four on wheat chromosome pairs 1B and 6B and two on rye chromosome pair 1R), whereas on alloauto-octoploid triticale eight rDNA sites (four on wheat-pairs 1B and 6B and four on rye chromosome pairs 1R) were observed.

As expected, the maximum number of active Ag-NORs per metaphase cell was coincident with the maximum number of nucleoli per interphase nucleus confirming that all and only the NORs functionally active during interphase are stained by silver at the next mitotic metaphase.

Comparison of the nucleolar activity between hexaploid and octoploid triticales analysed here indicates that the increase in 1R chromosomes from two to four does not change the suppression of rye nucleolar activity. This supports the suggestion that genomic interactions are under strong genetic control.

Key words: amphiploids, *in situ* hybridisation, nucleolar dominance, rDNA, silver staining, triticale.

INTRODUCTION

One of the most studied genomic interaction systems is the one concerning the dominance of one progenitor in the expression of ribosomal loci – nucleolar dominance or amphiplasty (Navashin, 1928). The phenomenon has been observed in many interspecific hybrids and their derivatives in plants and animals (see Lacadena *et al.* 1988 and Pikaard, 2000 for a review). In cereals, nucleolar dominance is a common feature in both intra- and intergeneric hybrids (see Lacadena *et al.* 1988).

Expression of the ribosomal RNA genes produces the nucleoli at interphase and, where the genes are intercalary on the chromosomes, leads to secondary constrictions at

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the nucleolar organizing regions (NORs) of metaphase chromosomes, forming the satellites.

rRNA gene location and expression can be assayed by a combination of methods including measurement of nucleolar volume and number (Martini and Flavell 1985), silver staining (Moreno *et al.* 1990; Neves *et al.* 1997, Lima-Brito *et al.* 1998), and *in situ* hybridisation (see Jiang and Gill 1994; Leitch and Heslop-Harrison 1992).

Analysis of rRNA gene expression by silver staining (Hubell 1985; Stack *et al.* 1991) is a powerful method to score active rDNA sites, while *in situ* hybridisation shows all sites of rRNA genes.

Within the *Triticeae*, the total number of rDNA loci per specie varies. Rye (*Secale cereale* L., 2n=2x=14, genome constitution RR), have a single pair of chromosomes with NORs, which are both normally expressed.

In bread wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD), rRNA genes have been found on chromosomes 1BS and 6BS (major sites), 5DS, 1AS, 7DL, 1BL, and 3DS (see Jiang and Gill 1994). Loci on the two satellite chromosome pairs, 1B and 6B, are large and strongly expressed, and the contribution of other loci to cellular rRNA is small (Martini and Flavell, 1985).

After the study of several hybrids, a hierarchy of expression of the rRNA genes was established (see Lacadena *et al.*, 1988). In wheat x rye F1 hybrids and in triticale, nucleolar dominance of wheat is observed, resulting in an almost total inactivation of rye origin rRNA genes (Cermeńo *et al.* 1984; Lacadena *et al.* 1988; Thomas and Kaltsikes, 1993; Neves *et al.* 1995, 1997). Consequently, in the large majority of cells, rye NOR activity is undetectable by the silver staining technique which reveals interphase nucleoli and stains metaphase NORs that where transcribed during the previous interphase (Hubel, 1985).

In bread wheat x *Aegilops umbellulata* (2n=2x=14, UU) hybrids and addition lines, rRNA gene activity of chromosomes 1U and 5U from *A. umbellulata* is dominant and the 1B and 6B rDNA is suppressed (Martini *et al.* 1982, Lacadena *et al.* 1988). Nucleolar dominance was also found in barley x rye hybrids (Ramsay and Dyer 1983), in wheat-barley addition lines (Santos *et al.* 1984), and in triticale x tritordeum hybrids (Lima-Brito *et al.*, 1998).

In the present work, we aimed to study the nucleolar activity in both hexaploid and alloauto-octoploid triticale and to localize the rRNA genes. For those purposes we used silver staining and *in situ* hybridisation techniques, respectively.

MATERIAL AND METHODS

The following species were used in the study: ten plants of regional rye 'Vila Pouca' (2n=14, RR), six plants of durum wheat 'Candial', (2n=28, AABB); five plants of triticale 'Douro' (2n=42, AABBRR), reselected in Department of Genetics and Biotechnology/University of Trás-os-Montes and Alto Douro; and ten plants of alloauto-octoploid triticale, (2n=56, AABBRRRR) obtained by Guedes-Pinto (1988) from crosses between triticale 'Corgo' (2n=42, AABBRR) and rye 'Vila Pouca' (2n=14, RR).

For root tip chromosome preparations, seeds were germinated on moist filter paper for 48 h at 25 °C. The 1.5-2cm excised root-tips were transferred to ice water for 24-30h

at 0 °C and then fixed in ethanol-acetic acid (3:1). Spread preparations were made as described by Schwarzacher and Heslop-Harrison, (2000). Fixed root-tips were partially digested with cellulase and pectinase before squashing in 45% acetic acid. Cover slips were removed after freezing with dry ice and slides air-dried.

To study nucleolar activity by scoring the number of silver stained nucleolar organizing regions (Ag-NORs) in metaphase cells and the number of nucleoli in interphase cells, we used the salt-nylon silver staining technique of Stack *et al.* (1991) with small modifications as described by Lima-Brito *et al.* (1998).

For *in situ* hybridisation technique, we followed the root-tip spread preparation protocol and the probe detection method described by Schwarzacher and Heslop-Harrison (2000). The probes used in hybridisation were: total genomic DNA from *Secale cereale* 'Petkus' labelled with digoxigenin and the ribosomal sequence pTa 71 (Gerlach and Bedbrook, 1979) labelled with TRITC (tetramethyl rhodamine isothiocyanate). *Triticum aestivum* 'Chinese Spring' DNA was used as blocking DNA to reduce cross hybridisation. The slides were counterstained with DAPI (4'-6' diamidino-2-phenylindole).

Then the slides were mounted in Citifluor-Glycerol and analysed on a Zeiss microscope. Photographs were taken on Fuji 400 colour print film, digitised to Photo CD and converted to grey scale in Adobe Photoshop with contrast optimisation affecting the whole image equally.

RESULTS

Table 1 summarizes the number of metaphase cells with different numbers of Ag-NORs, and the frequency of interphase cells with different numbers of nucleoli, after silver staining. The maximum number of nucleoli seen regularly at interphase is of greater significance than the distribution of numbers since nucleoli usually fuse during interphase. Therefore, statistical analysis of the distributions or maximum numbers of interphase nucleoli may reflect fusion frequencies as well as the numbers of active NORs.

In diploid rye 'Vila Pouca' all mitotic metaphase cells showed two Ag-NORs (Table 1) and the maximum number of nucleoli observed in interphase cells was also two. In durum wheat 'Candial', all mitotic metaphase cells showed four Ag-NORs (Table 1), the same as the maximum number of nucleoli observed in interphase cells. After silver staining, 88% of metaphase cells of hexaploid triticale 'Douro' showed four Ag-NORs (Table 1 and Fig. 1a). Five or six nucleoli were detected in only six interphase nuclei out of 2272 scored (Fig. 1b) giving good evidence for the suppression of the 1R rDNA loci. The same suppression could be detected in the alloauto-octoploid triticale in which some 89% of the metaphase cells analysed had four Ag-NORs (Table 1 and Fig. 1c). However, a very small percentage (7%) of metaphase cells showed more than four Ag-NORs. The number of nucleoli varies between one and eight (Fig. 1d), and the correspondence between the maximum number of Ag-NORs and nucleoli was also observed.

After *in situ* hybridisation using total genomic DNA from rye and the ribosomal sequence pTa 71 it was possible to identify clearly all the rye chromosomes (14 in 6x-triticale and 28 in the alloauto-octoploid triticale) present in metaphase cells as well

Table 1

Number of Ag-NORs in metaphase cells and frequency of interphase cells with different numbers of nucleoli in the lines studied.

Material	Genomic constitution	No. of metaphase cells with different nos. of Ag-NORs							Frequency of interphase cells with different numbers of nucleoli (%)								No. of interphase cells observed
		2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	
Rye 'Vila Pouca'	RR	-	-	125	-	-	-	-	80.52	19.48	-	-	-	-			3500
Durum wheat 'Candial'	AABB	-	-	90	-	-	-	-	19.80	52.65	21.43	6.12	-	-	-	-	3450
6x-Triticale 'Douro'	AABBRR	-	5	60	2	1	-	-	9.42	41.81	37.41	11.09	0.18	0.09	-	-	2272
AlloAuto-octoploid Triticale	AABBRRRR	1	7	199	3	11	-	2	12.00	40.74	35.30	11.74	0.14	0.04	0.02	0.02	16724

‘ - ‘ indicates none found

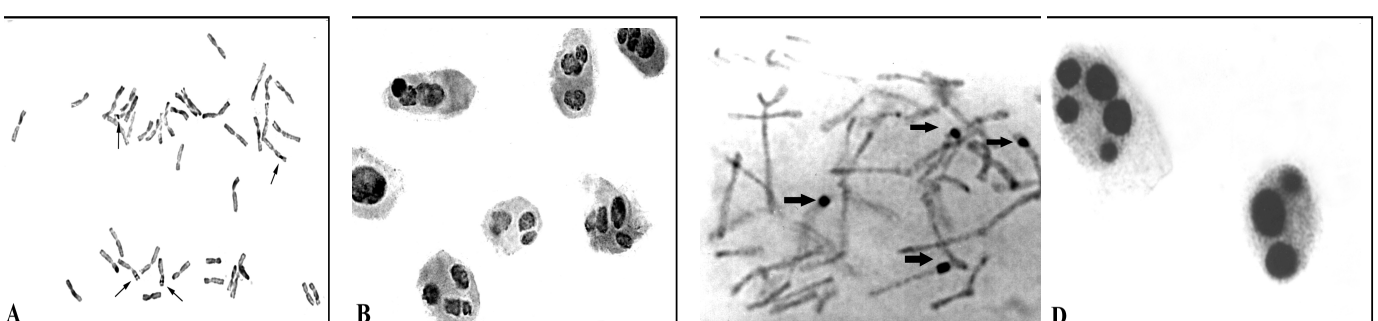

Fig. 1. The silver staining technique stains proteins with active rDNA in metaphase cells (Ag-NORs, arrows) and of nucleoli in interphase cells (A)- Hexaploide triticale ‘Douro’ showing four Ag-NORs from wheat chromosomes 1B and 6B. The rye-origin chromosome 1R NOR are suppressed. (B)- Interphase cells of 6x-triticale ‘Douro’ showing a variation from one to four nucleoli. (C)- Alloauto-octoploid triticale showing four Ag-NORs from wheat-origin chromosomes 1B and 6B. The four 1R NORs are suppressed. (D) – Interphase cells of 8x-triticale two interphase cell with three and five nucleoli.

as to localise the major satellite NOR chromosomes (6 in hexaploid triticale and 8 in the octoploid triticale), as can be seen in Fig. 2a and Fig. 2b.

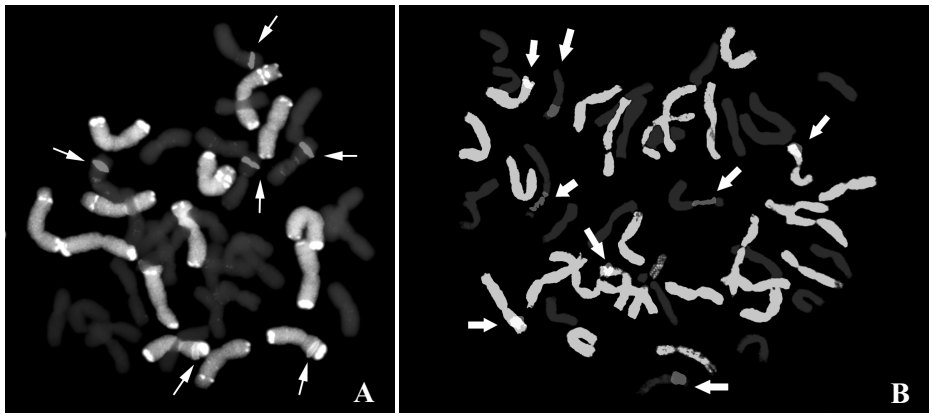

Fig. 2. Root tips metaphases after counterstained with DAPI and *in situ* hybridisation technique performed with total genomic DNA from rye (light grey) and ribosomal sequence pTa71 (arrows). (A) – 6x-triticale 'Douro' showing six rDNA sites, two on the rye chromosome pair 1R and four on wheat chromosome pairs 1B and 6B; (B) – Alloauto-octoploid triticale presenting eight rDNA sites, four on the rye-origin chromosome 1R and other four on wheat-origin chromosomes 1B and 6B

DISCUSSION

The results showed good correlation between the maximum number of active Ag-NORs per metaphase cell and the maximum number of nucleoli per interphase nucleus, confirming that all and only the NORs functionally active during interphase are stained by silver at the next mitotic metaphase (see Medina *et al.* 1986).

Previous reports showed that as ploidy level increases and, consequently, higher potentially active Ag-NORs are present, both the number of nucleoli and Ag-NORs per nucleus shows a wider range (see Lacadena *et al.*, 1984; Rangel-Figueiredo *et al.*, 1985). Our data also reveals that wider spectrum of occurrence of nucleoli per cell (Table 1) that can be explained by the possibility of nucleoli fusing into interphase nuclei.

The NOR in rye was found to be localised in the SAT chromosome 1R through *in situ* hybridisation experiments (see Appels *et al.*, 1980) and reconfirmed through silver staining and C-banding (see Lacadena *et al.*, 1984). Our results reveals that in diploid rye the number of Ag-NORs and nucleoli per cell were similar to those expected (see Rangel-Figueiredo *et al.*, 1985).

In durum wheat, dominance of NORs on chromosomes 1B and 6B over the minor rDNA sites was detected which confirms earlier results present by other authors (see Viegas and Mello-Sampayo, 1975; Lacadena *et al.*, 1988; Lima-Brito *et al.*, 1998).

The nucleolar activity of the chromosome 1R seems to be inactive in a wheat background (Viegas and Mello-Sampayo, 1975; Thomas and Kaltsikes, 1983, Cermeńo *et al.*, 1984; Rangel-Figueiredo *et al.*, 1985, Lacadena *et al.*, 1988; Vieira

et al., 1990a; Neves *et al.*, 1997; Lima-Brito *et al.*, 1998). The inactivation of rye NORs also occurs in barley x rye (Ramsay and Dyer 1983) and *H. chilense* x rye (Thomas and Pickering 1985; Pohler and Schrader 1988) hybrids, wheat-barley addition lines (Santos *et al.*, 1984), monosomic additions of *H. chilense* to rye (Linde-Laursen *et al.*, 1993), triticale x tritordeum F1 hybrids (Lima-Brito *et al.*, 1998), and other combinations in the Triticeae (see Lacadena *et al.*, 1988).

In the triticale cultivar 'Douro' studied here, most of the metaphase cells showed four Ag-NORs giving good evidence for the suppression of the 1R rDNA loci. It was also detected a very few cells with five or six Ag-NORs and nucleoli. However, there is no direct evidence in which chromosome the extra NOR is active.

After *in situ* hybridisation, six sites of 18S-25S rDNA were detected in hexaploid triticale AABBRR: four from wheat chromosome pairs 1B and 6B and two from rye chromosome pair 1R (see Fig. 2a). In the alloauto-octoploid triticale, AABBRRRR, two additional sites of 18S-25S rDNA were seen on rye chromosome pair 1R.

The analysis of the data obtained by silver staining both in AABBRR and AABBRRRR triticales showed that the Ag-NORs and nucleoli pattern are similar: four NORs and four nucleoli being the highest significant number observed. A few cells with six Ag-NORs were observed in both triticales and more nucleoli per cell are rare although they have been observed.

In both cases, the maximum number of NORs and nucleoli per cell are in accordance with the total maximum number of nucleolar regions present, six in hexaploid triticale and eight in octoploid triticale. However, it is clear that suppression of the rye nucleolar activity took place in both triticales, although a few rye NORs may be incompletely suppressed.

Comparison of the nucleolar activity between hexaploid and octoploid triticale analysed here indicates that the increase in 1R chromosomes from two to four does not change the suppression of rye nucleolar activity. This supports the suggestion that genomic interactions are under strong genetic control.

We now know that genes located on the long arm of rye chromosome 1R and on chromosome 2R influence the expression of 1R in triticale (Vieira *et al.*, 1990a, Neves *et al.* 1997).

Several factors influence the expression of rDNA genes including methylation (e.g. Heslop-Harrison 1990; Vieira *et al.* 1990b; Neves *et al.*, 1995; Castilho *et al.*, 1999) or length of sub-repeats in the intergenic spacer DNA of the rDNA (Sardana *et al.* 1992).

CONCLUSION

In situ hybridisation technique using the ribosomal sequence pTa71 allowed the unequivocal localisation of the rRNA genes on the wheat chromosome pairs 1B and 6B and on the rye chromosome pair 1R in both 6x- and 8- triticales analysed in this work. The study of nucleolar activity of these triticales, performed by silver staining technique, revealed the suppression of chromosome 1R in most of the mitotic metaphase and interphase cells scored.

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