Title: Variation of B chromosome associated with tissue culture in wheat-rye cross

Running title: B chromosome variation associated with tissue culture

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Abstract

In vitro variation of B chromosomes was studied by examining the callus cells derived from the immature embryos from a cross of Chinese Spring wheat (*Triticum aestivum* L.) and Fin 7416 rye (*Secale cereale* L.) carrying two B chromosomes. In 40-day-old callus cells, the numbers of B chromosomes ranged from 1 to 4 in 65.6% of the cells observed. The distribution of B chromosome numbers was associated with the ploidy levels of the normal chromosomes (A chromosomes). The frequency of the cells with high numbers of B chromosomes (i.e., three or four B chromosomes) in the amphiploid cells with 56 A chromosomes was greater than those in the haploid cells with 28 A chromosomes. Although structural changes in the rye A chromosomes were observed, cytological observation and genomic in situ hybridization demonstrated that the rye B chromosomes were conserved in morphological appearance following tissue culture.

**Key words:** B chromosome; *Secale cereale; Triticum aestivum*; amphiploid; tissue culture; genomic in situ hybridization.
B chromosomes occur in 10% to 15% of higher plant species (Jones 1995). The B chromosomes differ from the A chromosomes (normal chromosomes) because they are not necessary for plant growth and development, do not pair with the A chromosomes, and are inherited in a non-Mendelian inheritance mode (Bougourd and Jones 1997). Cultivated rye (*Secale cereale* L., 2n=2x=14, RR genome) is an important cereal species that has provided many useful traits for improvement of wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD). This out-crossing species often carries various numbers of B chromosomes across its geographical distribution, which offers a good system for studying phenotypic and genetic effects of B chromosomes (Jones and Puertas 1993; Niwa and Sakamoto 1995). The B chromosomes in rye always have subtelocentric positions of centromere, which are distinct from the centric or subcentric positions of centromere in the A chromosomes. The B chromosomes of various origins can be introduced from rye into common wheat or durum wheat (*T. durum* Desf.) genomes as B chromosome addition lines. These genetic stocks are genetically uniform populations, which avoid complications caused by the out-crossing nature of rye, and have been used to investigate the behavior of rye B chromosomes in hexaploid or tetraploid wheat (Lindström 1965; Nakata et al. 1991; Hossain et al. 1992; Niwa and Sakamoto 1995; Niwa et al. 1997).

The B chromosomes in rye may have adverse effects on plant growth and development and reduce fertility when present in large numbers, although they can have only minor effects on the host plants when they are present in small numbers (Jones 1995; Bougourd and Jones 1997). Inclusion of the rye B chromosomes in wheat genome resulted in irregular behaviour of the A chromosomes and reduction of pollen and selfed seed fertility in wheat (Niwa et al. 1997). But B chromosomes may increase recombination frequency of the A chromosomes (Alverez et al. 1991). B chromosomes of maize (*Zea mays* L.) resulted in non-disjunction and instability of A chromosome sets in tapetal cells, which regularly occurred in the programmed death of tapetal
cells (Chiavarino et al. 2000). The presence of B chromosomes was reported to be associated with numerical variation of A chromosomes in callus cells in certain species, such as rye and Crepis capillaries (L.) Waller (Asami et al. 1976; Maluszynska 1997). Instability of B chromosome numbers in vitro was also reported in maize (Zea mays L.) and Cymbopogon martini var. motia (Das and Widholm 1982; Screenath and Jagdishchandra 1988b). Variation of A chromosomes associated with tissue culture has been documented in wheat-rye crosses (Lapitan et al. 1984; Doré and Cauderon 1988). However, the impact of in vitro culture on variation of B chromosomes in wheat-rye cross is not known. This study was conducted to examine B chromosome changes in the callus cells and the progeny plants originating from a cross between wheat and B chromosome-carrying rye.

Results

Variation of B chromosomes in the callus cells

The B chromosomes of Fin 7416 rye were easily differentiated from the A chromosomes by their unique position of subterminal centromere (Fig. 1). In the callus cells of the CS × Fin 7416 (0B) control, no B chromosomes were observed in any cells (Table 1). In 40-day-old callus cells initiated from single embryo of the cross between CS wheat and Fin 7416 (2B) rye, the percentage of the cells with B chromosomes was 65.6%. The frequency of the cells with two B chromosomes was 33.1% of the total cells observed. The percentages of the cells with one, three or four B chromosomes were 17.5%, 5.6% or 9.4%, respectively. In 80-day-old callus cells, only a small percentage of the cells (1.6%) contained one B chromosome (Fig. 2). All the B chromosomes examined were present as typical subterminal centromeric chromosomes, and no structural changes were observed in B chromosomes in any callus cells.
Variation of B chromosomes associated with A chromosomes in the callus cells

Numerical variation of A chromosomes, which deviated from the normal cells with ABDR genomes (2n=28 A chromosomes), was observed in 40-day-old callus cells (Table 1). Twenty-four cells examined had the A chromosome numbers less than the normal cells (<28 A chromosomes). Nine of them contained one or two B chromosomes and one had four B chromosomes. Fifteen cells had A chromosome numbers ranging from 29 to 54, 4 of which carried one or three B chromosomes. In addition to aneuploid cells, 34 callus cells containing 2n=56 A chromosomes were observed. Comparison between the haploid callus cells (2n=28 As) and the amphiploid cells (2n=56 As) indicated that the numbers of B chromosome was associated with the ploidy levels of the A chromosomes (r=0.7816, P<0.01). The mean numbers of B chromosomes in an amphiploid cell (2.44±1.46) was significantly greater than the haploid cells (0.67±1.01) (P<0.01), as well as other categories of the cells with different A chromosome compositions (Fig. 3). Among the haploid cells, the predominant numbers of the B chromosomes were one or two, and only a small percentage of the cells contained three or four B chromosomes. In the amphiploid cells, the percentage of the cells that had three or four B chromosomes was greater than that of the cells that had one or two B chromosomes (Table 1).

Following an additional 40 d of culture, the amphiploid cells (2n=56 As) had disappeared in 80-day-old callus cells (Table 1). No cells contained A chromosomes greater than 2n=28, except for 2 cells with 2n=29 As. The callus cells with A chromosome numbers less than 2n=28 accounted for 29.4% of the total cells observed. B chromosomes occurred in only a small proportion of the callus cells examined (Fig. 2).

In 40-day-old callus cells, one to three B chromosomes were observed in 3 of the 4 cells that had telocentric A chromosomes or A chromosome segments. The mean numbers of B chromosomes included in the callus cell with A chromosome breakages was 1.50±1.29. In the
nine 80-day-old callus cells carrying telocentric chromosome, chromosome fragment, or dicentric chromosome, six contained one B chromosome (Table 1). The mean number of B chromosomes with structural alterations of A chromosomes was 0.67±0.50 (Fig. 3).

**B chromosome in the wheat-rye plants derived from tissue culture**

The calli of CS × Fin 7416 (2B) cross grew well once they were initiated, and many adventitious structures occurred from the calli during *in vitro* culture on the subculture medium. Many plantlets were produced from adventitious shoots and embryogenous calli that were grown on the medium for shoot formation. Two hundred plants were transplanted into field and all of them resembled F1 plants initiated from the hybrid seeds in their morphological performance.

Examination of chromosome numbers in 40 regenerated plants resulted in 38 plants with 2n=28 As and 2 plants with 2n=28 As+1 B. None of the 150 regenerated plants set any seed, but 2 out of 50 plants produced seeds following colchicine treatment. These seeds were increased for two additional generations. The chromosome numbers of the 14 plants chosen from the bulked F3 population ranged from 48 to 56. Two plants had 56 A chromosomes and one B chromosome with subterminal position of centromere.

**Genomic in situ hybridization (GISH) analysis using the rye probes prepared from the plants with or without B chromosomes.**

Using the genomic DNA prepared from the 0 B rye plant as the probe in the presence of CS genomic DNA as the blocker, GISH analysis on some callus cells of CS × Fin 7416 (2B) revealed 7 A chromosomes and 2 B chromosomes that were labelled green with the biotinylated fluorescin-FITC, demonstrating that they originated from rye. The other 21 A chromosomes were counterstained red by propidium iodide (Fig. 1A). The probe of rye DNA without B chromosome
stained the entire length of the B chromosomes, except for the terminal region of the long arm. A similar hybridization pattern in the B chromosomes was observed in the root tip cells derived from the F₁ hybrid seeds. When the rye genomic DNA from the plant with two B chromosomes was used as the probe, the hybridization signals were present over the entire B chromosome. The probe specific only to the chromosomes originated from rye detected 14 A chromosomes and one B chromosome in an F₃ plant that had the chromosome constitution of 2n=56 As+1 B. The remaining 42 chromosomes were red due to counterstaining with propidium iodide, indicating their wheat origin. The intensity of the hybridization signals was stronger in one or both ends of the rye chromosomes than in the interstitial parts of the chromosomes and the distal region of the long arm of the B chromosome was strongly stained (Fig. 1B).

Discussion

Accumulation of rye B chromosome numbers in the progeny plants of wheat-rye cross was realized through non-disjunction during formation of gametes (Niwa et al. 1997). In maize, variation of B chromosome numbers was associated with non-disjunction of B chromosomes at the second pollen mitosis, preferential fertilization of B chromosome-carrying gametes, and suppression of meiotic elimination of unpaired B chromosomes, which proved to be controlled by different genetic factors (Puertas 2002; González-Sánchez et al. 2003). Unlike the above polymorphism of B chromosome numbers, this study reported B chromosome variation due to tissue culture starting from wheat-B chromosome carrying rye hybrid embryo. Karyotype changes arising from tissue culture are common in plant species, which can be used as a source of genetic variation (Larkin and Scowcroft 1981; Lee and Phillips 1988; D’Amato 1995). Changes of B chromosome numbers were observed in the callus cells initiated from CS × Fin 7416 (2B). The variation of B chromosome numbers appeared to be associated with ploidy levels of A.
chromosomes. In the B chromosome-containing cells with 2n=28 As, the majority of cells carried one or two B chromosomes, and only a few cells consisted of three or four B chromosomes. However, high numbers (i.e., three or four) of B chromosomes were present more frequently in the cells with 2n=56 As than in the cells with 2n=28 As. The mean number of B chromosomes within an amphiploid cell (2n=56 As) was significantly greater than that within a haploid cell (2n=28 As).

Polyploidization of callus cells results in the formation of polyploidy cells, which has been observed in certain plant species during tissue culture (D’Amato 1995; Maluszynska 1997; Li et al. 2000). The presence of B chromosomes appeared to be associated with a high level of polyploidization in Crepis capillaries (L.) Waller (Maluszynska 1997). The frequency of amphiploid cells (2n=56 As) in the callus cells initiated from CS × Fin 7416 (2B) cross was higher than that in the callus cells derived from CS × Fin 7416 (0B) cross. In addition to amphiploid cells, aneuploid cells with chromosome numbers different from the expected 2n=28 As were observed in the callus cells (Table 1). Asami et al. (1976) investigated variation in the rye B chromosomes during in vitro culture and concluded that the presence of B chromosomes in callus cells was associated with large variation of A chromosome numbers. Endoreduplication was regarded as the main mechanism of polyploid cell formation (D’Amato 1985). Aneuploid cells may arise from amitosis, chromosome lagging at anaphase, and asymmetrical chromosome distribution (Bhojwani and Razdan 1983; Sree Ramulu et al. 1985; Lee and Phillips 1988; Hao et al. 2004). The hormones (such as 2,4-D) supplemented in medium for callus induction and subculture promoted mitotic irregularities and formation of amphiploid and aneuploid cells (Jha and Roy 1982; Li and Zhang 1991).

The B chromosomes were frequently visible in the initial stage of culture, but rarely observed in older aged callus cells. The callus cells with B chromosomes may be selected against
by tissue culture (Asami et al. 1976; Sceenath and Jagadishchandra 1988a). Cells with various
numbers of B chromosomes might be at a disadvantage and can be eliminated during \textit{in vitro}
propagation when competing with the normal cells. Similarly, amphiploid cells were present in
younger aged callus cells but were absent in older aged callus cells. None of the plants
regenerated were amphiploid. Diploid cells may have a selective advantage over polyploid cells
during propagation and differentiation (Maluszynska 1997). A programmed-cell-death-like
pathway was suggested to be responsible for elimination of polyploid cells in the calli that were
derived from diploid \textit{Citrus} spp. (Hao et al. 2004). These might explain that the polyploid cells
(2\textit{n}=56 \textit{As}) did not survive in the later stage of cell culture and they did not differentiate directly
into amphiploid plantlet in wheat-rye hybrid tissue culture. Since polyploid callus cells were
frequently observed in crosses between wheat and rye in the present study or between wheat and
\textit{T. durum}-\textit{Dasypyrnum villosum} \textit{L.} Candagy (Li et al. 2000), the detailed exclusion of polyploid
cells during cell propagation and differentiation warrants further study.

At the cytological level, the subterminal positions of centromere in the rye B chromosomes
were present in all the populations investigated (Niwa and Sakamoto 1995). Although structural
alterations, such as the formation of telocentric or dicentric chromosomes and chromosome
fragments, were observed in the A chromosomes in callus cells, the B chromosomes of rye that
had been exposed to tissue culture were conserved in their morphology. The subterminal
centromeric structure was observed in all the B chromosomes in the callus cells and the
regenerated plants derived from \textit{CS} × \textit{Fin 7416} (2B) cross. Further study is required to
demonstrate if alteration of DNA sequence occurred in the B chromosomes following \textit{in vitro}
culture.

\textbf{Materials and Methods}
Plant materials and callus culture

The 14-day-old embryos from cross between Chinese Spring (CS) wheat and Fin 7416 rye with the addition of 2 B chromosomes, hereafter referred to as Fin 7416 (2B), were surface sterilized in 70% ethanol for 1 min and in 0.1% corrosive mercuric chloride for 10 min, rinsed with sterile distilled water, and then placed on MS medium (Murashige and Skoog 1962) supplemented with 150 mg/L asparagine, 2.0 mg/L 2,4-D (2, 4-dichlorophenoxyacetic acid), and 50 g/L sucrose. Callus subculturing was performed on the medium containing MS salt, 150 mg/L asparagine, 2.0 mg/L 2, 4-D, and 20 g/L sucrose, which was changed at 20 to 25 d intervals. The medium for shoot formation was composed of half concentration of MS ingredients, 1.0 mg/L kinetin, 0.5 mg/L NAA (naphthalene acetic acid), and 50 mg/L sucrose. Shoots were maintained on MS medium containing 0.5 mg/L kinetin, 0.5 mg/L NAA, 3.0 mg/L MET (multi-effect triazole), and 80 g/L sucrose for 30 d to promote root formation. All the media were solidified with 7.5 g/L agar and adjusted to pH 5.8 before autoclaving. The plantlets were then stored in a refrigerator at 4ºC until field transplantation in late autumn. Fifty plants were treated with 0.2% colchicine and dimethylsulfoxide (w/v) at three- to four-leaf stage for 8 h at room temperature for chromosome doubling prior to transferring to field.

The calli initiated from the cross between CS wheat and Fin 7416 that lacked any B chromosomes, referred to as Fin 7416 (0B), were used as the control.

Cytological analysis

Chromosome analysis was performed on the callus cells initiated from the wheat-rye cross as previously described (Li et al. 2000). Compact calli (33-day-old following callus initiation) samples were separated into two parts and then transferred onto freshly prepared subculturing medium. One part was incubated for 7 d to make 40-day-old callus sample. The other part was...
subcultured to produce 80-day-old callus sample. Both samples were pretreated with ice-water at 4°C for 24 h and then fixed in 95% ethanol-glacial acetic acid (3:1). After staining with 1% acetocarmine, the fixed tissues were squashed in 45% acetic acid to spread the metaphase chromosomes on glass slides. Root tips were collected from the seeds that have been germinated on moist filter paper in a Petri dish. The ice-water treatment, fixation, and squashing methods for root tips were conducted following the same procedures as described above. The slides were stored at –80°C in a low temperature freezer until the cover slips were removed with a razor blade prior to genomic in situ hybridization (GISH) analysis. Fisher’s least significant difference (LSD, $P=0.05$) generated by SAS (SAS Institute, Raleigh, NC) was used to differentiate mean B chromosome numbers in different categories of A chromosome composition. Correlation analysis was conducted to demonstrate the association of B chromosome numbers with ploidy levels of A chromosomes.

**GISH analysis**

Genomic in situ hybridization was carried out on mitotic chromosome preparations following a published procedure (Li et al. 2000). The total genomic DNA extracted from the rye plants with or without B chromosomes was separately labelled with biotin-14-dATP via nick translation (BioNick™ Labelling System, Invitrogen Life Technologies, Carlsbad, CA) to be used as the probes. The total genomic DNA of CS wheat was sheared to prepare it as the blocking DNA. The hybridization signals were detected using fluorescein isothiocyanate (FITC)-avidin DN and biotinylated goat anti-avidin D (Vector Laboratories, Inc., Burlingame, CA). The wheat chromosomes were counterstained red with a propidium iodide solution. After mounting in a thin layer of antifade solution on the glass slides, the yellow-greenish FITC-labelled signals of the rye chromosomes and the red wheat chromosomes were visualized using a Zeiss fluorescent
microscope (Carl Zeiss, Oberkochen, Germany) with a digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

References


González-Sánchez M, González-Sánchez E, Molina F, Chiavarino AM, Rosato M, Puertas
MJ (2003). One gene determines maize B chromosome accumulation by preferential fertilization; another gene(s) determines their meiotic loss. Heredity 90, 122-129.


Lindström J (1965). Transfer to wheat of accessory chromosomes from rye. Hereditas 54,


Figure 1. Genomic in situ hybridization analysis of Chinese Spring × Fin 7416 (2B) cross using rye genomic DNA as the probe and Chinese Spring genomic DNA as the blocker. The rye chromosomes exhibit yellow-greenish fluorescent color due to labeling by FITC. The wheat chromosomes display red color as counterstained by propidium iodide.

(A) GISH analysis of a callus cell using 0 B genomic DNA as the probe. Seven A chromosomes and two B chromosomes of rye were detected. The terminal regions of the long arms of the B chromosomes (arrows) were not labeled. (B) GISH analysis of an amphiploid cell using 2 B genomic DNA as the probe. Fourteen A chromosomes and one B chromosome of rye were labelled yellow-green and 42 chromosomes of wheat were counterstained red. One or both ends of the rye chromosomes were strongly hybridized with the probe. The terminal region of the long arm of the B chromosome (arrow) was strongly stained. Bars represent 10 μm.

Figure 2. Frequency distributions of B chromosomes in the callus cells originating from Spring × Fin 7416 (2B) cross.

Figure 3. Mean numbers and standard deviations of B chromosomes in B-containing callus cells with various numbers of A chromosomes derived from Chinese Spring × Fin 7416 (2B) cross. CB: chromosome breakage, including telocentric chromosome, chromosome fragments and dicentric chromosome.
Table 1. Chromosomal changes in callus originating from the cross between Chinese Spring wheat and Fin 7416

<table>
<thead>
<tr>
<th>No. of cells observed</th>
<th>No. of B chromosomes</th>
<th>A chromosome number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chromosome breakage&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;28</td>
<td>28</td>
</tr>
<tr>
<td>CS × Fin 7416 (0B) 40-day-old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>97</td>
<td>0B</td>
<td>19 (19.6)&lt;sup&gt;c&lt;/sup&gt; 70 (72.1)</td>
<td>2 (2.1) 4 (4.1) 2 (2.1)</td>
</tr>
</tbody>
</table>

CS × Fin 7416 (2B) 40-day-old

<table>
<thead>
<tr>
<th></th>
<th>0B</th>
<th>14 (8.7) 20 (12.5) 11 (6.9) 6 (3.8) 1 (0.6) 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>6 (3.8) 17 (10.6) 3 (1.9) 2 (1.3) 1 (0.6) 0</td>
<td></td>
</tr>
<tr>
<td>2B</td>
<td>3 (1.9) 42 (26.3) 0 8 (5.0) 1 (0.6) 0</td>
<td></td>
</tr>
<tr>
<td>3B</td>
<td>0 1 (0.6) 1 (0.6) 7 (4.4) 1 (0.6) 0</td>
<td></td>
</tr>
<tr>
<td>4B</td>
<td>1 (0.6) 3 (1.9) 0 11 (6.9) 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>24 (15.0) 83 (51.9) 15 (9.4) 34 (21.3) 3 (1.8) 1 (0.6) 0</td>
<td></td>
</tr>
</tbody>
</table>

CS × Fin 7416 (2B) 80-day-old

<table>
<thead>
<tr>
<th></th>
<th>0B</th>
<th>73 (28.2) 170 (65.8) 2 (0.8) 0 3 (1.2) 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>3 (1.2) 1 (0.4) 0 0 1 (0.4) 2 (0.8) 3 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>76 (29.4) 171 (66.2) 2 (0.8) 0 4 (1.6) 2 (0.8) 3 (1.2)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The chromosome numbers shown in each column include only A chromosomes.

<sup>b</sup> T: telocentric chromosome; F: chromosome fragments; DC: dicentric chromosome.

<sup>c</sup> Number in brackets refers to the percentage of the total number of cells observed.
Figure 2

![Bar chart showing the frequency of different numbers of B chromosomes in 40-day-old and 80-day-old plants. The chart displays the following frequencies: 1B (17.5%), 2B (33.1%), 3B (5.6%), and 4B (9.4%). The chart indicates that 40-day-old plants have a higher frequency of 2B chromosomes compared to 80-day-old plants.](image-url)
Figure 3

![Bar chart showing the number of B chromosomes across different ranges of A chromosomes for 40-day-old and 80-day-old samples.](chart)

- **Number of B chromosomes**
  - <28: 0.67
  - 28: 0.04
  - 29-54: 0.01
  - 56: 0.00
  - CB: 0.00

- **Number of A chromosomes**
  - 40-day-old:
    - <28: 0.67
    - 28: 1.40
    - 29-54: 0.40
    - 56: 2.44
    - CB: 1.50
  - 80-day-old:
    - <28: 0.67