Chromosome Instabilities and Programmed Cell Death in Tapetal Cells of Maize With B Chromosomes and Effects on Pollen Viability

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ABSTRACT

B chromosomes (B's), knobbed chromosomes, and chromosome 6 (NOR) of maize undergo nondisjunction and micronucleus formation in binucleate tapetal cells. These chromosome instabilities are regular events in the program of tapetal cell death, but the B's strongly increase A chromosome instability. We studied 1B and 0B plants belonging to selected lines for high or low B transmission rate and their F_1 hybrids. These lines are characterized by meiotic conservation or loss of B chromosomes, respectively. The female B transmission (*fBt*^{h}) allele(s) for low B transmission is dominant, inducing micronucleus formation and B nondisjunction. We hypothesize that the *fBt*^{l} allele(s) induces knob instability. This instability would be sufficient to produce B loss in both meiocytes and binucleate tapetal cells. B instability could, in turn, produce instabilities in all chromosomes of maize complement. To establish whether the chromosomal instabilities are related to the tapetal programmed cell death (PCD) process, we applied the TUNEL technique. PCD, estimated as the frequency of binucleate tapetal cells with TUNEL label, was significantly correlated with the formation of micronuclei and the frequency of pollen abortion. It can be concluded that the observed chromosome instabilities are important to the PCD process and to the development of microspores to form viable pollen grains.

THE tapetum is the innermost cell layer that lines the anther locules. It is in direct contact with the pollen mother cells (PMCs). The tapetal tissue has a secretory role providing essential nutrients required for microspore and pollen grain development. The peculiar physiological and cytological features of tapetum development are well documented in a number of taxa (Pacini 1997, 2000; Raghavan 1997). In maize, after at least one mitosis with frequent abnormal chromatid segregation, cytokinesis is not completed and binucleate tapetal cells are formed when the PMCs are at meiotic prophase. The tapetal tissue disintegrates at the dehiscent pollen stage when the anther breaks down to allow pollination. Aberrant divisions of tapetal nuclei are an intrinsic characteristic of tapetal development, in such a way that disturbances of tapetal degeneration result in male sterility (Lee *et al.* 1979; Raghavan 1997).

The identification and functional characterization of genes expressed in the tapetum are critically important due to the diverse degrees of male sterility of their mutant phenotypes. For example, Cigan *et al.* (2001) found that in maize the MS45 protein is localized to the tapetum and expressed maximally during the early vacuolate microspore stage of development. Wilson *et al.* (2001) determined that the Arabidopsis *MS1* gene is a critical sporophytic controlling factor for anther and pollen development, with expression in the tapetum at the stage of microspore release from tetrads. Kapoor *et al.* (2002) reported a functional characterization of the tapetum-specific zinc-finger gene *TAZ1*, which causes premature degeneration and pollen abortion in petunia. Murray *et al.* (2003) studied the expression of the transcription factor HvGAMYB during early development of barley anthers. Sorensen *et al.* (2003) found that the *AMS* gene in Arabidopsis encodes a transcription factor playing a crucial role in tapetal cell and microspore development. Some of these mutations have been cytologically characterized. For example, Fei and Sawhney (2001) described the effects of *MS33* mutation on pollen development at the ultrastructural level. It interferes with intine formation and tryphine deposition in Arabidopsis.

Genes related to the deposition of pollen wall components from the tapetum are also being characterized. Some of these products play a role in the highly specific interactions at the pollen stigma interface during sexual reproduction. In maize pollen coats, the predominant protein is an endoxylanase whose mRNA is located in the tapetum. Wu *et al.* (2002) localized prexylanase in the tapetum and studied how it is transferred to the pollen coat. Karimi *et al.* (2002) found that genes coding for pollenins are expressed in the anthers and more specifically in the tapetum of Arabidopsis. Lai *et al.* (2002) identified the *AhSLF-S-2* gene related to the incompatibility multiallelic S locus in Antirrhinum. Wang *et al.* (2002) found that three *TAA1* homeologous genes in wheat express specifically within the tapetum cells.

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Their temporal expression matched the assembly of wall-impregnated sporopollenin.

Papini *et al.* (1999) and Wang *et al.* (1999) proposed that tapetal degeneration is a process of programmed cell death (PCD). Evidence that leads to the conclusion that anther development in angiosperms culminates in the PCD of specific tissues to facilitate the release of pollen has accumulated (reviewed in Wu and Cheung 2000).

A number of workers find morphological and biochemical similarities between PCD processes in plants and animals. For example, Jones (2000) states that the mitochondrion is a common factor that can serve in plant and animal cell death as a stress sensor and as a dispatcher of PCD. Similarly, Beers and McDowell (2001) indicate that the mitochondrion is a mediator of at least some forms of PCD. Moreover, Solomon *et al.* (1999) found cysteine proteases in soybean as the key enzymes in animal PCD, whereas de Jong *et al.* (2000) stated that caspase-like proteases are involved in plant PCD. Lincoln *et al.* (2002) indicate that plants possess a protease with substrate-site specificity that is functionally equivalent to certain animal caspases. However, plants display a unique PCD mechanism that depends on vacuolar lytic function (Kuriyama and Fukuda 2002).

In this and a previous article (Chiavarino *et al.* 2001), we use fluorescence *in situ* hybridization (FISH) to study binucleate tapetal cells in maize carrying B chromosomes. The FISH technique allowed us to determine that B chromosomes, knobbed chromosomes, and chromosome 6 (NOR) undergo nondisjunction and micronucleus formation. We concluded that these instabilities are regular events in tapetal cell death development, but B's strongly increase A chromosome instability.

In this work we extend that study to 1B and 0B plants belonging to selected lines for high (H) or low (L) B transmission rate and both HL and LH F_1 hybrids, to better determine the genetic control and significance of chromosome instabilities during anther development. In addition, to establish whether the chromosomal instabilities are related to the PCD process, we applied the TdT-mediated dUTP nick end labeling (TUNEL) technique to H, L, HL, LH, 0B, and 1B plants. We also compared pollen viability in the mature pollen grains of the same plants to determine how these alterations influence pollen development.

As far as we know, this is the first work in which the behavior of specific chromosomes is related to the PCD process.

MATERIALS AND METHODS

The materials used were 0B and 1B plants from selected lines of maize, *Zea mays* ssp. *mays*. The original material from which the selection was initiated belongs to the native race Pisingallo from northwest Argentina naturally carrying B chromosomes (Rosato *et al.* 1998). The selection process was carried out following the method described in Rosato *et al.* (1996), which consists of selecting plants with the highest and the lowest B transmission rate in female 1B × male 0B crosses. As the B's were always transmitted on the female side we called these lines H^f and L^f in previous works, but in this study we call them H and L for simplicity. F_1 hybrids between the lines were also obtained (González-Sánchez *et al.* 2003). HL F_1 hybrids were obtained by crossing 1B H × 0B L plants, whereas 1B L × 0B H plants were crossed to obtain LH F_1 hybrids.

Root tips were scored for B number, and then the plants were grown in an experimental field. Male inflorescences at meiosis were fixed in 3:1 ethanol:acetic acid and refrigerated at 4° until analyzed. Anther squashes for determining the convenient meiotic or pollen grain stage were made in 1% acetocarmine. Binucleate tapetal cells were scored in anthers from diplotene-metaphase I for chromosome instability studies using either Feulgen staining or FISH. For the TUNEL detection, male inflorescences were fixed in 0.1% paraformaldehyde. Anthers from pachytene tetrads were used. The last tapetal mitosis was found in anthers at zygotene.

The FISH procedure was the same as in Chiavarino *et al.* (2001), using the following repetitive DNA sequences as probes:

i. pZmBs, a clone containing the maize B chromosome-specific sequence (Alfenito and Birchler 1993), kindly provided by J. A. Birchler (Columbia, Missouri). This probe labels the B centromeric regions and, in especially good slides, a small B telomeric region is also labeled.
ii. pZm4-21, a clone containing the maize 180-bp knob repeat (Peacock *et al.* 1981), kindly provided by J. A. Birchler (Columbia, Missouri).
iii. pTa71, a clone containing the rDNA gene unit; the 5.8S, 18S, and 28S genes; and the intergenic spacer from *Triticum aestivum* (Gerlach and Bedbrook 1979).

The TUNEL technique was used to determine the relative stage of PCD. Binucleate tapetal cells were scored in anthers at three PMC stages: pachytene, metaphase I, and tetrads. *In situ* detection of DNA fragmentation was carried out with a modification of the TUNEL method (Tilly and Hsueh 1993; Surh and Sprent 1994). Squashed preparations were incubated in pepsin 0.1% for 4 min at 37°, fixed in 4% paraformaldehyde (Sigma, St. Louis) in 2× SSC for 30 min at room temperature, and then washed in 2× SSC. For labeling, slides were incubated for 60 min at 37° in the presence of a terminal deoxynucleotidyl transferase (TdT) with the *in situ* Cell Death Detection kit (Boehringer Mannheim, Mannheim, Germany), according to manufacturer's instructions. Controls were made without TdT. Slides were counterstained with 4′,6-diamidino-2-phenylindole (Boehringer Mannheim) and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Alexander staining was used as a conventional and simple technique to distinguish normal (fully stained) *vs.* aborted (empty) pollen grains. Anthers containing either bicellular or tricellular pollen grains were scored.

Photographs were made in an epifluorescence Olympus microscope equipped with a CCD camera. The images were optimized for best contrast and brightness using Adobe Photoshop 7.0.

RESULTS

Binucleate tapetal cells were scored for the presence of micronuclei with standard Feulgen staining in 0B and 1B plants of the H, HL, LH, and L genotypes, using

TABLE 1

Frequency of micronuclei in binucleate tapetal cells in 0B and 1B plants of the H, L, HL, and LH genotypes

B transmission line	B no.	No. of cells		
		Without Mn	With Mn	Total
H				
Three individuals	1B	2925 (99.19)	24 (0.81)	2949
Four individuals	0B	1998 (99.35)	13 (0.65)	2011
HL				
Four individuals	1B	1847 (92.49)	150 (7.51)	1997
Four individuals	0B	3535 (97.57)	88 (2.43)	3623
LH				
Five individuals	1B	2046 (92.87)	157 (7.13)	2203
Three individuals	0B	2180 (96.46)	80 (3.54)	2260
L				
Eight individuals	1B	2825 (94.64)	160 (5.39)	2985
Three individuals	0B	2086 (97.11)	62 (2.89)	2148

Percentages are shown in parentheses. Mn, micronucleus.

at least three individuals per genotype and at least three anthers per individual. Table 1 shows that H plants have fewer micronuclei than HL, LH, and L plants and that, in these three genotypes, 1B plants have more micronuclei than 0B plants (Figure 1A). A two-way ANOVA showed that there are significant differences among genotypes ($F = 5.73$; $P = 0.004$) and between 0B and 1B plants ($F = 10.63$; $P = 0.003$), the interaction being nonsignificant ($F = 0.93$; $P = 0.44$). The least significant difference test (LSD) *post hoc* test shows that there are nonsignificant differences among the HL, LH, and L genotypes, but the H genotypes are significantly different from the other three.

The pZmBs probe, specific to the maize B chromosomes, was used in FISH experiments to study the B behavior in binucleate tapetal cells in 1B plants of the four genotypes. The results are shown in Table 2. Unequal B distribution was observed, as one of the nuclei of the binucleate cell showed two labels and the other showed no label (Figure 2A). This indicates that B nondisjunction occurred in the mitotic anaphase preceding binucleate tapetal cell formation. One-way ANOVA showed that there are significant differences among the genotypes ($F = 6.75$; $P = 0.0025$), but the LSD *post hoc* test shows that there are nonsignificant differences among HL, LH, and L, whereas significantly less B nondisjunction occurs in the H line (Figure 1B).

From the total of 129 cells observed with micronuclei, 19 (14.73%) were labeled with the pZmBs probe (Table 2; Figure 2, B and C). This indicates not only that the B formed micronuclei in the tapetal cells, but also that other chromosomes of the normal complement suffered instabilities leading to their loss in the preceding anaphase. It is evident that the B is more unstable than the A's, because if all chromosomes had the same probability of forming micronuclei, the expected frequency of B micronuclei would be 1/21 (4.76%) and the expected frequency for any A chromosome would be 20/21 (95.24%). However, the observed relative proportion of B micronuclei is much higher (14.73%).

Double FISH was carried out with the pZmBs probe, specific to the maize B's, and the pZm4-21 probe, specific to the maize heterochromatic knobs, simultaneously. Previous data (Chiavarino *et al.* 2001) indicated that this maize race is polymorphic for the heterochromatic knobs, with five large and at least three small knobs. Due to this large number of knobs it is not possible to determine the exact number of knobs in each nucleus of the binucleate cells, but it is possible to distinguish between nuclei with apparently equal or unequal knob distribution (Figure 2D). Table 3 shows the frequency of binucleate tapetal cells with equal or unequal knob distribution in 0B and 1B plants of the four genotypes. A two-way ANOVA showed that there are significant differences among genotypes ($F = 5.31$; $P = 0.004$) and between 0B and 1B plants ($F = 7.49$; $P = 0.009$), the interaction being nonsignificant ($F = 0.39$; $P = 0.76$). In all cases 1B plants show more knob unequal distribution than 0B plants (Figure 1C). LSD *post hoc* test shows that in 1B plants the L genotype has significantly more unequal knob distribution than the other three. In 0B plants the H and L genotypes significantly differ, whereas the HL and LH genotypes are intermediate.

1B plants were studied simultaneously for the distribution of B's and knobs. In Table 4 the observed cells of the four genotypes are classified in four classes according to the normal disjunction or nondisjunction of the B's and the knobs (Figure 2D). Chi-square contingency tests were made to test if B disjunction *vs.* nondisjunction and equal *vs.* unequal knob distribution are independent events. In the H and LH lines the differences were significant ($\chi^2 = 15.72$, $P = 0.0001$ and $\chi^2 = 2.76$, $P = 0.097$, respectively), whereas in the HL and L lines the

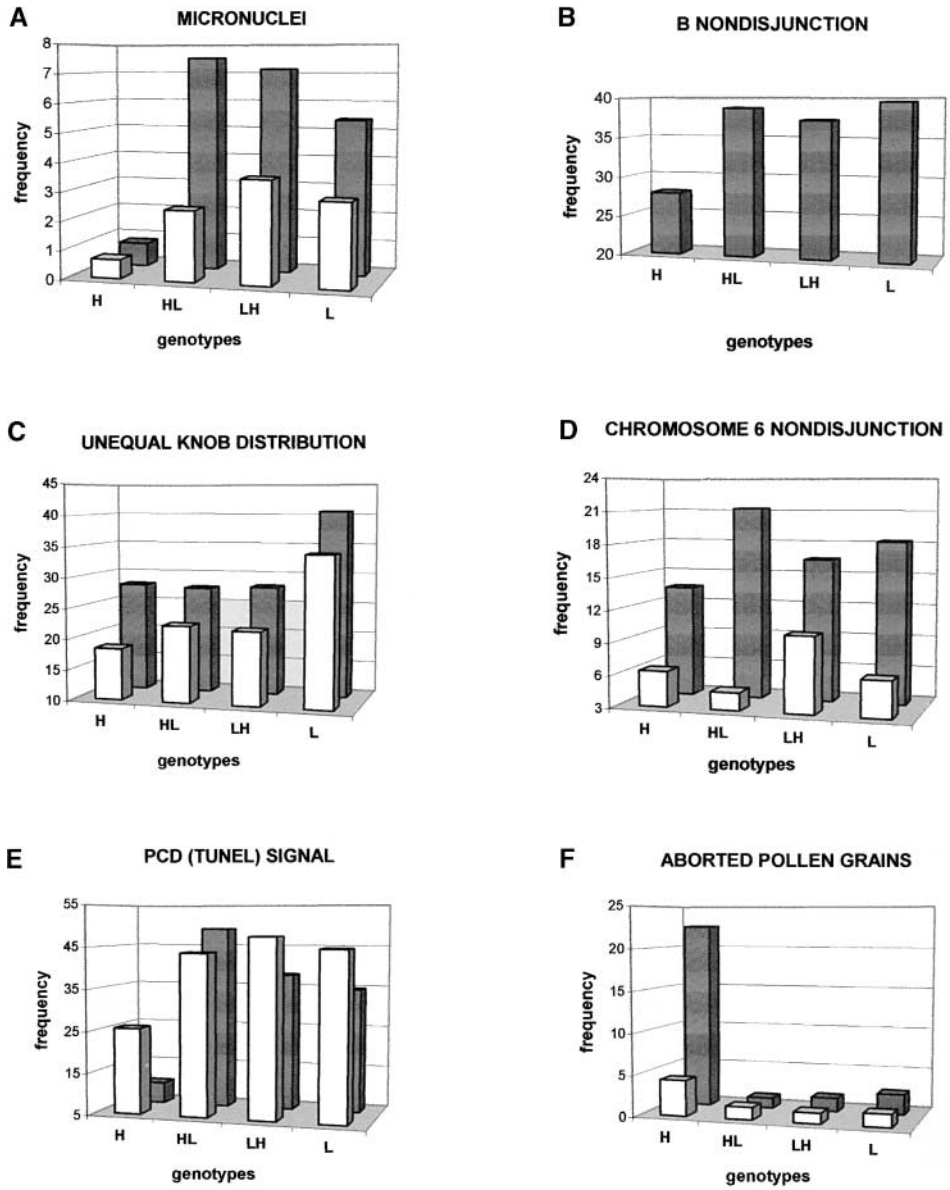

FIGURE 1.—(A–F) Mean frequency of micronuclei, B nondisjunction, unequal knob distribution, chromosome 6 nondisjunction, PCD, and aborted pollen grains in 0B (□) and 1B (■) plants of the H, HL, LH, and L genotypes.

differences were nonsignificant ($\chi^2 = 2.76$, $P = 0.09$ and $\chi^2 = 0.16$, $P = 0.69$, respectively). Contingency tables show that in the H, HL, and LH genotypes the observed number of normal distribution of both the B and the knobs and nondisjunction of the B and the knobs was higher than expected. Conversely, the expected number of cells showing unequal distribution of only one of the chromosomes was lower than expected.

The pTa71 probe was used in FISH experiments to study the behavior of chromosome 6 in binucleate tapetal cells in 0B and 1B plants of the four genotypes. This probe is specific to chromosome 6, which is the only maize chromosome carrying the nucleolar organizing region, located on the short arm. The results are shown in Table 5.

Nondisjunction of one or both chromosome 6's was observed (Figure 2E). The number of observed cells with nondisjunction of both chromosome 6's is small, but it seems that it is lower than that expected for randomness, which is the squared frequency of nondisjunction of one chromosome 6.

A two-way ANOVA showed that there are nonsignificant differences of chromosome 6 nondisjunction among genotypes ($F = 0.74$; $P = 0.53$), but significant differences between 0B and 1B plants ($F = 47.08$; $P = 0.0000$), the interaction being nonsignificant ($F = 1.96$; $P = 0.13$). In all cases 1B plants show a higher level of nondisjunction of chromosome 6 than do 0B plants (Figure 1D).

This probe also allowed observation of labeled micronuclei in the tapetal cells, demonstrating that chromosome 6 may be lost during the mitosis preceding binucleate tapetal cell formation (Figure 2F). The number of cells with labeled micronuclei is small, but labeled micronuclei were not observed in the H line, indicating again that the H line forms fewer micronuclei than the

TABLE 2

Types of binucleate tapetal cells observed with the pZmBs probe in 1B plants

	Types of cells						
	Without Mn		With Mn				
B transmission line	Normal	B nondisjunction	Unlabeled Mn	B nondisjunction and unlabeled Mn	Labeled Mn	Total B nondisjunction	Total cells
H, six individuals	448 (71.11)	173 (27.46)	4 (0.63)	3 (0.47)	2 (0.32)	176 (27.94)	630
HL, five individuals	453 (59.68)	287 (37.81)	12 (1.58)	7 (0.93)	0	294 (38.73)	759
LH, five individuals	426 (59.92)	254 (35.72)	14 (1.97)	10 (1.41)	7 (0.98)	264 (37.13)	711
L, eight individuals	735 (57.06)	483 (37.50)	34 (2.64)	26 (2.02)	10 (0.78)	509 (39.52)	1288

Percentages are shown in parentheses. Mn, micronucleus.

other three. It can also be observed that the frequency of labeled micronuclei is 18/83 (21.69%), which corresponds to 10.84 per chromosome 6. Comparing this frequency with the frequency of micronuclei with B label (19/129; 14.73%; Table 2), it can be concluded that the B is lost with a higher frequency. However, the frequency of chromosome 6 forming micronuclei is higher than the random probability for any chromosome to be lost (1/21; 4.76%).

The pTa71 and the pZmBs probes were simultaneously used in FISH experiments in 1B plants to study the distribution of both the B and the chromosome 6's (Table 6). Contingency tables show that in all cases the observed number of normal distribution of both the B and the 6 and nondisjunction of the B and the 6 was higher than expected. Conversely, the observed number of cells showing nondisjunction of only one of the chromosomes was lower than expected, but the differences were significant in three of the four cases. Chi-square contingency tests were made to test whether disjunction *vs.* nondisjunction of the B and chromosome 6 are independent events. In the H, HL, and L lines the differences were significant ($\chi^2 = 6.41$, $P = 0.0$; $\chi^2 = 11.94$, $P = 0.000$; and $\chi^2 = 14.96$, $P = 0.0001$, respectively), whereas in the LH line the differences were nonsignificant ($\chi^2 = 0.55$, $P = 0.46$).

The TUNEL technique was used to study the possible effect of these chromosome instabilities on the PCD process. The anthers were analyzed at three PMC stages: pachytene, metaphase I, and tetrads. The chromosomes in the PMCs are always labeled along their entire length, probably due to the DNA breakage produced at early meiotic stages related to crossing over. The binucleate tapetal cells were found either strongly labeled on the whole nuclei or slightly labeled on the surface (Figure 2, G and H) and the number of both types was scored.

Considering all genotypes as a whole, the frequency of binucleate tapetal cells showing strong TUNEL label increased from pachytene to tetrads, indicating that the normal PCD process progresses as the PMCs develop, although it never reached 100% at these stages. However, remarkable differences were found in some cases.

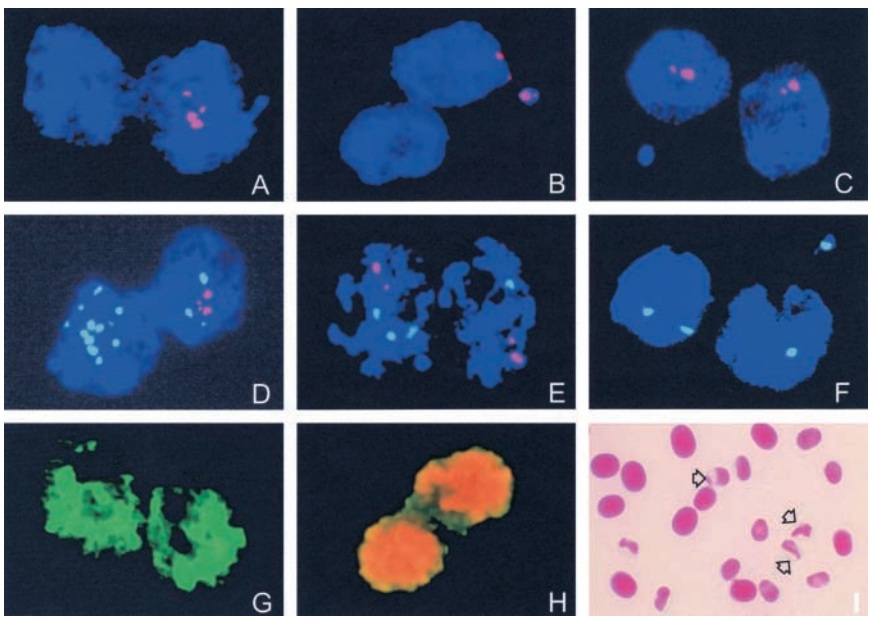

FIGURE 2.—(A–C) Localization of the maize B-specific probe (red) in binucleate tapetal cells. (A) B nondisjunction. The labels corresponding to the B centromere and telomere are side by side. (B) B in the micronucleus. (C) Normal disjunction of the B and unlabeled micronucleus. (D) Localization of the B-specific probe (red) and the knob-specific probe (green). B nondisjunction and unequal knob distribution is shown. (E) Localization of the B-specific probe (red) and the chromosome 6-specific probe (green). Normal distribution of the B and nondisjunction of chromosome 6 is shown. (F) Localization of the chromosome 6-specific probe (green). Chromosome 6 in the micronucleus is shown. (G and H) TUNEL labeling in binucleate tapetal cells. (G) Positive TUNEL, tapetal cell strongly labeled. (H) Negative TUNEL, cell slightly labeled on the surface. (I) Alexander staining in pollen grains of 1B H plants. Aborted pollen grains are not fully stained.

TABLE 3

Frequency of unequal knob distribution in 0B and 1B plants

B transmission line	B no.	Equal knob distribution	Unequal knob distribution	Total no. of cells
H				
Five individuals	1B	253 (72.08)	98 (27.92)	367
Five individuals	0B	270 (81.57)	61 (18.43)	331
HL				
Five individuals	1B	266 (72.48)	101 (27.52)	367
Five individuals	0B	285 (75.40)	93 (24.60)	378
LH				
Five individuals	1B	287 (72.11)	111 (27.89)	398
Five individuals	0B	270 (76.06)	85 (23.94)	355
L				
Six individuals	1B	214 (59.44)	146 (40.56)	360
Six individuals	0B	279 (65.65)	146 (34.35)	425

Percentages are shown in parentheses.

Table 7 shows the frequency of binucleate tapetal cells showing strong TUNEL label in 1B and 0B plants of the four genotypes when the PMCs are at metaphase I. A two-way ANOVA showed that there are nonsignificant differences of TUNEL label between 0B and 1B plants ($F = 4.48$; $P = 0.06$), but significant differences among genotypes ($F = 15.04$; $P = 0.0005$), the interaction being nonsignificant ($F = 2.95$; $P = 0.08$). However, an LSD *post hoc* test showed that there are significant differences between 1B and 0B plants of the H line and nonsignificant differences between 1B and 0B plants of HL, LH, and L lines. On the other hand, HL, LH, and L lines showed a higher number of cells with TUNEL label than did H lines (Figure 1E).

The frequency of normal and aborted pollen was estimated using the Alexander staining in 1B and 0B plants of the four genotypes (Table 8; Figure 2I). A two-way ANOVA showed that there are significant differences of pollen grain abortion among genotypes ($F = 7.88$; $P = 0.0001$) and between 0B and 1B plants ($F = 4.43$; $P = 0.038$), the interaction being also significant ($F = 4.52$; $P = 0.006$). The LSD *post hoc* test shows that the H line has more aborted pollen, and 1B plants of the H line have more aborted pollen than any other genotype (Figure 1F).

To determine whether the chromosome instabilities and micronuclei observed in the tapetal cells were related to the PCD process and pollen abortion, we studied the correlations between the following variables: micronucleus frequency, B nondisjunction frequency, knob unequal distribution, chromosome 6 nondisjunction, PCD (as frequency of TUNEL labeled cells), and pollen abortion. Since the variables related to B chromosomes can be studied only in 1B plants, we calculated the correlation coefficients in 1B and 0B plants separately. Table 9 shows the significant correlation coefficients, indicating that B nonsdisjunction, micronucleus frequency, and PCD are negatively correlated with pollen abortion.

DISCUSSION

B chromosomes are extra chromosomes that are not fully integrated into the normal behavior of the standard complement. In many plant species they are unstable at meiosis and undergo nondisjunction at pollen mitosis

TABLE 4

Types of binucleate tapetal cells observed with the pZmBs and the pZm4-21 probes in 1B plants

	Types of cells				
B transmission line	Normal	Normal for the B's unequal knob distribution	B nondisjunction equal knob distribution	B nondisjunction unequal knob distribution	Total
H, five individuals	200 (185.25)	57 (71.75)	53 (67.65)	41 (26.25)	351
HL, five individuals	170 (163.08)	55 (61.92)	96 (102.92)	46 (39.08)	367
LH, five individuals	188 (177.4)	58 (68.61)	99 (109.6)	53 (42.39)	398
L, six individuals	123 (124.8)	87 (85.17)	91 (89.2)	59 (60.83)	360

The number of cells expected under random assortment is shown in parentheses.

TABLE 5

Types of binucleate tapetal cells observed with the pTa71 probe

		Types of cells				
		Without micronucleus			With micronucleus	
B transmission line	B no.	Normal	Nondisjunction of one chromosome 6	Nondisjunction of both chromosome 6's	Chromosome 6 in micronucleus	Unlabeled micronucleus
H						
Seven individuals	1B	391 (86.51)	57 (12.61)	4 (0.88)	0	6
Six individuals	0B	401 (93.69)	26 (6.08)	1 (0.23)	0	1
HL						
Five individuals	1B	294 (78.82)	74 (19.84)	5 (1.34)	1	6
Five individuals	0B	328 (95.35)	16 (4.65)	0	3	3
LH						
Four individuals	1B	295 (83.57)	56 (15.86)	2 (0.57)	2	14
Five individuals	0B	304 (89.94)	31 (9.17)	3 (0.89)	2	10
L						
Six individuals	1B	393 (81.70)	87 (18.09)	1 (0.21)	5	18
Nine individuals	0B	1022 (93.59)	69 (6.32)	1 (0.09)	5	7

Percentages are shown in parentheses.

determining particular B transmission mechanisms. In maize, B nondisjunction regularly occurs at the second pollen mitosis (Randolph 1941; Carlson 1986), in the endosperm and in the tapetum. In these latter cases the B's may induce instabilities in the A's (Rhoades *et al.* 1967; Rhoades and Dempsey 1972, 1973; Alfenito and Birchler 1990; Chiavarino *et al.* 2001). These alterations might produce deleterious quantitative phenotypic effects, particularly related to fertility, because chromosome instabilities occur at various stages of sexual reproduction, whereas they are somatically stable. Remarkably, there are very few works on B maize effects on fitness variables in spite of the large literature on maize B chromosomes (Jones and Rees 1982).

Previous works of our laboratory have characterized the genetic control of maize B transmission rate (González-Sánchez *et al.* 2003). One gene located on the A chromosomes, which we call *mBt* (male B transmission), controls B preferential fertilization on the male side. Female B transmission is controlled by a gene(s) called *fBt* (female B transmission) also located on the A's. The H and L lines used in this work are the fBt^h fBt^h and the fBt^l fBt^l homozygous, respectively. In the L line and in the LH and HL F_1 hybrids (fBt^l fBt^h heterozygous) a significant loss of the B chromosome occurs both at meiosis of 1B plants and in the progeny of 1B × 0B crosses.

Chromosome instability is easily observed as micronucleus formation, which is a regular event in binucleate tapetal cells, because micronuclei appear in plants of all constitutions. The B chromosome is particularly unstable because it forms ~15% of the micronuclei, whereas 1/21 is expected. However, L, HL, and LH plants form more micronuclei than the H line does, showing, first, a genetic component in this process and, second, that the fBt^l allele(s) is dominant at this level. We think that micronucleus formation is also affected by environmental conditions because we found a significantly higher frequency of micronuclei in a previous study (Chiavarino *et al.* 2001).

The frequency of B nondisjunction in the binucleate tapetal cells is similarly related to the studied genotypes. Also in this case the fBt^l allele(s) behaves as a dominant, because H plants show significantly less B nondisjunction than do L, HL, and LH plants.

A parallel behavior of the B chromosome occurs in PMCs and in the tapetum. B univalents are conserved at male meiosis in the H line, whereas they are lost in a significant proportion of the microspores of the L line and the HL and LH hybrids (González-Sánchez *et al.* 2003). This shows that the fBt^l allele(s) is dominant and that there is not a maternal effect, because in both hybrids the B is lost both at meiosis and in its transmission with the same frequency as in the L line. We have no data on the B meiotic behavior on the female side because a quantitative study of maize female meiosis is unattainable. However, it is reasonable to accept that B behavior at female meiosis is similar to that of male meiosis because B loss or conservation at male meiosis and female B transmission are strongly correlated. Therefore, it can be suggested that the fBt^l allele(s) produces B instability in both the PMCs and the binucleate tapetal cells. Since the fBt^l allele(s) is dominant, it can also be suggested that its expression is sporophytic and occurring at the diploid level, before the reduction of chromosome number.

The similarity between PMCs and tapetal cells is remarkable. Both the sporogenous and tapetal cells have

TABLE 6

Types of binucleate tapetal cells observed with the pZmBs and the pTa71 probes in 1B plants

B transmission line	Normal	Normal for the B's, nondisjunction of one chromosome 6	B nondisjunction, normal for chromosome 6	B and one chromosome 6 nondisjunction	Normal for the Bs, nondisjunction of both chromosome 6's	B and both chromosome 6's nondisjunction
H, seven individuals	275 (266.43)	29 (41.56)[a]	116 (124.6)	28 (19.43)[a]	4	0
HL, five individuals	193 (179.71)	34 (48.3)[a]	101 (114.3)	40 (30.7)[a]	1	4
LH, four individuals	193 (190.54)	34 (37.46)[a]	102 (104.46)	22 (20.54)[a]	1	1
L, six individuals	257 (241.03)	38 (53.97)[a]	136 (151.97)	49 (34.03)[a]	0	1

The number of cells expected under random assortment is shown in parentheses.

[a] The chi square was calculated by adding the number of cells shown in columns 3 to 6 and 4 to 7.

a common origin from the archesporial cells (Echlin 1973; Canales *et al.* 2002) and remain in close contact during their development. Yang *et al.* (1999), analyzing the *SPL* mutation in Arabidopsis, suggested that the development of the anther walls, the tapetum, and the microsporocytes is tightly coupled. Interestingly, Aragón-Alcaide *et al.* (1997) found association of homologous chromosomes simultaneously both in premeiotic PMCs and in the surrounding tapetal cells of wheat. It may be hypothesized that sporophytic to gametophytic transition is initiated with a signal terminating in tapetal PCD on one side and pollen grain maturation on the other.

Knobbed chromosomes also show instabilities in binucleate tapetal cells. In all cases 1B plants show more knob unequal distribution than 0B plants, and L plants show more unequal distribution than H plants, but HL and LH behave as intermediates. On the other hand, in H, HL, and LH the observed number of normal distribution of both the B and the knobs and nondisjunction of both the B and the knobs was higher than expected. This suggests that instability is extended to all chromosomes when either the B's or the knobbed chromosomes become unstable.

Our hypothesis is that the *fBt*^{l} allele is directly related to a differential knob constitution present in the L line and absent in the H line. If the *fBt*^{l} allele(s) induces knob instability, the B, which also carries a knob, is also affected. Probably, the B would be affected more than any other chromosome because of its own special constitution. This instability would be sufficient to induce B loss in many cells, both PMCs and binucleate tapetal cells. B instability could, in turn, produce instabilities in all of the chromosomes of the maize complement. This hypothesis would also explain the intermediate behavior of the HL and LH F_1 hybrids for the unequal knob distribution, because the L line would have two of these unstable knobs, whereas the hybrids would have only one.

This hypothesis is in agreement with the instabilities observed in chromosome 6. In this case nondisjunction of chromosome 6 is not related to the H, L, HL, and LH genotypes, but 1B plants show more instabilities than 0B plants. In all cases, the observed number of normal distribution of both the B and the 6 and nondisjunction of both the B and the 6 was higher than the nondisjunction of only one of the chromosomes. This is again in agreement with the hypothesis that B instability induces instability in the remaining chromosomes, particularly in knobbed chromosomes like chromosome 6. In this case, chromosome 6 is not affected by the *fBt*^{l} allele.

It is interesting to study whether these chromosome instabilities are related to the PCD process, which is an essential feature of the binucleate tapetal cells. Granell (1999), Buckner *et al.* (1998, 2000), Wu and Cheung (2000), and Giuliani (2002) reported reviews of the PCD program in plants where the TUNEL technique is mainly

TABLE 7

Types of binucleate tapetal cells observed with the TUNEL technique in 0B and 1B plants

B transmission line	B no.	Types of cells		Total
		With TUNEL label	Without TUNEL label	
H				
Two individuals	1B	51 (10.02)	458 (89.98)	509
Two individuals	0B	94 (25.61)	273 (74.39)	367
HL				
Two individuals	1B	167 (49.12)	173 (50.88)	340
Two individuals	0B	107 (43.67)	138 (56.33)	245
LH				
Two individuals	1B	174 (38.00)	284 (62.01)	458
Two individuals	0B	88 (47.57)	97 (52.43)	185
L				
Three individuals	1B	270 (34.66)	509 (65.34)	779
Three individuals	0B	205 (44.86)	252 (55.14)	457

Percentages are shown in parentheses.

used (Jones 2001), but FISH is not generally used in PCD studies to reveal the fate of specific chromosomes during cell death. In our laboratory both TUNEL and FISH have been used, FISH being particularly useful to show chromosome instabilities in binucleate tapetal cells when the PMCs are at meiotic stages.

A higher frequency of cells showing TUNEL label was found in L, HL, and LH plants than in the H line at metaphase I. This result is not surprising because one would expect that higher levels of chromosome instabilities would result in acceleration of the PCD process. It is, however, unexpected that 1B plants of the H line suffer a significant delay with respect to 0B plants because 1B plants are always more unstable.

Interestingly, the PCD process, estimated as the frequency of binucleate tapetal cells with TUNEL label, is significantly correlated with the frequency of pollen abortion. L, HL, and LH plants show very low frequency of pollen abortion, whereas the plants of the H line show significantly higher pollen abortion, particularly 1B ones. It can be concluded that the chromosome instabilities observed in the L, HL, and LH plants are important to the PCD process and to the development of microspores to form viable pollen grains. We do not have sufficient information to determine whether or not the *fBt* gene(s) is directly related to the PCD process. However, the death signal transduction occurs via pleiotropic signaling pathways (Kuriyama and Fukuda 2002) and PCD seems to be affected by the expression of *fBt*.

The fBt^{I} dominant allele(s), inducing knobbed chromosome instabilities, increases plant fitness because plants with this allele(s) have nearly 100% of viable

TABLE 8

Normal and aborted pollen grains in 0B and 1B plants of the four genotypes

B transmission line	B no.	No. of normal pollen grains	No. of aborted pollen grains	Total
H				
Four individuals	1	2789 (77.47)	811 (22.53)	3600
Four individuals	0	3445 (95.69)	155 (4.31)	3600
HL				
Four individuals	1	2963 (98.77)	37 (1.23)	3000
Three individuals	0	2069 (98.52)	31 (1.48)	2100
LH				
Three individuals	1	2066 (98.38)	34 (1.62)	2100
Four individuals	0	2666 (98.74)	34 (1.26)	2700
L				
Three individuals	1	2634 (97.56)	66 (2.44)	2700
Four individuals	0	3544 (98.44)	56 (1.56)	3600

Percentages are shown in parentheses.

TABLE 9

Significant correlation coefficients between the variables studied

	1B plants		0B plants	
Variables	*r*	*P*	*r*	*P*
B nondisjunction and aborted pollen	−0.9788	0.0212		
Micronuclei and PCD	0.9737	0.0263	0.9777	0.0222
Micronuclei and aborted pollen	−0.9579	0.0421	−0.9519	0.0480
PCD and aborted pollen	−0.9418	0.058	−0.9949	0.0051

pollen grains and tend to lose the B's. Conversely, the *fBt*^{h} recessive allele produces chromosome stability and, consequently, lack of B meiotic loss in 1B plants. It is possible that B presence during microspore development produces deleterious effects, the frequency of pollen aborted reaching 22.5%. Unfortunately, we have no data showing a possible reduction of fertility on the female side in 1B plants.

As instabilities of knobbed chromosomes and chromosome 6 are not correlated with TUNEL label frequency or pollen abortion, it can be deduced that A chromosome instabilities induced by B chromosomes do not affect normal pollen development. This indicates the slight parasitic nature of maize B chromosomes and reinforces the idea that the *fBt*^{l} allele(s) provides the defense of the A complement against the B chromosome attack (González-Sánchez *et al.* 2003).

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