Chromosome characterization in *Thinopyrum ponticum* (Triticeae, Poaceae) using *in situ* hybridization with different DNA sequences

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Abstract

*Thinopyrum ponticum* (2n = 10x = 70, JJJJ's) belongs to the Triticeae tribe, and is currently used as a source of pathogen resistance genes in wheat breeding. In order to characterize its chromosomes, the number and position of 45S and 5S rDNA sites, as well as the distribution of the repetitive DNA sequences pAs1 and pSc119.2, were identified by fluorescent *in situ* hybridization. The number of nucleoli and NORs was also recorded after silver nitrate staining. Seventeen 45S and twenty 5S rDNA sites were observed on the short arms of 17 chromosomes, the 45S rDNA was always located terminally. On three other chromosomes, only the 5S rDNA site was observed. Silver staining revealed a high number of Ag-NORs (14 to 17) on metaphase chromosomes, whereas on interphase nuclei there was a large variation in number of nucleoli (one to 15), most of them (82.8%) ranging between four and nine. The pAs1 probe hybridized to the terminal region of both arms of all 70 chromosomes. In addition, a disperse labeling was observed throughout the chromosomes, except in centromeric and most pericentromeric regions. When the pSc119.2 sequence was used as a probe, terminal labeling was observed on the short arms of 17 chromosomes and on the long arms of five others. The relative position of 45S and 5S rDNA sites, together with the hybridization pattern of pAs1 and pSc119.2 probes, should allow whole chromosomes or chromosome segments of *Th. ponticum* to be identified in inbred lines of wheat *x Th. ponticum*.

Key words: *Thinopyrum*, silver staining, 45S and 5S rDNA, pSc119.2, pAs1.

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Introduction

*Thinopyrum ponticum* (Podp.) Barkworth & D. R. Dewey, previously named *Agropyron elongatum* auct. non (Host) P. Beauv., *Elytrigia pontica* (Podp.) Holub. or *Lophopyrum ponticum* (Podp.) Á. Löve, is an important source of genes for wheat, especially for leaf and stem rust resistance (McIntosh et al., 1998). Because of its decaploid nature (2n = 10x = 70) and chromosome pairing complexity, various genome formulas have been reported for this species (Muramatsu, 1990; Wang et al., 1991; Zhang et al., 1996). More recently, Chen et al. (1998) suggested, by using genome *in situ* hybridization, that *Th. ponticum* consists of five closely related genomes represented by the formula JJJJ's, and that J, J's, E and St genomes from the *Thinopyrum* species are closely related.

Due to the importance of *Th. ponticum* genes in wheat breeding, cytological markers associated with chromosomes or chromosome segments have been used in gene introgression programs. The use of fluorescent *in situ* hybridization (FISH) has made it possible to locate several specific DNA sequences throughout the chromosomes of various species of Triticeae, allowing alien chromatin to be identified in interspecific hybrids. Probes corresponding to multigenes, such as 45S and 5S rDNA, and highly repetitive DNA sequences, are the most commonly used markers of specific chromosomes or genome regions. For example, in *Triticum aestivum* L., Mukai et al. (1990; 1991) labeled five chromosome pairs with 45S rDNA sites (1AS, 1BS, 6BS, 5DS, 7DL) and six pairs with 5S rDNA sites (1AS, 1BS, 1DS, 5AS, 5BS e 5DS). The pAs1 probe, originally obtained from *Aegilops tauschii* Coss (D genome), allowed the D genome chromosomes (Rayburn and Gill, 1986) to be identified, along with small sites on chromosomes 1A, 4A, 1B, 3B, 6B and 7B in wheat (Mukai et al., 1993). Similarly,
the pSc119.2 probe from Secale cereale L. (R genome) dis
tinguished all B genome chromosomes and 4A, 5A, 2D, 3D, 4D e 5D chromosomes. The simultaneous use of these
two probes allowed 17 out of 21 chromosome pairs of T. aestivum to be identified (Mukai et al., 1993). More re-
cently, Cuadrado et al. (2000) characterized the seven pairs of the B genome and some chromosomes of the A and D
genomes of T. aestivum using two simple sequence repeats (SSR), (AAC)3 and (AAG)3.

Besides the FISH, another procedure used to charac-
terize chromosomes has been silver staining, which was de-
veloped to detect nucleoli in interphase cells and nucleolus organizer regions (NORs) in metaphase chromosomes (Fernandez-Gomez et al., 1969; Hizume et al., 1980). The activity of 45S rDNA genes is usually associated with
NORs and secondary constrictions, and has been studied at
cytological level by recording the number and volume of
nucleoli in interphase cells and NORs in metaphase chro-
mosomes by silver staining (Martini and Flavell, 1985;
Vieira et al., 1990; Lima-Brito et al., 1998).

In the genus Thinopyrum, the localization of rDNA sites
has been reported in some species. In Th. distichum
(Thunb.) Á. Löve (2n = 4x = 28, JJ)1, two 45S rDNA sites
have been located in homeologous groups 5 and 6, and two
SS rDNA sites have been found in homeologous group 5
and in a non-defined group (Fominaya et al., 1997). In Th. elongatum (Host) D. R. Dewey [= Agropyron elongatum
(Host) P. Beauv., Lophopyrum elongatum (Host) Nevski,
Lophopyrum elongatum (Host) Á. Löve] (2n = 2x = 14, E),
the 5S rDNA sites were associated with chromosomes 1E
and, possibly, 5E (Dvorák et al., 1989). Similarly to related
diploid species, the decaploid Th. ponticum has two 45S
rDNA loci per monoploid genome, in all a total of 20 sites
(Li and Zhang, 2002). Highly repetitive DNA sequences,
such as pSc119.2 cloned from rye (Lapitan et al., 1987) and
pLeUCD2 of Th. elongatum (Zhang and Dvorák, 1990;
Kim et al., 1993), have been isolated and used in the charac-
terization of Thinopyrum chromosomes and for the iden-
tification of introgressed alien chromatin in wheat x
Thinopyrum hybrids. In the present study, FISH was used
to identify the number and position of 45S and 5S rDNA se-
dquences, as well as the distribution of the highly repetitive
DNA sequences pAs1 and pSc119.2 in the Th. ponticum
genome. The number of nucleoli and NORs was also
recorded to elucidate whether all 45S rDNA loci were active
in this allopolyploid.

Material and Methods

Plant material and chromosome preparation

The clone of Th. ponticum used in this study was the
PF Ag. el. 84001 accession, maintained under cultivation at
Embrapa Trigo, Passo Fundo (Rio Grande do Sul, Brazil).

1. Genome designation of Thinopyrum species was based on Chen et al.

Root tips were pretreated in ice-cold water for 24 h,
fixed in ethanol: acetic acid (3:1, v/v) and then stored at
-20 °C. The material was digested in an enzyme mixture
containing 2% (w/v) cellulase (Onozuka R10) and 20%
(v/v) pectinase (Sigma) for 2 to 3 h at 37 °C. Afterward,
each root tip was squashed in a drop of 45% acetic acid
and frozen in liquid nitrogen.

DNA probes and labeling

Four DNA probes were used for FISH analysis: (1) clone pTa71, containing the repeat unit of 18S-5.8S-26S
rDNA from T. aestivum (Gerlach and Bedbrook, 1979); (2)
clone pTa794, which corresponds to the complete SS gene
unit from T. aestivum (Gerlach and Dyer, 1980); (3) clone
pSc119.2, containing the 120-pb repeat unit of a tandemly
arranged DNA family derived from S. cereale (McIntyre et al.,
1990); and (4) clone pAs1, including a 1 kb repetitive
DNA sequence from Ae. tauschii (Rayburn and Gill, 1986).
Probes were labeled with digoxigenin-11-dUTP (Roche),
biotin-11-dUTP (Sigma) or rhodamine-5-dUTP (Amersham)
using nick translation for pTa71 or the poly-
merase chain reaction for pTa794, pSc119.2 and pAs1.

Fluorescent in situ hybridization (FISH)

FISH was performed essentially as described by
Heslop-Harrison et al. (1991) at 85% stringency. No
immunocytochemistry was necessary to detect the direct
rhodamine labeling. Biotin-labeled probes were detected
using avidin-rhodamine conjugate (Vector), while digoxi-
genin-labeled probes were detected using anti-
digoxigenin-fluorescein isothiocyanate (FITC) conjugate
(Roche). All preparations were counterstained with
2 µg/mL 4′, 6-diamidino-2-phenylindole (DAPI) and
mounted in Vectashield H-1000 (Vector). Photographs
were taken on Fujicolor Super HG or Kodak Ultra 400 print
film, and scanned at 300 dpi. The images were optimized
for best contrast and brightness with Adobe Photoshop 6.0.

Silver staining

The silver staining technique employed was that de-
scribed by Vieira et al. (1990). After cold treatment, roots
were fixed in 50% ethanol, glacial acetic acid and 37%
formaldehyde (18:1:1, v/v/v) for a minimum of 4 h at room
temperature and a maximum of three days at 0 °C to 2 °C.
Fixed roots were washed several times in distilled water to
eliminate any residues of fixative. Once clean, they were
immersed overnight in an aqueous solution of 20% AgNO3
at 60 °C. Residual silver was eliminated by washing with
distilled water and the staining developed with 1%
hydroquinone and 10% formaldehyde (1:1, w/v) for one to
10 min, depending on the intensity required. After a further
wash with distilled water, the root tips were squashed in
45% acetic acid containing a drop of 1% carmine, which
aids viewing in normal light microscopy.

The number of nucleoli was recorded in 1,064
interphase nuclei and the number of active NORs was ob-
served in 42 metaphases. Best cells were photographed on Kodak Imagelink ASA 25 print film, and scanned at 300 dpi. The images were optimized for best contrast and brightness with Adobe Photoshop 6.0.

Results

In situ hybridization

Both simultaneous and sequential FISH with a two-by-two combination of probes were used to study the metaphase chromosomes of *Th. ponticum*. The 5S and the 45S rDNA genes were located simultaneously using pTa794 and pTa71 probes, respectively. For both sequences, minimal differences of intensities were detected between loci. Twenty 5S rDNA sites were observed at interstitial position in the short arms of 20 chromosomes. Seventeen 45S rDNA were located at a terminal position in the short arms of 17 out of the 20 chromosomes carrying the 5S rDNA, on a double labeling pattern (Figures 1a, b). Considering the number of rDNA sites, as well as the ploidy level (2n = 10x = 70) and the chromosome morphology of *Th. ponticum*, double labeling seemed to be restricted to chromosomes of two homoeologous groups, both submetacentrics.

The pAs1 probe produced terminal labels on both arms of all 70 chromosomes. In addition, dispersed labeling was observed throughout the chromosomes, except for centromeric and most pericentromeric regions (Figure 1c). The pSc119.2 probe produced labeling on terminal positions of the short arms of 17 chromosomes. Two of them, with different morphologies, also showed telomeric labels on the long arm. In addition, pSc119.2 labels were observed on the long arm of three chromosomes with 45S rDNA sites (Figure 1d).

Silver staining

An attempt was made to analyze the activity of different NORs in cells undergoing interphase to metaphase. Silver staining revealed a high number of Ag-NOR sites (14 to 17). Seventeen were observed in 76% of the 46 metaphases studied (Figure 2a). However, in the 1,064 interphase nuclei studied, the number of nucleoli varied from one to 15, most (82.8%) showing four to nine nucleoli (Figure 2b).

Discussion

Ribosomal genes

The species of the Triticeae tribe vary greatly in number of 45S ribosomal DNA sites. For example, *Ae. comosa*...
Sibth. & Sm. ssp. comosa shows nine pairs of rDNA loci distributed among their seven chromosome pairs, whereas S. cereale has only one pair of these loci (Appels et al., 1980). Nevertheless, the main 45S rDNA loci occur preferentially in the homoeologous groups 1, 5 and 6 (Lacadena et al., 1988; Badaeva et al., 1996). In Th. elongatum (2n = 2x = 14, E) and in Th. distichum (2n = 4x = 28, JJ), the 45S rDNA loci are located on the short arms of two chromosome pairs named as 5 and 6 (Dvorák et al., 1984; Fominaya et al., 1997). In Th. ponticum analyzed, the 45S rDNA sites were located in a similar position in 17 chromosomes, suggesting that, except in three chromosomes, the ribosomal genes are present in two chromosome pairs of each one of the five genomes (JJJ'J'). The occurrence of these sites in 17 instead of 20 chromosomes, as observed by Li and Zhang (2002), most likely indicates a reduction in the number of repeats or a deletion of sites from this accession. A similar reduction has already been described in other polyploids (Linde-Laursen et al., 1992) and it may be more frequent in higher polyploids. In common wheat, the elimination of DNA sequences is one of the main responses to allopolyploidization, occurring after the formation of the polyploid and involving a large fraction of the genome, including 45S rDNA repetitive units (Shaked et al., 2001). For example, in the chromosome 1AS of T. monococcum L., the number of repetitive units of 45S rDNA was largely reduced after its incorporation into the polyploid wheats (Jiang and Gill, 1994). In addition, the terminal position of this locus in Th. ponticum might facilitate its partial or total elimination in some sites (Pestsova et al., 1998).

The number of Ag-NOR sites of Th. ponticum revealed after silver staining was generally similar to that of 45S rDNA labeled after FISH (17). However, the mean number of nucleoli per nucleus was much lower. This indicates that all or nearly all rDNA sites are necessary for normal cell function. At interphase, the Ag-NOR sites tend to coalesce, as suggested by Lacadena et al. (1988).

In Triticeae, the 5S rDNA sites consist of a 120 bp coding sequence alternating with non-transcribed spacers, which are classified into two types according to their length and sequence (Gerlach and Dyer, 1980). In most genomes the sites with short spacers are located in homeologous group 1, whereas those with long spacers are located in group 5 (Reddy and Appels, 1989; Dvorák et al., 1989). In Th. elongatum, the presence of 5S rDNA in the short arms of chromosomes of the homeologous group 1 and probably 5 has been suggested (Scoles et al., 1988; Dvorák et al., 1989). In Th. ponticum, we found two 5S rDNA sites per haploid genome. Considering the distribution of these sites in other species, and the proximity of these sites to the 45S rDNA sites, it is tempting to conclude that these chromosomes belong to homeologous groups 1 and 5.

In Th. ponticum, the 45S rDNA locus was always distally located in relation to the 5S rDNA locus. This disposition is similar to that found in homoeologous group 5 of the D genome of T. aestivum (Mukai et al., 1990; Mukai et al., 1991), of Ae. umbellulata Zhuk. (Castilho and Heslop-Harrison, 1995) and of Th. distichum (Fominaya et al., 1997). However, in homoeologous group 1 of the B genome of T. aestivum (Mukai et al., 1990; Mukai et al., 1991) and Ae. umbellulata (Castilho and Heslop-Harrison, 1995), these sites are located in an inverted position. In Th. elongatum, one pair of 45S and 5S rDNA loci are separately located in chromosomes 6E and 1E, respectively (Dvorák et al., 1984; Dvorák et al., 1989).

pSc119.2 and pAs1 repetitive sequences

The sequence pSc119.2 from S. cereale has been widely used in FISH experiments due to its high copy number and polymorphic location in many species of the Triticeae. Lapitan et al. (1987), studying the distribution of

**Figure 2** - Analysis of NOR activity, using the silver-staining procedure. a. Partial metaphase showing the maximum number of Ag-NORs found in a single cell (17 sites). b. Interphase nuclei with six nucleoli. Bar = 10 μm.
this sequence in three species of Thinopyrum, found 10 terminal and only one subterminal site in Th. bessarabicum (Savul & Rayss) Á. Løve (2n = 2x = 14, J genome). In Th. elongatum (2n = 2x = 14, E genome), these authors found 10 terminal sites plus other sites distributed throughout the entire length of all chromosomes, except in the telomeres of one chromosome. On the other hand, in the hexaploid Th. intermedium (Host) Barkworth & D.R. Dewey (JJSI), the pSc119.2 sequence hybridized to a few chromosomes. Similarly, in the decaploid Th. ponticum studied in the present work, a reduction in the number of pSc119.2 sites was found, when compared to those of the two diploid species referred to above.

The pAs1 probe has also been widely used in the Triticeae. In T. aestivum, the identification of all chromosomes was possible when this probe was associated with a GAA-rich satellite sequence. The authors observed 48 pAs1 bands (Pedersen and Langridge, 1997) and most large sites were located on the terminal region of D genome chromosomes, as described by Rayburn and Gill (1986). In fact, most large blocks of tandem repetitive sequences are usually located in a similar position in all the chromosomes of a given species. It is possible that these chromosome regions favor or show greater tolerance to the presence of such tandem repetitions (Flavell, 1982). In the present study, Th. ponticum showed terminal bands on both arms of the 70 chromosomes. Another characteristic of this probe in Th. ponticum was the disperse labeling throughout all the chromosomes, except in the centromeric and most of the pericentromeric regions. This diffuse labeling in Th. ponticum, unlike that found in T. aestivum, makes this sequence an important tool for chromosome differentiation in T. aestivum x Th. ponticum hybrids.

These data show that the number and position of the sites revealed by the probes pTa71, pTa794, pAs1 and pSc119.2 are valuable markers to characterize and identify the chromosomes of Th. ponticum in inbred lines of wheat x Th. ponticum.

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