RESEARCH ARTICLES

Rye Bs Disclose Ancestral Sequences in Cereal Genomes with a Potential Role in Gametophyte Chromatid Segregation

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Two sequence families, E3900 and D1100, are amplified on the subtelomeric domain of the long arm of rye B chromosomes, the region that controls its drive mechanism. In this work, polymerase chain reaction (PCR) with a number of primers spanning E3900 shows that the organization and nucleotide sequence of E3900-related portions are present and highly conserved on rye A chromosomes as well as in other cereals. Quantitative Real-Time PCR estimates two E3900 sequences to be represented in 100–150 copies on Bs and at least as single copies on As. A novel E3900-related sequence, with a deletion that results in a frameshift and subsequently an open reading frame with putative DNA binding motifs, is identified. Expression analysis of E3900 indicates identical transcription levels in leaves from plants with and without Bs, showing that the expression of these sequences must be silenced on Bs and tightly regulated on As in leaves. In contrast, E3900 transcription is upregulated during meiosis exclusively in plants with Bs, maintaining a high level of transcription in the gametophyte. Interestingly, Bs not only influence their own chromatid segregation but also that of the regular chromosome complement of both rye and wheat. There is a drastic increase in frequency of disrupted metaphase and anaphase cells in the first mitosis of pollen grains carrying Bs, which appears to be due to anomalous adherences between sister chromatids. Taken together, this work provides insight into how E3900 sequences are potentially associated with important evolutionary mechanisms involved in basic cellular processes.

Introduction

B chromosomes (Bs) were discovered just over one century ago (Wilson 1907) and later defined as "A supernumerary and dispensable chromosome which does not recombine with the normal chromosome complement (A chromosomes, As) and follows its own evolutionary pathway" (Beukeboom 1994). Since their discovery, Bs have been identified in a variety of fungi, plant, and animal out crossing species, with specific forms for each species and sequence variation at a frequency compatible to the mutation rate. Although Bs contain many sequences derived from As, the origin of the structure and function of these enigmatic chromosomes remains to be determined (Jones and Houben 2003). In general, Bs have been considered to be a type of selfish DNA, behaving like nuclear parasites with autonomous descent. Interestingly, these chromosomes show a non-mendelian mode of transmission, with some form of accumulation mechanism, or drive, having been described in approximately 60% of all the organisms that carry them. It is currently thought that Bs are maintained in populations by an equilibrium between their drive mechanism and the negative effects they provoke when in high numbers (Jones et al. 2008).

Bs exist to varying degrees in most natural rye populations (Jones and Puertas 1993) having been identified throughout the world with numbers varying from 0 to 8 per plant (for a review, see Jones and Houben 2003). In contrast to A chromosomes, Bs are supernumerary and dispensable. There is extensive literature on the cytogenetic and molecular structure of rye Bs, which have a number of functional domains including centromeric and telomeric sequences that are also found on As (reviewed in Jones and Houben 2003; Jones et al. 2008). The rye B is easily recognizable by its morphology at metaphase, having roughly half the length of As and a subterminal centromere that defines a long arm that is five times longer than the short arm. Their size is approximately 850×10^6 bp, representing roughly 10% of the haploid genome. Although rye Bs tend to have a neutral phenotype in reduced numbers, there are evident deleterious effects as their numbers increase, including reduced plant fertility (Jiménez et al. 1994). Bs display normal mitotic behavior in the sporophyte but not in the first mitosis postmeiosis. As initially described by Hasegawa (1934), Bs have a tendency to accumulate due to nondisjunction of sister chromatids and subsequent delay in their separation, thereby resulting in joint inclusion in the generative nucleus (Jones 1991). Lima-de-Faria (1962) proposed the drive process to be controlled by the terminal region of the long arm of Bs. This region has since been shown to be essential for nondisjunction of B pericentromeric chromatin domains by causing sister chromatid adherence (Jones and Houben 2003).

Analysis of chromosomal behavior of wheat lines with introgressed fragments of rye Bs has recently established that nondisjunction is controlled by two sequence families that localize to the subtelomeric domain of the B long arm (Endo et al. 2008). These two sequence families, namely, D1100 (Sandery et al. 1990) and E3900 (Blunden et al. 1993), were identified by comparative analysis of restriction-digested genomic DNA from plants with and without Bs. Fluorescent in situ hybridizations (FISH) with probes specific for D1100 label two zones in the subtelomeric region that are separated by an interstitial space where there is less signal. On the other hand, E3900-specific probes result in a more homogeneous and distal signal that overlaps the D1100 domain closer to the telomere (Wilkes et al. 1995).

Key words: E3900 rye B repeat, developmentally regulated transcription, chromosome segregation.

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Although these two sequences make up the heterochromatic block of Bs at the terminal region of the long arm, portions of this chromatin are decondensed and transcriptionally active (Langdon et al. 2000; Carchilan et al. 2007).

The complete sequence of E3900 became available in 2000 from an experimental rye population (Langdon et al. 2000). In this comprehensive study, a few E3900-related sequences were identified, suggesting that E3900-related sequences are not all arranged in the same manner. The most complete sequence was 3,984 nt long and has a number of interesting characteristics. These include uneven methylation patterns throughout the sequence, a few small duplications that may represent instability motifs as well as a partial open reading frame for a Gag protein from a Ty3/*gypsy* type retrotransposon. This *gag* domain holds high homology to the *crwydryn* retroelement, which has colonized the centromeric domains of the Poaceae (Presting et al. 1998). The role of the *gag* domain in E3900 remains to be determined, as does the precise role of E3900 on the maintenance and persistence of Bs. It was recently shown that the E3900 domain of Bs undergoes decondensation during interphase and that, unlike heterochromatic regions of As, it is simultaneously marked by trimethylated H3K4 and by trimethylated H3K27 (Carchilan et al. 2007). This is an unusual combination of apparently conflicting histone modifications. Also, the 3′ portion of E3900 was found to be poly-adenylated and transcriptionally active in a tissue-specific manner. It was therefore suggested that E3900 RNA has structural or catalytic functions and is probably involved in B nondisjunction.

The present study provides original molecular and cytogenetic insight into the evolution and function of E3900-related sequences. Our results show a high degree of conservation in the organization of these sequences in various plants, including distinct rye populations, a domesticated rye variety, and wheat, which is an autogamic species. A novel E3900-related fragment with a deletion of 1,133 nt with respect to the published sequence is identified, and the nucleotide sequence of this novel E3900 variant is nearly identical between As and Bs. Expression analysis clearly indicates that this novel sequence is equally transcribed in leaves from plants with 1B, 2Bs, and no Bs. The corresponding conceptually translated cDNA sequence contains a frameshift that results in an open reading frame for a *gag* domain with putative DNA binding motifs. Conversely, transcription regulation of E3900-related sequences in meiocytes differs drastically to that of leaves, where upregulation of all E3900-related sequences occurs exclusively in plants carrying Bs. Direct evidence that Bs affect chromosome behavior is observed as a dosage-dependent increase in the frequency of abnormal first mitosis of pollen grains in rye and wheat plants carrying 2Bs and 4Bs. The role of E3900 sequences on B persistence is discussed as well as their possible function in cereal genomes.

Materials and Methods

Plant Material and DNA Isolation

Rye plants with and without Bs were utilized for DNA extraction. These included plants from distinct populations carrying Bs (*Secale cereale*, $2n = 2x = 14 + Bs$), namely, EP (experimental population with isogenic Bs established in Aberystwyth by Professor N. R. Jones), JNK (Japanese origin), Paldang (Korean origin), and Puyo (Korean origin), as well as the domesticated variety Imperial that does not carry Bs (*S. cereale*, $2n = 2x = 14$). The autogamic species hexaploid wheat (*Triticum aestivum* cv Chinese spring, $2n = 6x = 42$), and the synthetic octaploid triticale (*T. aestivum* "Chinese Spring" × *S. cereale* "Imperial," $2n = 8x$, AABBDDRR) were also utilized. The synthetic wheat line Lindström with introgressed rye Bs (*T. aestivum*, $2n = 6x = 42 + S.$ *cereale* Bs, Müntzing 1973) was utilized exclusively for pollen grain cytological analysis. Individual plants were germinated and grown in controlled conditions at a 16 h light (20 °C)/8 h dark (20 °C) cycle. The number of Bs was determined for each plant by inducing c-metaphases and counting Bs, as previously described (Delgado et al. 2004). Plants with 2Bs were utilized for DNA and RNA analysis of +B plants, unless otherwise specified. For genomic DNA extraction, fresh, young leaves were collected, frozen in liquid nitrogen, and kept at −80 °C until use. DNA was extracted from at least two plants of each genotype using the modified cetyltrimethylammonium bromide method (Saghaimaroof et al. 1984).

Table 1
Primers Utilized for E3900 Analysis and Actin Controls

Primer (Position in bp[a])	Primer Sequence
1F (85)	5′-TAGCCCGTCGTCCTATTTCCGTGTAA
2F (1,973)	5′-ATTAGTCACGTGTGATCAACGTGG
2F1 (2,181)	5′-TAAAGGCATCGTCCTGGGTCTTGT
2F2 (2,846)	5′-TTGATGACATGATCGCCGTTTGGC
3F (3,911)	5′-AACTGCCGTGTATGTGGAGACAG
0R (297)	5′-GATGCAATCGATCTGGAATTGCC
1R (1,976)	5′-CCGGCCACGTTGATCACACGTGAC
2R1 (2,582)	5′-ATGCATCTGCATGTGCGTCCTTTG
2R2 (3,048)	5′-TGCCGGAGAACGAAGGGATTGAAA
2R (3,497)	5′-TGACTCCAGTCGTTGCCCAGCTG
3R (3,911)	5′-ACTGTCTCCACATACACGGCAGTT
Actin2-F	5′-GCTGGATTCTGGTGATGGTGTGAG
Actin2-R	5′-CAATGAGAGATGGCTGGAAGAGGAC
Secalin-F	5′-ATGAAGACCTTACTCATGCTTGCAA
Secalin-R	5′-TCAGTGGCCAACAATACCAGTG

[a] Position on E3900 published sequence.

Polymerase Chain Reaction (PCR) for Analysis of E3900 in Plants with and without Bs

PCRs were performed with various primers spanning the published E3900 sequence (accession number AF222021) in order to analyze the genomic organization and transcription activity of E3900 in rye lines with and without Bs. Primer names and sequences are shown in table 1, and the expected genomic DNA amplification products for each primer combination are shown in figure 1*A*. PCR reactions were repeated at least three times for each primer combination. Each 20 μl PCR mix contained 20 ng of genomic DNA (or cDNA), 1.5 mM $MgCl_2$, 0.1 pM of each primer, 0.25 mM dNTP (deoxyribonucleotide triphosphates), and 1 U Taq polymerase. Genomic DNA (or cDNA) was amplified for 25 cycles (95 °C–5 min, 25 cycles of 95 °C, 1 min; 65 °C, 1 min; and 72 °C, 1 min, and a final

A

Expected Fragments

primer comb.	size	primer comb.	size
1F/0R	236 bp	**2F/2R1**	634 bp
1F/1R	1916 bp	**2F/2R2**	1100 bp
1F/2R	3436 bp	**2F1/2R1**	426 bp
1F/3R	3851 bp	**2F1/2R2**	892 bp
2F/2R	1548 bp	**2F2/2R2**	227 bp
2F/3R	1963 bp	**3F/0R**	393 bp

B

Obtained Fragments

primer comb.	genome	size	accession number	Comparison to E3900 (AF222021) size difference	sequence similarity
1F/1R	JNK 0B	1875 bp	EU938383	-41 bp	97%
1F/2R	JNK +B	2267 bp	EU938384	**-1169 bp***	98%
2F/2R	JNK +B	1549 bp	EU938389	1 bp	>99%
	JNK 0B	416 bp	EU938385	**-1132 bp***	>99%
	JNK +B	416 bp	EU938386	**-1132 bp***	>99%
	Triticale	415 bp	EU938387	**-1133 bp***	>99%
	Wheat	416 bp	EU938388	**-1132 bp***	>99%
2F/3R	JNK +B	830 bp	EU938390	**-1133 bp***	98%
3F/0R	JNK 0B	377 bp	EU938391	-16 bp	95%

C

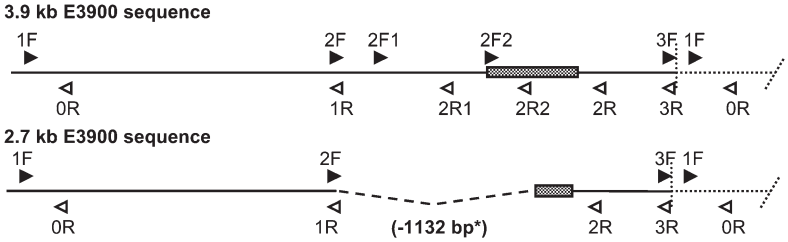


FIG. 1.—PCR products of E3900-related sequences. (*A*) Sizes of expected amplification products for all primer combination are shown. Primer combinations are demonstrated on the left and the expected fragment sizes in base pairs on the right. (*B*) Major bands obtained with each primer combination from +B and 0B plants were gel isolated and sequenced, and consensus sequences were compared with the published E3900 sequence (AF222021). Primer combination, plants for which sequencing data were introduced to the databases and respective accession numbers are shown for each fragment. In comparison with the published sequence, there is a deletion of 1,132 bp (corresponding to 1,133 bp in our sequencing results) in 1F2R, 2F2R, and 2F3R fragments (shown by *). Imperial Rye, EP, and JNK, A and B chromosomes sequences are highly similar to the corresponding region of the published sequence (>95%). (*C*) The two E3900 size variants are graphically shown with forward primers (black arrowheads) and reverse primers (white arrowheads) in their respective positions. The thick gray boxes represent the E3900 *gag* domain, and dotted lines show E3900 sequences arranged in tandem. Dashed line illustrates the 1,132-bp deletion identified by 2R and 3R primers, as shown in *B* (*).

elongation step of 72 °C for 5 min). PCR products were separated by 1.5% agarose gel electrophoresis and detected by Ethidium Bromide staining. PCRs with E3900-specific primers without genomic DNA template were performed as negative controls.

Sequence Analysis of E3900 Fragments

Fragments of interest were gel purified, and sequenced in both directions with the appropriate primers (Automatic Sequencer ABI3700, Applied Biosystems). When there were problems with sequencing, the gel-isolated fragment was cloned with the Blunt-Ended PCR Cloning Kit, following manufacturers' instructions (GE Healthcare, Cat#: RPN5110). Consensus sequences were determined by performing alignments with at least three sequencing reactions and the consensus sequences used for Blast.

Quantitative Real-Time PCR

In order to determine the number of copies of E3900-related sequences on Bs and As, qRT-PCR was performed utilizing the BIO-RAD IQ 5 Multicolor Real-Time PCR detection System. The BIO-RAD IQ SYBR Green Supermix (BIO-RAD Cat# 170-8880S) was utilized for PCR with E3900-specific primers on genomic DNA from EP and JNK plants with 2Bs and without Bs as well as on Imperial rye, following manufacturers' instructions. Titration curves were calculated for plants with and without Bs with the Actin2 gene and at least three replicates of four DNA dilutions. In plants without Bs, 0.8, 4, 8, and 16 ng of genomic DNA was utilized, which correspond to approximately 100; 500; 1,000; 2,000 copies of the haploid genome, respectively. In plants with 2Bs, 0.9, 4.5, 9, and 18 ng of genomic DNA was utilized, corresponding to approximately 100; 500; 1,000; and 2,000 copies of the haploid genome, respectively. Mean C_t for each dilution was plotted on the x axis and the log number of copies on the y axis, and the resulting linear graph used to calculate the regression line (log#copies = x(mean C_t) + a, R^2 was always >than 0.9). For analysis of 2F/2R and 3F/0R E3900 fragments, the mean C_t of at least three replicates of each genomic dilution was utilized and the number of copies estimated by fitting the mean C_t into the Actin2 regression equations. As negative controls, Real-Time PCR reactions without genomic DNA template were performed with primer combinations 2F/2R and 3F/0R.

Fluorescence In Situ Hybridization

PCR products obtained with primer combinations 2F/2R and 2F1/2R1 were used as probes for in situ hybridization. The reaction conditions and amplification program were as described above, except that 1 μl (1 nmol/μl) of digoxigenin–dUTP or biotin–dUTP (deoxyuridine triphosphate) (Roche, Gipf-Oberfrick, Switzerland) was added to

the reaction mixture in order to label PCR products. Root tips were prepared as previously described for cytological analysis (Schwarzacher and Heslop-Harrison 2000), with the following modifications. Two JNK + B and two EP + B plants were utilized for cytogenetic analysis. Fixed root tips were digested with pectinase/cellulase in 1× EB (Enzyme Buffer) for 2 h 15 min at 37 °C, and squashes were performed in 45% glacial acetic acid. Meiotic chromosome squashes from EP influorescences were prepared as previously described (Carchilan et al. 2007). Nuclei and chromosomes were counterstained with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) in Citifluor antifade mounting medium (AF1; Agar Scientific). Samples were examined using a Zeiss Axioskop 2 epifluorescence microscope, and images were obtained using a Zeiss AxioCam digital camera. Digital images were processed using PHOTOSHOP (Adobe Systems).

cDNA Isolation and Quantitative PCR

To determine E3900 transcriptional activity in leaves, total RNA was extracted from leaves of at least two JNK and EP plants with and without Bs. To determine transcriptional activity in meiocytes, individual ears were staged for premeiosis, meiosis, and pollen grains undergoing the first mitotic division. Anthers of a single ear from two distinct EP plants with and without Bs were utilized, and RNA was isolated with the Qiagen RNeasy Mini Kit (Cat# 74904), following manufactures' instructions. After verifying concentration and integrity, 3 μg of total RNA was utilized for RNase free DNase digestion, and first strand cDNA synthesis was completed with reverse transcriptase Superscript II and random primers (dN_9), following manufacturers' instructions (Invitrogen, cat no 18064-014). The resulting cDNA was utilized for semiquantitative PCR with primer combinations 1F/0R, 2F/2R, 2F1/2R1, and Actin2 as control. Gel separated fragments were isolated and sequenced. To ensure lack of genomic DNA contamination in semiquantitative RT-PCR experiments, cDNA isolated from leaves and anthers of 0B and +B plants was utilized for PCR with primers specific for the 75K γ-secalin genes of rye, which are exclusively expressed in the grain (Chen et al. 2008).

Transcription activity of E3900 sequences in 0B and +B plants was further analyzed by qRT-PCR with the BIO-RAD IQ SYBR Green Supermix (BIO-RAD Cat# 170-8880S). To ensure that genomic DNA was completely absent prior to cDNA synthesis, PCRs were performed with Actin2 primers and 250 ng of DNase digested RNA. PCR on cDNA with primer combinations 2F/2R and 2F1/2R1, and Actin2 gene were conducted, and all PCR experiments were repeated three times with at least three replicates per genotype/primer combination in each experiment. All comparisons of expression levels were performed on identical cDNA dilutions. Melt curves were observed to ensure correct amplification products, and 2F/2R or 2F1/2R1 threshold cycles (C_t) equilibrated with mean Actin C_t to calculate ΔC_t (ΔC_t = C_t of interest − mean Actin2 C_t). 2F2R-400 and 2F12R1 expression levels were analyzed by calculating $\Delta\Delta C_t$ ($\Delta\Delta C_t = \Delta C_t a$ − mean $\Delta C_t b$, where a and b are being compared), which in turn was used to determine mean fold change ($2^{-\Delta\Delta Ct}$) ± standard deviation between genotypes, developmental stages, and/or E3900-related sequences.

Quantification of Abnormal Metaphases in Rye and Wheat Pollen Grains Carrying Bs

Anthers were selected for the first mitotic division of the pollen grain from JNK rye as and Lindström wheat with 0Bs, 2Bs, or 4Bs. Anthers were fixed in ethanol:glacial acetic acid (3:1) and DNA staining was performed prior to pollen grain dispersal. Three anthers from two individual plants of each genotype were utilized for cytological analysis. Due to natural autofluorescence of pollen grain coats, classical Feulgen DNA staining was utilized to visualize chromosomes without disrupting the pollen grain coat and chromosome arrangement.

Results

Genomic Organization of E3900 Is Highly Conserved

Figure 1*A* indicates the expected amplification product(s) for each primer combination based on the E3900 published sequence (accession number AF222021). Negative controls for PCR reactions are shown in supplementary figure S1A, Supplementary Material online. For the sake of clarity, the various primer combinations are designated by each primer separated with a slash and the resulting amplification products by the respective primer names without separation. Gel electrophoresis of PCR products show that most primer combinations result in identical banding profiles in +B and 0B plants, although much less product is amplified from 0B plants. Taken together, the banding profiles obtained for all primer combinations show very little variation in the genomic organization of E3900-related sequences between rye populations. Primer combinations 1F/1R (fig. 2*A*), 3F/0R (supplementary fig. S2A, Supplementary Material online), 1F/0R, and 3F/1R (data not shown), result in major bands of the expected sizes in plants with and without Bs in all populations. This indicates that the arrangement of sequences attributable to the 5′ half and the final 3′ portion of E3900 are highly conserved. On the other hand, PCR involving primers 2R and 3R define a variable interstitial domain, attributable to a deletion of approximately 1 kb between 2F and 2R in respect to the published sequence, as explained in detail below and illustrated in figure 1*B* and *C*.

Primer Combinations 1F/2R and 1F/3R

PCR results obtained with primer combination 1F/2R are shown in supplementary figure S2B, Supplementary Material online. Banding profiles reveal a major band approximately 1 kb less than expected in all plants with Bs, of roughly 2,500 bp. The expected 3,500-bp product is also visible in plants with Bs from all populations as is a smaller 1,800-bp fragment in Puyo with Bs. Primer combination 1F/3R also results in major amplification products approximately 1 kb less than expected, evident as a major band with roughly 3 kb in plants with Bs from all populations.

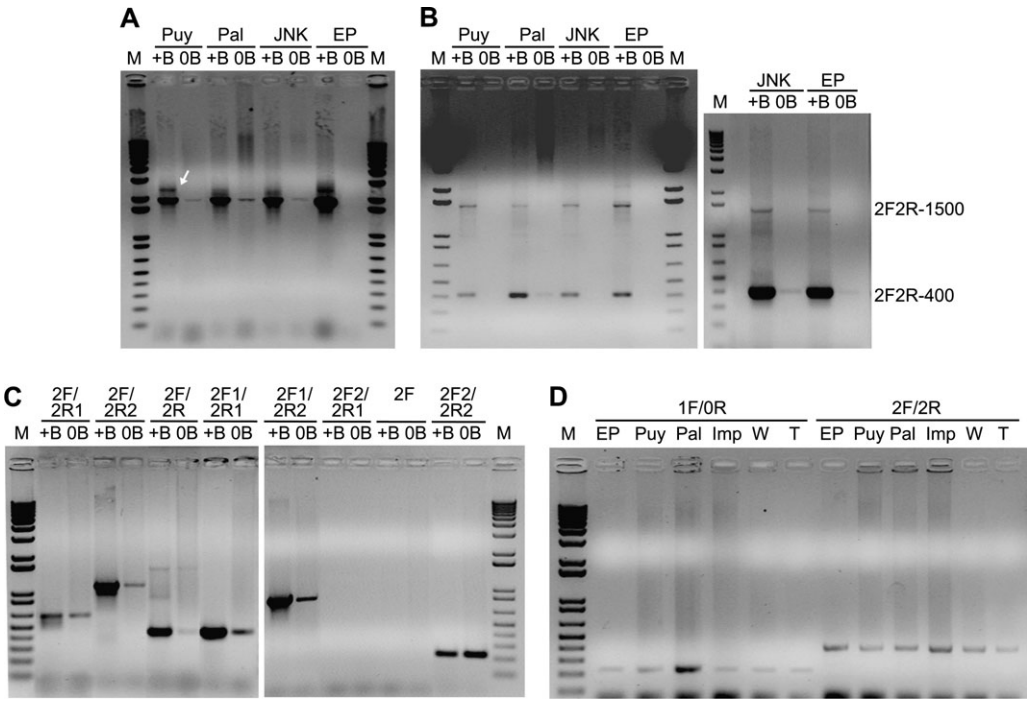


FIG. 2.—E3900-specific primers amplify identical fragments in 0B and +B plants. (*A*) PCR banding profiles with genomic DNA with primer combination 1F/1R from rye populations Puyo (Puy), Paldang (Pal), JNK, and EP with and without Bs show nearly identical major bands of approximately 2 kb in +B and 0B plants. Minor amplification products were found to differ between populations in +B plants (white arrow). (*B*) PCR banding profiles with genomic DNA from rye populations Puyo (Puy), Paldang (Pal), JNK, and EP with and without Bs and primer combination 2F/2R show two bands in all +B plants, one with the expected 1.5 kb, and a smaller more intense band of approximately 400 bp. The gel on the right shows that a large volume of 2F/2R PCR product (100 μl, 5× standard PCR reaction) permits the visualization of the major 2F/2R product (2F2R-400) in 0B plants. (*C*) PCR banding profiles with genomic DNA from JNK +B and 0B and primer combinations spanning the 2F and 2R genomic region. Primer combinations 2F/2R1, 2F/2R2, 2F/2R, 2F1/2R1, 2F1/2R2, 2F2/2R1, 2F, and 2F2/2R2 are shown. Bands of the expected sizes are obtained for all primer combinations, and no products are amplified with single primers or when primer combinations with forward primers downstream of reverse primers are used. (*D*) PCR products with primer combination 1F/0R (genomic DNA from EP, Puyo, Paldang, Imperial rye, wheat cv. Chinese Spring, and triticale), and with primer combination 2F/2R (genomic DNA from EP, JNK, Puyo, Paldang, wheat cv. Chinese Spring, and triticale). Both primer combinations result in the expected amplification products for all genotypes. M is the molecular weight marker (1 kb^+) for all gels.

This primer combination also amplifies a minor product of the expected size just under 4 kb.

Primer Combination 2F/2R

PCRs show a major product of approximately 400 bp, and a minor product with the 1.5 kb expected size, as demonstrated in figure 2*B*. Although it appears that there is no amplification product in plants without Bs, the smaller fragment is visible in 0B plants when more PCR product is loaded on the gel, as illustrated in the right gel of figure 2*B*. For the purpose of clarity, this smaller 2F2R fragment will be referred to as 2F2R-400 and the larger as 2F2R-1,500. A more in-depth analysis of the 2F2R-400 and 2F2R-1,500 fragments is discussed below and in the following sections.

Internal 2F/2R Region

Primer combinations 2F/2R1, 2F/2R2, 2F1/2R1, 2F1/2R2, 2F2/2R1, 2F2/2R2, 2F, and 2R were utilized to better understand the genomic region between primers 2F and 2R. PCRs with JNK and EP genomic DNA result in fragments of expected size when compared with the published sequence, as shown in figure 2*C*. As with other primer combinations, identical sized fragments are also amplified in plants without Bs with all these primer combinations. Unlike other primer pairs, 2F2/2R2 amplifies the same amount of product in 0B and +B plants, likely due to amplification within *gag* sequences that are not E3900-related. Importantly, this result provides evidence that the other primers utilized in this study are in fact specific to E3900-associated sequences. No bands are observed with primer combination 2F2/2R1, indicating that this region is not repeated in tandem. Also, because no product is obtained with primers 2F (fig. 2*C*) and 2R (data not shown) alone, we can conclude that there are no inversions within this genomic region of the E3900 repeat.

Primer Combination 2F/3R

This results in two major amplification products, one with the expected 2 kb size and one of approximately 850 bp, again 1 kb smaller than expected (supplementary fig. S2C, Supplementary Material online). A minor fragment of approximately 1,600 bp is also amplified in all plants with Bs. As with primer combination 2F/2R, 2F/3R amplification products are not detected in 0B plants unless more PCR product is loaded on the gel (data not shown).

E3900 in Other Cereal Species

Primer combinations 1F/0R and 2F/2R amplify identical sized fragments from genomic DNA of rye plants without Bs, rye cv. Imperial, wheat cv. Chinese Spring as well as synthetic octaploid triticale (*T. aestivum* Chinese Spring × *S. cereale* "Imperial") (fig. 2*D*), showing that E3900-related sequences are maintained intact since rye and wheat genus divergence as well as through natural (wheat) and synthetic (triticale) allopolyploidization.

E3900-Related Sequences Are Arranged Head to Tail

To check the genomic arrangement of E3900-associated sequences, PCRs with each primer utilized in this study were performed on plants with and without Bs (fig. 2*C* shows the results for 2F; results for the remaining primers are not shown). PCRs with single primers result in no amplification products, indicating there are no E3900-related sequences arranged head to head or tail to tail. On the other hand, all primer combinations that do not span the deletion defined by 2F2R-400 result in a single major amplification product in +B plants (1F/1R and 3F/0R PCR results are shown in fig. 2*A* and supplementary fig. S2A, Supplementary Material online, respectively), which is compatible with E3900 sequences being arranged in a head to tail fashion on the B chromosome domain.

In conclusion, major amplification products obtained with 2R and 3R as reverse primers are approximately 1 kb smaller than expected (fig. 2*B* and supplementary fig. S2B and C, Supplementary Material online). Interestingly, identical products are amplified in rye plants without Bs as well as in other cereal species with primer pairs that amplify segments associated with the 5′ (1F/0R) and 3′ (2F/2R) halves of the published E3900 sequence.

E3900 Sequences Are Present on the As

Major bands obtained with each primer combination from +B and 0B plants were sequenced and compared with the published E3900 sequence (AF222021), as summarized in figure 1. Consensus sequences were determined from at least three sequencing reactions. Due to high percent sequence similarity at the nucleotide level, sequencing data for each fragment was uploaded to the databases for JNK with 0Bs, unless otherwise indicated. Genomic sequence data from this article can be found in the EMBL/GenBank databases with the accession numbers EU938383–EU938391 (fig. 1*B*). Sequencing results permitted for the identification of two forms of E3900, one that is highly similar to the published sequence. The other E3900 variant is approximately 1 kb smaller (fig. 1*B* and graphical representation in fig. 1*C*). The nucleotide sequence of each E3900-related segment is described below.

1F1R sequence is a 1,875-bp fragment that is >99% identical between JNK with and without Bs and EP with Bs, with 97% sequence similarity to the equivalent portion of the E3900 published sequence.

1F2R resulted in a 2,267-bp sequence composed of 1F1R and 2F2R-400 with high sequence similarity to the corresponding portions of the published sequence (see 1F1R and 2F2R-400 for percentage similarities) and which is nearly identical between JNK and EP. Consensus sequence of JNK with Bs was added to the databases. As shown on the alignment with the published E3900 sequence, the smaller than expected size of this fragment can be attributed to the deletion of 1,132 nt corresponding to positions 2063–3194 of the published sequence.

2F2R-400 was gel isolated and sequenced for EP, JNK, Puyo, Paldang, with and without Bs as well as for rye cv. Imperial, wheat cv. Chinese Spring, and triticale. Data were added to the databases for JNK with and without Bs, as well as for wheat and triticale. The 416-bp JNK sequence is identical in 0B and +B plants as well as astonishingly similar between cultivars and species, with only 2-nt differences between all the genomes analyzed as well as in comparison to the corresponding region of the published sequence. As seen above for the smaller than expected 1F2R fragment, sequence alignment with the published data shows that 2F2R-400 contains a deletion of 1,132 bp in respect to the published sequence, with the breakpoint just upstream and including approximately half of the gag domain. Interestingly, comparing 2F2R-400 with 2F2R-1500 described below or the published E3900 sequence shows the presence of a GCTA nucleotide sequence flanking the 5′ and 3′ deletion breakpoints. One GCTA sequence is lost in the 2F2R-400 sequence.

2F2R-1500 was gel isolated and cloned from JNK with Bs. Sequencing data showed >99% sequence similarity between the 1,549-bp fragment and the published E3900 sequence (our sequence has one extra T at nucleotide position 2082 of the published sequence, and two further nucleotide changes in a total of 1,549 bp). When compared with the 2F2R-400 sequence described above, the deletion in 2F2R-400 is 1,133 nt long.

2F3R major product was gel isolated and sequenced for EP and JNK with Bs, resulting in a 830-bp sequence that has >99% nucleotide identity between the two populations. The 5′ portion of this sequence is identical to the corresponding 2F2R-400 fragment. The remaining 414-bp sequence aligns downstream of the 2R primer on the published sequence, and has 1nt less and a total of 8 nt changes 3′ of nucleotide position 640 of 2F3R.

3F0R was gel isolated and sequenced for EP with Bs as well as JNK with and without Bs. The JNK 0B 377-bp sequence shows >99% sequence similarity to that obtained from JNK + B and 95% to the corresponding 393 bp on the published sequence.

The sequence data show almost complete lack of variation in E3900 sequences between As and Bs. In all cases, the percentage sequence similarity between JNK 0B and +B plants is greater (>99% for 1F1R, 2F2R-400, and 3F0R) than that observed in comparison to the published sequence (97% for 1F1R, >99% for 2F2R-400, and 95% for 3F0R).

E3900-Related Sequences Are Present in Approximately 100 Copies on Bs

Quantitative Real-Time PCR on plants with 0Bs and 2Bs was utilized to estimate the number of copies of

E3900-related sequences on each B chromosome as well as on the haploid A complement. The mean number of copies ± standard errors for each primer combination and genome analyzed are shown in supplementary figure S3, Supplementary Material online. The results of the quantitative curve of numerous genomic DNA dilutions estimate 2F2R-400 sequences to be present on average in 150 and 120 copies on Bs from EP and JNK, respectively, and at least as single copies on the As of EP, JNK and Imperial rye (supplementary fig. S3, Supplementary Material online). 3F0R sequences are estimated to be present on average in 100 and 120 copies on Bs of EP and JNK, respectively, and at least as single copies on As from EP, JNK, and Imperial (supplementary fig. S3, Supplementary Material online). The variation in the mean number of copies of E3900-related sequences in EP and JNK plants with Bs can be explained by the fact that E3900 is repeated on the B domain, and therefore likely to vary between Bs. The melt curves for various genomic dilutions and primer combinations 2F/2R and 3F/0R are shown in supplementary figure S3, Supplementary Material online. Each primer combination amplifies PCR products with distinct melt curves, with 2F/2R having a lower melt temperature than 3F/0R. Importantly, each primer combination results in the melt curves with single peaks (supplementary fig. S3, Supplementary Material online), which supports the quantification of a single 2F/2R or 3F/0R amplification product. Graphs of threshold cycles (C_t) for three replicates of genomic dilution corresponding to 500 copies of the haploid genome for EP and JNK with and without Bs as well as the domesticated variety Imperial are demonstrated in supplementary figure S3, Supplementary Material online. Quantitative Real-Time PCR with 2F/2R and 3F/0R primer combinations do not result in amplification products, as confirmed in supplementary figure S1A, Supplementary Material online.

Chromosome Mapping of E3900 Segments

In order to compare the distribution of the two E3900 variants, probes corresponding to 2F2R-400 and 2F1R1 were utilized for FISH. 2F2R-400 represents the shorter variant of E3900, whereas 2F12R1 exclusively labels the longer E3900 variant. Chromosome spreads of root tip cells show the expected chromosomal localization for E3900 labeling with both probes (fig. 3). 2F2R and 2F12R1 labeled domains are indistinguishable in somatic interphase, as well as in prophase and metaphase chromosomes. Although there is progressive condensation of the E3900 domain throughout the cell cycle, both probes show complete overlap on the terminal region of the long arm of Bs (fig. 3*A*–*C*). At meiosis, extended pachytene chromosomes provide better resolution of the E3900 chromosome domain. Overall, 2F2R-400 and 2F12R1 probes map to identical domains on Bs. However, detailed comparison of the two probes clearly reveals that 2F12R1 mostly colocalizes with 2F2R-400, but 2F2R-400 has a more widespread distribution, indicating that this sequence is present at a higher frequency on the E3900 B chromosome domain (fig. 3*D*).

E3900 Sequences Are Transcriptionally Regulated in 0B Plants

Semiquantitative RT-PCR was performed with primer combinations 2F/2R, 1F/0R, and 2F1/2R1 on cDNA isolated with random primers from leaves of EP and JNK plants with and without Bs. Negative controls as well as controls for genomic DNA contamination of cDNA are shown in supplementary figure S1B, Supplementary Material online. The transcription levels 2F2R-400 appear identical in leaves from EP and JNK plants regardless of the number of Bs (fig. 4*A*). cDNA sequences for 1F0R and 2F2R-400 from JNK and EP with and without Bs can be found in the EMBL/GenBank databases (accession numbers EU938392–EU938399). Quantitative RT-PCR of 2F2R-400 and 2F12R1 shows that these sequences are expressed at very low levels, regardless of the presence of Bs. A minimum of 150 ng of cDNA per reaction is necessary to obtain amplification products with measurable threshold cycles. Nevertheless, the results show a slight increase in the expression of these sequences in leaves from plants with 2Bs compared with those within 0B, with mean fold changes ± standard deviation of 1.7 ± 0.23 and 1.6 ± 0.11 for 2F12R1 and 2F2R, respectively (fig. 4*C*-*I*).

Sequencing analysis of 2F2R-400 (EU938396–EU938399) shows greater than 99% sequence similarity at the nucleotide level between the two populations as well as to the corresponding 2F2R-400 genomic DNA sequence. Interestingly, all the differences between 2F2R-400 cDNA sequences lie between nucleotides 38 and 49, upstream of the potential translation start site discussed below. The other E3900-associated sequences are also transcribed in leaves of all plants, with 1F0R showing slight variation in transcription levels between plants regardless of the number of Bs. There are also very few differences in 1F0R cDNA sequences between EP and JNK plants with and without Bs (EU938392–EU938395), although the observed approximately 90% sequence similarity is less than that for 2F2R-400. Considering the relatively higher proportion of E3900-related sequences on Bs, it is likely that 2F2R-400 and 1F0R transcripts are due to expression of E3900-related sequences residing on As.

Differential Expression of E3900 Sequences in Meiocytes and Pollen Grains

In contrast to the results obtained with cDNA from leaves, the transcription of E3900-related sequences are upregulated in meiocytes and pollen grains exclusively in plants with Bs (fig. 4*B* and *C*). Amplification of cDNA with primer combinations 1F/0R and 2F1/2R1 shows transcription in anthers of +B plants with meiocytes at premeiosis, undergoing meiosis and at pollen grain developmental stages, with slightly more product at meiosis (fig. 4*B*). On the other hand, 2F2R-400 shows a more defined peak of transcriptional activity at meiosis (fig. 4*B*).

Quantitative RT-PCR with various cDNA dilutions shows that 2F2R and 2F12R1 are expressed at very low levels at premeiosis and meiosis in 0B plants and that these sequences are differentially regulated in anthers of +B

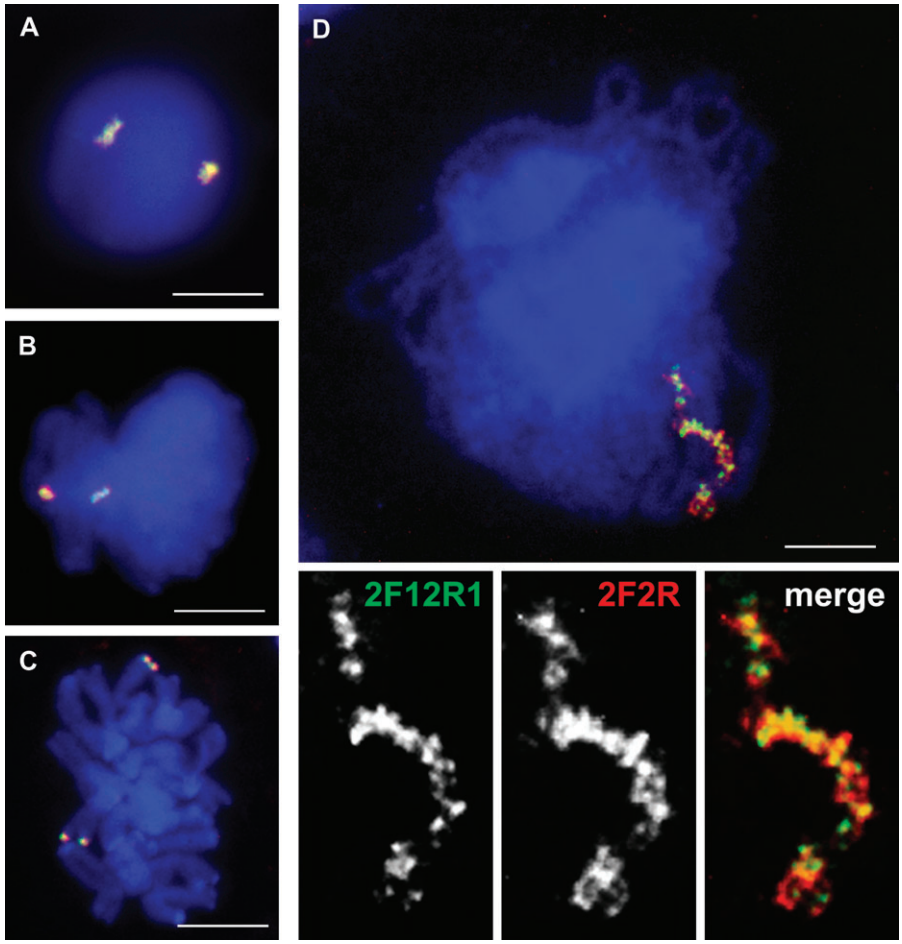


FIG. 3.—In situ hybridization of E3900 fragments in distinct cell types. FISH with probes corresponding to E3900 fragments obtained with primer combinations 2F1/2R1 (green) and 2F/2R (red) on EP + 2Bs somatic cells, yellow signals indicate probe overlap. (*A–C*) Mitotic cells show nearly complete overlap of both probes throughout the cell cycle as well as in differentiated cells, visualized as yellow signals. (*A*) Meristematic interphase cell with partial decondensation of the E3900 B domain. (*B*) Prophase cell demonstrating increased condensation of the E3900 domain. (*C*) Metaphase cell showing typical highly condensed E3900 labeling on the long arm of Bs, visualized on individual chromatids as double dots. (*D*) Pachytene cell with 2Bs paired at the E3900 domain. Both probes label the entire domain, although the higher resolution provided by extended pachytene chromosomes illustrates a proportionally wider distribution of 2F2R relatively to 2F12R1. The bottom panels show enlarged and separate probe labels in black and white as well as the colored merged image of both probes. Bars = 10 μm.

plants. In 0B plants at premeiosis, 2F12R1 showed similar expression levels to those observed in leaves, whereas 2F2R expression is approximately 40% in comparison to leaves (fig. 4*C*-II). As observed with semiquantitative PCR, 2F12R1 expression is upregulated in a similar level at premeiosis and meiosis from +B plants (fig. 4*C*-III). On the other hand, comparisons of expression levels between the two sequences clearly show that 2F2R is expressed at greater levels than 2F12R1. Importantly, in relation to 2F12R1, 2F2R-400 expression levels increase by 3.2 ± 0.65 at premeiosis and 8.34 ± 0.84 at meiosis (fig. 4*C*-IV).

2F2R-400 Contains an Open Reading Frame for a Gag Domain with a Nucleotide Binding Motif

The conceptually translated 1F0R, 2F12R1, and 2F2R-400 cDNA sequences were checked for potential open reading frames as well as functional amino acid domains. The putative 1F0R and 2F12R1 translation products did not show any significant homology to proteins in the databases. On the other hand, the 2F2R-400 cDNA sequence (accession number EU938399) provided interesting results, as shown in detail in figure 5. In order to determine how 2F2R-400 and flanking sequences are associated with transcription regulation, the genomic DNA sequences 100 nt upstream of the 2F primer (accession number EU938383) and between 2R and 3R primers (accession number EU938390) were also utilized. Prediction analysis of protein-coding gene structure was performed on the 941 bp with the WebGene Gene Builder tool (http://www.itb.cnr.it/sun/webgene/). The results show a potential TATA box (CAAATAAGCC) 25 bp upstream of the 2F primer, corresponding to position 1948 of the published sequence. There are also a number of putative poly-adenylation prediction sites, including two at nucleotide positions 740 (TCGAGACACG) and nucleotide 873 (AAAATAAAGA), corresponding to positions 3742 and 3876 of the published sequence, respectively. Importantly, the 1,133-bp deletion that gives rise to the genomic sequence that encodes

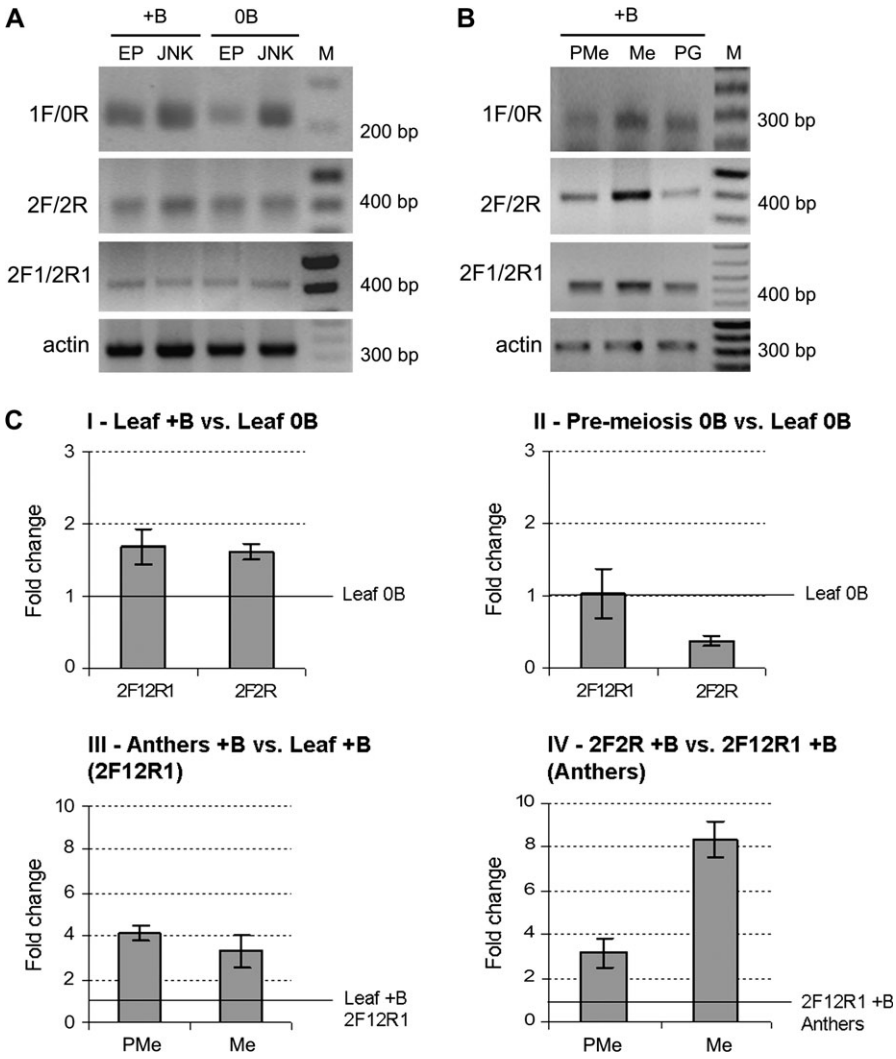


FIG. 4.—Transcription of E3900-related sequences are regulated on As and Bs. (*A*,*B*) Semiquantitative PCR results with primer combinations shown on the right. M is the molecular weight marker (1 kb^+, band molecular weights shown on the right), and actin controls are shown for all samples. (*A*) cDNA from JNK and EP +B and 0B leaves. Equal transcription levels of 2F2R-400 and 2F12R1 are observed in all individuals tested, regardless of the presence or absence of Bs. cDNA sequencing analysis results in 100% similarity at the nucleotide level between populations as well as to the 2F2R-400 genomic DNA sequence. Primer combination 1F/0R shows more variable levels of expression, regardless of the number of Bs. (*B*) cDNA from EP +B anthers staged for premeiosis (PMe), meiosis (Me), and pollen grain undergoing the first mitotic division (PG). Although all E3900 related sequences are slightly more expressed during meiosis for all primer combinations, 2F/2R shows a marked peak of transcription. (*C*) Comparative analysis of 2F2R-400 and 2F12R1 expression between genotypes, primer combinations, tissue types, and/or developmental stages by Quantitative Real-Time PCR. Results are shown as relative fold changes in transcription levels ($2^{-\Delta\Delta Ct}$) ± standard deviation. In all cases, the variable used as standard was attributed a fold change value of 1 (solid horizontal line). (*I*) 2F12R1 and 2F2R-400 expression levels increase slightly +B plants in comparison to 0B plants. (*II*) Comparison of 2F12R1 and 2F2R-400 expression levels between leaves and premeiosis in 0B plants indicates that although 2F12R1 is transcribed equally in both tissues, 2F2R-400 expression decreases at premeiosis. (*III*) In +B plants, 2F12R1 expression is greater at premeiosis (PMe) and meiosis (Me) than in leaves, although there is no significant difference in transcription levels between the two developmental stages. (*IV*) 2F2R-400 is transcribed at greater levels than 2F12R1 in anthers of +B plants. 2F12R1 expression at premeiosis and meiosis were used as standards. In comparison with 2F12R1, there is a 3-fold increase in 2F2R-400 expression at premeiosis (PMe) and an 8-fold increase at meiosis (Me). To calculate fold changes, the following mean ΔC_t ± standard deviations were utilized; 6.54 ± 0.35 for 2F12R1 and 1.63 ± 0.072 for F2R-400 in 0B leaves (used in *I* and *II*), 5.79 ± 0.20 for 2F12R1 in +B leaves (used in *III*), and 3.76 ± 0.12 for 2F12R1 at premeiosis and 4.10 ± 0.34 at meiosis in +B plants (used in *IV*).

2F2R-400 cDNA results in a frameshift when compared with the putative translation product of the published sequence or the 2F2R-1500 genomic sequence. This frameshift is imperative for the existence of an open reading frame, where the 2F2R-400 conceptually translated product has a putative start codon at nucleotide position 52 and a putative stop codon 70 nt downstream of the 2R primer. Analysis of the 2F2R-400 translated sequence against the pfam database (http://pfam.sanger.ac.uk/) supports the existence of a *gag* retrotransposon domain at amino acids 24–88 (E = 4.4 e −12), of which three amino acids originate from 9 nt that lie upstream of the 1,133-bp deletion. Within the *gag* domain, there is an internal sequence of SVDEYYKEM at peptide position 76–84 with homology to a hand domain (E = 0.047). This domain, encoded by nucleotide sequence AGTGTTGATGAATATTACAAGGAGATG at position

FIG. 5.—Translated 2F2R-400 has an open reading frame. Translation product of 2F2R-400 is shown in respect to its highly conserved genomic and cDNA sequences. Due to the deletion of 1,133 nt involving the repeat gcta (shown in open box), 2F2R-400 reveals a novel truncated sequence. Putative open reading frame (amino acids in bold and italic) shows a hand motif (dark gray box) and a partial gag domain. 2F and 2R primers are underlined, stop codons are shown in light gray boxes, and poly-A sites are in strikethrough.

3339 on the published sequence, is believed to have DNA and nucleosome-binding properties (Grune et al. 2003).

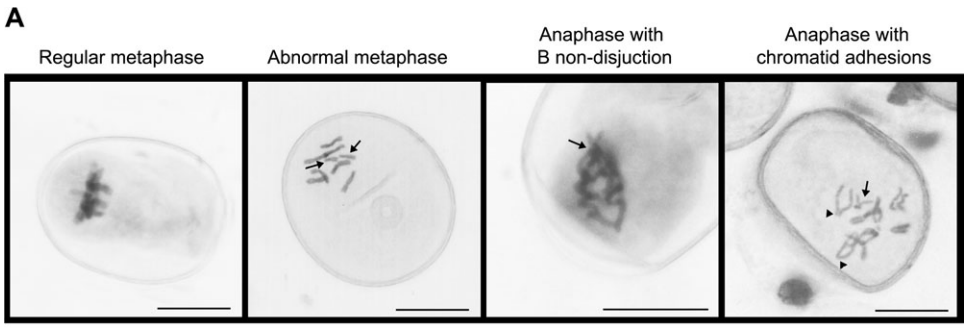
B Chromosomes Affect A Chromosome Behavior in a Dosage-Dependent Manner

Rye (JNK) and wheat (Lindström) plants with and without Bs were utilized to evaluate the effects of Bs on chromosome behavior in the first pollen grain division. Lindström wheat is a synthetic line where introgressed rye Bs are maintained through the characteristic chromatid nondisjunction drive mechanism (Jenkins and Jones 2004). Cytological analysis of the first mitotic division of pollen grains was performed with classical Feulgen staining in order to allow chromosome visualization within the pollen grain without disturbing the pollen coat, thereby minimizing disruption of chromosome arrangement. In the typical first pollen grain mitotic metaphase, condensed chromosomes group on a nonequatorial metaphase plate. Aligned chromosomes are located toward one pole of the pollen grain, indicating an asymmetric metaphase spindle (fig. 6*A*, first panel). Analysis of numerous pollen grain cells shows that Bs induce disruption of the metaphase plate in the first mitotic division postmeiosis. Highly condensed chromosomes are visibly dispersed, resembling the effects of induced spindle disassembly. However, in contrast to what characterizes a chemically induced c-metaphase, chromosomes maintain a polarized position in the cell and sister chromatids show no evident loss of adherence at the chromosome arms (fig. 6*A*, second panel). The frequencies of disrupted metaphase plates in pollen grains isolated from rye and wheat mother plants with 4Bs, 2Bs, and without Bs are shown in table 2. Interestingly, the vast majority of dispersed metaphases observed had visible Bs. Furthermore, the frequency of anomalous metaphases increases significantly in JNK rye and Lindström wheat plants with 4Bs compared with those with 2Bs, indicating a dosage-dependent effect of Bs on As.

Metaphase progresses to anaphase as expected in wheat and rye plants from the same lines without Bs, whereas anomalous A chromosome behavior is observed in approximately half of the anaphases in plants with Bs (fig. 6*A*, third and fourth panels). From a total of 86 anaphases scored in JNK rye plants with 2Bs, 36% show regular chromosome behavior and 16% show evident nondisjunction of Bs (fig. 6*A*, third panel). The remaining pollen grain cells (48%) reveal some form of abnormal A chromosome behavior. These include chromosomes unattached to the spindle, chromosome grouping, and/or failure of complete chromatid disassociation due to persistence of attachment regions outside the centromeric and pericentromeric domains (fig. 6*A*, fourth panel). Chromosomal position is maintained toward one pole of the cell in abnormal metaphase and anaphase cells (fig. 6*A*), clearly indicating that the anomalies observed are not due to spindle assembly.

Discussion

E3900 belongs to a sequence family, which together with D1100, has been defined as being specific to rye Bs (Sandery et al. 1990; Houben et al. 1996; Langdon et al. 2000; Carchilan et al. 2007; Endo et al. 2008). Both these sequences accumulate at the subterminal region of the long arm of Bs and are believed to have a role in their drive mechanism. In this work, we provide a number of novel insights into the genetic nature of the E3900 sequence on Bs as well as its role on their particular mode of inheritance, mediated by B sister chromatid nondisjunction in the first mitosis of the pollen grain. We show that E3900 is highly conserved on rye Bs as well as on A genomes across



B

	Sporophyte	Gametophyte	
2F2R, 2F12R1 transcription	0B = +B	0B < +B	
Mitotic phenotype	0B = +B	0B	+B
	Regular chromatid separation	Regular chromatid separation	Increased chromatid adhesion

FIG. 6.—Effects of Bs on mitotic chromatid segregation in the male gametophyte. (*A*) Pollen Grains from rye plants carrying Bs after Feulgen staining. The first panel shows a regular rye pollen grain first metaphase, with chromosomes typically aligned at the polarized metaphase plate. The second panel demonstrates a B chromosome induced abnormal metaphase with highly condensed chromosomes dispersed and positioned toward the cell pole; 2Bs are visible (arrows). The third panel shows nondisjunction of B chromatids (arrow) at anaphase. In the fourth panel, an abnormal anaphase with evident noncentromeric A chromatid adhesions (arrowheads) are shown; B chromosome is indicated by arrow. Bars = 10 μm. (*B*) Relation between 2F2R and 2F12R1 transcription levels and mitotic chromatid behavior. In the sporophyte, plants with and without Bs show identical levels of transcription of 2F2R and 2F12R1 sequences and cells progress normally through mitosis. In the gametophyte from plants without Bs, regular mitotic progression is also observed in the first mitotic division. Transcription levels of the two sequences increase in the meiocyte and gametophyte exclusively from +B plants. Gametophytes from these plants have abnormal metaphases, as well as increased sister chromatid adhesion at anaphase.

species and is differentially expressed in a tissue-specific manner, as summarized in figure 6*B*. Transcription regulation of E3900-related sequences is drastically different in leaves than in anthers. Furthermore, there is an effect of Bs on A chromosome segregation in the first mitotic division of the pollen grain, due to obvious adherences between sister chromatids.

E3900 on As and Bs Is Ancient and Highly Conserved

The published E3900 sequence is 3,984 bp long and has been extensively analyzed (accession number AF222021), having been defined as the largest predicted size of canonical organization of E3900 on rye Bs (Langdon et al. 2000). Our results support that the E3900 domain on Bs is composed of a family of sequences, as previously demonstrated by Langdon et al. (2000). However, in contrast with the generally accepted notion that this sequence is specific to rye Bs, our results indisputably show that E3900-related sequences are present in the regular chromosome complement of rye and other cereals. The discrepancy between our results and previous data indicating that E3900 sequences are exclusive to Bs (Sandery et al. 1990; Houben et al. 1996; Langdon et al. 2000; Carchilan et al. 2007) can be justified by the high level of amplification of this sequences on Bs compared with As and the fact that previous analysis were based on Southern blotting and FISH. The repetitive nature of E3900-related fragments on Bs results in a high signal in Southern blotting in +B plants compared with a very low or relatively nonexisting signal in 0B plants, and FISH is unable to detect single or low copy number loci. The PCR-based technique used in the present work establishes the presence of E3900 sequences rye, wheat, and triticale A genomes.

Our PCR banding profiles and sequence data show hardly any variation in fragment size and nucleotide sequence for all plants tested, regardless of the presence or absence of Bs. This demonstrates that the organization and nucleotide sequence of the segments that make up this domain are highly conserved between rye populations and species, as well as between As and Bs. Expectedly, quantitative real-time PCR, shows a 100- to 150-fold enrichment of two E3900-related sequences (2F2R-400, 3F0R) on Bs compared with As, supporting accumulation of these sequences on rye Bs. Also, our PCR results did not detect E3900-related fragments arranged head to head or tail to tail, indicating the absence of inversions of the repeat or related sequences on their B domain. Although there are a number of repetitive sequences shared between As and Bs, to our knowledge E3900 represents the first sequence

Table 2
Effect of B Chromosomes on Metaphase in the First Mitotic Division of the Pollen Grain

	% of Anomalous Metaphases (Total Number of Pollen Grains Scored)		
Genotype	0B	2B	4B
JNK rye	1.4 (69)	21.0 (218)	36 (327)
Lindström wheat	0 (56)	19.1 (42)	42 (249)

that is present in a repetitive manner on Bs and in single or low copy number on the A genome (for a review, see Puertas 2002, Jones et al. 2008).

Besides the expected products, we uncover a novel E3900-related sequence (named 2F2R-400), which is highly conserved, evident as virtually 100% nucleotide sequence similarity between populations and species. 2F2R-400 has a deletion of 1,133 nt corresponding to positions 2063–3190 of the published sequence, giving rise to a 416-bp fragment. This form of E3900 was likely responsible for previously isolated clones that defined canonical E3900-specific families with 2.7 kb (Blunden et al. 1993; Langdon et al. 2000). Which of the two variants is the most ancestral E3900 sequence remains to be determined, because both can be explained by either an insertion in the smaller sequence represented by 2F2R-400 or a deletion in the larger 2F2R-1500 variant. Nucleotides flanking the deletion or insertion breakpoints provide important clues as to how the two variants may have occurred. Previous studies have shown that methylation is not equal throughout the sequence, with the first half, or 5′ end of the sequence being methylated to a greater degree. Of specific significance is a nonmethylated HpaII site at position 1920 of the published sequence, which is approximately 140-bp upstream of the deletion/insertion breakpoint (Langdon et al. 2000). Regarding the nucleotide sequence in the region of the insertion/deletion, there are potential instability motifs immediately upstream and downstream of the *gag* domain. Especially interesting is a 4-nt sequence (GCTA) that flanks each deletion/insertion breakpoint, with one GCTA being absent in 2F2R-400. Primers within the 1,133-bp deletion that define 2F2R-400 amplify identical products of the expected size in 0B and +B plants, showing that both variants of E3900 are present on Bs and As. FISHs on extended pachytene chromosomes show that 2F2R-400 is more represented throughout the E3900-specific B domain than 2F12R1, which lies within the deletion that defines 2F2R-400.

The lack of evident divergence in E3900 between rye populations and wheat establishes that E3900 sequences are ancestral and highly conserved. Although many repetitive sequences are found across related species, they characteristically present some level of inter or intraspecies variability. A similar approach to that used in the present work on the major component of rye heterochromatin pSc119.2 revealed the presence of homologous sequences in related species but with variable levels of nucleotide variation (Contento et al. 2005). The high level of conservation of E3900 provides insight into how this sequence accumulated on Bs. Considering that E3900 contains a retrotransposon *gag* domain, it is possible that these sequences accumulated on Bs by the "copy and paste" mechanism typical of retrotransposons. However, the low fidelity of reverse transcription associated with accelerated retroelement evolution (Gabriel and Mules 1999) would likely result in relatively higher sequence divergence than what is observed. Furthermore, the deletion uncovered by 2F2R-400 is better explained by unequal homologous recombination involving instability motifs and the GCTA nucleotide sequence that flanks the deletion breakpoints.

It has been previously shown that Bs originated prior to rye speciation, as interspecies meiotic B chromosome pairing has been observed (Niwa and Sakamoto 1995). Our results demonstrate that E3900-related sequences that accumulate on Bs have been maintained on A chromosomes since before *Triticum* and *Secale* genus divergence as well as through wheat and triticale allopolyploidization. E3900 on Bs is therefore not representative of a typical repetitive sequence. Rather, it is composed of sequences that are highly conserved and present in single or low copy numbers in cereal A genomes. Furthermore, the lack of variation in nucleotide sequence and arrangement of E3900-related sequences on Bs shows constraints on the amplification process specific to the long arm of rye Bs. This strongly suggests that these sequences have an important functional role in cereals.

Transcription and Functional Analysis of E3900

In this work, transcriptional analysis shows that three E3900-related sequences (1F0R, 2F12R1, and 2F2R-400) are expressed in leaves of 0B and +B plants. The transcription levels of 1F0R are variable, whereas 2F12R1 and 2F2R-400 are transcribed at similar levels in all plants, regardless of the number of Bs. Because A chromosomes have approximately one copy of these sequences compared with 100–150 copies in Bs, the data strongly suggest that E3900 sequences are partially silenced in Bs and transcriptionally active on As. Previous analysis of E3900 transcription involving Northern Blots with probes that hybridize to the entire E3900 sequence as well as to the 3′ portion of E3900 showed in a strong hybridization signal just below 3 kb (Carchilan et al. 2007). This smaller variant of E3900 likely represents E3900 with the 1,133-bp deletion identified by 2F2R-400.

The transcription of the same sequences is completely different in anthers, where there is upregulation of all E3900 sequences exclusively in plants with Bs. The sequence 2F2R-400 shows a peak of expression at meiosis compared with the premeiosis and pollen grain stages, which is less significant in the other two sequences. In contrast to expression in leaves, this is better explained by transcription of B specific sequences. Transcription of E3900 sequences in anthers was previously reported (Carchilan et al. 2007), although none of the primers combinations utilized amplified E3900 sequences exclusively derived from the deleted form of E3900. The cDNA analysis in the earlier work failed to reveal transcription in leaves of 0B or +B plants, which can be explained by the extremely low levels of 2F2R-400 and 2F12R1 transcription in leaves demonstrated here.

In comparison with the expression levels of sequences from the E3900 central region, cDNA analysis and Northern Blots demonstrated high levels of transcription of E3900 sequences that map 3′ of nucleotide 3231 in plants with Bs (Carchilan et al. 2007). These terminal sequences did not show significant differences in transcription levels between roots, leaves, or anthers, which suggests that they may not be regulated in a tissue-specific manner. Our results of 1F0R expression, which maps to the 5′ portion

of E3900, also show that this initial segment of E3900 is not transcriptionally regulated in the same manner as 2F2R-400 or 2F12R1. Taken together, both studies support differential regulation of E3900 transcription along its length. The results shown here establish transcriptional activity and regulation of E3900 in plants without Bs, with particularly tight tissue and developmental regulation of sequences involved in insertion/deletion variant defined by 2F2R-400. Despite the fact that we cannot determine whether there is pre or posttranscriptional regulation of E3900 sequences, we can conclude that the expression of these are upregulated in meiocytes from plants with Bs and that this regulation occurs in a stage-specific manner.

Other than 2F2R-400, cDNA conceptually translated sequences do not reveal any significant homology to the databases and may play a structural role as previously suggested (Carchilan et al. 2007). Conversely, the conceptually translated 2F2R-400 cDNA encodes an open reading frame for a partial *gag* domain of plant retrotransposons as well as signals in the flanking genomic regions that are associated with transcription initiation and poly-adenylation (illustrated in fig. 5). Of particular interest is the effect of the 1,132 (according to published data) or 1,133 (according to our data) base pair deletion that defines the 2F2R-400 fragment on the translation reading frame. This deletion causes a frameshift that results in a translation initiation codon (ATG) as well as a putative stop codon 70 nt downstream of the 2R primer. In contrast, the translation product of the larger 2F2R-1500 fragment does not show a translation initiation codon upstream of the *gag* domain.

Retrotransposons are known to play an important role in centromeric function and kinetochore assembly in cereals, where centromeres are composed of satellite repeats interspersed with centromeric retrotransposons (for a review see Ma et al. 2007). Interestingly, the *gag* domain within E3900 has a high homology to *crwydryn*, which has colonized the centromeres of Poaceae (Presting et al. 1998; Langdon et al. 2000). Most of what is known about Gag proteins comes from the study of retroviruses, where it plays a role in virion size, budding, and packaging of other viral components (Swanstrom and Wills 1997). Gag is usually translated as one open reading frame and is then processed by the viral protease into at least three major products, the matrix, capsid, and nucleocapsid. The two latter products, which are derived from the center and the carboxyl end of the amino acid sequence, are believed to have reverse transcriptase and RNA-binding functions (Teysset et al. 2003). In the human HIV-1 retrovirus, individual Gag domains have numerous and complex roles, including nucleic acid binding and chaperone properties (Freed 1998; Cruceanu et al. 2006). When the 2F2R-400 nucleic acid sequence is aligned to HIV-1 *gag* (accession number EU581828.1), most similarity occurs in the 3′ region, where DNA-binding domains reside, albeit at low percentage similarity (47.2%). The translation product of 2F2R-400 also shows a partial Hand DNA binding motif (Grune et al. 2003), often associated with chromatin remodeling proteins (fig. 5).

Although E3900 contains a *gag* domain with putative DNA binding properties, there are marked differences between this sequence and retrotransposons or retroviruses. Firstly, the gag domain does not encode the typical retroelement zinc knuckle motif (CX2CX4HX4C where X can be any amino acid). Secondly, our results show a very tight regulation on the expression of E3900-related sequences, whereas retrotransposon transcription is widespread in plants, especially in the grasses (Vicient et al. 2001).

B Chromosomes Affect A Chromosome Behavior

Bs display a well-studied drive mechanism, involving nondisjunction of sister chromatids in the first gametophyte mitosis and subsequent accumulation in the generative nucleus (for a review, see Jones 1991). The precise genetic process that causes nondisjunction remains to be determined, as genes have not been found in the regions involved. The importance of the E3900 and D1100 in this process was recently established by analysis of chromosomal behavior of wheat lines with introgressed fragments of rye Bs (Endo et al. 2008). Our results show a developmental and tissue-specific expression of E3900-related sequences and upregulation of E3900 transcription in meiotic cells and pollen grains in plants with Bs. It has been previously shown that Bs affect A chromosome behavior, such as rDNA condensation patterns (Morais-Cecílio et al. 1997; Delgado et al. 2004). Considering that the highly conserved E3900 on As and Bs has a functional role on chromatid adhesion related to B drive, the observed drastic increase of E3900 transcription in anthers from +B plants may well influence A chromatid segregation. In fact, our cytological analyses of cells from plants with and without Bs show that Bs affect wheat as well as rye A chromosomes during pollen grain mitosis, when B nondisjunction occurs. This is evident as highly condensed and dispersed metaphase chromosomes, and anaphases with anomalous adherences between sister chromatids that are not mediated by the centromere. Curiously, some effects of Bs on rye A chromosome behavior were described by Hakansson (1957) but virtually never referred to since. Our results further demonstrate that the effects of Bs on chromatid cohesion are observed in rye as well as wheat and increase proportionally with the number of Bs in the mother plant. Aberrant mitotic products resulting from the effects of Bs on As may explain the reduced fertility in plants with increasing numbers of Bs. Of particular relevance is the 20% and 40% observed decrease in fertility, measured as seed setting, observed in seeds from plants with 2Bs (Müntzing 1963; Jiménez et al. 1994) and 4Bs (Müntzing 1963), respectively. These percentages are remarkably similar to the percentages of abnormal metaphases scored in wheat and rye plants with 2Bs and 4Bs.

It is unlikely that the observed effects of Bs are due to microtubule disassembly, as chromosome congregate at their typical polarized position in the cell during metaphase. Also, centromere separation is observed at anaphase, indicating spindle action. The cellular phenotypes seen here have notable similarities with human cells deficient for proteins involved in sister chromatid separation due to RNAi depletion of separase (Giménez-Abián et al. 2005). Separase-depleted cells form congressed spindles with disorganized metaphase plates as well as excessive chromosome

condensation and chromosome arm stickiness at anaphase. Our observations strongly suggest that the B drive mechanism is mediated by a deregulation of a functional process controlling general sister–chromatid disjunction.

Final Remarks

Taken together, this work presents a number of novel insights into how Bs evolved and are maintained. E3900 is highly conserved between As and Bs of rye populations as well as other cereal species, establishing an ancestral origin and strongly suggesting a functional role. Of utmost interest is a novel form of E3900, namely, 2F2R-400, which encodes the only putative protein product with an open reading frame for a partial retrotransposon *gag* domain and a partial hand DNA binding motif. It is tempting to propose a model in which E3900-related protein and/or RNA products are directly involved in sister chromatid pairing and adherence in general. In this model, the overexpression of a putative protein (2F2R-400) as well as RNA sequences (1F0R, 2F12R1) in meiocytes and pollen grains carrying Bs are not only involved in B nondisjunction but also on sister chromatid adherence on A chromosomes. It is important to note that the observed tight regulation of E3900 transcription on As and silencing on Bs in leaves is likely essential for plant growth and survival, as the overexpression of these RNAs would result in anomalous chromosome adhesions and unsuccessful cell division. In conclusion, rye Bs not only provide a useful system to study chromosome behavior but are also a source of functional genomic sequences with a role on chromatid segregation.

Supplementary Material

Supplementary figures S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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