Chromosome Res (2016) 24:183–195
DOI 10.1007/s10577-015-9516-2

Cytomolecular characterization and origin of de novo formed maize B chromosome variants

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Received: 2 November 2015 / Revised: 20 December 2015 / Accepted: 29 December 2015 / Published online: 9 January 2016

Abstract B chromosomes are dispensable elements that occur in many species, including maize. The maize B chromosome is acrocentric and highly heterochromatic and undergoes nondisjunction during the second pollen mitosis. In this study, we determined the genetic behavior and organization of two naturally occurring B chromosome variants (designated B^{ta} and B^{tb}). The morphology and genetic behavior of the B^{ta} chromosome were similar to those of the typical B chromosome, but the B^{ta} chromosome contained a deletion in the first heterochromatin region and had higher transmission frequencies through both male and female parents. The B^{tb} chromosome was reduced in size, consisted primarily of heterochromatin, and had a lower transmission frequency. The B^{tb} chromosome lacked nondisjunctional behavior, which was restored by the presence of normal B chromosomes in the cell. Furthermore, the B^{tb} chromosome contained two centromeric regions, only one of which was active. The organization of these two naturally occurring B chromosome variants was also determined using fluorescence in situ hybridization with B-associated sequences and by amplification of B-specific molecular markers to create possible evolutionary models.

Keywords B chromosome · FISH · maize · variant · dicentric · centromere

Abbreviations

BFB	Breakage-fusion-bridge
CK	Centromeric knob
DE	Distal euchromatin
DH	Distal heterochromatin
FISH	Fluorescence in situ hybridization
PE	Proximal euchromatin
SCAR	Sequence-characterized amplified region

Introduction

Supernumerary B chromosomes are dispensable chromosomes that contain large selfish DNA elements. B chromosomes were first described more than a century ago (Wilson 1907) and have been identified in approximately 15 % of eukaryotic species (Jones and Rees 1982; Camacho 2005). Generally, B chromosomes do not pair or recombine with any of the normal

Responsible Editor: Jiming Jiang.

Electronic supplementary material The online version of this article (doi:10.1007/s10577-015-9516-2) contains supplementary material, which is available to authorized users.

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chromosomes (A chromosomes) during meiosis, are often heterochromatic, lack detectable genetic effects on individuals, and are transmitted in a non-Mendelian manner (Jones et al. 2008). Although the genetic features and molecular compositions of B chromosomes have been revealed, their origin and evolution remain a mystery (Jones and Houben 2003; Jones et al. 2008; Houben et al. 2014). Due to their nonessential nature, B chromosome polymorphisms are expected among populations. Indeed, several B chromosome structural variants have been identified in plants such as *Brachycome dichromosomatica* (Houben et al. 1999), *Scilla autumnalis* (Parker et al. 1991), and the rye *Secale cereale* (Marques et al. 2012), as well as in animals such as the grasshopper *Eyprepocnemis plorans* (Bakkali et al. 1999). These studies indicate that most B chromosome variants have a monophyletic origin from a unique type of ancestral B chromosome.

The maize B chromosome was identified a century ago (Kuwada 1915). During the pachytene stage of meiotic prophase I, the typical B chromosome contains a diminutive short arm and a long arm with a heterochromatic knob adjacent to the centromere, a proximal euchromatic region, a large block of distal heterochromatin, and a distal euchromatic region (McClintock 1933). To ensure its survival, the maize B chromosome has evolved several mechanisms, such as nondisjunction during the second pollen mitosis (Longley 1927; Roman 1947) and preferential fertilization of the egg by sperm carrying B chromosomes (Roman 1948). The nondisjunction mechanism requires *trans*-acting elements located in the proximal and distal euchromatic regions of the B chromosome long arm (Ward 1973; Lin 1978). To obtain insight into the nature and origin of the maize B chromosome, numerous molecular approaches have been applied to evaluate the organization and transcription of its DNA. The results have led researchers to isolate repetitive elements and molecular markers specific to the maize B chromosome (Alfenito and Birchler 1993; Stark et al. 1996; Lin and Chou 1997; Cheng and Lin 2004; Peng et al. 2005; Lamb et al. 2007; Chien et al. 2014; Lin et al. 2014; Kao et al. 2015). However, most of the identified maize B chromosomal DNA is similar to A chromosomal DNA (Chilton and McCarthy 1973; Alfenito and Birchler 1993; Stark et al. 1996; Cheng and Lin 2003; Lamb et al. 2005; Lo et al. 2009).

In maize, the genetic behavior and mitotic structure of four types of B chromosome variants derived from the typical B chromosome have been documented (Randolph 1941). However, a detailed model of the possible origin of these variants has not been proposed. Moreover, a collection of small maize B chromosomes that vary in size was identified during the breakage-fusion-bridge (BFB) cycle (McClintock 1939, 1941) of a translocation between the B chromosome and the short arm of chromosome 9 (Zheng et al. 1999). The structure and transmission rates of these B chromosome variants, as well as their pairing, disjunction and sister chromatid cohesion during meiosis, have been reported (Kato et al. 2005; Han et al. 2007). Furthermore, engineered minichromosomes were constructed by modifying the maize B chromosome using telomere sequence-mediated chromosome truncation and were characterized (Yu et al. 2007; Masonbrink et al. 2013). These minichromosomes offer enormous potential for the development of artificial chromosomes for use in plant breeding and biotechnology (Birchler and Han 2013; Houben et al. 2013).

To gain greater understanding of the origin of the naturally occurring B chromosome variants, we identified and characterized two structural B chromosome variants derived from the typical maize B chromosome. We found that both B chromosome variants displayed different transmission characteristics compared with those of the typical B chromosome, and that one of them lost its nondisjunctional ability. Using fluorescence in situ hybridization with B-associated sequences and amplification assays of B-specific molecular markers, the organization of these two B chromosome variants was determined, and accordingly, possible models of their origin were proposed.

Materials and methods

Plant materials

The maize inbred line L289, which carries typical B chromosomes, was propagated by crossing euploid L289 plants in our laboratory for decades. Two progeny plants containing B chromosome variants (designated B^{ta} and B^{tb}) that differ in morphology from that of the typical B chromosome were identified and characterized. The B^{ta} chromosome was discovered in a progeny

plant derived from an L289 plant containing two B chromosomes, and the B^{tb} chromosome was discovered in a progeny plant derived from an L289 plant containing one B chromosome.

Cytogenetic procedures

Chromosomal constitutions were determined in Feulgen-stained root-tip cells as described by Lin (1977). To determine the nondisjunction and transmission frequencies of the B variants, L289 plants with one B, B^{ta}, or B^{tb} chromosome were crossed as males and females with euploid L289 plants. To test the nondisjunction of the B^{tb} chromosome in the presence of the normal B chromosome, an L289 plant carrying two B and one B^{tb} chromosomes was crossed as the male to a euploid L289 plant. The chromosomal constitutions of the resulting progeny were identified in root-tip cells. Chromosomes at different meiotic stages were prepared in pollen mother cells from L289 plants bearing two B, B^{ta}, or B^{tb} chromosomes following the standard protocol (Cheng and Lin 2003). The lengths of the pachytene chromosomes were measured using an imaging system (DP2-BSW ver. 2.1, Olympus Corp., Tokyo, Japan).

Fluorescence in situ hybridization (FISH) was performed according to the method of Cheng (2010). The B-repeat probe specific to the B chromosome centromere was generated using the clone B1.1a (Cheng 2010). The CL-repeat probe was generated using the clone pCLa1 (Cheng and Lin 2004). The Stark repeat probe, generated using the clone pStark-1, was provided by J. A. Birchler (Lamb et al. 2007). The CentC probe was derived from clone CC0B-1 (Peng and Cheng 2011). The 180-bp knob repeat was derived from clone pBPC21 (Cheng and Lin 2003). The probe directed against the telomere was cloned as described in Wang and Chen (2003), and the probe directed against the long terminal repeats of the maize retroelement CentA was cloned as described in Mroczek and Dawe (2003), except that a pGEM-T easy vector (Promega, Madison, WI, USA) was used. The identity of the DNA insert was confirmed by sequencing. The fluorescence signals were captured using a cooled charge-coupled device camera (DP73, Olympus Corp.) on an Olympus BX51 fluorescence microscope and were processed using Photoshop (Adobe, San Jose, CA, USA).

Genomic DNA isolation and B-specific molecular marker amplification

Maize genomic DNA was isolated according to Lin and Chou (1997). Primers for six maize B-specific molecular markers were used, including CL-repeat (Cheng and Lin 2004), Stark (Lamb et al. 2007), SCAR313, SCAR345, SCAR349, and SCAR426 (Kao et al. 2015). PCR was performed in a 25-μl reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 M betaine (Sigma-Aldrich, MO, USA), 100 μM of each dNTP, 0.25 μM of each primer (see Supplemental Table 1), 100 ng of genomic DNA, and 0.5 units of Pro-*Taq* DNA polymerase (Protech, Taipei, Taiwan). Amplification was performed in a Perkin Elmer GeneAmp 2400 Thermal Cycler under the following conditions: 5 min at 94 °C; 30 cycles of 30 s at 94 °C; 30 s at the annealing temperature (Supplemental Table 1) and 90 s at 72 °C; and a final extension for 10 min at 72 °C. The resulting products were separated via 1 % agarose gel electrophoresis. A pair of maize *actin* gene-specific amplification primers was used to produce an internal control, according to the method of Kao et al. (2015).

Results

Chromosomal structures of B chromosome variants

During mitotic metaphase, the morphology of the B^{ta} chromosome was similar to that of the typical B chromosome (Fig. 1a, b), but the B^{tb} chromosome was reduced in size (Fig. 1c). According to FISH using probes directed against the centromere-specific sequence CentC (Ananiev et al. 1998) and the B-centromere-specific sequence B-repeat (Alfenito and Birchler 1993), both the B and B^{ta} chromosomes exhibited B-repeat signals adjacent to the CentC signals at one end, as well as CentC signals along the long arm (Fig. 1d, e). However, the B^{tb} chromosome displayed centromere-specific signals at both ends, suggesting it was a dicentric chromosome (Fig. 1f). The presence of B-repeat signals confirmed that the two B variants were derived from the typical B chromosome. To determine the fine structures of the B variants, bivalent chromosomes were observed during the pachytene stage. The pachytene B chromosome consists of a short arm, a centromeric knob (CK), proximal euchromatin (PE),

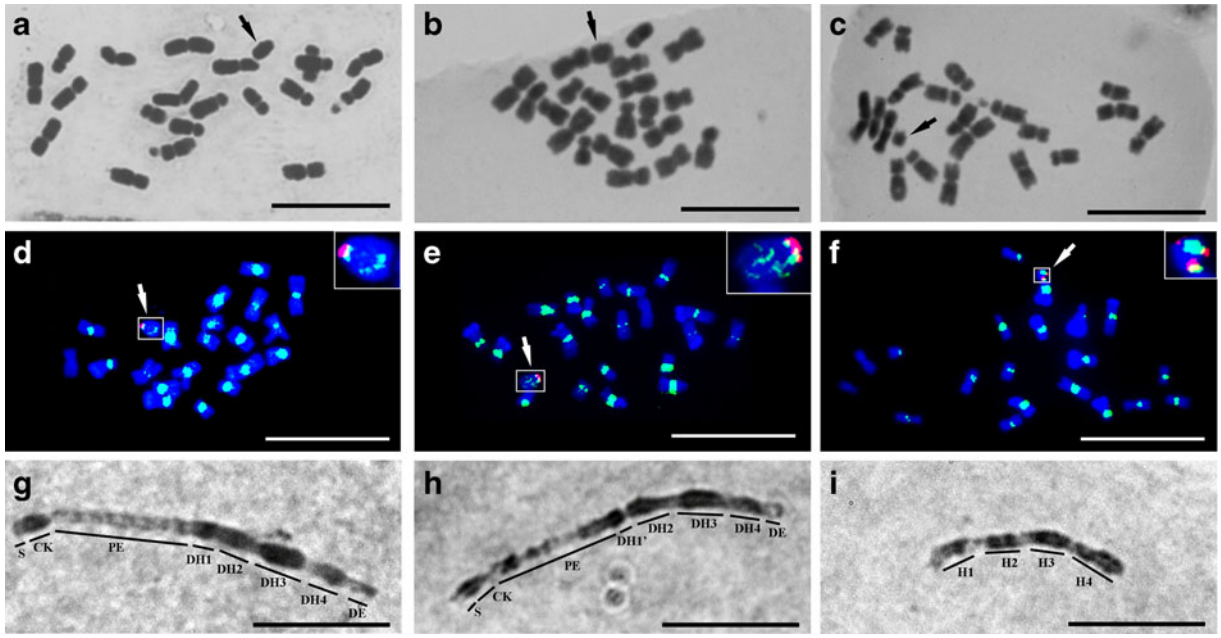

Fig. 1 Chromosomal structures of B chromosome variants. Mitotic metaphase cells containing the typical B (**a**, **d**), B^{ta} (**b**, **e**), or B^{tb} (**c**, **f**) chromosome. *Arrows* indicate the B chromosome and B variants. The maize centromere-specific CentC probe (*green*) and the B-chromosome-specific B-repeat probe (*red*) were used to detect the B and A chromosome centromeres (**d**–**f**). The pachytene B chromosome consists of a short arm (*S*), a centromeric knob (*CK*), proximal euchromatin (*PE*), four blocks of distal heterochromatin (*DH1–DH4*), and distal euchromatin (*DE*) (**g**). The pachytene B^{ta} chromosome is similar to the B chromosome but contains a block of distal heterochromatin (*DH1'*) that is smaller than the DH1 block of the B chromosome (**h**). The pachytene B^{tb} chromosome consists of four blocks of heterochromatin (*H1–H4*) (**i**). *Scale bars* are all equal to 10 μm

four blocks of distal heterochromatin (DH1–DH4), and distal euchromatin (DE) (Fig. 1g). The pachytene B^{ta} chromosome was similar to the B chromosome but contained a smaller first heterochromatin region (DH1') than that of the B chromosome (Fig. 1h). The ratio between lengths of the DH1 region and the B chromosome was 0.08 ± 0.1 ($n = 10$), and that of the DH1' region and the B^{ta} chromosome was 0.05 ± 0.1 ($n = 10$; Supplemental Table 2). In contrast, the pachytene B^{tb} chromosome only carried four blocks of heterochromatin (H1–H4) (Fig. 1i).

Homolog pairing, transmission, and nondisjunction of B chromosome variants

To determine the behavior of the B chromosome variants during cell division, mitotic cells containing a single B, B^{ta}, or B^{tb} chromosome were hybridized with the B-repeat and CentC probes. The results showed that all three B chromosome types moved normally during metaphase, anaphase, and telophase, suggesting that each of the B variants had a functional centromere (Supplemental Figure S1). During the diakinesis of pollen mother cells carrying two B, B^{ta}, or B^{tb} chromosomes, 100 % ($n = 81$) of the observed cells containing the typical B chromosome formed bivalents, whereas 10 % ($n = 131$) of those containing the B^{ta} chromosome and 62 % ($n = 148$) of those containing the B^{tb} chromosome formed two unpaired univalents (Supplemental Table 3 and Fig. 2). These results support a positive correlation between chromosome size and homolog pairing, as described by Han et al. (2007).

The transmission frequencies of L289 plants carrying one B variant chromosome through their male and female gametes were 31.6 and 25 % for the B chromosome, 41.7 and 43.4 % for the B^{ta} chromosome, and 22.4 and 20 % for the B^{tb} chromosome, respectively (Table 1). The nondisjunction frequencies of the B and B^{ta} chromosomes during the second pollen mitosis were determined to be 54 and 57.2 %, respectively. The B^{tb} chromosome lacked the ability to undergo nondisjunction. However, when normal B chromosomes were also present, nondisjunction of the B^{tb} chromosome was restored (Table 2). This result was expected, given that the PE and DE regions of the B chromosome are

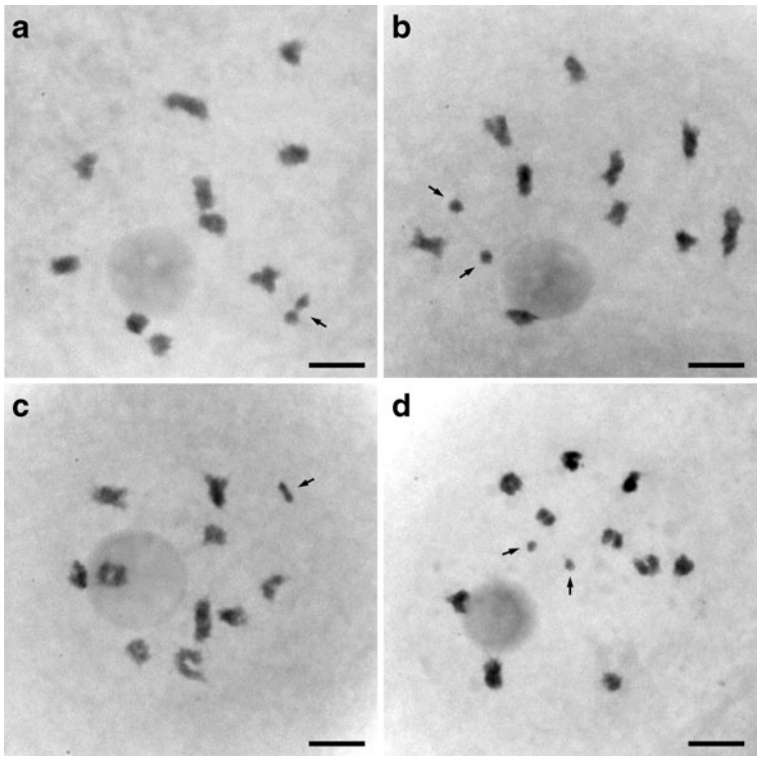

Fig. 2 Homolog pairing of B chromosome variants. Pollen mother cells with two copies of the B^{ta} (**a**, **b**) or B^{tb} (**c**, **d**) chromosome were observed during diakinesis. *Arrows* show bivalents (**a**, **c**) or univalents (**b**, **d**) of B chromosome variants. *Scale bars* are all equal to 10 μm

essential for nondisjunction (Ward 1973; Lin 1978). Moreover, novel structural B variants that differed in size and morphology from the B^{ta} or B^{tb} chromosome were observed in seven progeny (Tables 1 and 2). The two progenies of plants carrying the B^{ta} chromosome contained B variants that were smaller than the B^{ta} chromosome, and the four progenies of plants carrying the B^{tb} chromosome contained B variants that were larger than the B^{tb} chromosome. The remaining one B variant derived from the B^{tb} chromosome was a metacentric chromosome (data not shown).

Distribution of maize repetitive elements on B chromosome variants

To investigate the organization of the B chromosome variants in detail, maize repetitive elements that have been localized to the B chromosome, including the B-

Table 1 Transmission and nondisjunction frequencies of B chromosome variants

Female parent	Male parent	B variant frequency in progeny			Total (γ)	Transmission frequency (%)[a]	Nondisjunction frequency (%)[b]
		0	1 (α)	2 (β)			
L289	L289 + 1B	39	10	8	57	31.6	54.0
L289 + 1B	L289	45	15	0	60	25.0	0
L289	L289 + $1B^{ta}$	42	16 (2)[c]	14	72	41.7	57.2
L289 + $1B^{ta}$	L289	56	43	0	99	43.4	0
L289	L289 + $1B^{tb}$	38	11 (3)	0	49	22.4	0
L289 + $1B^{tb}$	L289	49	13 (1)	0	62	20.0	0

[a] Transmission frequency = $(\alpha + \beta)/\gamma$

[b] Nondisjunction frequency = $(\beta/0.655)/(\alpha + \beta/0.655)$. The value for progeny with two B variants was corrected to account for approximately 65.5 % preferential fertilization of sperm carrying two B chromosomes (Carlson 1969)

[c] The number of progeny that exhibited a novel B variant that differed in size and morphology from the B^{ta} or B^{tb} chromosome

Table 2 The occurrence of B^{tb} nondisjunction in the presence of a normal B chromosome

Female parent	Male parent	B variant frequency in progeny							Total
		0	2B	1B + 2B^{tb}	2B + 1B^{tb}	2B + 2B^{tb}	1B^{tb}	2B^{tb}	
L289	L289 + 2B + 1B^{tb}	20	17	1	2 (1)[a]	3	2	1	46

[a] The number of progeny that exhibited a novel B variant that differed in size and morphology from the B^{tb} chromosome

repeat, telomere repeat, 180-bp knob repeat, CL-repeat, Stark repeat, CentC, and CentA (Lamb et al. 2005), were used as FISH probes for hybridization of pachytene bivalents of the B variants (Fig. 3). B-repeat signals were observed at the distal half of the CK on the B and B^{ta} chromosomes (Fig. 3a, b) but were detected at both terminals of the B^{tb} chromosome (Fig. 3c). Telomere signals were detected at both distal ends of all three

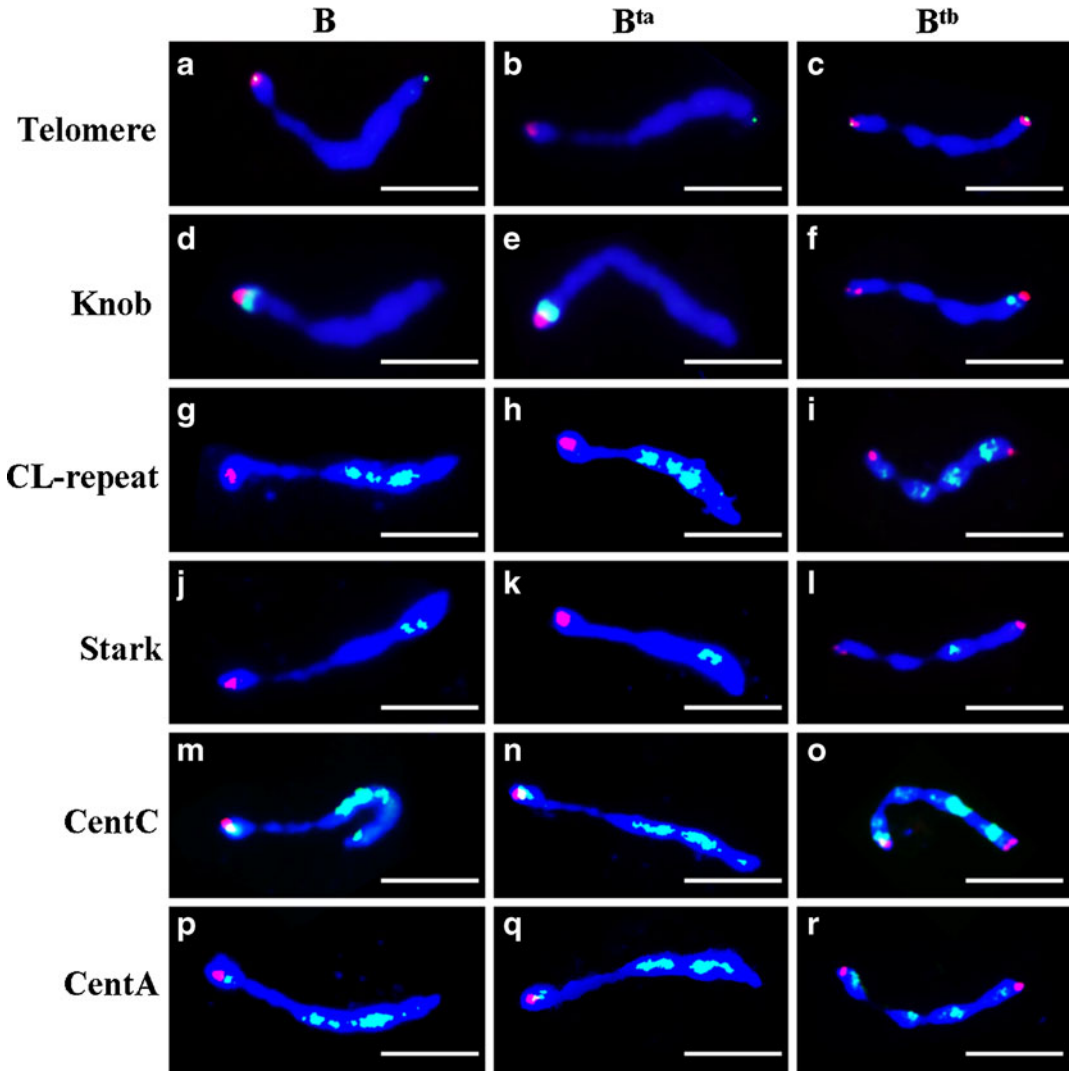

Fig. 3 FISH analysis of B chromosome variants. Pachytene bivalents of the B, B^{ta}, and B^{tb} chromosomes were hybridized with FISH probes specific for various maize repetitive elements (green), including a telomere repeat (**a**–**c**), 180-bp knob repeat (**d**–**f**), CL-repeat (**g**–**i**), Stark repeat (**j**–**l**), CentC repeat (**m**–**o**), and CentA repeat (**p**–**r**). Chromosomes were stained with DAPI (*blue*) and labeled with the B-repeat probe (*red*). *Scale bars* are all equal to 10 μm

chromosomes (Fig. 3a–c). The 180-bp knob repeat signals were found at the proximal half of the CK of the B and B^{ta} chromosomes (Fig. 3d, e). On the B^{tb} chromosome, the 180-bp knob repeat signals were present near the B-repeat signals at the distal end of the H4 block (Fig. 3f). The CL-repeat probe hybridized to the first three heterochromatin regions of the B and B^{ta} chromosomes (Fig. 3g, h) and to all four heterochromatin regions of the B^{tb} chromosome (Fig. 3i). The Stark repeat was detected specifically at the DH3 block of the B and B^{ta} chromosomes (Fig. 3j, k) and at the H3 block of the B^{tb} chromosome (Fig. 3i). CentC signals were observed in multiple regions along the length of the B and B^{ta} chromosomes, including a CK region that colocalized with a region of intense B-repeat signaling and the four blocks of heterochromatin (Fig. 3m, n). On the B^{tb} chromosome, the CentC signals colocalized with the B-repeat signals at the distal half of the H1 block and were located at the proximal half of the H1 block, as well as at the other three blocks of heterochromatin (Fig. 3o). The CentA signals on the B and B^{ta} chromosomes were located at the CK near the B-repeat signals and at the four heterochromatin regions (Fig. 3p, q). On the B^{tb} chromosome, CentA signals were detected at the proximal half of the H1 block as well as at the other three blocks of heterochromatin (Fig. 3r). The above FISH results are graphically presented in Fig. 4.

The active centromere of B chromosome variants

To determine the position of the active centromere on B variants, we used the B-repeat and the 180-bp knob repeat as FISH probes to analyze the B variants during meiotic metaphase I. On the B and B^{ta} chromosomes, the 180-bp knob repeat signals were located behind the B-repeat signals at the most poleward positions (Fig. 5a, b), indicating that the CK regions of the B and B^{ta} chromosomes contained active centromeres (Fig. 4a, b). On the dicentric B^{tb} chromosome, a sole B-repeat signal was observed at the most poleward position, and another B-repeat signal that colocalized with the 180-bp knob repeat signals was observed at the equatorial plate (Fig. 5c). Accordingly, the active centromere of the dicentric B^{tb} chromosome was located in the distal half of the H1 region, whereas the centromere at the end of the H4 region was inactivated (Figs. 3f and 4c).

Amplification of B-specific molecular markers of B chromosome variants

To determine the fine structural rearrangements that occurred on the B variants, primer pairs that amplify six B-specific markers that have been mapped to definitive B chromosome regions were used for PCR of the

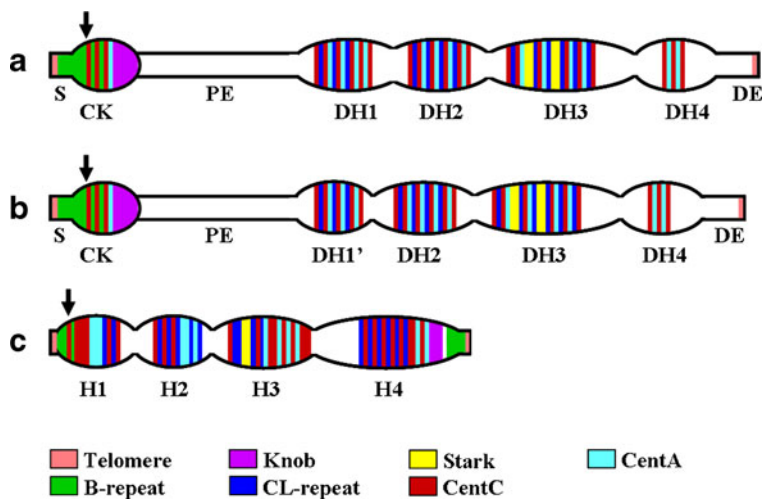

Fig. 4 Hybridization patterns of maize repetitive elements on B chromosome variants. The *diagrams* illustrate the hybridization patterns of probes for various repetitive elements on the B (**a**), B^{ta} (**b**), and B^{tb} (**c**) chromosomes. The pachytene B chromosome consists of a short arm (*S*), a centromeric knob (*CK*), proximal euchromatin (*PE*), four blocks of distal heterochromatin (*DH1–DH4*), and distal euchromatin (*DE*). The pachytene B^{ta} chromosome is similar to the B chromosome but contains a first block of distal heterochromatin (*DH1'*) that is smaller than the DH1 block of the B chromosome. The pachytene B^{tb} chromosome carries four blocks of heterochromatin (*H1–H4*). *Arrows* indicate the position of the active centromere

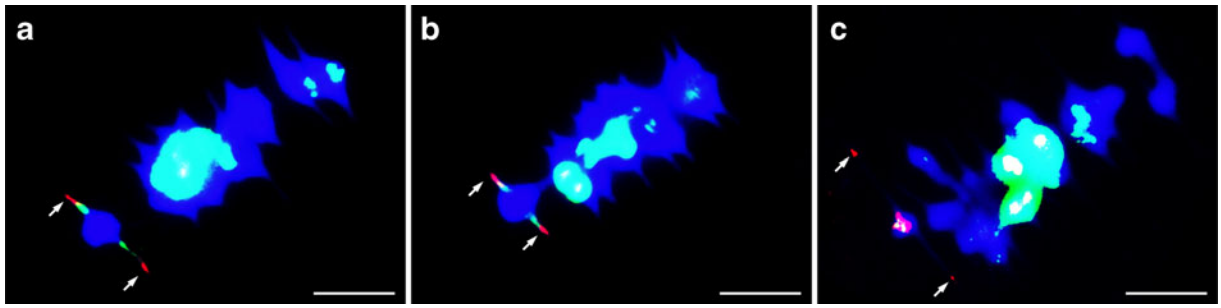

Fig. 5 Determination of the functional centromere of B chromosome variants. Meiotic metaphase I cells containing bivalent B (**a**), B^{ta} (**b**), or B^{tb} (**c**) chromosomes were hybridized with the 180-bp knob repeat (*green*) and the B-repeat (*red*) probes. Chromosomes were stained with DAPI (*blue*). *White arrows* indicate the B-repeat signals at the most poleward positions. *Scale bars* are all equal to 10 μm

genomic DNA of plants carrying the three B chromosome types. As shown in Table 3 and Supplemental Figure S2, the CL-repeat marker was located at the CK and the first three heterochromatin regions of the B chromosome (Cheng 2010). The Stark marker was mapped to the DH3 region of the B chromosome (Lamb et al. 2007). The four B-specific sequence-characterized amplified region (SCAR) markers were located throughout the four distal heterochromatin regions of the B chromosome, as follows: SCAR426 at DH1; SCAR313 at DH1 and DH2; SCAR345 at DH1 and DH3; and SCAR349 at DH1 and DH4 (Kao et al. 2015). Genomic DNA from L289 plants lacking the B chromosome (0B) and containing one B (1B), B^{ta}, or B^{tb} chromosome was used as the template to amplify the six B-specific molecular markers. As shown in Table 3 and Supplemental Figure S3, PCR products corresponding to each marker were present in the 1B genomic DNA but were absent in the 0B genomic DNA, supporting the B chromosome specificity of these markers. Identical amplification patterns of the CL-repeat and Stark markers were obtained using 1B, B^{ta}, and B^{tb} genomic DNA. PCR for the four SCAR markers yielded similar amplification patterns, with lower levels of the products produced using B^{ta} genomic DNA as the template and only the SCAR345 product obtained using B^{tb} genomic DNA as the template.

Table 3 Amplification of six B-specific molecular markers from B chromosome variants

Marker	Position[a]	0B[b]	1B	B^{ta}	B^{tb}
CL-repeat	CK, DH1, DH2, DH3	–[c]	+	+	+
Stark	DH3	–	+	+	+
SCAR426	DH1	–	+	(+)	–
SCAR313	DH1, DH2	–	+	(+)	–
SCAR345	DH1, DH3	–	+	(+)	+
SCAR349	DH1, DH4	–	+	(+)	–

CK centromeric knob

DH1–DH4, four blocks of distal heterochromatin

[a] Mapped positions of the six B-specific molecular markers on the B chromosome according to Lamb et al. (2007), Cheng (2010), and Kao et al. (2015)

[b] Genomic DNA from L289 plants lacking the B chromosome (0B) and containing one B (1B), B^{ta}, or B^{tb} chromosome

[c] "+" and "–" represent the presence and absence of the B-specific marker, respectively. (+) represents a reduced level of PCR product compared with that of the one B PCR product

Discussion

Few studies have been published regarding the naturally occurring B chromosome derivatives in maize. Randolph (1941) described the four types of B chromosome derivatives, called the C, D, E, and F chromosomes, which are progressively smaller fragments of the typical B chromosome. The D chromosome, which resembles the B^{tb} chromosome, is spherical, with a diameter roughly equivalent to that of an ordinary chromosome. Longley (1956) proposed that these chromosomes originated from a foldback of a univalent B chromosome occurring during mid-prophase of meiosis, as described by McClintock (1933). The foldback configuration may have been due to homologous pairing between the different regions of the B chromosome, with subsequent exchanges in these regions leading to the formation of diminutive B chromosomes.

Based on the results of this study and those of Lamb et al. (2005), repetitive-element sequences are abundant in the CK and the four distal heterochromatin regions of the B chromosome (Figs. 3 and 4); these repetitive elements provide the foundation for homologous pairing between the different regions of the B chromosome. Accordingly, we can propose several possible processes that may have shaped the formation of the B^{ta} and B^{tb} chromosomes during meiosis. The B^{ta} chromosome may have arisen from an unequal crossing over in the DH1 region of the B chromosome (Fig. 6a). Based on the smaller size of the DH1' region in the B^{ta} chromosome (Supplemental Table 2 and Fig. 1h) and the reduced level of amplification of PCR products representing the four DH1-localized SCAR markers obtained using B^{ta} genomic DNA (Table 3 and Supplemental Figure S3), a single unequal crossing over may have occurred between the DH1 regions of two homologous B chromosomes, resulting in the formation of the DH1' region of the B^{ta} chromosome and the deletion of the distal portion of the DH1 region, the site of the four B-specific SCAR markers.

The formation of the B^{tb} chromosome from the B chromosome is more complicated. Longley (1956) proposed that the dyscentric pairing of a univalent B chromosome with an X-shaped or U-shaped exchange produced the diminutive B chromosome. Therefore, a univalent B chromosome could pair dyscentrically, followed by exchanges between different chromosomal regions and misdivision of the centromere during meiosis I to generate a dicentric chromosome. This dicentric chromosome may subsequently suffer the loss of function of one centromere, the amplification of heterochromatin, and the addition of telomeric repeats to produce the B^{tb} chromosome (Fig. 6b). The B^{tb} chromosome apparently lacks the short arm, possibly due to the misdivision of the centromere of a univalent B chromosome during meiosis I, which would result in the formation of telocentric chromosomes, as described by Kaszás and Birchler (1996). The broken ends were healed via the addition of telomeric repeats to stabilize this chromosome (Fig. 3c).

Furthermore, FISH analysis showed that the H1 region of the B^{tb} chromosome exhibited B-repeat signals that colocalized with CentC signals at the distal half and signals for three repetitive elements, CL-repeat, CentC, and CentA, at the proximal half (Fig. 4c). This signal organization in the H1 region could be derived from a U-shaped exchange between the CentC sequences at the centromeric region of the CK and the proximal half of the DH2 region in the dyscentric pairing configuration of the B chromosome that caused the loss of the CK, PE, DH1, and partial DH2 fragments from the dicentric chromosome (Fig. 6b), as shown by the absence of the 180-bp knob repeat at the H1 region of the B^{tb} chromosome (Figs. 3f and 4c) and the absence of the three SCAR markers in the DH1 or DH2 regions in the B^{tb} genomic DNA (Table 3 and Supplemental Figure S3). Similarly, another U-shaped exchange could occur between the 180-bp knob sequences at the CK and the DH4 region (Lamb et al. 2005) that would result in the loss of the DE and of partial DH4 fragments from the dicentric chromosome (Fig. 6b). This possibility was supported by the absence of SCAR349 from the DH4 region in the B^{tb} genomic DNA (Table 3 and Supplemental Figure S3) and the loss of nondisjunction, which requires the DE of the B chromosome (Ward 1973), by the B^{tb} chromosome (Table 1). The exchange also led to the reduction of the 180-bp knob repeats at the H4 region of the B^{tb} chromosome (Fig. 3f).

To be stable, a dicentric chromosome must have one active and one inactive centromere; otherwise, two active centromeres will lead to chromosome breakage during cell division. In maize, several stable dicentric chromosomes have been identified based on the B-A translocation of chromosomes undergoing the chromosome type BFB cycle (Han et al. 2006; Liu et al. 2015). In the dicentric B^{tb} chromosome, the centromere in the distal half of the H1 region is active, but the centromere at the end of the H4 region has become inactive (Figs. 4c and 5c). CentC repeats are the key elements of maize centromeres (Jin et al. 2004), and the functional centromere of the B chromosome is a small, CentC-rich domain that is embedded with a large array of B - repeats (Jin et al. 2005). Thus, it is rational to conclude that the centromere in the distal half of the H1 region is active because CentC signals colocalized with the B - repeat in this region but not at the end of the H4 region (Figs. 3o and 4c).

Heterochromatin amplification has been proposed to occur in chromosomes in in vitro plant cultures (Lapitan et al. 1984; Johnson et al. 1987) and during the evolution of B chromosomes in *Plantago lagopus* (Dhar et al. 2002) and maize (Cheng 2010). Subsequently, heterochromatin amplification may occur on the dicentric chromosome to form the B^{tb} chromosome, which carries

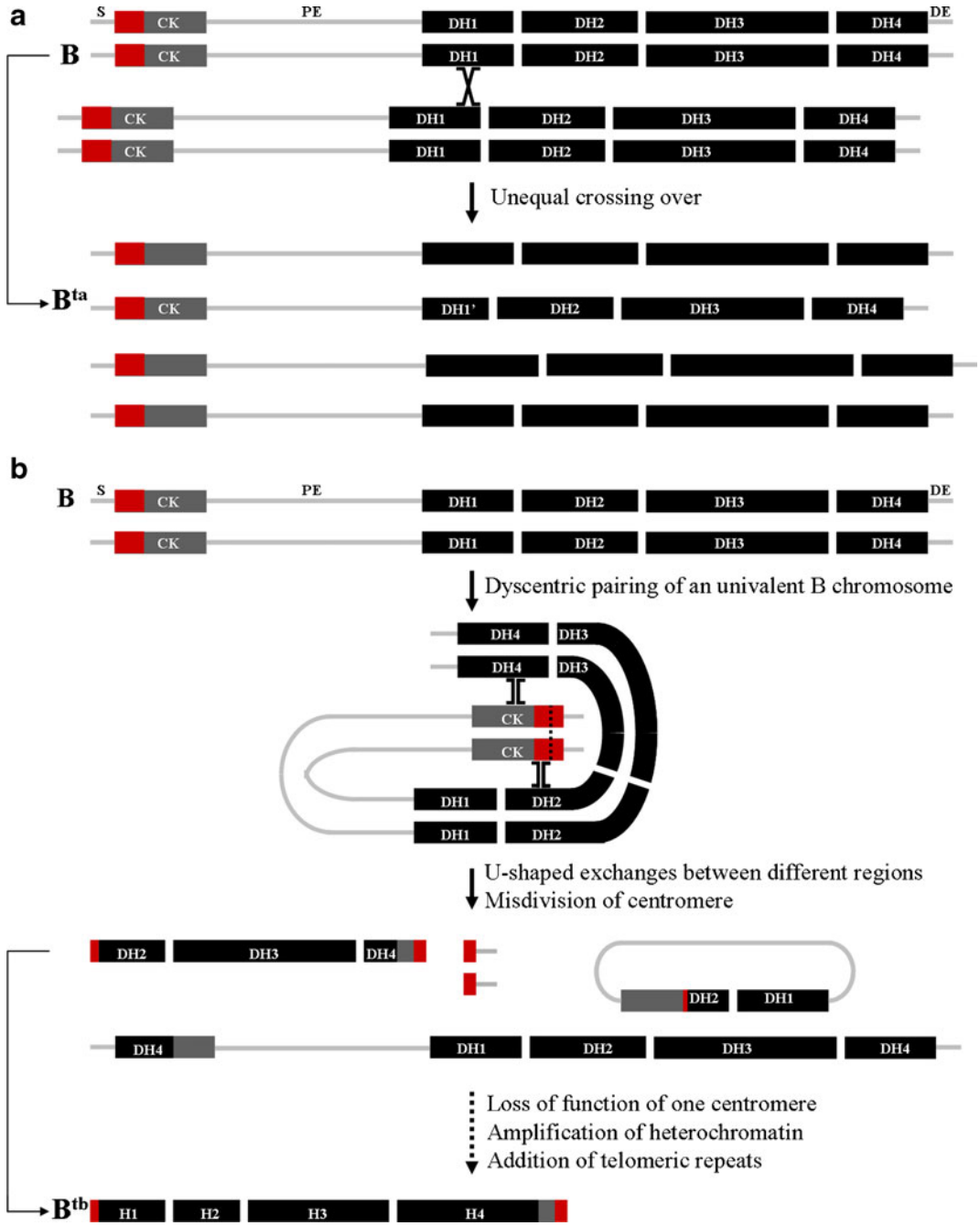

Fig. 6 Possible modes of origin of the B^{ta} and B^{tb} chromosomes. The typical B chromosome consists of a short arm (*S*), a centromeric knob (*CK*), proximal euchromatin (*PE*), four blocks of distal heterochromatin (*DH1–DH4*), and distal euchromatin (*DE*). **a** A B^{ta} chromosome carrying a smaller *DH1*' region can be generated via an unequal crossing over (*black lines*) between the DH1 regions of two homologous B chromosomes during meiosis. **b** Dyscentric pairing of a univalent B chromosome followed by U-shaped exchanges (*black lines*) between the CK and DH2 regions and between the CK and DH4 regions, and misdivision (*dashed line*) of the centromere will produce a dicentric chromosome, two telocentric chromosomes, a ring chromosome, and an acentric fragment. Then, the loss of function of one centromere, amplification of heterochromatin, and addition of telomeric repeats on the dicentric chromosome will produce a B^{tb} chromosome containing four blocks of heterochromatin (*H1–H4*). *Red boxes* indicate regions of B-repeats, and *gray boxes* indicate regions of 180-bp knob repeats. Euchromatin regions are indicated with *gray lines*, and heterochromatin regions are indicated with *black boxes*

four blocks of heterochromatin (Fig. 6b). The H3 fragments on the B^{tb} chromosome were predominantly derived from the DH3 region of the B chromosome based on the observation of the DH3-specific Stark signals at the H3 region of the B^{tb} chromosome (Fig. 3l). Fragments of the other three heterochromatic blocks found on the B^{tb} chromosome may be derived from the distal portion of the DH2 region, the proximal portion of the DH4 region, or the portion of the DH3 region that lacks the Stark sequences.

Several distinct properties have been documented in the Gramineae by which B chromosomes enhance their transmission and accumulate in nature (Jones 1995). In rye, the B chromosomes are maintained in populations through nondisjunction during the first pollen mitosis and the first egg cell mitosis and by then directing themselves in an unreduced number into the sperm and egg nuclei (Houben et al. 2014). The frequency of nondisjunction in pollen is consistently high, and a *trans*-acting signal controlling nondisjunction has been mapped to the distal part of the long arm of rye B chromosomes (Lima-De-Faria 1962). In maize, the B chromosome ensures its own survival by nondisjunction during the second pollen mitosis, together with the preferential fertilization of the egg by the sperm carrying the B chromosomes (Longley 1927; Roman 1948) which result in a higher frequency of transmission through the male parent (Table 1; Randolph 1941). In our study, the nondisjunction frequency of the B^{ta} chromosomes was similar to that of the typical B chromosome. However, interestingly, the male and female transmission frequencies of this chromosome were similar, and both were higher than that of the typical B chromosome (Table 1). The deletion in the DH1' region or other undetectable rearrangements on the B^{ta} chromosome should account for its unusual transmission characteristics. The B^{tb} chromosome lost the ability for nondisjunction due to the lack of PE and DE regions, which contain *trans*-acting elements essential for nondisjunction of the B chromosome (Ward 1973; Lin 1978). However, if a typical B chromosome is present in a cell containing a B^{tb} chromosome, nondisjunction of both types of chromosome occurs (Table 2). Furthermore, the small B^{tb} chromosome showed lower transmission frequencies through both parents, comparable to the transmission behavior of mini B chromosomes generated by other means (Kato et al. 2005; Yu et al. 2007).

Several de novo structural B chromosome variants were observed in the progeny of L289 plants carrying the B^{ta} or B^{tb} chromosome (Tables 1 and 2). These variant chromosomes differed in size and morphology from the B^{ta} and B^{tb} chromosomes, and they might have been generated via scenarios similar to those described above (Fig. 6). In maize, minichromosomes generated by transgene-mediated telomere seeding in the B chromosome (Yu et al. 2007) and BFB cycles of a translocated B chromosome (Zheng et al. 1999) have been analyzed and could be used to develop chromosome-based vector systems (Kato et al. 2005; Han et al. 2007; Birchler and Han 2013; Houben et al. 2013; Masonbrink et al. 2013; Graham et al. 2015). Structural B variants derived from naturally occurring B^{ta} or B^{tb} chromosomes have the potential to function as new sources of minichromosomes for the development of plant artificial chromosomes in the future.

Acknowledgments This study was supported by grants from the National Science Council grant of Taiwan (NSC 102-2311-B-005-001) and the Ministry of Science and Technology of Taiwan (MOST 104-2311-B-005-012-MY3).

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