

Transcriptionally Active Heterochromatin in Rye B Chromosomes ^W

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B chromosomes (Bs) are dispensable components of the genomes of numerous species. Thus far, there is a lack of evidence for any transcripts of Bs in plants, with the exception of some rDNA sequences. Here, we show that the Giemsa banding-positive heterochromatic subterminal domain of rye (*Secale cereale*) Bs undergoes decondensation during interphase. Contrary to the heterochromatic regions of A chromosomes, this domain is simultaneously marked by trimethylated H3K4 and by trimethylated H3K27, an unusual combination of apparently conflicting histone modifications. Notably, both types of B-specific high copy repeat families (E3900 and D1100) of the subterminal domain are transcriptionally active, although with different tissue type-dependent activity. No small RNAs were detected specifically for the presence of Bs. The lack of any significant open reading frame and the highly heterogeneous size of mainly polyadenylated transcripts indicate that the noncoding RNA may function as structural or catalytic RNA.

INTRODUCTION

B chromosomes (Bs) are dispensable components of the genomes of numerous species of both plants and animals. They do not pair with any of the standard A chromosomes at meiosis, by definition, and have irregular modes of inheritance (reviewed in Jones and Houben, 2003). In rye (*Secale cereale*), there is a widespread polymorphism for Bs across all geographic regions where this species grows as wild or semiwild populations (Jones and Puertas, 1993), and the Bs are structurally identical at the cytological level among geographically distinct populations. The absence of Bs from some individuals, by definition, and their presence in variable numbers in others provide a unique experimental system to investigate their apparent genetic emptiness. A unique feature of the rye B is that it undergoes a directed nondisjunction at both the first pollen grain and first egg cell mitosis, based on sticking of sensitive sites on either side of the centromere, which delay separation of sister chromatids, and then directs them into the cells destined to become gametes. The accumulation process is controlled by a *trans*-acting genetic element located in the distal part of the long arm of the B chromosome. This is known because deficient Bs, which lack this terminal region of the long arm, undergo normal disjunction,

unless a standard form of the B chromosome is present in the same cell. Numerical accumulation is counterbalanced by loss of unpaired Bs at meiosis and by the deleterious effects of the Bs on physiological and reproductive fitness (Jones, 1995).

This particular aspect of the transmission of the rye B has triggered a number of investigations aiming to identify and isolate the genetic element controlling nondisjunction in gametophytes, but thus far no nondisjunction element has been identified (Langdon et al., 2000).

In terms of structural organization, the rye B is mainly composed of DNA sequences in common with those of the A chromosomes (As) (Rimpau and Flavell, 1975; Timmis et al., 1975), with the exception of the terminal part of the long arm (Tsujiimoto and Niwa, 1992; Wilkes et al., 1995; Houben et al., 1996). Two B-specific families of high-copy repetitive DNA, D1100 (Sandery et al., 1990) and E3900 (Blunden et al., 1993), have been isolated and mapped to this terminal domain. Both families are organized in a complex manner, and neither represents a simple monotonous array of tandem repeats (Langdon et al., 2000). In situ hybridization shows that at c-metaphase the D1100 displays two adjacent clusters with a small gap between them, with the more homogeneous E3900 positioned toward the telomere (Wilkes et al., 1995; Langdon et al., 2000). This B-specific domain is characterized as the B heterochromatic block, which replicates late in S phase and also corresponds to the most prominent and conserved Giemsa-banding positive band discriminated by 4',6'-diamidino-2-phenylindole (DAPI) staining at metaphase (Jones and Puertas, 1993; Houben et al., 1996; Langdon et al., 2000) (Figure 1A). Thus far, there is a lack of evidence for any active genes in the Bs of rye, or of maize (*Zea mays*) or other plants, with the exception of some rDNA sequences of *Crepis capillaris* (Leach et al., 2005).

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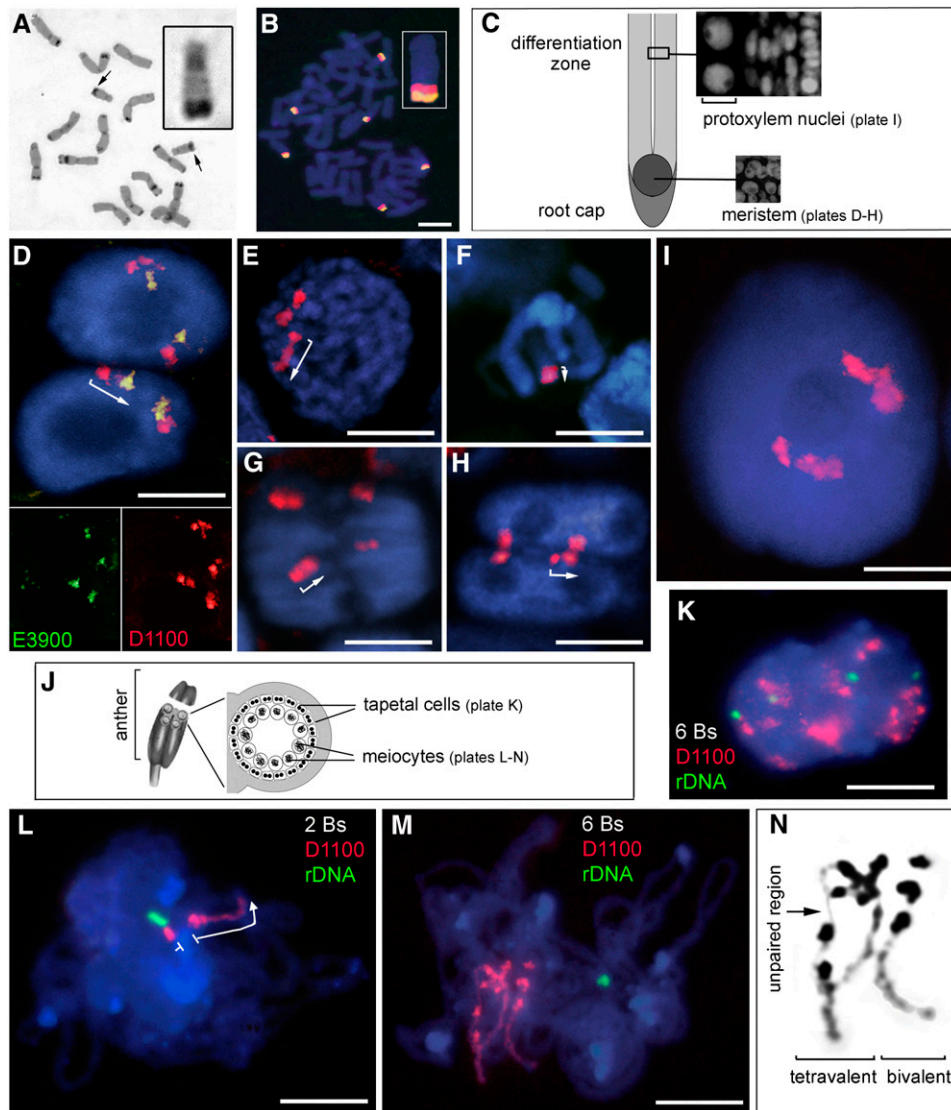


Figure 1. Cytological Organization of the B-Specific Domain during Development.

(A) Giemsa C-banded metaphase chromosomes of rye with Bs (arrows and inset).

(B) Root tip meristematic c-metaphase cell of Lindström wheat with six Bs hybridized with D1100 (red) and E3900 (green) probes. At this stage, the D1100 terminal domain displays a high level of condensation, and the B-specific domain is defined as two contiguous blocks (one centromere-proximal and the other distal); E3900 labels only the distal block (yellow signal due to superimposition of red and green channels).

(C) Organization of the root tip showing the location of meristematic and protoxylem nuclei.

(D) and **(E)** Heterogeneous organization of the B-specific domain defined by D1100 (red), showing condensed and diffuse regions in 2B meristematic cells. The start of the arrow indicates the start of the B domain, and the end of the arrow indicates the telomeric end of the domain.

(D) Two interphase nuclei hybridized with D1100 and E3900 (green; yellow signal is due to superimposition with D1100 red signal). An unlabeled gap frequently separates the condensed and decondensed regions. The bottom of the figure shows the single channel images of the same nuclei for E3900 (left) and D1100 (right).

(E) to **(H)** B-specific domain during mitoses in meristematic cells with two Bs: **(E)** at prophase, **(F)** at metaphase showing the B-specific domain fully condensed (only one B is visible), **(G)** at anaphase, and **(H)** at telophase (only one of the Bs is visible over its full length due to the orientation of projection).

(I) D1100 pattern in a nucleus from a developing xylem cell (see **[C]** for location of cell type).

(J) to **(N)** organization of the B-specific domain in anther tissues: **(J)** location of meiocytes and tapetal cells, **(K)** binucleate tapetal nuclei, **(L)** and **(M)** pachytene nuclei with two Bs fully paired **(L)** and six Bs with irregular pairing **(M)**. The curved arrow in **(L)** indicates the full length of B-specific domain, and the intercalary cut shows the extended gap in D1100 labeling. **(N)** shows the magnification of the B-specific domain from the six B pachytene cells in **(M)**, showing two fully paired Bs forming a bivalent (right) and four Bs with irregular pairing forming a tetravalent. DAPI staining for DNA is in blue. Bars = 10 μ m.

Heterochromatin was first defined cytologically by Heitz (1928) as the genome fraction that maintains a high level of condensation throughout the cell cycle. It was later associated with genome regions that replicate late in the S phase and are mainly composed of repetitive DNA sequences with low gene density. More recent advances in chromatin characterization, in terms of epigenetic marks, showed the involvement of DNA methylation and posttranslational histone modifications (reviewed in Richards and Elgin, 2002; Craig, 2005; Kouzarides, 2007). Although there has been great progress in the discovery of several residue-specific histone methylations and other histone modifiers involved in chromatin packaging (reviewed in Martin and Zhang, 2005), methylation of histone H3 at Lys residues 4, 9, and 27 has become the best studied. Whereas the euchromatin-specific methylation of H3K4 is highly conserved among eukaryotes, heterochromatin indexing by methylation marks at H3K9/27 and H4K20 is more variable (Fuchs et al., 2006). Several studies have shown that modification of the histone H3 tail by methylation of Lys residues 9 (H3K9me) and 27 (H3K27me) negatively regulate transcription by promoting a compact chromatin structure (Bannister et al., 2001; Jacobs et al., 2001; Cao et al., 2002; Czermin et al., 2002; Francis et al., 2004). By contrast, euchromatin is marked by the same type of modification at Lys residue 4 (H3K4me) (Noma et al., 2001; Santos-Rosa et al., 2002; Zegerman et al., 2002). The identification of a H3K4-specific histone demethylase (Shi et al., 2004) challenged the view that histone methylations are permanent and nonreversible.

In this study, through *in situ* localization of rye B-specific repetitive DNA families D1100 and E3900, we disclose that the B heterochromatic domain is consistently decondensed at interphase in two distinct genomic backgrounds of rye and also in a wheat (*Triticum aestivum*)–rye B addition line. Furthermore, we show that this chromatin conformation is maintained in different cell types, namely meristematic, differentiated, and meiocytes. Immunodetection of epigenetic marks revealed that the atypical behavior of this B-specific heterochromatic domain is accompanied by enrichment in the euchromatic mark H3K4me₃, but no distinctive features were obtained for the heterochromatic marks H3methylK9/K27 and H4K20me or for methylated cytosine residues. In addition, transcriptional activity of the E3900 and D1100 repeats in somatic and meiotic tissue has been demonstrated.

RESULTS

The Heterogeneous Organization of the B-Specific Domain Is Maintained in Different Genomic Environments and Distinct Cell Types

At c-metaphase the B-specific domain is characterized by a condensed Giemsa-banding positive region (Figure 1A, arrows) that corresponds to the location of the two B-specific sequences D1100 and E3900. D1100 occupies the entire domain, and E3900 is restricted to a more terminal position (Figure 1B). In meristematic interphase cells, the organization of the domain, defined by the presence of the D1100 repeat family, was evaluated in both rye and wheat root meristematic cells through confocal microscopy after *in situ* hybridization, using structurally preserved root tip tissue sections (Figure 1C). The B domain

forms a well-defined region displaying a heterogeneous internal organization. There are two distinct regions: one is tightly condensed and the other is more diffuse (Figure 1D), as previously described (Morais-Cecílio et al., 1996; Langdon et al., 2000). Simultaneous labeling with the E3900 repeat reveals that the decondensed D1100 region is the more distal of the two since it colocalizes with the E3900 region, which is subterminal (Figures 1B and 1D). Decondensation in the subterminal part of the domain is observed not only at interphase but also at prophase and to a lesser extent in anaphase and telophase of mitosis, but no decondensation was observed in metaphase (Figures 1E to 1H). A gap in D1100-positive regions (Figure 1D) is frequently observed separating the condensed from the decondensed regions. In untreated metaphases (Figure 1F) the B-terminal domain has a condensed appearance, likewise observed in c-metaphases (Figures 1A and 1B). Although no major differences in the B domain organization were detected between rye and wheat, the level of decondensation of the subterminal region at interphase is higher in rye than in wheat (data not shown) and increases with the number of Bs present (Table 1). The D1100 *Dra*I restriction pattern shows six identical bands, for B-carrying wheat and rye (see Supplemental Figure 1 online), although two additional bands (1.6 and 0.65 kb) were revealed in this analysis that were not seen previously (Sandery et al., 1990). The *Eco*RI restriction pattern for the E3900 sequence in both rye and wheat gave the same results (data not shown) as previously obtained by Blunden et al. (1993) in rye.

To establish whether the decondensed interphase structure of the B-specific domain is a general feature, further cell types were analyzed, such as differentiated root cells and anther cells (meiocytes at pachytene and tapetal cells) (Figures 1C and 1I to 1N).

Structurally preserved sections of roots in the differentiation zone allowed the analysis of two distinct cell types: parenchyma and developing xylem vessel cells. Protoxylem vessels are easily recognized as a central row of large cells due to endoreduplication that occurs as part of the differentiation process, and, as expected, they present enlarged nuclei accompanied by a correspondingly extended D1100 domain (Figures 1C and 1I). In these endopolyploid cells, as in parenchyma (data not shown), the same basic organization of the B-specific domain is maintained, with a condensed region in the centromere proximal end and a pronounced decondensation toward the telomeric end. In anther cells (Figures 1J to 1N), the level of decondensation is

Table 1. Average Length (μm) of D1100- and E3900-Labeled Regions in Interphase Nuclei from JNK Rye Plants with 2Bs or 4Bs

Domain	2Bs	4Bs	<i>t</i> Test ^a
D1100	2.37	3.53	P = 0.0026
E3900	1.30	1.66	P = 0.0030

The measurements were performed in the longitudinal axis of the B-specific domain defined by the centromere proximal condensed D1100 block and by the distal region labeled by both D1100 and E3900. Mean values were obtained from at least 30 nuclei from three different plants.

^aP is the probability associated with a two-tailed Student's *t* test.

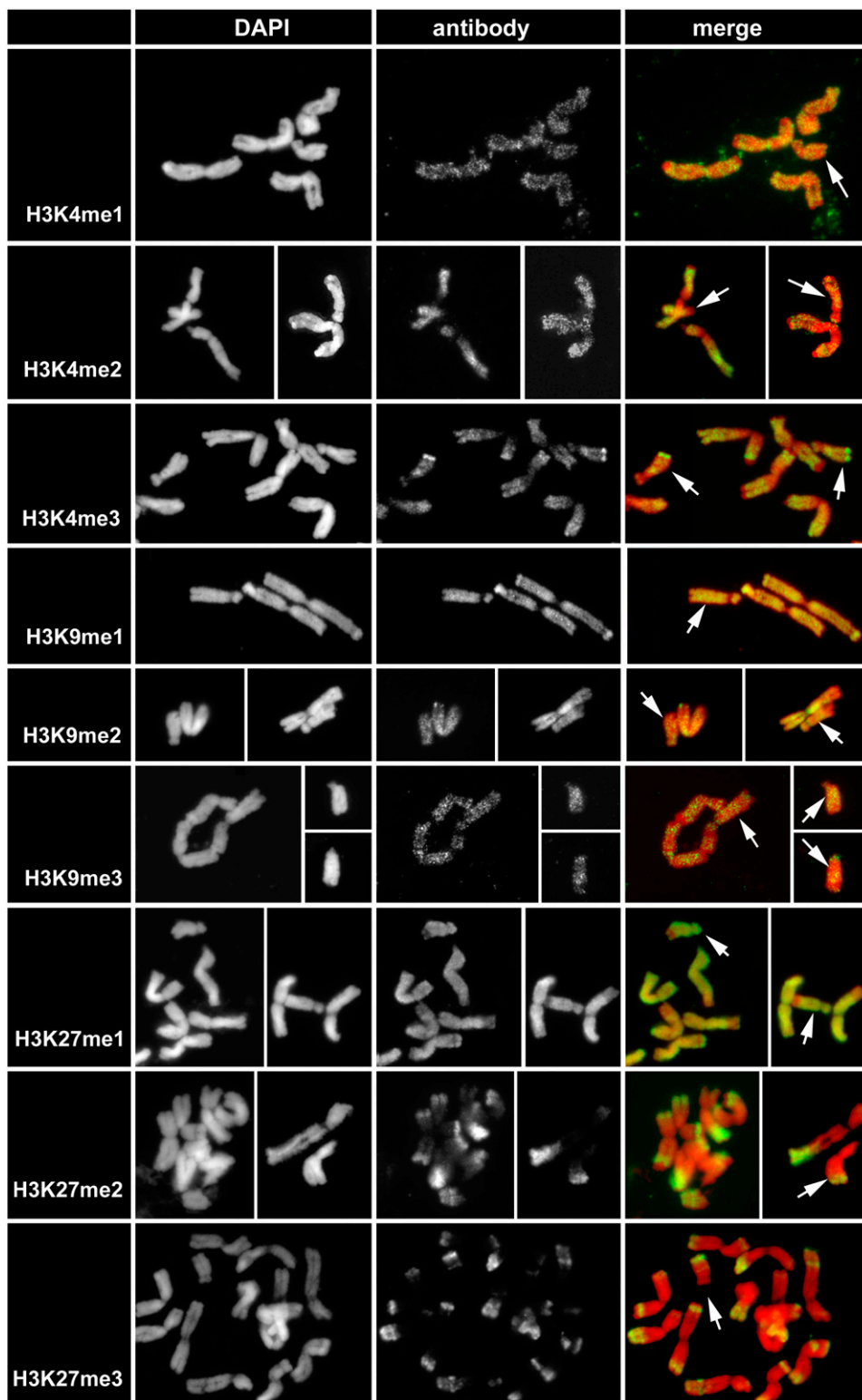


Figure 2. Histone Marks of As and Bs.

Metaphase cells of rye with Bs (arrows) after immunostaining with antibodies specific for H3K4me1,2,3; H3K9me1,2,3; and H3K27me1,2,3.

greatly increased. In binucleated tapetal cells (Figure 1K), the distal region of the domain assumes a cloudy appearance. At pachytene, the D1100 domain is particularly extended, although a more condensed zone toward the centromere is still recognized (Figures 1L to 1N). In this cell type, however, the decondensed distal region is particularly extensive relative to the condensed proximal block, which was always detected and usually far apart from the rest of the domain, leaving a very pronounced gap (Figure 1L). This high level of decondensation contrasts with strong condensation observed in the 45S rDNA cluster visualized as a single block due to chromosome pairing. The pairing pattern of the B-specific region varies with B number: in most 2B meiocytes, only one D1100 domain was observed, indicating close association of these domains (Figure 1L). Conversely, a more complex arrangement was detected in 6B meiocytes (Figures 1M and 1N). Although the structural organization of the B-specific domain is maintained, various configurations were detected with both incomplete pairing and multivalent formation (Figure 1N). The more decondensed organization of the B-specific domain is, however, a generalized feature of meiocytes, and it is not directly related with levels of chromosome pairing since it is observed both in cells with regular or abnormal pairing at this region.

The B-Specific Domain Has a Unique Distribution of Histone H3 Trimethylated at Lys Residues 4 and 27

As the B-specific heterochromatic domain undergoes a cell cycle-dependent decondensation process, we characterized its structure in terms of epigenetic marks by examining the methylation status of certain Lys residues in the N-terminal tails of histones H3 and H4. Cells were stained with antisera that discriminated between mono-, di-, and trimethylated Lys residues (Lys-4, Lys-9, and Lys-27 of H3 and Lys-20 of H4; Figure 2, Table 2).

Table 2. Spatial Relationship of a Variety of Histone H3/H4 and DNA Modifications with the Mitotic Rye B Chromosome

	B-Terminal Domain	B without Terminal Domain
H3K4me1	Dispersed	Dispersed, except pericentromere
H3K4me2	Dispersed	Dispersed, except pericentromere
H3K4me3	Strongly increased	Dispersed, except pericentromere
H3K9me1	Dispersed	Dispersed, except pericentromere
H3K9me2	Dispersed	Dispersed
H3K9me3	Weakly dispersed	Weakly dispersed
H3K27me1	Deficient	Dispersed
H3K27me2	Dispersed	Deficient
H3K27me3	Dispersed	Deficient
H4K20me1	Weakly dispersed	Weakly dispersed
H4K20me2	Weakly dispersed	Weakly dispersed
H4K20me3	Weakly dispersed	Weakly dispersed
DNA methylation	Dispersed	Dispersed

The chromosomal distribution patterns observed for the heterochromatin marks H3K9me1 and 3 were as reported for H3K9me2, which is characteristic for plants with large genomes (Houben et al., 2003), for both As and Bs, with a uniform distribution throughout chromosome arms. While the terminal heterochromatic regions of As and Bs were H3K27me1 deficient, the same chromosomal regions were enriched in di- and trimethylated H3K27. Staining specific for H4K20me1,2,3 resulted in a weak and disperse labeling (see Supplemental Figure 2 online). The distribution pattern of 5-methylcytosine DNA residues showed a punctuated and uniform pattern along both the As and Bs, without any particular sites of accumulation (see Supplemental Figure 3 online). These patterns are independent of the number of Bs present.

The highly conserved euchromatin-specific methylation mark at Lys-4 of H3 revealed a B domain-specific distribution pattern. H3K4me1,2 preferentially mark euchromatin of A and B chromosomes, although the heterochromatic B subterminal domain displays some level of labeling. Immunodetection of histone H3 trimethylated at Lys-4 revealed the same distribution pattern of signals for the As both in rye (Figures 3A and 3B) and wheat (Figures 3C and 3D). There is a higher density of labeling in chromosome arms, a decrease in pericentromeric regions, and an absence of signal in heterochromatin. This is particularly evident in the telomeric heterochromatic blocks of rye As that are brightly stained with DAPI (Figure 3A). Surprisingly, the largest signal of H3K4me3 was seen in the terminal part of the long arm of the Bs. To be more precise, it superimposes with the distal block of the D1100/E3900-positive region that forms the most prominent DAPI-stained region in these chromosomes (Figures 3B and 3G). This immunostaining pattern is dependent on the presence of the B-specific domain, since morphological variants of the B chromosome that lack the terminal part of the long arm do not show any particular enrichment in H3K4me3 (Figure 3G). At interphase, no pronounced H3K4me3 labeling was found, most likely due to decondensation and therefore reduced intensity of immunosignals (Figures 3E and 3F). Alternatively, but less likely, a cell cycle-dependent B domain-specific demethylation of H3K4me3 occurs. This H3K4me3 distribution pattern is identical for plants with different numbers of Bs in rye and wheat.

The E3900 and D1100 B-Specific Repeats Are Transcriptionally Active

The abundance of trimethylated Lys-4 of histone H3 and the decondensed structure at interphase of the B subtelomeric domain prompted us to investigate whether transcriptional activity of this region could be detected. The B-terminal region is mainly composed of arrays of E3900 and D1100 repeats (Langdon et al., 2000), and these were used as probes in an RNA gel blot analysis of total RNAs prepared from plants with and without Bs. As shown in Figure 4A, probe E3900-0N, which covers the entire length of E3900, detected a continuum of transcripts ranging in size from 6.5 to <0.2 kb that were mainly present in plants containing Bs. In addition, bands of 2, 3, and 5 kb were detected over the smear. Weak hybridizing bands in 0B material could be explained by cross-hybridization with homologous transcribed

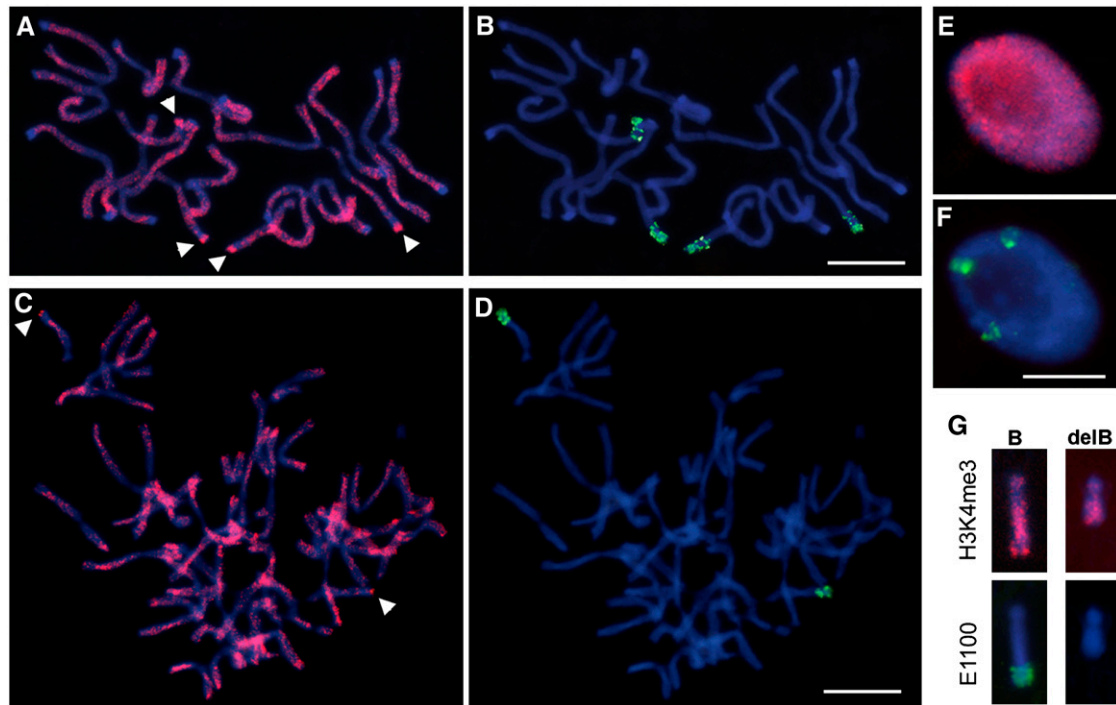


Figure 3. Comparative Analysis of B Variants.

(A) to (F) Root tip meristematic metaphase cells of JNK rye (A) and (B) with four Bs (arrowheads) and of Lindström wheat (C) and (D) with two Bs (arrowheads) and an interphase nucleus of JNK rye with three Bs (E) and (F) after immunodetection of H3K4me3 (A), (C), (E) in red following in situ hybridization with the B-specific D1100 repeat (B), (D), and (F) in green. In (A), note the absence of H3K4me3 in the heterochromatic subtelomeric blocks of rye As. Bars = 10 μ m.

(G) Individual standard B and deleted B (delB) after immunodetection of H3K4me3 (red) and in situ hybridization of D1100 (green). DNA is stained with DAPI (blue).

A sequences. To analyze whether the entire E3900 repeat is transcriptionally active in total, or only parts of it, five subregions of E3900 were used as RNA gel blot probes. All regions revealed cross-hybridization with RNAs of small size (<200 bases; Figure 4A, arrows) derived from anthers with Bs, with the highest level of transcription at the end of the 3900 repeat (region 5N). Weak hybridizing bands in 0B material could be explained by cross-hybridization of short microsatellite sequences located within the 3'-region of E3900 (region 5N) (Langdon et al., 2000). Anther- and B-specific hybridization of small size transcripts were also found for D1100 (Figure 4B, arrow).

Therefore, to further characterize the transcribed sequences, RT-PCR experiments were conducted on total RNA isolated from roots, leaves, and anthers of 0B and +B rye and wheat Lindström plants. E3900 transcripts were amplified from all subregions of the repeat (Figure 5A). The primer pair D1100-1RT allows the amplification of a D1100-specific fragment of expected size (Figure 5B). In each cDNA sample derived from RNA of B-containing plants, a product of the expected size, which was not present in any sample derived from the RNA of plants lacking Bs, was amplified. As indicated by RNA gel blot hybridization (Figure 4), there were differences in yield in many cases, mostly showing a higher abundance of B transcripts in anthers rather than in roots and leaves.

As control, RT-PCR with primers specific for the elongation factor eEF1- α showed a comparable yield in all three tissues, and the differences were not due to unequal RNA loading. Notably, although the same number of PCR cycles were used to amplify the different E3900 subregions, as was demonstrated by RNA gel blot hybridization (Figure 4), regions RT4 to RT6 revealed the highest level of transcription with less tissue type specificity.

Control experiments (Figure 5C) with RNA of all samples without an initial reverse transcription step (lanes -RT) and on genomic template DNA (lane g) confirmed that the PCR products are unequivocally dependent on reverse transcription of RNA molecules originating from transcribed E3900/D1100 repeats on the Bs. This result confirms that the B-located E3900/D1100 repeats are transcribed. Hence, both types of B-specific repeats are transcriptionally active, although with different tissue type-dependent activity.

RT-PCR products amplified from anthers were sequenced. All of the sequences obtained shared a high similarity (92 to 99%) with either D1100 or E3900 repeats (see Supplemental Table 2 online). Both repeat transcripts are at least partly polyadenylated, since all D1100/E3900 RT-PCR products were obtained from cDNA synthesized with poly(dT) primers. In addition, RNA gel blot hybridization of both repeats showed cross-hybridization with fractions enriched for polyadenylated RNA, although the

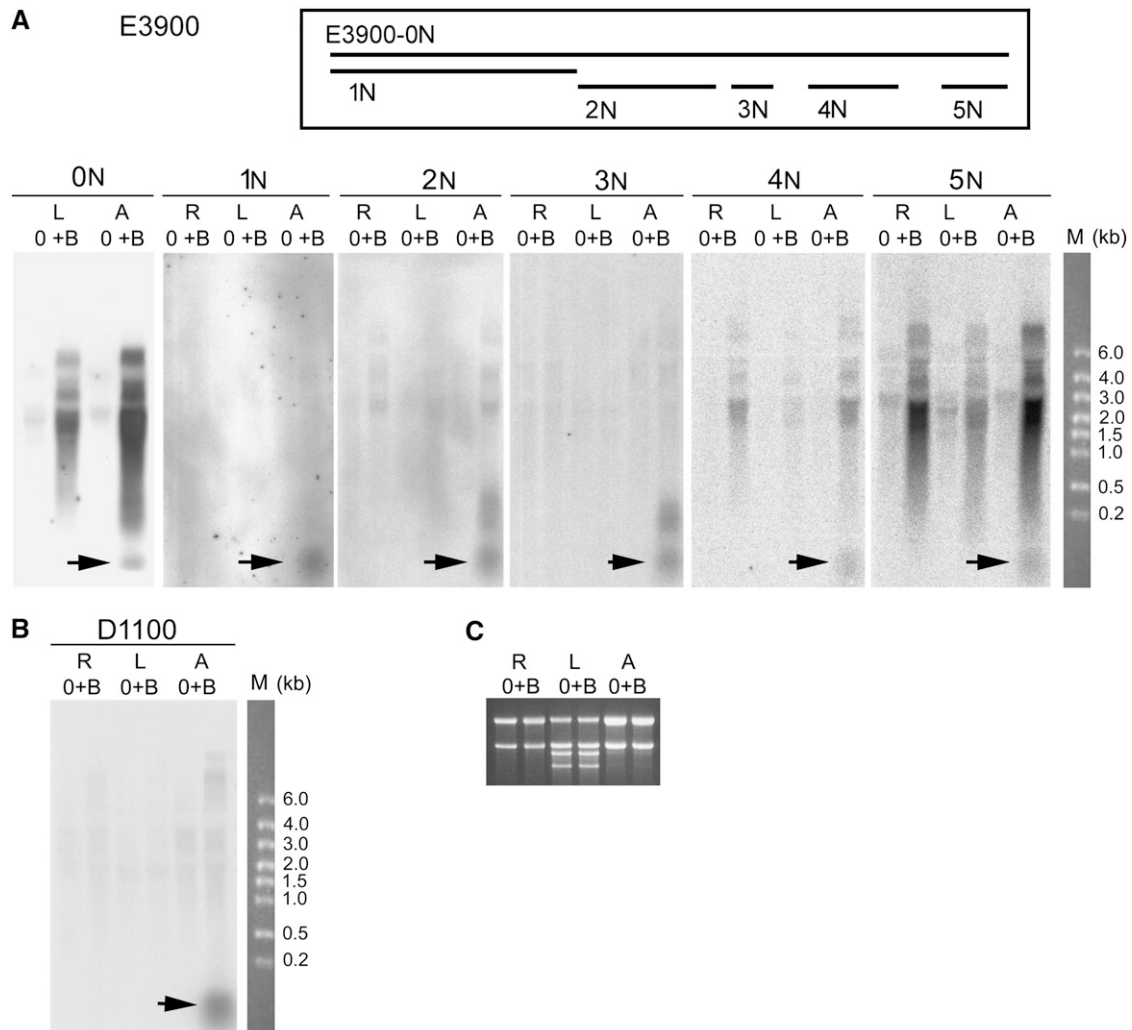


Figure 4. Transcriptional Analysis of E3900 and D1100.

(A) and **(B)** RNA gel blot analysis of E3900 **(A)** and D1100 **(B)** transcripts isolated from roots (R), leaves (L), and anthers (A) of 0B/+B rye. Schemata indicates the different regions (0N to 5N) of E3900 used as hybridization probes. Arrows indicate B-specific signals of small size (<200 bases). **(C)** Ethidium bromide-stained RNA gel that was used as a loading control. Size marker is the RiboRuler RNA ladder (Fermentas).

E3900 revealed a significantly stronger signal in the polyadenylated fraction than the D1100 (Figure 6).

To determine whether the D1100 and E3900 transcripts are processed into small RNA (smRNA), we conducted RNA gel blot hybridizations using low molecular weight RNA isolated from anther and leaf tissue of plants with and without Bs. The blots were hybridized with the E3900-specific probe 3N and the cloned RT-PCR product of D1100. Neither probe detected any B-specific smRNAs but gave hybridization signals in the size range of 21 to 24 nucleotides in RNA from anthers, compared with leaves, and independent of the presence or absence of Bs (see Supplemental Figure 4 online). This result implies that the majority of D1100 and E3900 transcripts escaped processing by the RNA interference machinery in leaf and anther tissue. To check probe quality, the D1100 probe was hybridized with artificially generated smRNAs, and hybridization of 21 nucle-

tides in length was found, depending of the amount of control smRNA loaded (see Supplemental Figure 4A online, arrow).

DISCUSSION

In the two genomic backgrounds analyzed, both meristematic nuclei and those from differentiated and meiotic cells showed cell cycle-dependent decondensation of the distal zone of the B-terminal region, whereas the proximal zone remains condensed, as previously described (Morais-Cecílio et al., 1996; Langdon et al., 2000). The level of decondensation of the distal zone increases with the number of Bs, although to a greater extent in rye than in wheat. These genome-associated differences may be related with the availability of chromatin remodeling factors. Notwithstanding some variation, decondensation of the subterminal domain is a consistent feature, independent of

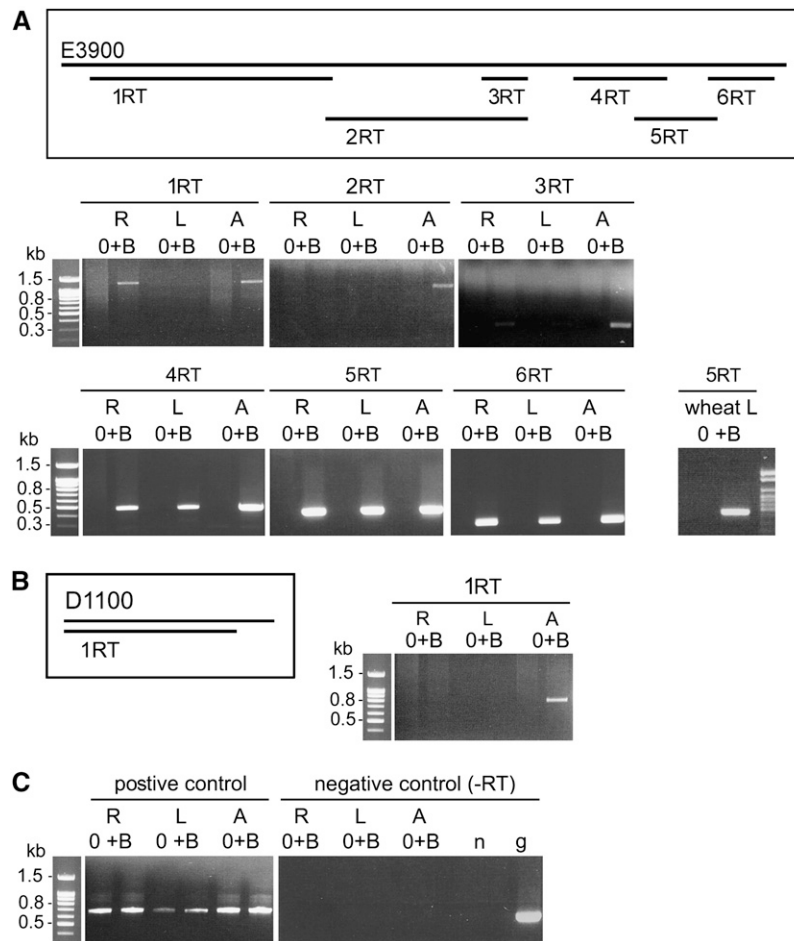


Figure 5. Transcriptional Analysis of E3900 and D1100 Reveals Tissue Type–Specific Activity.

(A) and **(B)** E3900 and D1100 RNA abundance assessed by semiquantitative RT-PCR on root (R), leaf (L), and anther (A) tissue of 0B/+B rye and 0B/+B wheat. Schematas indicate the different regions of E3900 and D1100 amplified by RT-PCR.

(C) Controls: positive control using B-independent primers (eEF1- α elongation factor). Negative control using RNA of all samples without an initial reverse transcription step to demonstrate the absence of genomic DNA contamination, (n) PCR without template DNA and (g) PCR on genomic +B DNA. Each sample contained approximately the same amount of RNA.

genotype and cell type, which is an unexpected result since this domain represents otherwise classic constitutive heterochromatin. C-banding procedures reveal that at mitosis rye Bs have a large heterochromatic band in the distal part of their long arm, where the two B-specific repetitive DNA families are clustered. This segment was also found to replicate later than the rest of the B, in the last part of the S-phase (Lima-de-Faria and Jaworska, 1972), but our results are in conflict with the established knowledge about the heterochromatic nature of the distal end of the rye B. Based on our findings, we propose that the B-subterminal domain forms inconsistent heterochromatin. We also note that the meristematic metaphase pattern of the B-specific domain matches perfectly between rye and wheat, indicating that chromosome location of that fraction is maintained after the introgression of the B into an alien species. This is further confirmed by the identical restriction patterns obtained in both species for the D1100 and for E3900 repeat families. The restriction pattern obtained here for the D1100 sequence in wheat and rye differs

slightly from the one found by Sandery et al. (1990), with two additional *Dra*I fragments of 1.6 and 0.650 kb. The fact that the cloned D1100 fragment used as probe belongs to a DNA family that displays heterogeneity between its members (Sandery et al., 1990; Langdon et al., 2000) may account for the differences observed between the two sets of results.

H3K27 methylation shows a species-specific chromosomal distribution. The euchromatic regions of rye As and Bs are uniformly H3K27me1 labeled. By contrast, the same modification in *Arabidopsis thaliana* and barley (*Hordeum vulgare*) seems to be a heterochromatin mark (Fuchs et al., 2006). H3K27me2 is typical for heterochromatin in *Arabidopsis* and rye but characteristic of euchromatin in barley, while H3K27me3 is euchromatin specific in *Arabidopsis* and barley but clusters at a certain heterochromatic position in *Vicia faba* (Fuchs et al., 2006) and rye As and Bs.

The peculiarity of the terminal B region lies in the fact that, contrary to the Giemsa-positive telomeric heterochromatic regions

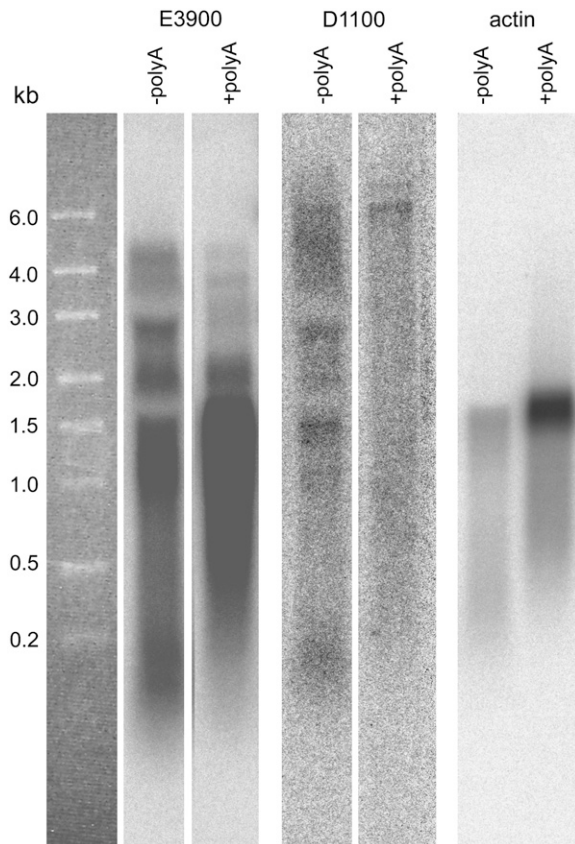


Figure 6. D1100 and E3900 Undergo Polyadenylation.

RNA gel blot hybridization of D1100 and E3900 with total RNA separated into poly(A)⁺ and poly(A)⁻ fractions. A probe specific for actin was used as a poly(A)⁺ positive probe.

of As, this domain is simultaneously marked by trimethylated H3K4 and methylated H3K27, an unusual combination of apparently conflicting chromatin modifications. Conversely, detection of mono-, di-, or trimethylated H3K9, H4K20, and methylated cytosine residues show no discrimination for the B-terminal domain. Methylation of histone H3 at Lys residues 4, 9, and 27 has become one of the most consistent epigenetic marks to differentiate euchromatin and heterochromatin across a wide range of species (reviewed in Martin and Zhang, 2005). Histone Lys residues may be mono-, di-, or trimethylated *in vivo*, and the fully functional significance of these three states remains unclear (Rice et al., 2003). Both dimethyl and trimethyl states of H3K4 have been described as being associated with active euchromatic regions, although the trimethylated, rather than the dimethylated, form of K4 in H3 marks the transcription start site of eukaryotic genes. However, only the H3K4 trimethylated state has been related to gene transcription in yeast (Santos-Rosa et al., 2002) and mammals (Miao and Natarajan, 2005). The presence of H3K4me3 in a chromatin fraction enriched in heterochromatic histone modifications and DNA methylation was only recently reported for the first time in the urochordate *Oikopleura dioica* (Spada et al., 2005). The occurrence of H3K4me3 in

domains with heterochromatic features might therefore be a more common situation than hitherto realized. Moreover, the terminal domain of the rye B seems to have a high specificity for the trimethylation status of H3K4, since no enhanced labeling was found with antibodies directed against the mono- and dimethylated states of H3K4.

Further work is required to resolve whether nonrepressive (H3K4me) and repressive (H3K27me) histone modifications co-exist within the same nucleosome or whether they occupy alternate nucleosomes of the terminal heterochromatic, but transcriptionally active, B region.

The fact that only the subtelomeric domain is highly enriched in trimethylated H3K4 shows a direct correlation with dynamic chromatin decondensation. The decondensation of that chromosome domain was consistently observed in all cell types analyzed and is particularly striking in pachytene cells in contrast with the behavior of other repetitive DNA sequences observed in the same cells, namely, the subtelomeric heterochromatic blocks in the As and the rDNA cluster, that remain tightly condensed at this stage (Cunado et al., 2000). The maintenance of a condensed state during meiotic prophase and low levels of recombination are general features of repetitive DNA sequences (Schwarzacher, 2003). Interestingly, an extensive analysis at the molecular level revealed a high degree of instability in this region and a potential for amplification especially associated with the E3900 sequence (Langdon et al., 2000). These authors suggest that sequence polymorphism may be involved in the B transmission equilibrium, since small changes in relative sequence amount may alter the balance between condensed and decondensed forms affecting meiotic pairing. Variation in the B ability for forming bivalents at metaphase I is one of the main features modulating their transmission rate (M.M. Jiménez et al., 1997; G. Jiménez et al., 2000), since the mitotic drive affected by nondisjunction occurs at a constant and high frequency. This strongly supports the hypothesis that the chromatin conformation of the B-terminal domain is a crucial feature for B transmission and for the maintenance of rye Bs in natural populations.

An intriguing result of our analyses is the identification of transcripts arising from the B-specific tandem repeats in the terminal region. Satellite DNA is generally considered not to be transcribed. However, various examples of transcribed tandem repeats have recently been reported in several organisms, including plants (May et al., 2005; Zhang et al., 2005). These examples suggest an active role for tandem repeat transcripts in several regulatory layers from chromatin modulation to transcription, RNA maturation, and translation (reviewed in Ugarkovic, 2005) and even to centromere function (Bouzinba-Segard et al., 2006).

The function of B transcripts and the mechanism of transcription of B-tandem repeats are unknown at present. Their transcription may be due to readthrough from other active sequences, such as mobile elements. It has been shown that transcription of centromeric satellite DNA (May et al., 2005), or heterochromatin-located genes, is driven by adjacent regulatory elements of retroelements (Dimitri et al., 2005). Both B repeats undergo polyadenylation, at least in part, as also shown for noncoding RNA transcribed in stressed human cells (Rizzi et al., 2004), and the highest transcription activity was found in anthers.

The size heterogeneity and the lack of any significant open frame is compatible with the idea that these molecules are in fact composed partly of a variable number of repeats of E3900 and D1100 sequences, arguing against a possible coding function. The size heterogeneity of B transcripts could also be explained by the fact that the members of the E3900 and D1100 sequence families are not organized as a simple monotonous array. Members of the two B repeat families are often contiguous, and more than one size class of *EcoRI* fragments was identified as derived from D1100 and E3900 families (Langdon et al., 2000). We hypothesize that these transcripts could serve some structural function in the organization and regulation of Bs. It is tempting to speculate that the unique chromatin conformation and transcription activity of the B-terminal region could be involved in the *trans*-acting mechanism of directed nondisjunction of the rye B at pollen mitosis. Although no controlling element for this process has been identified in the B-specific domain, the deletion of this region leads to a loss of the nondisjunction, indicating its direct involvement in chromatid nondisjunction (reviewed in Jones, 1995). The process of nondisjunction is highly regulated and only occurs in the gametophytes, but we cannot exclude the hypothesis that it might be related with the presence of a unique combination of chromatin marks and noncoding RNA in diploid cells.

METHODS

Plant Material

Seeds of rye (*Secale cereale* cv JNK; $2n = 2x = 14 + Bs$) and wheat (*Triticum aestivum* cv Lindström; $2n = 6x = 42 + Bs$), both known to carry rye Bs, and a JNK rye line selected for the presence of deleted Bs (structural variants that lack the terminal part of the long arm (Ribeiro et al., 2004), were germinated on moist filter paper at 24°C.

Some of the primary root tips were immersed in ice-cold water for 24 h to induce c-metaphases, while others were without this treatment to avoid metaphase chromosome condensation. All root tips were fixed in 4% (w/v) formaldehyde. For meiotic preparations, immature spikes were collected, and anthers selected for the pachytene stage were fixed in fresh ethanol:glacial acetic acid (3:1 [v/v]).

Indirect Immunostaining, Giemsa C-Banding, and Fluorescence in Situ Hybridization

Chromosome preparation and immunolocalization of modified histones were done according to published methods (Houben et al., 1996). The primary antibodies (Abcam and Upstate Biotechnology) were used in the following dilutions: 1:2000 (anti-H3K4me3), 1:500 (anti-H3K9me2), 1:200 (anti-H3K4me1,2, anti-H3K9me1,3, and anti-H3K27me1,2,3), and 1:100 (anti-H4K20me1,2,3) in PBS and 1% BSA. Secondary antibodies were conjugated to Cy3, and DNA was counterstained with DAPI. Immunolocalization of 5-methylcytosine residues and Giemsa C-banding were performed according to published methods (Schlegel and Gill, 1984; Castilho et al., 1999). Following histone immunostaining, fluorescence in situ hybridization was performed on chromosome spreads according to (Schwarzacher and Heslop-Harrison, 2000). Meiotic pachytene spreads and root tip longitudinal sections (~30 μm) obtained with a vibratome (Santos et al., 2002) were also used for fluorescence in situ hybridization. The rye-specific probes pJRB1100, containing an *EcoRI* fragment of

1.1 kb from the repetitive family D1100 (Sandery et al., 1990); pTZE3900, containing an *EcoRI* fragment of 3.9 kb from the repetitive family E3900 (Blunden et al., 1993); and pTa71, containing 45S rDNA gene sequences, were labeled using a nick-translation kit (Roche).

Cell Analysis

The number of Bs (from 0 to 6) was determined in squashed c-metaphases. Mitotic and meiotic cell spreads were analyzed with epifluorescence microscopy (Zeiss Axioskop 2; Olympus BX61). Root sections were observed by confocal microscopy (Bio-Rad MRC-1000 UV). Optical section images were assembled as composite images using Photoshop (Adobe Systems) and Confocal Assistant 4.02 (Bio-Rad).

RNA Extraction, RT-PCR, and RNA Gel Blot Hybridization

Total RNA was isolated from young roots, young leaves, and anthers (microscopically staged between meiosis and development of mature pollen) using the Trizol method (Chomczynski and Sacchi, 1987). To remove genomic DNA contamination, DNase (Roche) digestion was performed. DNase was inactivated by heating for 10 min at 70°C and then removed by phenol:chloroform treatment. The absence of genomic DNA contamination was confirmed by PCR with specific primers for a region of E3900 on DNase-treated RNA. cDNA was synthesized from 1 μg RNA (Clontech). Separation of total RNA into poly(A)⁺ and poly(A)⁻ fractions was performed with the Dynabeads mRNA purification kit (Invitrogen).

The RT-PCR mix contained 75 ng of cDNA from rye and wheat Lindström material with and without Bs, 1 μM of each primer (see Supplemental Table 1 online), buffer, deoxynucleotide triphosphate, and 1 unit of *Taq* polymerase. Thirty amplification cycles (45 s at 95°C, 1 min at 64°C, and 2 min at 72°C) were run for the amplification of E3900 and D1100 transcripts. Sequences of cloned RT-PCR products were deposited in the GenBank database under accession numbers EF566937, EF566938, EF566939, EF566940, and EF538668.

For RNA gel blot experiments, 20 μg of RNA was blotted onto a Hybond-N⁺ membrane (Amersham). Prehybridization and hybridization were performed in Church buffer (7% SDS, 10 mM EDTA, and 0.5 M phosphate buffer, pH 7.2) at 64°C. Equal loading of RNA samples after spectrophotometric measurement was monitored by gel electrophoresis and ethidium bromide staining. The probes either generated from the clones D1100 and E3900 or by PCR amplification using primer pairs specific for E3900 regions 1N-5N (see Supplemental Table 1 online) were labeled using the HexaLabel DNA labeling kit (Fermentas).

After overnight hybridization, the membranes were washed twice in 40 mM phosphate buffer, pH 7.0, containing 1% SDS and 2 mM EDTA at 65°C; they were then exposed for 1 h for probes 3900-4 and -5 or 48 h for all other probes. The barley actin EST clone (BU 990587; Zhang et al., 2004) was used as hybridization control.

Analysis of smRNA

RNA was isolated from leaf or anthers using the Trizol method (Chomczynski and Sacchi, 1987). smRNAs were enriched and analyzed according to Mette et al. (2005). RNA (60 μg) was resolved on denaturing 15% acrylamide gel and then transferred to a Zeta-Probe nylon membrane (Bio-Rad). Decade RNA markers (Ambion) were used as size markers. To equalize the migration speed of markers and probes, 60 μg of *Escherichia coli* tRNA was added to markers. RNA probes were synthesized according to an RNA in vitro transcription kit (Fermentas) and labeled with [α -³²P]UTP (Amersham). The hybridization was performed overnight in 125 mM sodium phosphate buffer, pH 7.2, containing 250 mM NaCl, 7% SDS, and 50% deionized formamide. After hybridization, the membranes were washed two times for 30 min in 2× SSC and 0.2% SDS at 42°C.

To remove any unspecific hybridization, membranes were treated with RNase A and then exposed to an x-ray film for 5 d. smRNA of D1100 as a hybridization positive control was prepared using the ShortCut RNAi kit (New England Biolabs).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EF566937, EF566938, EF566939, EF566940, and EF538668.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Metaphase Cells of Rye with Bs after Immunostaining with Antibodies Specific for H4K20me1,2,3.

Supplemental Figure 2. DNA Gel Blot Hybridization Showing *Dral* Restriction Patterns.

Supplemental Figure 3. Metaphase Cell of Rye with Bs after Immunostaining with Antibody Specific for 5-Methylcytosine DNA Residues.

Supplemental Figure 4. Small RNA Analysis.

Supplemental Table 1. List of Primers.

Supplemental Table 2. Sequence Comparison between Genomic DNA and Transcribed Sequences.

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