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# Nucleolar organizer activity and competition in Tricepiro Don René INTA, a synthetic forage crop

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**Abstract** — The nucleolar organizer activity and the presence of rDNA zones were studied in Tricepiro Don René INTA, a synthetic forage crop (2n=42, 6x, AABBRR with introgression of *Thinopyrum* in 6A chromosome). The use of pTa71 probe revealed the presence of six rDNA zones but Ag-NOR indicated the presence of only four NORs (nucleolus organizer regions). Differential amphiplasty phenomenon could explain the inactivity of some rDNA zones. Simultaneous use of FISH (pTa71 probe) and DAPI banding analysis indicated that the chromosomes with rDNA zones belong to two rye and to four wheat chromosomes. The maximum number of nucleoli observed in somatic cells and the number of chromosomes with secondary constrictions agreed with the four active NORs detected with the Ag-staining method. The use of the pSc119.2 probe allowed to recognize that chromosomes with secondary constrictions are the wheat chromosomes 1B and 6 B.
The results obtained using classical and molecular cytogenetics lead us to conclude that this crop has a similar behavior to that observed in hexaploid triticales in relation to the phenomenon of amphiplasty. Furthermore, the complexity of genetic interactions between wheat and rye genomes described in triticales, are not modified by the presence of *Thinopyron* introgression in Tricepiro Don René INTA.

**Key words:** Ag-NORs, Amphiplasty, Nucleolar competition, pTa71, pSc119.2, Tricepiro

## INTRODUCTION

Tricepiro Don René INTA is a synthetic cereal of high forage value obtained by G. Covas in 1972 in Argentina by crossing an hexaploid triticale (2n=6x=42, AABBRR) and an octoploid trigopiro (2n=8x=56, AABBDDJJ) (Covas 1976; Covas *et al*. 1980; Covas 1989; 1995; Ferrari 2004; Ferrari *et al.* 2005). Tricepiro Don René INTA is composed of 14 rye chromosomes and 28 wheat chromosomes (genomes A and B), with introgression of a region from *Thinopyrum* in the 6A chromosome pair belonging to the A wheat genome (Ferrari *et al.* 2005). It has a genome constitution similar to hexaploid triticale with introgression from *Thinopyrum*.

The nucleolus organizer regions (*Nor* loci) of wheat consist of tandem arrays of numerous ribosomal RNA (rRNA) gene units. The nucleolus itself is a product of rRNA gene expression. In bread wheat*, Triticum aestivum* (2n = 6x = 42, AABBDD), NORs are on the short arms of chromosomes 1A, 1B, 6B, and 5D (Mukai *et al*. 1991; Luo *et al.* 1998). Most nucleolar activity in this species (90%) is due to the major NORs located on chromosomes 1B (NOR1) and 6B (NOR2) (Jordan *et al.* 1982; Martini and Flavell 1985; Luo *et al.* 1998).

The NORs are associated with a nucleolus during interphase, and are usually cytologically visible in mitotic chromosomes as a secondary constriction delimiting a distal satellite. Silver staining is the cytogenetic method commonly used to detect the position, on metaphase chromosomes, of NORs that were functionally active during the preceding interphase (Miller *et al.* 1976a;b; Lacadena *et al.* 1984a,b; Cermeno *et al.*

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1984; 1987; JIMÉNEZ *et al* 1988; MORENO *et al.* 1990; CARPETA *et al.* 2002).

Ribosomal genes (rDNA) have been identified with the pTa71 probe containing the18S-5.8S-25S ribosomal sequences from *Triticum aestivum* (GERLACH and BEDBROOK 1979; MORAIS-CECILIO *et al.* 2000; CARPETA *et al.* 2002; CUADRADO *et al.* 2004).

Differential amphiplasty (changes that affect individual chromosomes of the complement, according to RIEGER *et al.* 1991) has been reported in many interespecific hybrids and also in artificial amphiploids (CERMENO *et al.* 1985; LACADENA *et al.* 1988; PIKAARD 1999; 2000; LEWIS and PIKAARD 2001; KASHKUSH *et al* 2002; PONTES *et al.* 2003). In the wheat x rye amphiploid triticale, containing 28 chromosomes of wheat and 14 from rye, rDNA of rye origin (on chromosome 1R) is not expressed, while the 1B and 6B rDNA from wheat origin, show strong expression (THOMAS and KALTSIKES 1983; LACADENA *et al.* 1984a; CERMENO *et al.* 1984; CERMENO *et al.* 1987; LACADENA *et al.* 1988; NEVES *et al.* 1997).

Tricepiro Don René INTA has a genome composition similar to triticale in which besides the A, B and R genomes introgression of *Th. ponticum* is also present. This crop is an interesting model to analize the amphiplasty phenomenon and the dominance relationship between nucleolus organizer chromosomes from wheat and rye in presence of alien DNA from *Thinopyrum*.

## MATERIALS AND METHODS

*Plant material* - Seeds of Tricepiro Don René INTA were provided by Ing G. COVAS and Ing H. PACCAPELO. Some individuals of these materials were cultivated in the greenhouse of the Instituto Fitotécnico de Santa Catalina, Llavallol, Prov. de Buenos Aires, Argentina.

*Meiotic studies* - Immature flowers were fixed in 3:1 (absolute alcohol: acetic acid) fixative under homogeneous experimental conditions. The anthers were squashed in 2% acetic hematoxylin as stain and 1% ferric citrate as mordant (NUNEZ 1968). Slides were made permanent by freezing with dry ice, removing the cover slip, dehydrating in absolute alcohol and mounting in Euparal.

*Mitotic studies* - Roots 1 cm long were pre-treated in ice-cold water for 36 hours and fixed in 3:1 (absolute alcohol: acetic acid) during 24 hours at room temperature and stored at -20°C.

*Feulgen Reaction* - After fixation, root tips were rinsed for 50 minutes in distilled water and Feulgen Reaction was carried out according to TITO *et al.* (1991).

*Silver staining* - Silver staining technique was carried out according to NEVES *et al.* (1997).

*In situ hybridization procedures* - Fixed roots in 3:1 (absolute alcohol: acetic acid) were washed in 0.01 M citric acid-sodium citrate, pH 4.6 buffer to remove fixative, and transferred to an enzyme solution containing 2% cellulase and 20% liquid pectinase. The softened material was again washed in the buffer solution mentioned above. Finally, chromosomes were squashed onto slides in a drop of 45% acetic acid. Preparations showing well spread metaphase cells were selected by phase contrast light microscopy. After removal of the cover slips by freezing the slides, the latter were subjected to air-drying.

The following probes were used for *in situ* hybridization: pTa71 contains 9 kilobase (kb) *Eco*R1 repeat unit of 18S-5.8S-25S rDNA genes and spacers isolated from wheat, *Triticum aestivum* (GERLACH and BEDBROOK 1979) and pSc119.2, isolated from *S. cereale,* containing the 120-pb family subclone obtained by MCINTYRE *et al.* (1990). JONES and FLAVELL (1982) reported the predominantly telomeric distribution of the 120-bp family in rye, with some minor interstitial sites, and the presence of some cross-hibridization to wheat (MUKAI *et al*. 1991). Probes were labelled by nick translation with biotin 14-dUTP (Bionick Labelling System, GIBCO BRL). The *in situ* technique was carried out according to CUADRADO and JOUVE (1995) with minor modifications.

To detect biotin-labelled probes, slides were treated with conjugate Streptavidin-Cy3 (red). Slides were counterstained with 4'6-diamidino-2-phenylindole (DAPI) and subsequently mounted in antifade solution. Slides were examined with a Carl Zeiss Axiophot epifluorescence microscope with appropriate Carl Zeiss filters. Photographs were taken using Kodak Ultra 400 colour film.

## RESULTS

Mitotic cells of Tricepiro Don René INTA presented 42 chromosomes and two pairs with secondary constrictions. Silver staining performed on mitotic metaphase revealed the presence of four Ag-NORs (Fig. 1a); moreover, the evaluation

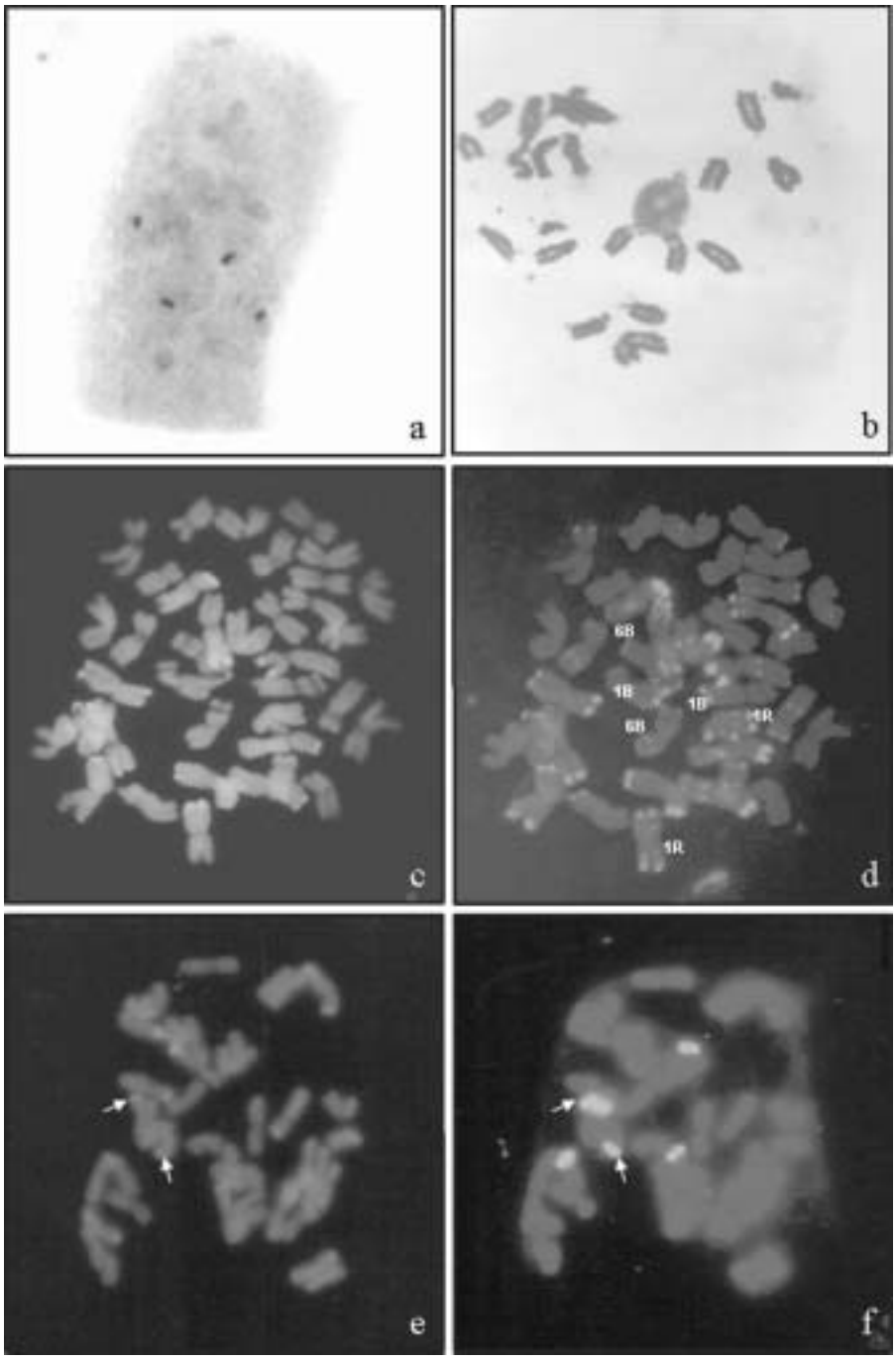


Fig. 1 — Tricepiro Don René INTA. **(a)** Silver-stained somatic metaphase. **(b)** Diakinesis stained with Feulgen; note the presence of only one nucleolus. Two bivalents are associated to the nucleolus. **(c, d)** Identification of 1B, 6B and 1R chromosomes after FISH with pSc119.2 detected with Streptavidin-Cy3 (bright signal) **(c)** and DAPI counterstaining **(d)**. **(e, f)** FISH using pTa71 detected with conjugate Streptavidin-Cy3 **(f)** and DAPI counterstaining **(e)** (arrows show the two rye chromosomes).

of 1276 interphase cells allowed to detect one and up to four nucleoli per cell. Meiotic cells showed only one nucleolus per cell (n= 500 pachytene cells) and two bivalents were associated to the nucleolus in all the diplotene cells studied (n= 47 cells) (Fig. 1b). The Figures 1c and 1d showed FISH analysis with pSc119.2 and DAPI counter staining. DAPI shows chromosomes with secondary constrictions, while pSc119.2 reveals that those chromosomes are 1B and 6B.

On the other hand, *in situ* hybridization with the probe pTa71 indicated the presence of 6 rDNA zones (Fig. 1f). DAPI counterstaining revealed that two of these rDNA zones belonged to chromosomes with intense DAPI bright bands in the telomeric region, characteristic of *Secale cereale,* while the other four chromosomes showed no bands as it is characteristic of wheat chromosomes (Fig. 1e).

## DISCUSSION

Tricepiro was obtained by crossing an hexaploid, triticale (2n=6x=42), and an octoploid, trigopiro (2n=8x=56). Several years of self breeding resulted in a line, 3-40, that was registered as a cultivar under the name Tricepiro Don René INTA (Covas 1976; 1989; 1995; Tosso *et al.* 1997). This cultivar is composed by 14 rye chromosomes, 28 wheat chromosomes and it has *Thinopyrum* introgression zones on wheat chromosomes (Ferrari et a*l.* 2005). The existence in Tricepiro Don René of morphological, agronomical and biochemical characteristics resembling *Thinopyrum* suggests that the *Thinopyrum* introgression would be more extensive than that revealed by GISH (Ferrari 2004; Ferrari *et al.* 2001; 2005).

There are a lot of evidences for the existence of wheat-rye nucleolar competition (amphiplasty) in triticales (Lacadena *et al.* 1984a; Cermeno *et al.* 1984). Similar cases of amphiplasty were observed in other amphiploids: *Hordeum vulgare - S. cereale* (Ramsay and Dyer 1983) and in the hybrids *Aegilops ventricosa x Secale cereale* (Orellana *et al.* 1984), *Ae. triuncialis x S. cereale, Ae. variabilis x S. cereale, Ae. biuncialis x S. cereale, Ae. juvenalis x S. cereale* (Cermeno and Lacadena 1985). Moreover, in other cases, such as in the amphiploid *Triticum aestivum - Agropiro elongatum,* rDNA zones of both species are generally expressed (Lacadena 1984b).

Tricepiro Don René INTA genomic formula is very similar to that of hexaploid triticales but it has *Thinopyrum* introgression that provides its own characteristics on phenotypic traits (Ferrari 2004; Ferrari *et al.* 2001).

The observations performed on cells at prophase I of meiosis together with the consideration of the number of chromosomes with secondary constrictions and the number of nucleoli detected at somatic interphase cells suggest the occurrence of two chromosome pairs bearing nucleolus organizing regions.

The silver procedure used to visualize gene functionality during the preceding interphase also confirmed the presence of four NORs genetically active.

The probe pSc119.2, revealed that the chromosomes with satellites correspond to chromosomes 1B and 6B, while 1R chromosome pair do not show secondary constrictions.

In the hexaploid triticales the rye NOR on the short arm of chromosome 1R is generally supprесed and the wheat NORs are active (Thomas and Kaltsikes 1983; lacadena *et al.*1984a). Neves *et al.* (1997) presented models for chromosomal interactions in triticales that hold control on rDNA expression. They showed that substitution of 2R rye chromosome by 2D chromosome from hexaploid wheat (triticale-2D(2R)) leads to activity of all six major rDNA loci. Therefore, the suppression of the amphiplasty phenomenon in 2D(2R) hexaploid triticales is associated with the absence of chromosome 2R. On the other hand, Vieira *et al.* (1990) observed that the loss of the rye 1R long arm is responsible for rye NOR expression.

Pikaard (1999) rewiewed these results and concluded that complex chromosomal interactions affect nucleolus expression in triticale.

In the present work the *in situ* hybridization using pTa 71 as a probe showed 6 rDNA zones in tricepiro Don René INTA. Two of them would belong to two rye chromosomes, and the other four to wheat chromosomes. This observation is an evidence of the presence of differential amphiplasty in this crop.

The results obtained using classical (karyotype, meiotic behaviour, DAPI and Ag-NOR bands) and molecular cytogenetics (FISH) lead us to conclude that this crop of trigeneric origin (*Triticum, Secale* and *Thinopyrum*) has a similar behaviour to the hexaploid triticales in relation to the phenomenon of amphiplasty.

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