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# Nucleolar activity in *Triticum* x *Thinopyrum* hybrids with different ploidy level

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**Abstract**

The activity of nucleolar organizer regions (NORs) and the presence of ribosomal DNA (rDNA) zones were studied in two *Triticum* x *Thinopyrum* hybrids: a hexaploid hybrid with 2n = 42 chromosomes, named trigopiro SH16 INTA, and a decaploid hybrid with 2n = 56 chromosomes, named trigopiro Don Noé INTA. The use of the pTa71 probe revealed the presence of 10 rDNA signals in both hybrids, whereas the Ag-NORs technique showed 10 signals in SH16 and 8 in Don Noé. We concluded that all trigopiro SH16 INTA NORs are active and that the activity of one NOR pair of trigopiro Don Noé INTA is suppressed. Therefore, the amphiplasty phenomenon is present in trigopiro Don Noé INTA but not in trigopiro SH16 INTA.

**Keywords:** Triticum, Thinopyrum, *Ag-NOR, pTa71, in situ hybridization*

## Introduction

Nucleolar organizer regions (NORs) are comprised of tandem arrays of ribosomal genes (rDNA) that are transcribed by RNA polymerase I (Pol I) and ultimately result in the formation of a nucleolus (Prieto & MacStay 2007). The nucleolus is the site of ribosome assembly where the rDNA genes are active. NORs that are active during interphase remain relatively decondensed at metaphase, forming the so-called secondary constrictions (McKeown & Shaw 2009). The rDNA genes are regulated and expressed according to the physiological need for ribosomes. One of the levels at which the rDNA genes are regulated is an epigenetic on/off switch that controls the number of active rDNA genes. In plants and other higher eukaryotic organisms, DNA hypomethylation and histone hyperacetylation are correlated with transcriptional activity, and DNA hypermethylation and histone hypoacetylation are correlated with transcriptional silencing (Lawrence & Pikaard 2004).

Nucleolar dominance, initially known as differential amphiplasty, is an epigenetic phenomenon that describes the formation of the nucleolus around the rDNA genes inherited from only one of the progenitors of an interspecific hybrid or allopolyploid (Silva et al. 2008; Tucker et al. 2010). This has been explained as a heterochromatization phenomenon of a parental set of rDNA genes that are clustered in NORs (Silva et al. 2008). Preuss and Pikaard (2007) studied hybrids between *Brassica* species and proposed that a dosage control system would be also responsible for the chromatin-mediated silencing of one parental set of rDNA genes.

Amphiplasty can be observed in some hybrids and allopolyploids obtained by crossing species of the tribe Triticeae (Ramsay & Dyer 1983; Orellana et al. 1984; Cermeño & Lacadena, 1985; Cermeño et al. 1987; Lacadena et al. 1988; Pikaard 1999, 2000; Lewis & Pikaard 2001; Pontes et al. 2003; Ferrari et al. 2006; Silva et al. 2008). Both the total number of rDNA loci per genome and the number of genes expressed at each locus vary between the species of this tribe. The study of several hybrids of the Triticeae has shown a hierarchy of expression for the rDNA genes, which is indicative of the complex genome and gene interactions (Lima-Brito et al. 1998). Many different species of *Thinopyrum* (2n = 70, 2n = 42, or 2n = 14) and *Triticum* (2n = 42 or 2n = 28) have been hybridized and results of these crosses, named trigopiros, play an

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important role in wheat improvement (Cai et al. 1998).

The allohexaploid bread wheat (*T. aestivum*) (2n = 6x = 42, AABBDD) has four chromosome pairs (1B, 6B, 5D, 1A) with NOR's, all of which are in the short chromosome arms (Sepsi et al. 2008). Brasileiro-Vidal et al. (2003) studied *Thinopyrum ponticum* (2n = 10x = 70, JJJJsJs) and determined the presence of 17 45S rDNA sites located on the terminal position of the short arms of 17 chromosomes. These authors also recognized the presence of a high number of Ag-NORs (14–17) on metaphase chromosomes, whereas on interphase nuclei they observed a large variation in the number of nucleoli (1–15).

Trigopiro SH16 INTA and trigopiro Don Noé INTA are two *Triticum–Thinopyrum* amphiploids cultivated in Argentina. *In situ* hybridization using different probes (genomic DNA, pSc119.2, and pAs1) has allowed us to conclude that the chromosome number of trigopiro SH16 INTA is 2n = 42 and that the genome composition would be: 14 chromosomes of the B genome, the 2D and 4D chromosome pairs of wheat, 14 chromosomes of the J genome of *Thinopyrum*, and the remaining chromosomes probably of the A genome of wheat (Fradkin et al. 2011). Trigopiro Don Noé INTA has 2n = 56, with 42 chromosomes of *Triticum* (14 belonging to the A genome, 14 to the B genome, and 14 to the D genome) and, 14 chromosomes of *Thinopyrum* (J genome) (Fradkin et al. 2014). According to Brasileiro-Vidal et al. (2005b), *Triticum* x *Thinopyrum* allopolyploids are generally stabilized in 2n = 8x = 56 or 2n = 6x = 42 chromosomes, but mitotic instability may produce chimerical tissues with different chromosome numbers and uneven proportion of wheat and *Thinopyrum* chromosomes.

The aims of the present work were to determine the number of rDNA regions in trigopiro SH16 INTA and trigopiro Don Noé INTA and to analyze the dominance relationship between NORs from *Triticum* and *Thinopyrum* in both trigopiros, with different ploidy level.

## Materials and methods

### *Plant material*

Seeds of trigopiro Don Noé INTA and SH16 INTA were kindly provided by Ing H. Paccapelo and Ing V. Ferreira, respectively.

### *Methods*

One-centimeter-long roots were pre-treated in ice-cold water for 48 h and fixed in 3:1 (absolute alcohol: acetic acid) during 24 h at room temperature and stored at −5°C or FAA [1:1:18(v/v) formaldehyde (37%)–ethanol (50%)–glacial acetic acid], and store for 2–3 days at 4°C.

### *Ag-NOR procedures*

Pre-treated roots that were fixed in FAA were stained with NOR staining technique according to Neves et al. (1997) with minor modifications: silver-stained roots were washed in 0.01 M citric acid–sodium citrate, pH 4.6 buffer, 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.

### *In situ hybridization procedures*

Pre-treated roots that were fixed in 3:1 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 were subjected to air-drying. The following probes were used for *in situ* hybridization: pTa71 contains 9 kilobase (kb) EcoRI repeated unit of 18S–5.8S–25S rDNA genes and spacer isolated from wheat, *Triticum aestivum* (Gerlach & Bedbrook 1979).

The images were captured with a Leica LMDB epifluorescence microscope equipped with a digital camera (Leica DFC 350 FX) using the Leica IM50 version 4.0 program (Leica Microsystem, Cambridge, UK). Images were analyzed using Adobe Photoshop CS6 software.

## Results

Trigopiro Don Noé INTA showed mitotic instability and its chromosome numbers varied from 2n = 37 to 56.

Figure 1a and b shows mitotic metaphase cells of trigopiro SH16 INTA with 42 chromosomes. Ten chromosomes present Ag-NOR bands. Arrow heads show strong terminal bands on the short arms of four chromosomes, while the arrows show weaker terminal signals in the short arms of six chromosomes.

Figure 1e and f shows mitotic metaphase cells of trigopiro Don Noé INTA. Figure 1e shows 50 chromosomes, while Figure 1f shows 42 chromosomes.

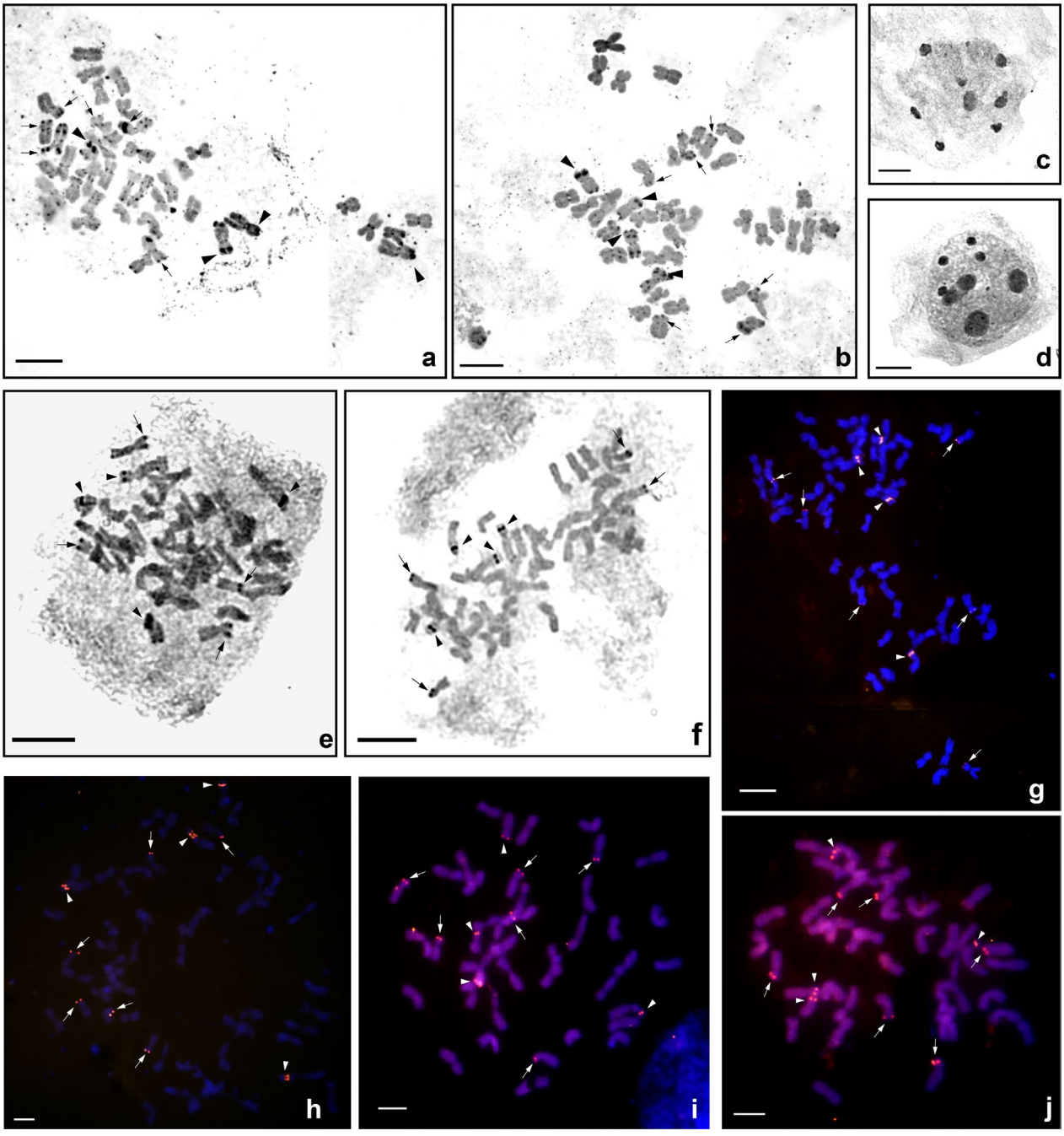


Figure 1. Ag-NOR and FISH techniques on trigopiro SH16 INTA and trigopiro Don Noé INTA. (a, b) Silver staining in mitotic metaphase cells of trigopiro SH16 INTA (2n = 42). Ten Ag-NORs signals are present in this hybrid. Arrow heads correspond to strong terminal bands on short arms of chromosome pairs 6B and 1B. Arrows show weaker terminal bands on short arms of six chromosomes. (c, d) Interphase nuclei of trigopiro Don Noé INTA show eight nucleoli. (e, f) Silver staining in mitotic metaphase cells of trigopiro Don Noé INTA. (e) Cell with 2n = 50 chromosomes with eight Ag-NORs signals. (f) Cell with 42 chromosomes, showing eight Ag-NORs signals. Arrow heads on both metaphase cells correspond to chromosome pairs 6B and 1B with strong signals and arrows on the other four chromosomes are weaker signals. (g, h) FISH on mitotic metaphase cells of trigopiro Don Noé INTA using the pTa71 probe. Ten signals were observed. (i, j) FISH on trigopiro SH16 INTA using the pTa71 probe. Ten signals were observed. Bars = 10 μm.

In both cases, a maximum of eight Ag-NOR bands are present. Arrow heads show strong terminal bands on the short arms of four chromosomes, and arrows show weaker terminal bands on the short arms of four chromosomes. Due to the mitotic instability the chromosome numbers in trigopiro Don Noé varied from 2n = 37 to 56. We confirmed the number of NORs functionally active by analyzing the interphase nuclei and a maximum of eight nucleoli were observed (Figure 1c and d).

The hybridization patterns obtained in mitotic metaphase cells of trigopiro Don Noé (2n = 56)

using the pTa71 probe are shown in Figure 1c and h, whereas those corresponding to trigopiro SH16 INTA (2n = 42) are shown in Figure 1i and j. The presence of 10 rDNA signals was observed in both trigopiros.

## Discussion

Early wheat–*Thinopyrum* amphiploids, developed for producing perennial wheat, resulted in the development of numerous wheat–*Thinopyrum* complexes. So far, a variety of derivatives, including hexaploids, octoploids, partial amphiploids, and derived addition lines, have been obtained (Chen 2005; Fedak & Han 2005). Wheat–*Thinopyrum* amphiploids generally carry a complete set of the wheat genomes (AABBDD) and one set of the alien genome and show ploidy stabilization. However, sometimes wheat chromosomes are substituted by *Thinopyrum* chromosomes or *vice versa* (Fedak et al. 2000; Sepsi et al. 2008).

In the present study, we analyzed two wheat–*Thinopyrum* amphiploids: trigopiro SH16 INTA with 2n = 42 chromosomes, and trigopiro Don Noé INTA with 2n = 56 chromosomes. Don Noé INTA showed mitotic instability, and its chromosome number varied from 2n = 37 to 56. Many authors have reported this phenomenon in wheat and wheat–*Thinopyrum* amphiploids (Guerra & Moraes-Fernandez 1977; Brasileiro-Vidal et al. 2003; Brasileiro-Vidal et al. 2005a, 2005b).

The distribution and activity of rDNA genes have been studied in the two progenitor species of trigopiro by different authors. *T. aestivum* (AABBDD) is a hexaploid wheat that has four chromosome pairs with rDNA sites (1B, 6B, 5D, 1A). All these rDNA sites are present in the terminal zone of short chromosome arms (Sepsi et al. 2008). Chromosomes 1B and 6B have 90% of the total rDNA genes and, according to Lacadena et al. (1988), the activity of the nucleolar zones can be ordered according to the following series: 6B > 1B >> 5D >> 1A. Localization of rDNA sites has been reported in some species of the genus *Thinopyrum* (Brasileiro-Vidal et al. 2003; Mirzaghaderi et al. 2010). Brasileiro-Vidal et al. (2003) characterized *Thinopyrum ponticum* (2n = 10x = 70, JJJJsJs) chromosomes and identified 17 45S rDNA at the terminal position of the short chromosome arms. These authors hypothesized that except in three chromosomes, the rDNA are present in two chromosome pairs of each one of the five genomes (JJJJsJs). Mirzaghaderi et al. (2010) studied *Thinopyrum bessarabicum* (2n = 2x = 14, EbEb) and found two pairs of chromosomes with rDNA genes.

In the present study, using the pTa71 probe, we identified the rDNA genes containing the18S–5.8S–25S ribosomal sequences from *Triticum aestivum* (Gerlach & Bedbrook 1979; Morais-Cecilio et al. 2000; Carpeta et al. 2002; Cuadrado et al. 2004). We observed that trigopiro SH16 INTA and trigopiro Don Noé INTA, both with different ploidy level, showed 10 signals. Besides, both amphiploids presented four strong signals, which we propose correspond to chromosome pairs 6B and 1B according to Lacadena et al. (1988) description. The remaining six chromosomes would correspond to wheat and *Thinopyrum* genomes.

Using the probe pTa71, some authors have observed that wheat–*Thinopyrum* amphiploids with 2n = 56 chromosomes show between 10 and 12 hybridization signals (Brasileiro-Vidal et al. 2005a; Georgieva et al. 2011).

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 (Cermeño et al. 1984, 1987; Lacadena et al. 1984b; Carpeta et al. 2002).

In the present study, we observed a maximum of 10 Ag-NOR signals in trigopiro SH16 INTA and 8 signals in trigopiro Don Noé INTA. In both cases, we concluded that the strong signals would correspond to chromosomes1B and 6B, which, according to Lacadena et al. (1988), are those with higher activity.

In trigopiro SH16 INTA, the number of Ag-NOR signals revealed after silver staining was the same as that revealed with the pTa71 probe. In contrast, in trigopiro Don Noé INTA, the number of Ag-NOR signals revealed by the two techniques was different (10 vs 8, respectively). The existence of nucleolar competition between *Triticum* and *Thinopyrum* has been studied by different authors, who observed that the behavior of the NORs of wheat is variable in the presence of *Thinopyrum* chromosomes. Armstrong et al. (1991) determined that in *T. durum* x *Th. distichum* rDNA zones are both expressed in their amphiploid. However, Lacadena (1984a) observed that in *T. aestivum* x *Agropyron elongatum* (=*Thinopyrum elongatum*) the activity of chromosomes 6 and 7 of *Thinopyrum* are suppressed. Taking into account the results of these authors, we suggest that all trigopiro SH16 INTA NORs are active and that the activity of one pair of *Thinopyrum* NOR is silenced in trigopiro Don Noé INTA. Therefore, the amphiplasty phenomenon is present in trigopiro Don Noé INTA but not in trigopiro SH16 INTA.

Chen and Pikaard (1997) suggested that inactive rRNA genes subjected to nucleolar dominance are silenced reversibly by a mechanism operating, at least

in part, at the level of chromatin structure. Inhibiting either cytosine methylation or histone deacetylation is sufficient for the derepression of the inactive set of rRNA genes, suggesting that these processes are partners required together for rRNA gene silencing.

In wheat, inactive rRNA genes have been found to be methylated somewhat more heavily than the active genes, suggesting that methylation is correlated directly with nucleolar dominance (Flavell et al. 1988).

Chen and Pikaard (1997) proposed a model for nucleolar dominance that involves two sets of mechanisms: those that initially discriminate dominant from under-dominant rRNA genes in a hybrid genome and those that enforce dominance and silencing. Their experiments provide insights into the likely enforcement mechanism. As for a likely discrimination mechanism, the enhancer imbalance hypothesis remains a viable possibility. The latter (Reeder 1985) can be combined with rRNA gene silencing into a scenario similar to that proposed by Flavell (1986). Perhaps a limiting transcription factor is titrated initially by the dominant genes, and the quiescent genes become packaged into a repressive chromatin structure that maintains their silence.

These mechanisms could explain the observations revealed in our study, where wheat–*Thinopyrum* hybrids with different ploidy level have the same number of rDNA genes but different number of active NORs.

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