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Structural features of two major nucleolar organizer regions (NORs), *Nor-B1* and *Nor-B2*, and chromosome-specific rRNA gene expression in wheat

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SUMMARY

The reference genome sequence of wheat 'Chinese Spring' (CS) is now available (IWGSC RefSeq v1.0), but the core sequences defining the nucleolar organizer regions (NORs) have not been characterized. We estimated that the total copy number of the rDNA units in the wheat genome is 11 160, of which 30.5%, 60.9% and 8.6% are located on *Nor-B1* (1B), *Nor-B2* (6B) and other NORs, respectively. The total length of the NORs is estimated to be 100 Mb, corresponding to approximately 10% of the unassembled portion of the genome not represented in RefSeq v1.0. Four subtypes (S1–S4) of the rDNA units were identified based on differences within the 3' external transcribed spacer regions in *Nor-B1* and *Nor-B2*, and quantitative PCR indicated locus-specific variation in rDNA subtype contents. Expression analyses of rDNA subtypes revealed that S1 was predominantly expressed and S2 weakly expressed, in contrast to the relative abundance of rDNA subtypes in the wheat genome. These results suggest a regulation mechanism of differential rDNA expression based on sequence differences. S3 expression increased in the ditelosomic lines Dt1BL and Dt6BL, suggesting that S3 is subjected to chromosome-mediated silencing. Structural differences were detected in the regions surrounding the NOR among homoeologous chromosomes of groups 1 and 6. The adjacent regions distal to the major NORs were expanded compared with their homoeologous counterparts, and the gene density of these expanded regions was relatively low. We provide evidence that these regions are likely to be important for autoregulation of the associated major NORs as well as silencing of minor NORs.

Keywords: nucleolar organizer region, rDNA unit subtypes, nucleolar dominance, gene expression, sequence structure, *Triticum aestivum* L.

INTRODUCTION

Eukaryotic cells have hundreds or thousands of copies of rRNA genes clustered in long tandem arrays at chromosomal loci, and some of these loci form nucleolar organizer regions (NORs), which were first described by McClintock (1934). Only loci with active rRNA transcription and processing can form a nucleolus during the interphase of each cell cycle (Ritossa and Spiegelman, 1965; Phillips *et al.*, 1971). Ribosomal RNA genes are transcribed by RNA

polymerase I into a large primary precursor, which is then processed into the 18S, 5.8S and 26S rRNAs of the catalytic core in cytoplasmic ribosomes (Turowski and Tollervey, 2015). The amount of active rRNAs varies with cellular demand for ribosome production and protein synthesis and is regulated in two ways: coarse and fine regulation. First, the number of active rRNAs is controlled by the on-off state (dosage control) via epigenetic regulatory mechanisms (coarse regulation) and then by modulation

of the initiation frequency of RNA polymerase I for the transcription of each active gene (fine regulation) (Grummt and Pikaard, 2003). In interspecific hybrids, the NORs of one species can be dominant over the NORs of another species, which is referred to as 'nucleolar dominance' (for review see McStay, 2006; Tucker *et al.*, 2010). Nucleolar dominance is an epigenetic phenomenon reported not only for interspecific hybrids but also for allopolyploid species (Jiang and Gill, 1994; Pikaard, 2000). Ribosomal RNA gene silencing in nucleolar dominance results from concerted changes in DNA methylation and histone modification patterns, which are directed in part by non-coding siRNAs (Pontvianne *et al.*, 2012).

In hexaploid wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD), two major NORs have been identified on the short arms of chromosomes 1B and 6B in addition to minor sites on chromosome arms 1AS, 5DS and 7DL (Mukai *et al.*, 1991). These NORs have been designated *Nor-B1*, *Nor-B2*, *Nor-A1*, *Nor-D3* and *Nor-D4*, respectively. The major NORs, *Nor-B1* and *Nor-B2*, are cytologically readily observed at mitotic metaphase as secondary constrictions, indicating that these two NORs are transcriptionally active, as confirmed by silver staining (Cermeño *et al.*, 1984). The chromosomal locations of NORs were also confirmed by DNA/rRNA hybridization (Liang *et al.*, 1977) and *in situ* hybridization (Appels *et al.*, 1980; Mukai *et al.*, 1991; Jiang and Gill, 1994). The copy number of rRNA genes in wheat 'Chinese Spring' (CS) has been estimated to be 9150; most of these (60%) are located on 6B (*Nor-B2*), with approximately 30% on 1B (*Nor-B1*) and the remaining 10% on several chromosome arms, as estimated from signal intensities in hybridization studies (Flavell and O'Dell, 1976). Nucleolar dominances were observed in several interspecific hybrids between species in the tribe Triticeae (Neves *et al.*, 1995; Guo and Han, 2014; Mirzaghader *et al.*, 2017). However, the genomic architecture of NORs in wheat and their sequence-based analysis, including gene expression, remain unexplored because most previous studies on NORs in wheat and related species were based on cytological techniques such as silver staining and fluorescent *in situ* hybridization (FISH).

The International Wheat Genome Sequencing Consortium (IWGSC) has successfully constructed a reference genome sequence (IWGSC RefSeq v1.0) for the hexaploid wheat cultivar CS (IWGSC, 2018) which allows us to analyze the genomic structures of NORs on wheat chromosomes at the nucleotide sequence level. In this work we provide insights into the number of rRNA genes on wheat chromosomes, the genomic structures of the regions surrounding NORs, the subtypes of rRNA gene (rDNA) units and the expression status of these subtypes through characterization of two major NORs in the wheat genome, *Nor-B1* and *Nor-B2*, at the genomic sequence level. This information also enables us to discuss the subgenomic

nucleolar dominance associated with polyploidization during the evolution of hexaploid wheat.

RESULTS

Identification of the rDNA loci in the reference genome sequence IWGSC RefSeq v1.0

Using the rRNA gene sequences (Gerlach and Bedbrook, 1979) as queries, a BLAST search was conducted against the IWGSC RefSeq v1.0 chromosomes 1B and 6B pseudomolecules, on which two major NORs, *Nor-B1* and *Nor-B2*, are respectively located. We could not find any large stretches of rDNA arrays on either pseudomolecule, indicating that the IWGSC RefSeq v1.0 does not completely cover the core sequences of both NORs. However, we identified several copies of intact rDNA units, as well as incomplete rDNA units or rRNA genes interrupted by other genomic sequences in the 80.6–80.9 and 111.0–111.2 Mb regions of chromosome 1B and in the 111.6–123.8 Mb region of 6B (Tables S1 and S2, Figure S1 in the online Supporting Information). In a previous study (Kobayashi *et al.*, 2015) we mapped *Nor-B2* on the chromosome 6B bacterial artificial chromosome (BAC) physical map, using radiation hybrid mapping with several DNA markers including the intergenic spacer marker between the 18S and 26S rRNA genes. By comparing the sequence of the BAC contig CTG151 located just proximal to *Nor-B2* with RefSeq v1.0, we found that region spanning the 111.9–112.5 Mb interval on chromosome 6B was the position of *Nor-B2*.

Detailed analysis of chromosome 1B deletion lines (Endo and Gill, 1996) by FISH revealed that the 1BS-02 line had a deletion breakpoint proximal to *Nor-B1* and that the 1BS-23 line had a distal breakpoint (Figure S2). To determine the position corresponding to the deletion bins in the 1B pseudomolecule, we designed PCR markers in the 80.6–80.9 and 111–111.2 Mb regions (Table S3) and screened the 1B deletion lines. ISBP1B0003, ISBP1B0004, ISBP1B87300-1 and ISBP1B87300-4 located in the 80.6–80.9 Mb region, were mapped between the 1BS-02 and 1BS-23 breakpoints, while ISBP1B0005, ISBP1B0007, ISBP1B0008 and ISBP1B0009 in the 111–111.2 Mb region were found to be proximal to the 1BS-02 breakpoint (Figure S2). These analyses unequivocally showed that the position of *Nor-B1* was distal to the breakpoints of 1BS-02, which ruled out the possible *Nor-B1* locus being in the 111–111.2 Mb region. From this, we concluded that the position of *Nor-B1* is in the 80.6–80.9 Mb region of chromosome 1B.

Estimation of the rDNA unit copy number in the wheat genome by quantitative PCR and FISH

To estimate the copy number of rDNA units in the wheat genome, we conducted quantitative PCR (qPCR) with the

26S, 18S and 5.8S rRNA sequences. The estimated number of copies for 18S and 5.8S were 13 200 and 6450, respectively (Tables 1 and 2). Based on the 26S rDNA results, which showed an intermediate value among the three rDNAs, we estimated that the copy number of the rDNA units in the wheat genome was approximately 11 160, which is 20% greater than that found in the previous study of Flavell and O'Dell (1976) (namely 9150). Furthermore, we calculated the rDNA unit numbers for two NORs, *Nor-B1* and *Nor-B2*, on chromosomes 1B and 6B, respectively, using the qPCR values obtained from the ditelosomic lines Dt1BL and Dt6BL, respectively. As shown in Tables 1 and 2, *Nor-B1*, *Nor-B2* and other NORs possessed approximately 3400, 6800 and 960 rDNA units, which corresponded to 30.5%, 60.9% and 8.6% of total rDNA units, respectively. These estimated values fit well with those in a previous study (Flavell and O'Dell, 1976).

In addition to qPCR, we performed FISH analysis on the metaphase cells to estimate the proportion of rDNA units in the wheat genome using the internal transcribed spacer (ITS) region as a probe (Figure S3). Chromosomes 1B, 4B and 6B were identified by the diagnostic signals of the pSc119.2 probe. Because no NOR sites were reported on chromosome 4B in the wheat genome, we used the chromosome 4B signal as a background threshold. Based on the quantification of FISH signals from 61 well-spread metaphase cells, we estimated the proportion of *Nor-B1* to be 30.7% and that of *Nor-B2* to be 61.2%, supporting the results of qPCR shown above.

Structural features of regions surrounding the NOR on chromosomes 1B and 6B

To characterize the structural features of the regions around major NORs in the wheat genome we compared the gene positions on the chromosomes in homoeologous groups 1 (1A, 1B and 1D) and 6 (6A, 6B and 6D). To this end we selected 60 Mb and 45 Mb NOR-surrounding regions on chromosomes 1B and 6B, respectively, hereafter referred to as the *Nor-B1* and *Nor-B2* regions (Table S4). In the *Nor-B1* region, 304 high-confidence (HC) genes were annotated (IWGSC, 2018), and homology searches using these HC genes identified 39 Mb and

Table 2 Estimated number of rDNA units in the nucleolar organizer regions (NORs) of Chinese Spring

NORs ^a	Whole genome	<i>Nor-B1</i>	<i>Nor-B2</i>	Others
rDNA units in NOR	11 159	3401	6800	958
% in whole genome	100.0	30.5	60.9	8.6
Calculated length (kb) ^b	100 431	30 609	61 200	8622

^a*Nor-B1*, chromosome 1B; *Nor-B2*, chromosome 6B; Others, includes *Nor-A1* (1A), *Nor-D3* (5D) and others.

^bOne rDNA unit calculated as 9 kb.

37 Mb syntenic regions on chromosomes 1A and 1D, containing 306 and 307 HC genes, respectively (Table S4). Among these HC genes, 192 gene loci were identified as homologous between 1A and 1B, and 192 loci were identified as homologous between 1B and 1D (Table S5). Comparison of the chromosomal positions of these genes showed a clear co-linearity among group 1 chromosomes (Figure 1). However, we identified a 9 Mb segment (the 74.2–83.2 Mb region) distal to the *Nor-B1* locus that lacked synteny with the homoeologous counterparts of chromosomes 1A and 1D, and fewer genes were located in this segment (Figure 1).

Within the *Nor-B2* region on chromosome 6B, 269 HC genes were annotated (IWGSC, 2018). We estimated that regions of 23 Mb and 19 Mb on 6A and 6D were syntenic to the *Nor-B2* region and included 253 and 183 HC genes, respectively (Table S4). We determined that 160 loci and 118 loci were homologous between 6A and 6B and between 6B and 6D, respectively (Table S5). The gene orders were basically conserved among the three homoeologous chromosomes (Figure 1). However, we identified a 16 Mb segment distal to *Nor-B2* (the 96.9–113.3 Mb region) that was completely missing from chromosome 6D. In contrast, several genes located in this segment were common to chromosomes 6A and 6B, but the gene order was disrupted due to inversion and translocation and the intergenic distances were larger on 6B than on 6A (Figure 1).

On the rice genome we mapped 268 and 229 HC genes in the *Nor-B1* and *Nor-B2* regions, respectively, to study the synteny between wheat and rice in these regions. Eventually, 107 and 93 HC genes in the *Nor-B1* and *Nor-B2* regions were mapped on the rice chromosomes 5 and 2, respectively, as expected from previous results (Moore et al., 1995). Although the ratios of mapped HC genes were not high (39.9% and 40.6%, respectively), their distribution showed co-linearities in the NOR regions between wheat 1B and rice 5, and wheat 6B and rice 2 (Figure S4). The segments with lower gene density mentioned above were less syntenic than the surrounding regions.

Table 1 Copy number of rDNA sequences in the Chinese Spring (CS) genome estimated by quantitative PCR

Lines used as a template ^a	CS	Dt1BL	Dt6BL
18S rDNA	13 195 ± 180	9028 ± 484	4850 ± 216
26S rDNA	11 159 ± 573	7758 ± 459	4359 ± 108
5.8S rDNA	6447 ± 116	3959 ± 10	2448 ± 120

^aDt1BL, ditelosomic 1BL line of CS; Dt6BL, ditelosomic 6BL line of CS.

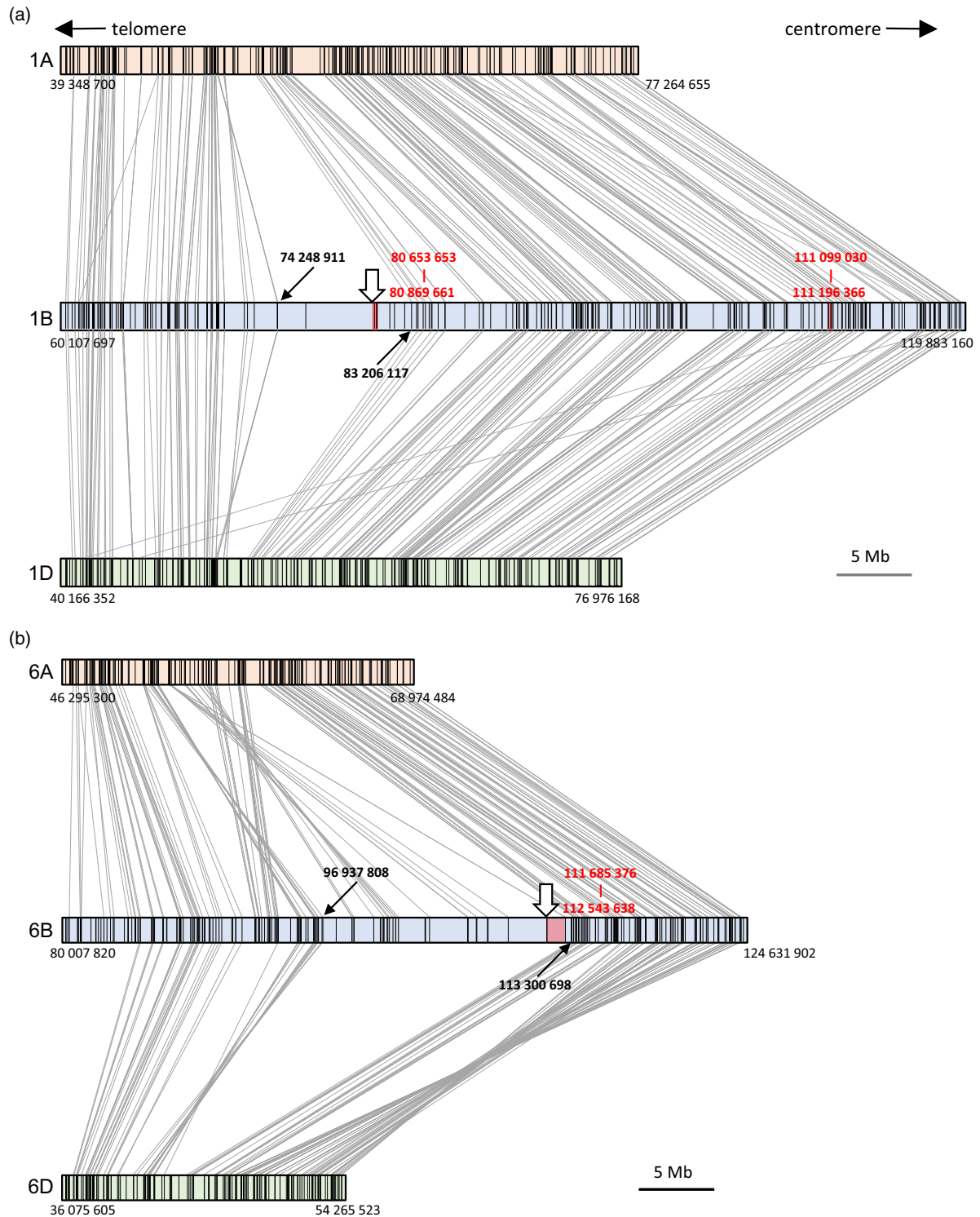


Figure 1. Position and order of homoeologous gene loci among the region peripheral to the nucleolar organizer region (NOR) and corresponding region of the chromosomes in homoeologous groups 1 and 6. Comparison of the position and order of homoeologous gene loci among the region peripheral to the NOR and corresponding region of the chromosomes in homoeologous groups 1 (a) and 6 (b). The position of the high-confidence gene locus is indicated with a vertical line on each chromosome. The line between chromosomes represents the homoeologous relationship of gene loci. The red segment on chromosomes 1B and 6B represents a genomic region containing rRNA gene sequences and the white arrow indicates the position of the NOR. Detailed positions for each segment are indicated by the nucleotide positions (Mb) in the pseudomolecules for each chromosome.

Characterization of the non-syntenic segments distal to the NORs *Nor-B1* and *Nor-B2*

As mentioned above, we identified segments with lower gene density in adjacent regions distal to the two major NORs (Figure 1). First, we speculated that these segments could have an origin in other locations of the wheat genome and could have been duplicated or translocated upstream of NOR loci. To rule out this possibility, we analyzed the contents and distributions of low-confidence (LC) genes in both segments. We found 58 and 122 LC genes on the segments with fewer genes on 1B and 6B, respectively (Table S6). A homology search of LC genes against all HC genes in RefSeq v1.0 revealed that approximately two-thirds of LC genes on the segments had a similarity to HC genes, but the best homologues of LC genes were distributed over all IWGSC chromosomes, and we could not assign the specific regions on wheat chromosomes corresponding to either segment with fewer genes (Table S6). These results indicated that the sequence of segments with fewer HC genes adjacent to NOR loci on 1B and 6B did not originate from simple insertion from other parts of the wheat genome; these will therefore be referred to as 'non-syntenic segments' in this article.

To further characterize these non-syntenic segments, we investigated repeat contents in the regions surrounding the NORs, including non-syntenic segments and their counterparts on homoeologous chromosomes, using the IWGSC annotation (https://urgi.versailles.inra.fr/download/iwggsc/IWGSC_RefSeq_Annotations/v1.0/iwggsc_refseqv1.0_TransposableElements_2017Mar13.gff3.zip; IWGSC, 2018). The proportions of repetitive sequences in the 1B and 6B segments were 93.3% and 89.0%, respectively, which were higher than those in whole chromosomes 1B and 6B, at 86.5% and 87.1%, respectively (Figure 2, Table S7). On the other hand, the repeat contents in homoeologous counterparts were lower than or same as those in whole chromosomes (Figure 2, Table S7), which contrasts with the higher repeat proportions observed in chromosomes 1B and 6B. However, while these segments in 1B and 6B were richer in repeats than were the overall chromosomes, the relative proportions of the main families of transposable elements were no different from those of the regions surrounding the NOR or the chromosomes (Figure S5).

Identification and differential expression of the rDNA unit subtypes in *Nor-B1* and *Nor-B2*

Within an rDNA unit, the coding sequences for three rRNA genes are highly conserved, but spacer sequences (spacers between rRNA genes or rDNA units) are much less conserved. In previous studies in wheat (Appels and Dvořák, 1982a,b; Dvořák and Appels, 1982; Barker *et al.*, 1988), rDNA unit spacers were characterized and used for studying the differentiation of *Triticum* and related species. To

study sequence diversification of the rDNA unit, we extracted and compared sequences of rDNA unit spacers (Tables S1 and S2). Most of the spacer regions are disrupted with other sequence insertions and/or truncated by the sequence gaps. This might be due to the absence of the core NOR regions within the IWGSC RefSeq v1.0, as previously described. Consequently, we could not use the rDNA unit spacers for the diversification study.

Instead of the rDNA unit spacers, we used the 3' ETS (the ETS region downstream of the 26S rRNA gene) from two NORs, *Nor-B1* and *Nor-B2* (Tables S1 and S2). Based on the nucleotide differences and insertions/deletions within the 3' ETS, we identified four subtypes of rDNA units (Figure S6). The distributions and the amounts of these subtypes in the wheat genome were revealed by PCR amplification using genomic DNA of CS and its ditelosomic lines Dt1BL and Dt6BL, with primer pairs in the 3'-ETS variable region (Figure 3). Subtype 1 (S1) of the rDNA unit was the most abundant in the wheat genome, with two-thirds and one-third of this subtype located in *Nor-B2* and *Nor-B1*, respectively. The same distribution pattern was observed for subtype 2 (S2), which was shown to be the second most abundant subtype in the wheat genome. Subtype 4 (S4) was the least abundant among the four rDNA subtypes and was found to be specific to *Nor-B2* on chromosome 6B. Indeed, as no amplified products from Dt6BL were detected, while amplification was detected from Dt1BL (Figure 3). In contrast, no significant changes in the relative amount of subtype 3 (S3) were observed in the two ditelosomic lines compared with the amount of S3 in CS, suggesting that this subunit was not located on chromosomes 6B and 1B. This result was confirmed by PCR amplification using a series of ditelosomic and nullisomic-tetrasomic lines for other chromosomes, which showed that most of S3 was present on chromosome 5D, i.e. at the *Nor-D3* locus (Table S8).

To determine the expression profiles of four rDNA unit subtypes, we performed RT-PCR analyses using the same primer pairs as used for the genome analysis in Figure 3. As shown in Figure 4, the expression pattern of rDNA unit subtypes was different from the relative abundance ratio of the rDNA unit subtypes in the wheat genome (Figure 3). Subtype 1 was preferentially expressed, while the expression of S2 was detected at a very low level and we hardly detected the expression of S3 or S4.

However, when we used Dt1BL and Dt6BL, which lack *Nor-B1* and *Nor-B2*, respectively, the expression patterns of rDNA unit subtypes were considerably changed (Figure 4). The expression of S1 was dominant in both ditelosomic lines (as in CS), although the relative expression rates were slightly increased in ditelosomic lines. The expression of S2 in Dt6BL was similar to that in CS, but increased in Dt1BL. The most striking changes were observed for the expression of S3. In contrast to CS where

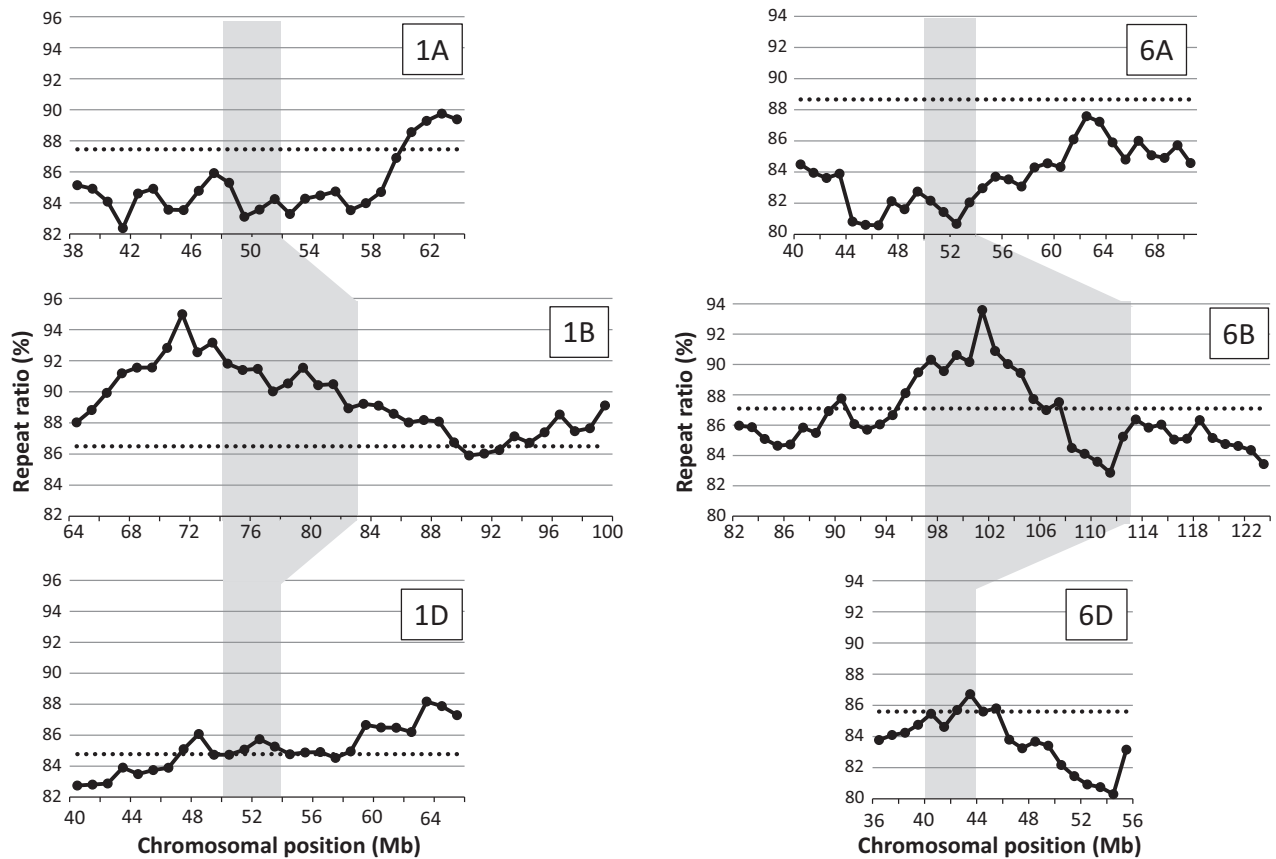


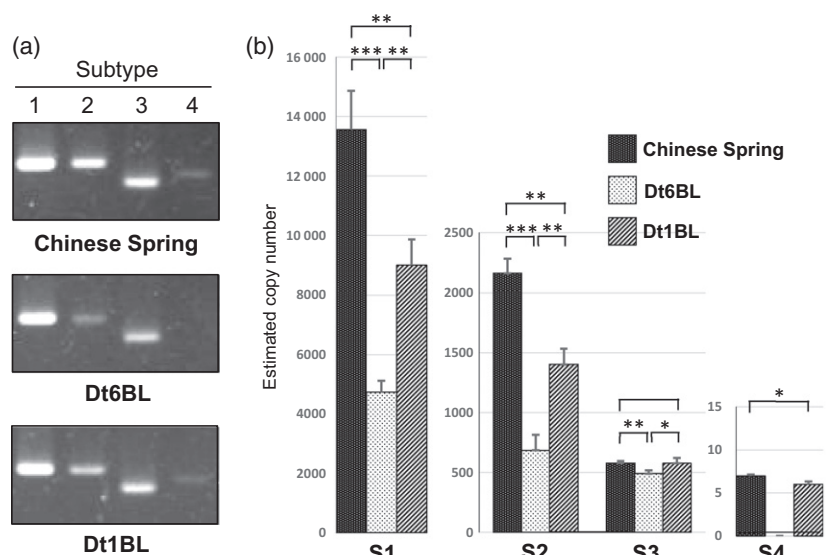
Figure 2. Repetitive element frequencies in the regions surrounding the nucleolar organizer region (NOR) in chromosomes 1B and 6B, and their homoeologous counterparts.

The non-synthetic segments and the corresponding regions in the homologous counterparts are indicated by shaded boxes. Chromosomal positions on the pseudomolecules of RefSeq v1.0 are indicated at the bottom of the figure. All results were subjected to sliding window analysis of 10 Mb in overlapping steps of 1 Mb. Dashed lines indicate the average repeat frequencies for the whole chromosome.

Figure 3. Altered rDNA unit subtype content in Chinese Spring and its ditelosomic lines Dt6BL and Dt1BL.

(a) Gel images showing genomic DNA PCR amplification products for each subtype 1–4.

(b) Estimated copy number of each subtype by quantitative PCR. S1–S4 represent subtypes 1–4, respectively. Asterisk(s) indicate levels of statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) between samples.



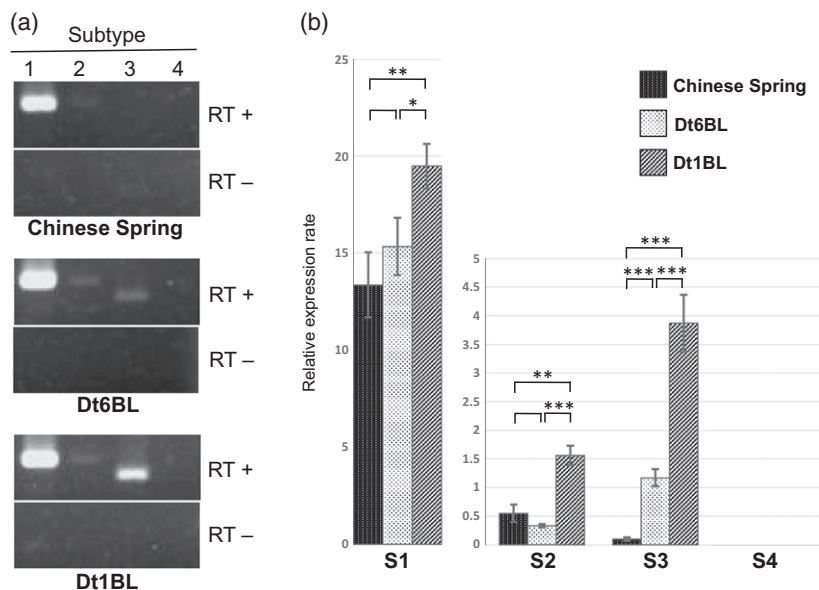


Figure 4. Altered rDNA unit subtype expression in Chinese Spring and its ditelosomic lines, Dt6BL and Dt1BL.

(a) Gel images showing semiquantitative RT-PCR amplification products for each subtype 1–4.

(b) Relative expression rate of each subtype by quantitative (q)RT-PCR. S1–S4 represent subtypes 1–4, respectively. RT+ and RT– represent cDNA templates used for qRT-PCR with and without reverse transcriptase in its first strand syntheses, respectively. Asterisk(s) indicate levels of statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) between samples.

S3 expression could hardly be detected, both ditelosomic lines showed significant increases in S3 expression, especially Dt1BL, where its expression was 40 times higher than that in CS (Figure 4).

Epigenetic status of regions surrounding NORs on chromosomes 1B and 6B

To investigate epigenetic status, we analyzed three DNA methylations (CG, CHG and CHH) as well as four histone marks (H3K27me3, H3K4me4, H3K36me3 and H3K9ac) in the regions surrounding the NOR on chromosomes 1B and 6B and their homoeologous regions on chromosomes 1A, 1D, 6A and 6D.

The CG methylations for the NOR-surrounding regions, including the non-syntenic segments of all six chromosomes, are shown in Figure S7 and Table S9. Compared with the CG methylation ratios of whole chromosomes 1B and 6B (95.7% and 95.9%, respectively), the methylation ratios of the NOR-surrounding regions in 1B and 6B were higher, and in the non-syntenic segments of 1B and 6B the methylation ratios reached 98.6% and 98.2%, respectively (Figure S7, Table S9). On the other hand, the CG methylation rates in the homoeologous counterparts were lower than or the same as those for whole chromosomes, which contrasts with the higher CG methylations observed in chromosomes 1B and 6B. This pattern was similar to that of the repetitive element frequency. In all chromosomes investigated, the frequency of CHG and CHH methylations in the regions surrounding the NOR was lower than that in the whole chromosomes (Table S9), and we could not find any differences among chromosomes as was found for CG methylation.

We detected DNA sequences associated with four histone modifications [H3K4 trimethylation (me3), H3K27 trimethylation (me3), H3K36 trimethylation (me3), and H3K9 acetylation (ac)] for chromosomes 1B and 6B, as well as their homoeologous chromosomes 1A, 1D, 6A and 6D (Figure 5). In general, histone marks were stable in the NOR-surrounding regions of all six chromosomes except for chromosome 1B; the number of peaks per HC gene of H3K36me3, H3K27me3 and H3K9ac was between 0.5 and 1.0, and the number of peaks of H3K4me3 was between 1.0 and 1.5. However, in the NOR-surrounding region of chromosome 1B, we found that all histone marks were commonly increased in the adjacent region distal to the non-syntenic segment and in the distal half of the non-syntenic segment, especially H3K4me3 and H3K9ac, whose peaks per HC gene reached 3.0 and 2.5 (Figure 5).

DISCUSSION

In a previous study (Kobayashi *et al.*, 2015) we developed a BAC-based physical map for wheat chromosome 6B and defined the position of *Nor-B2* on this map. However, our physical map covered only the border regions of *Nor-B2* and did not include the BAC contigs with the core region of the NOR, i.e. large stretches of rDNA arrays. This could be explained by the absence of a *HindIII* site within the rDNA unit, preventing the core region with highly repeated rDNA units from being cloned into BACs.

The recently released reference genome sequence of wheat, IWGSC RefSeq v1.0 (IWGSC, 2018), could provide an opportunity to perform an in-depth analysis of rDNAs at the sequence level. However, it appeared that the core regions of the NORs were not included in any of the

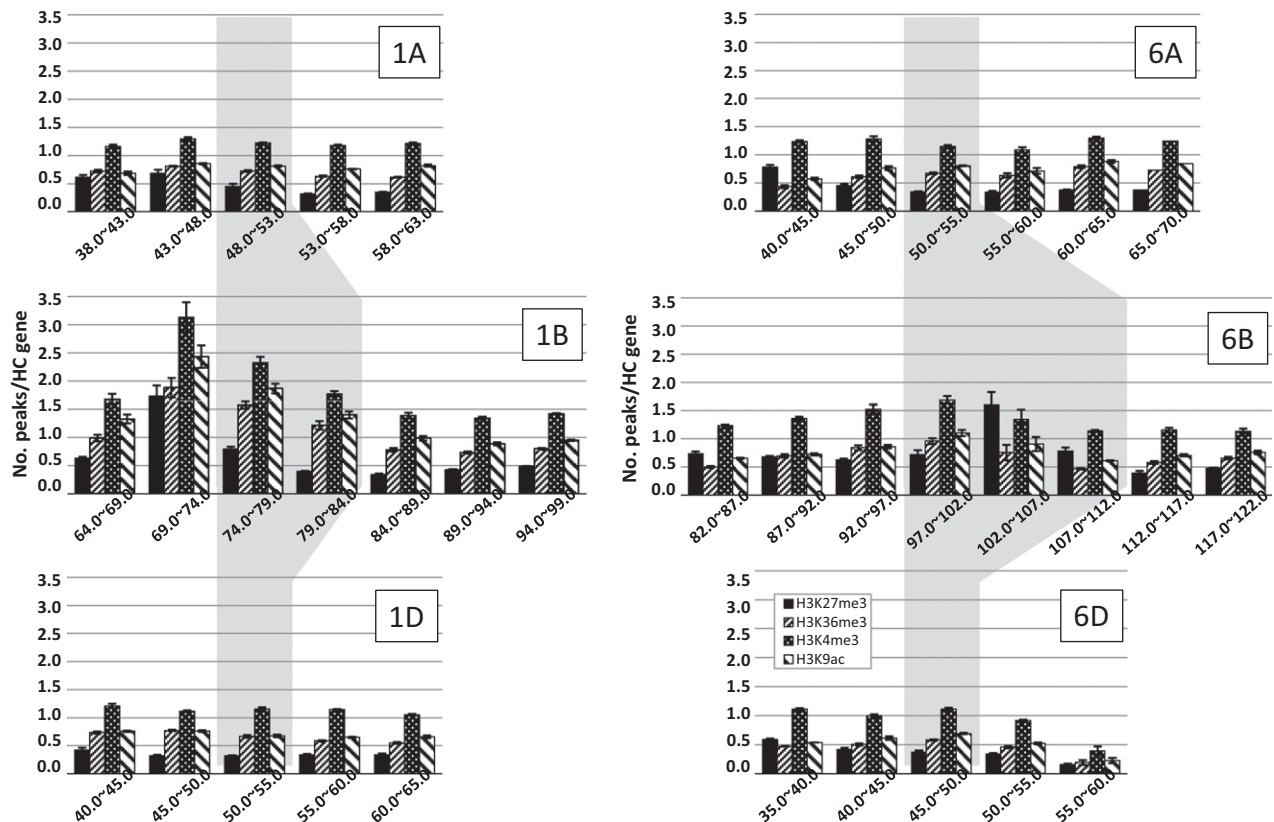


Figure 5. Histone modification frequency in the regions surrounding the nucleolar organizer region (NOR) of chromosomes 1B and 6B and their homoeologous counterparts. The non-synthetic segments are indicated by shaded boxes. Chromosomal positions on the pseudomolecules of RefSeq v1.0 are indicated at the bottom of the figure. There are four types of histone modification: H3K27me3, H3K36me3, H3K4me3 and H3K9ac. For each modification, detected peaks were counted and then divided by the number of high-confidence (HC) genes in a sliding window. All results were subjected to sliding window analysis. Each window was 10 Mb long and overlapped for 9 Mb with the preceding sliding window. In each 5 Mb length, the average and standard deviations of the peak number per HC gene were calculated.

chromosome pseudomolecules, probably due to difficulties in the sequence assembly of highly repetitive regions such as NORs. To estimate the size of the NORs that are missing in the RefSeq v1.0 pseudomolecules, we conducted qPCR and FISH analyses (Tables 1 and 2, Figure S3). The total copy number of the rDNA units estimated in the wheat genome is approximately 11 160, of which 30.5, 60.9 and 8.6% are located on *Nor-B1* (chromosome 1B), *Nor-B2* (chromosome 6B) and other NORs, respectively. These estimations and their proportion in the wheat genome are consistent with those in a previous study (Flavell and O'Dell, 1976). Considering an average size of 9 kb per rDNA unit, the sequence lengths of NORs missing in RefSeq v1.0 are estimated to be approximately 61.2 Mb and 30.6 Mb on chromosomes 6B (*Nor-B2*) and 1B (*Nor-B1*), respectively (Tables 1 and 2). The total length of IWGSC RefSeq v1.0 is 14.5 Gb, which represents as much as 94% of the wheat genome (less than 1 Gb is not covered by RefSeq v1.0) (IWGSC, 2018). The NORs identified in this study comprise approximately 100 Mb in total, which

corresponds to approximately 10% of the unassembled portion of RefSeq v1.0.

Although the core regions of NORs on chromosomes 1B and 6B were not represented in RefSeq v1.0, we identified four subtypes (S1–S4) of rDNA units on the 1B and 6B pseudomolecules based on nucleotide differences and insertions/deletions within the 3' ETS (Tables S1 and S2, Figure S6). Quantitative PCR and qRT-PCR using several cytological stocks as materials provided information about chromosome-specific variation in rDNA subtype content and expression. The major subtypes S1 and S2 are common to both major NORs, *Nor-B1* and *Nor-B2*, but minor subtype S4 is specific to *Nor-B2* on 6B (Figure 3). Subtype 3 is located in *Nor-D3* on chromosome 5D rather than in *Nor-B1* and *Nor-B2* (Table S8). These results indicate the chromosome- (or NOR-) specific composition of rDNA subtype in the wheat genome. A similar pattern was observed in *Arabidopsis thaliana*, in which four rDNA unit subtypes were identified (Mohannath *et al.*, 2016). Indeed, two of these subtypes were mapped

to *NOR2* on chromosome 2, and the remaining two were mapped to *NOR4* of chromosome 4. Interestingly, all silenced rDNA unit subtypes mapped to *NOR2*, and all active rDNA unit subtypes mapped to *NOR4*. In contrast, in the mutant line in which the 2–3 Mb sequence of the end of the short arm of chromosome 4 was replaced by the corresponding region of chromosome 2, including much of *NOR2*, rRNA genes derived from *NOR2* escaped from silencing. These results suggest that selective rRNA gene silencing is not regulated in a gene-by-gene manner based on gene sequence variation but is a consequence of their positions on the chromosomes (Chandrasekhara *et al.*, 2016; Mohannath *et al.*, 2016). Our results for rRNA gene expression in wheat contrasted with those for *Ara*-bidopsis stated above. In wheat, the expression of S1 was dominant, while S2 was weakly expressed (Figure 4), which was not in accordance with the relative abundance ratio of the rDNA subtypes in the wheat genome shown in Figure 3. These results indicate that the expression of these two subtypes, S1 and S2, might be differentially regulated based on the sequence differences between them because these subtypes are located in the same active NORs, *Nor-B1* and *Nor-B2*. On the other hand, the positional effect might regulate the expression of S3 on *Nor-D3* because the activity of this minor NOR seems to be regulated by the existence of the major active NORs *Nor-B1* and *Nor-B2* (Figure 4).

What regions are responsible for the regulation of rRNA expression? In terms of nucleolar dominance in the wheat \times rye amphiploid triticale, wheat NORs are dominant over the rye NOR on the short arm of chromosome 1R, but substitution of rye chromosome 2R by wheat chromosome 2D prevents suppression of rye-origin rDNA (Neves *et al.*, 1997). Furthermore, in translocation of the short arm of rye 1R onto the long arm of wheat homoeologous group 1 chromosomes (1RS/1BL translocation), the rye NOR becomes co-dominant with wheat NORs (Vieira *et al.*, 1990). These results indicate the presence of two types of regulatory sequences, *cis*- and *trans*-acting, to suppress the rye NOR on the long arm of rye chromosomes 1R and on chromosome 2R, respectively. As mentioned above, in *A. thaliana*, when the rDNA subtypes silent in the *NOR2* context are engineered into *NOR4* they become active (Chandrasekhara *et al.*, 2016; Mohannath *et al.*, 2016). This result also shows that the chromosomal context, i.e. the DNA sequences outside the NORs, is important for the regulation of rRNA gene expression. Chandrasekhara *et al.* (2016) proposed that sequences adjacent to the NOR on their centromere-proximal sides are important for the selective silencing of *NOR2*, and this 60-kb region is composed of transposable elements and transposon remnants and appears to be packaged as transcriptionally repressed heterochromatin. We found that the deletion of one major NOR activated rRNA gene expression in *Nor-D3* (Figure 4),

which indicates *trans*-acting regulation of rRNA gene expression by DNA sequences outside the NOR.

From the sequence comparisons of the regions surrounding the NORs on 1B and 6B with their homoeologous counterparts of the A and D genomes, we found non-syntenic segments in the adjacent regions distal to the NORs (Figure 1). Both segments on 1B and 6B were physically longer and encoded fewer HC genes than the corresponding regions of their homoeologous counterparts and their syntenic rice chromosomes. We suspect that these distal regions, including non-syntenic segments, are possible candidates for the *trans*-regulation of *Nor-D3* and for the autoregulation of the associated NORs, such as those in *A. thaliana*.

It is known that epigenetic conditions such as DNA methylation and histone modifications are coordinated to regulate changes in gene expression for rRNA genes on NORs (for review see McStay, 2006; Tucker *et al.*, 2010). Therefore, we analyzed the sequence contents, DNA methylation and histone marks for the NOR-surrounding regions, including the non-syntenic segments on chromosomes 1B and 6B. The NOR-surrounding regions, especially the non-syntenic segments of 1B and 6B, comprise a higher portion of transposable elements and have more CG methylation than the whole chromosomes (Figures 2 and S7), characteristics that are not observed in the homoeologous counterparts in the chromosomes of the A and D genomes. These results seem to indicate that the accumulation of transposable elements is one of the causes of the formation of non-syntenic segments and that CG methylation is introduced to silence the transposon. However, CHG methylation (Table S9) and the negative histone marker H3K27me3 (Figure 5) in the NOR-surrounding regions of 1B and 6B did not correspond to the repeat content or CG methylation. Although the four histone marks investigated here were at the same level in the NOR-surrounding regions of all chromosomes, we found that all histone marks increased in the NOR-surrounding region of chromosome 1B (Figure 5). In particular, the relative values of H3K4me3 and H3K9ac seem to be higher than in other chromosome regions. H3K4me3 and H3K9ac are positive marks for gene expression, which suggests a positive functional implication regarding the expression of the genes located in this region. At present, our data for epigenetic status in the NOR-surrounding regions are very limited, and we cannot make conclusions about the role of epigenetic status in regulating the maintenance of NORs and rRNA gene expression. It will be interesting to see which genes in the NOR-surrounding regions are regulated by these epigenetic marks and are involved in NOR maintenance and rRNA gene expression.

Nucleolar dominance associated with selective rRNA gene silencing has been reported in wheat and related

species. In the wheat \times rye triticale, wheat NORs are dominant, and the rye NOR on the short arm of chromosome 1R was silenced (Neves *et al.*, 1997). In synthetic allotetraploid wheat both sets of parental NORs were expressed just after hybridization, but one parental NOR was immediately silenced after chromosome doubling (Guo and Han, 2014). C-subgenome NOR loci were dominant in wheat \times *Aegilops* interspecific hybrids (Mirzaghader *et al.*, 2017). In addition, Guo and Han (2014) reported that allopolyploidization was a critical step for NOR silencing that could not be reversed by haploidization in wheat. They also showed that the copy number of rDNA units on the recessive NORs decreased upon loss of rRNA gene expression. Here, we identified a minor NOR, *Nor-D3*, on chromosome 5D that is silenced in wheat, possibly as a result of nucleolar dominance. However, in contrast to the results of Guo and Han (2014), we also demonstrated that this silencing could be reversed upon deletion of one of the major NORs, *Nor-B1* or *Nor-B2*, leading to a reactivation of *Nor-D3* (Figure 4). Although we do not have the data for the change in copy number on *Nor-D3* that occurred after interspecific hybridization for hexaploid wheat, the remaining rDNA units on *Nor-D3* retain the ability to express rRNA genes, even after silencing by nucleolar dominance.

Our results also indicate that there is a difference in suppression ability between *Nor-B1* and *Nor-B2* against *Nor-D3*. The deletion of *Nor-B1* has a three-fold greater effect on the reactivation of *Nor-D3* than does the deletion of *Nor-B2* (Figure 4B). It is interesting to note that the suppression ability is inconsistent with the size of the NOR, because *Nor-B2* is twice as large as *Nor-B1* (Tables 1 and 2). As described above, we speculated that the non-syntenic segments of *Nor-B1* and *Nor-B2* might have opposite functions for gene expression, negative and positive respectively. If the non-syntenic segment of *Nor-B1* has a negative effect on gene expression, its deletion from the genome is more effective for the reactivation of gene expression on the silenced NOR, *Nor-D3*. This hypothesis could be applied to gene expression on active NORs such as *Nor-B2* on chromosome 6B, because the deletion of *Nor-B1* induces higher expression of subtypes S1 and S2, which are located on 6B (Figure 4).

Overall, our results provide significant evidence that the nucleolar dominance in wheat can be reversible if the suppressor (the dominant NOR) is removed from the genome. Further studies are necessary to reveal the regulatory mechanisms of rRNA gene expression that lead to nucleolar dominance in wheat, perhaps using combined approaches with molecular characterization, including epigenetic status, and specific cytological materials such as chromosome-engineered lines, including ditelosomic and/or chromosome deletion lines.

EXPERIMENTAL PROCEDURES

Plant materials

Wheat (*T. aestivum* L.) CS and its nullitetrasonic and ditelosomic lines (Sears, 1966; Sears and Sears, 1978) were used in this study. In addition, deletion lines of CS (Endo and Gill, 1996) for chromosome 1BS (1BS-02, 1BS-05, 1BS-06, 1BS-08, 1BS-09, 1BS-10, 1BS-16, 1BS-18, 1BS-19, 1BS-22, 1BS-23 and 1BS-24) were employed to determine the location of *Nor-B1*. All plant materials were obtained from the NBRP-Wheat, Japan (<http://www.shigen.nig.ac.jp/wheat/komugi/>).

DNA sequence analyses

The reference sequence of the bread wheat CS, IWGSC RefSeq v1.0 (IWGSC, 2018), was downloaded from the INRA URGI site (<https://urgi.versailles.inra.fr/download/iwgs/iwgs/iwgs/iwgs/RefSeq/Annotations/v1.0/>), and used for subsequent analyses. To identify homoeologous relationships between annotated gene sequences, a BLASTP search (Altschul *et al.*, 1997) was reciprocally performed with a threshold of E-value less than 10^{-5} between the gene sequences on chromosomes of homoeologous group 1 (1A, 1B and 1D) or homoeologous group 6 (6A, 6B and 6D), respectively. High-confidence gene sequences were obtained from the IWGSC RefSeq v1.0 annotation (IWGSC, 2018) and used as queries or subjects. We compared the positions of genes on homoeologous chromosomes and drew the relationships of homoeologous genes as graphical displays using the software MapChart (Voorrips, 2002). To detect repeat regions, we used Censor 4.2.28 (Jurka *et al.*, 1996) with the option 'mode norm' using IWGSC repeat annotation data (iwgsc_refseqv1.0_TransposableElements_2017Mar13.gff3) from URGI.

The PCR assays

Conventional (semiquantitative) PCR and qPCR to amplify several parts of the rDNA unit and to estimate the copy number of the rDNA unit in the wheat genome were performed using 20 ng (conventional PCR) and 10 ng (qPCR) of the genomic DNAs of CS, nullitetrasonic and ditelosomic lines. The primer pairs used for PCR assays are listed in Table S3. Conventional PCR amplification with GoTaq (Promega, <http://www.promega.com/>) was performed under the following conditions: 1 min at 96°C; 32 cycles of 30 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C; and 5 min at 72°C. Quantitative PCR was conducted under the following conditions using a Light-Cycler Nano (Roche Diagnostics, <https://www.roche.com/>) and KAPA Library Quantification Kits (Roche Diagnostics): 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 10 sec at 58°C and 20 sec at 60°C. The qPCR results reflect the results of three independent experiments using two DNA preparations for each sample.

The RT-PCR assays

The total RNA was isolated from the youngest leaves of 13-week-old plants of CS, nullitetrasonic and ditelosomic lines grown in a greenhouse using an RNeasy Plant Mini Kit (Qiagen, <http://www.qiagen.com/>). The RNA was further treated with RNase-free DNase (TaKaRa, <http://www.takara-bio.com/>) to remove residual DNA. Complementary DNA was generated using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen, Thermo Fisher, <https://www.thermofisher.com/>). For conventional (semi-quantitative) RT-PCR, cDNA corresponding to 5 ng of total RNA was used as a template, and the PCR conditions were the same as for the conventional PCR described above, except that there were 34 cycles. Quantitative RT-PCR was conducted using cDNA

samples corresponding to 50 ng of total RNA as a template, and amplification was performed in a LightCycler Nano using KAPA Library Quantification Kits under the following conditions: 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 10 sec at 56°C, and 20 sec at 60°C. The qRT-PCR results reflect the results of three independent experiments using two cDNA preparations for each sample. The primer pairs used for PCR assays are listed in Table S3.

Fluorescent *in situ* hybridization imaging

For chromosome identification, the B-genome-specific repetitive sequence pSc119.2 (McIntyre *et al.*, 1990) was detected by the synthesized oligonucleotide probe (Invitrogen, Thermo Fisher) 5'-end-labeled with 5-(and-6)-carboxytetramethylrhodamine (TAMRA). The sequence was as follows: TAMRA-5'-CCGTT TTGTG GACTA TTACT CACCG CTTTG GGGTC CCATA GCTAT-3'. To quantify NOR signals, the ITS sequence was amplified from the genomic DNA of CS. First, we calculated the total signal intensities of the given chromosome preparation ($2n = 42$) as the summation of the number of pixels multiplied by the signal intensities and then determined the proportion of the signal intensities at the *Nor-B1* and *Nor-B2* loci by dividing their signal intensities by the total intensities of a given chromosome spread. To map the position of *Nor-B1* using deletion stocks, we used the ETS sequence of rDNA unit subtype S1, which was amplified from the nulli-6B tetra-6A line. The amplified fragments were labeled directly with digoxigenin (DIG) using DIG High Prime (Roche Diagnostics) or indirectly labeled by Biotin High Prime (Roche Diagnostics) according to the manufacturer's recommendations. The primers and their sequences are given in Table S3. For chromosome preparation and FISH, we followed the methods of Sakai *et al.* (2009). The FISH images were observed using an Olympus BX61 microscope equipped with a CCD camera (Olympus DP30BW, <https://www.olympus-global.com/>) and were captured individually by three colors. Images and quantification of signals were merged out using ImageJ 1.51n in the Fiji package (Schindelin *et al.*, 2012).

DNA methylation assays

Cytosine methylation was profiled in DNA extracted from 2-week-old CS leaf tissue. Libraries prepared after conversion of unmethylated cytosine based on sodium bisulfite (WGBS) were constructed from purified nuclei prepared using the methods described by Zhang *et al.* (1995). Input DNA was quantified using the Qubit High-sensitivity DNA Kit, and 270 pg of lambda DNA was added to 500 ng of nuclear DNA to assess the conversion efficiency obtained using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corp., <https://www.zymoresearch.com/>). The WGBS libraries were prepared using the TruSeq DNA Kit (Illumina, <https://www.illumina.com/>), and 2×125 bp paired-end sequence reads were generated using the Illumina HiSeq 2500 v4 platform (Genome Quebec, <http://www.genomequebec.com/>). Sequence quality assessment and adaptor removal were performed using Trim_galore_v0.4.1 (Lindgreen, 2012). High-quality paired-end sequence reads were aligned to the CS genome using Bismark version 0.16.1 (Krueger and Andrews, 2011), ensuring the removal of duplicate reads and only retaining unique unambiguous read alignments. The data were processed to exclude regions with low coverage using a binomial test.

Histone mark analyses

To study histone modifications, we used chromatin immunoprecipitation sequencing (ChIP-seq) data from IWGSC (2018). The ChIP assays were performed using anti-H3K9ac (07-352, Merck Millipore, <http://www.merckmillipore.com/>), anti-H3K27me3 (07-449, Merck Millipore), anti-H3K4me3 (07-473, Merck Millipore) and

anti-H3K36me3 (ab9050, Abcam, <https://www.abcam.com/>) antibodies, using a procedure adapted from Veluchamy *et al.* (2016). Ten-day-old *in vitro* seedlings were used as a starting material for isolation of nuclei. Protein/DNA complexes from cross-linked chromatin were immunoprecipitated with antibodies. The ChIPed DNA was recovered and subjected to ChIP-Seq library construction using the NEB-Next Ultra II DNA Library Prep Kit for Illumina according to the manufacturer's recommendations, and the libraries were sequenced using 2×75 bp pair-end reads on the NextSeq 500 platform (Illumina). Paired-end reads were aligned against IWGSC RefSeq v1.0 using Bowtie2 v2.3.3 (Langmead and Salzberg, 2012), and enrichment peaks were called with MACS2 v2.1.1. The ChIP-seq input data were used as a control. The peaks were annotated according to their position and overlap with HC genes (iwgsc_refseqv1.0_HighConf_2017Mar13.gff3). Original ChIP-seq data are available under SRA study PRJNA420988 (SRP126222), and can be accessed using the SRA RunSelector: <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP126222>.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Consensus sequence of the rDNA unit in *Nor-B1* and *Nor-B2*.

Figure S2. A diagram of the short arm of chromosome 1B indicating the positions of the breakpoints of deletion lines and fluorescent *in situ* hybridization images of the deletion lines.

Figure S3. Representative fluorescent *in situ* hybridization images used to analyze copy numbers at the *Nor-B1* and *Nor-B2* loci in hexaploid wheat.

Figure S4. Relationship of the high-confidence gene positions between wheat and rice chromosomes.

Figure S5. Distribution of repetitive elements on regions surrounding the nucleolar organizer regions on chromosomes 1B and 6B.

Figure S6. Consensus sequences of four subtypes for the 3' external transcribed spacer region of rDNA units.

Figure S7. CG DNA methylation ratios in the regions surrounding the nucleolar organizer regions of chromosomes 1B and 6B and their homoeologous counterparts.

Table S1. BLAST analysis of rDNAs against chromosome 1B on IWGSC RefSeq v1.0.

Table S2. BLAST analysis of rDNAs against chromosome 6B on IWGSC RefSeq v1.0.

Table S3. Sequences of primer pairs used in this study.

Table S4. Comparison of high-confidence gene contents among nucleolar organizer regions and their homoeologous regions of groups 1 and 6 chromosomes.

Table S5. Homoeologous relationships of high-confidence genes between homoeologous chromosomal regions.

Table S6. Distribution of high-confidence genes with a similarity to the low-confidence genes on the non-syntenic segments the

regions surrounding the nucleolar organizer regions on chromosomes 1B and 6B.

Table S7. Average repeat frequency of the regions surrounding the nucleolar organizer regions on chromosomes 1B and 6B and their homoeologous counterparts.

Table S8. Copy numbers of the rDNA unit subtype 3 by quantitative PCR in the genomes of nullisomic-tetrasomic and ditelosomic lines.

Table S9. Average DNA methylation ratio of the regions surrounding the nucleolar organizer regions on chromosomes 1B and 6B and their homoeologous counterparts.

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