1 **Original Article**

2 **Anatomy, transcription dynamics and evolution of wheat**
3 **ribosomal RNA loci deciphered by a multi-omics approach**

4 **Zuzana Tulpová¹, Aleš Kovařík², Helena Toegelová¹, Pavla Navrátilová¹, Veronika**
5 **Kapustová¹, Eva Hřibová¹, Jan Vrána¹, Jiří Macas³, Jaroslav Doležel¹, Hana Šimková^{1*}**

6 ¹ Institute of Experimental Botany of the Czech Academy of Sciences, Centre of the Region
7 Haná for Biotechnological and Agricultural Research, Olomouc, Czech Republic

8 ² Czech Academy of Sciences, Institute of Biophysics, Brno, Czech Republic

9 ³ Biology Centre, Czech Academy of Sciences, Institute of Plant Molecular Biology, České
10 Budějovice, Czech Republic

11 Running title: Bread wheat rRNA loci – structure and transcription

12 *** Correspondence:**

13 Hana Šimková simkovah@ueb.cas.cz

14

15

16

17

18

Abstract

- **Background and Aims** Three out of four RNA components of ribosomes are encoded by 45S rDNA loci, whose transcripts are processed into 18S, 5.8S and 26S ribosomal RNAs. The loci are organized as long head-to-tail tandem arrays of nearly identical units spanning over several megabases of sequence. Due to this peculiar structure, the number of rRNA genes, their sequence composition and expression status remain unclear, especially in complex polyploid genomes harbouring multiple loci. Here we conducted a complex study to decipher structure and activity of both major and minor rRNA loci in hexaploid bread wheat (*Triticum aestivum*).
- **Methods** We employed an original, multi-omics approach, combining chromosome flow sorting and optical mapping with transcriptome and methylome sequencing.
- **Key Results** The former two techniques enabled unbiased quantification of rDNA units in particular loci of the wheat genome. Total number of rRNA genes organized in tandem arrays was 4388, with 64.1, 31.4, 3.9 and 0.7% located in short arms of chromosomes 6B, 1B, 5D and 1A, respectively. At the expression level, only 1B and 6B loci contributed to transcription at roughly 2:1 ratio. The 1B:6B ratio varied among five analysed tissues (embryo, coleoptile, root tip, primary leaf, mature leaf), being the highest (2.64:1) in mature leaf and lowest (1.72:1) in coleoptile. Cytosine methylation was considerably higher in CHG contexts in the silenced 5D locus compared to the active 1B and 6B loci.
- **Conclusions** A fine genomic organization and tissue-specific expression of rRNA loci were deciphered, for the first time, in a complex polyploid species. We documented various mechanisms of rRNA dosage control, including gene elimination and stable inactivation related to nucleolar subdominance of A and D-genome loci, and a subtle,

1 developmentally regulated silencing of one of the major loci. The results are discussed
2 in the context of wheat evolution and transcription regulation.

3 **Keywords**

4 *Triticum aestivum*, bread wheat, 45S ribosomal DNA, ribosomal RNA, nucleolus organizer
5 region, flow cytometric sorting, optical mapping, RNA-seq, cytosine methylation, chloroplast
6 rRNA, mitochondrial rRNA

7

8

9

10

11

12

13

14

15

16

17

18

19

1 INTRODUCTION

2 Ribosomal DNA (rDNA) is a general term for genes encoding several types of ribosomal
3 RNA (rRNA), which are crucial components of ribosomes. 45S rDNA locus, specific for
4 eukaryotic genomes, is associated with the nucleolus and is termed nucleolus organizer region
5 (NOR). 5S rRNA gene loci are located apart from the NOR locus in a majority of organisms.
6 Both nuclear 5S and 45S rDNA units are mostly organized as long head-to-tail tandem arrays.
7 The 45S rDNA unit is composed of conserved genes for 18S, 5.8S and 26S rRNA, separated
8 by internal transcribed spacers ITS1 and ITS2. A smaller part of the unit is a non-transcribed
9 intergenic spacer (IGS) composed of several types of repeats of variable size and sequence.
10 The 45S rDNA is transcribed by polymerase I into a large precursor, which is processed into
11 the three types of rRNA. The 5S rRNA genes are approximately 120 bp long and, interspersed
12 by non-transcribed spacers of a few hundred base pairs, they form arrays spanning over
13 several kilobases, which makes them accessible to long-read sequencing technologies such as
14 PacBio (Symonová *et al.*, 2017). On the contrary, 45S rDNA unit size ranges from 8 to 13 kb
15 across yeast, plant and animal genomes (Gerlach and Bedbrook, 1979; Pruitt and Meyerowitz,
16 1986; Nelson *et al.*, 2019) and its arrays span megabases of sequence. This precludes their
17 complete assembling from whole-genome next-generation-sequencing data and was only
18 possible when coupling clone-by-clone approach with long-read nanopore sequencing (Sims
19 *et al.*, 2021).

20 The absence of the 45S rDNA arrays from genome assemblies hampers their analysis, which
21 is especially challenging in complex and polyploid genomes comprising multiple rRNA gene
22 loci, as is the case of bread wheat. Bread wheat (*Triticum aestivum* L.) is an allohexaploid
23 species with a genome of 16.58 Gb (Doležel *et al.*, 2018), composed of three subgenomes
24 ($2n=6x=42$, AABBDD). The huge genome size, polyploidy and, consequently, a lack of a
25 genome sequence hampered genome analysis for a long time. On the other hand, the large

1 chromosomes and complex genome composition attracted the attention of cytogeneticists and
2 stimulated research on wheat NORs by microscopic and other techniques. These studies made
3 use of a collection of aneuploid lines generated for *T. aestivum* cv. Chinese Spring (Sears
4 1954) that enabled assigning NORs to particular chromosomes and studying their
5 transcription by cytogenetic techniques. As early as in late 1950's, Crosby (1957) reported
6 that there were at least four different chromosomes in hexaploid wheat, 1A, 1B, 6B and 5D,
7 able to form nucleoli. In *T. aestivum* 'Chinese Spring' (CS), large nucleoli were assigned to
8 chromosomes 1B and 6B, while smaller ones, micronucleoli, were associated with other
9 chromosomes, mostly the 5D (Darvey and Driscoll, 1972). Nevertheless, the activity of the
10 minor loci has not been broadly confirmed.

11 First estimates of numbers of rRNA gene copies contained within the bread wheat genome
12 were done by Flavell and co-workers (summarized in Flavell and O'Dell, 1976). Using
13 rRNA/DNA hybridization assays, they estimated 9150 copies of 18S-25S rRNA genes per 2C
14 nucleus of CS wheat, out of which 5500 (60%), 2700 (30%) and 950 (10%) were assigned to
15 6B, 1B and other chromosomes (predominantly 5D and 1A), respectively. Surprisingly, rRNA
16 gene number in 1B and 6B correlated negatively with nucleolar volume in root tip cells of CS
17 wheat where the volume of nucleolus on chromosome 1B was twice of that on chromosome
18 6B (Martini and Flavell, 1985), indicating double transcription activity of the 1B NOR
19 compared to that of 6B. This study also demonstrated that the minor NORs on 5D and 1A
20 increased their activity when the major NORs were deleted. In the most recent study on
21 wheat NORs, Handa *et al.* (2018) used quantitative PCR (qPCR) and fluorescence *in situ*
22 hybridization to quantify rDNA units in the bread wheat genome. Their estimate for the total
23 copy number of rDNA units per CS wheat genome (1C) was 11,160 (100 Mb sequence),
24 which was more than double amount compared to the study by Flavell and O'Dell, 1976.
25 Besides, the authors compared rDNA units extracted from the reference genome of bread

1 wheat, the International Wheat Genome Sequencing Consortium (IWGSC) RefSeq v1.0
2 (IWGSC, 2018), and identified four rDNA subtypes and analysed their expression, which was
3 not proportional to their representation in the genome. They also positioned the major 45S
4 rDNA loci on chromosomes 1B and 6B, taking the presence of rDNA clusters in the sequence
5 as a clue.

6 Our BLAST search for 45S rDNA in the IWGSC RefSeq v1.0 genome, carried out in
7 Ensembl Plants (https://plants.ensembl.org/Triticum_aestivum/Tools/Blast), revealed several
8 additional clusters of rDNA (Fig. 1A), mainly on short arms of chromosomes 1A (1AS), 1B
9 (1BS), 5D (5DS), 7D (7DS) and long arms of 1B (1BL) and 4A (4AL), most of which likely
10 correspond to minor loci identified previously by *in situ* hybridization (Mukai *et al.*, 1991).
11 However, none of the clusters in the IWGSC RefSeq, including those assigned to *Nor-B1* and
12 *Nor-B2*, comprised a regular array of rDNA units. This virtual absence of genomic
13 information on the bread wheat NORs, together with discrepancies in rDNA copy-number
14 estimates and only vague information on transcriptional activity of particular loci, limited
15 mostly to root tissues investigated by the cytogeneticists, call for a detailed study to resolve
16 sequence organization and tissue-specific expression of rRNA genes from individual loci.

17 We showed previously (Kapustová *et al.*, 2019) that Bionano optical mapping, a technology
18 that labels and visualizes short sequence motifs along 150-kb to 1-Mb long DNA molecules
19 (Lam *et al.*, 2012), is suitable for identification of rDNA arrays in a genomic context thanks to
20 the presence of the labelled motifs in the 45S rDNA unit. Here, we used this technology,
21 combined with separation of particular rDNA-bearing wheat chromosome arms by flow
22 cytometry (Staňková *et al.*, 2016), to search for the presence of rDNA arrays in the
23 anticipated positions in 1BS and 6BS chromosome arms as well as in 1AS and 5DS, which
24 are expected to bear minor rDNA loci. Additionally, analysis of optical-map raw data for the
25 occurrence of tandemly organized repeats provided a novel approach to quantify rDNA units

1 that discriminates potentially functional units organized in arrays from dispersed and
2 fragmented ones, which are likely non-functional. Engagement of the major and the minor
3 loci in rRNA synthesis was assessed in five tissues by analysing RNA sequencing data for
4 chromosome-specific sequence variants in 26S rRNA and the transcriptional activity of
5 particular loci was compared with cytosine methylation level in the rDNA units. Moreover,
6 the high-throughput RNA-seq data provided information on the dynamic contribution of
7 particular organelles to the overall rRNA production. Based on the data obtained, we outline a
8 scenario for the evolutionary histories of rDNA loci in three wheat subgenomes and propose a
9 model for control of rRNA amount through genetic and epigenetic mechanisms.

10 MATERIALS AND METHODS

11 *Plant material*

12 Seeds of double ditelosomic stocks of bread wheat (*Triticum aestivum*, L.) cv. Chinese
13 Spring, dDt1AS, dDt1BS, dDt6BS and dDt5DS (Sears and Sears, 1978), were kindly
14 provided by Prof. Bikram Gill (Kansas State University, Manhattan, USA). Seeds of *T.*
15 *aestivum* cv. Chinese Spring with a standard karyotype were provided by Dr. Pierre Sourdille
16 (INRAE, Clermont-Ferrand, France).

17 *Bionano optical mapping*

18 Chromosome arms 1AS, 1BS, 6BS and 5DS were purified in million amounts by flow sorting
19 from corresponding telosomic lines according to Kubaláková *et al.* (2002) and used for HMW
20 DNA preparation as described in Staňková *et al.* (2016). Bionano optical maps (OMs) of 1AS,
21 1BS and 6BS were constructed using NLRS labelling chemistry (Nt.*BspQI* enzyme) and data
22 were generated on the Irys platform (Bionano Genomics, San Diego, USA). The 5DS arm was
23 labelled by DLS chemistry (DLE-1 enzyme) and molecules were analysed on the Saphyr

1 platform (Bionano Genomics). DNA labelling and data collection followed protocols of
 2 Bionano Genomics (<https://bionanogenomics.com/products/bionano-prep-kits/>) with minor
 3 modifications. Optical map of the 1AS was assembled by Bionano IrysSolve 2.1.1 software,
 4 using optArguments_human.xml file. Assemblies of 1BS, 6BS and 5DS OM were done by
 5 Bionano Solve 3.4 applying cmaps generated from fasta files of 1BS, 6BS and 5DS
 6 pseudomolecules of the IWGSC RefSeq v1.0 (IWGSC, 2018) as a reference. Assembly
 7 parameters specified by optArguments_nonhaplotype_noEs_noCut_irys.xml file were used
 8 for 1BS and 6BS map assembly whereas
 9 optArguments_nonhaplotype_noEs_noCut_DLE_saphyr.xml file were used for the 5DS OM.
 10 Information about input DNA amount, labelling chemistry, quantity and quality of data used
 11 for the OM assembly are stated in Table S1 [**Supplementary Information**]. The optical maps
 12 were aligned to the wheat reference using Bionano Solve v3.4 with RefAligner v9232 and
 13 pValue threshold of $1e-15$ was applied for the query-to-anchor comparison. The alignments
 14 were visualized in Bionano Access v1.5. The OM of 1BS and 6BS showed good
 15 concordance between distances in the map and in the sequence (length differences <1%),
 16 while alignment of the 5DS map revealed ~8% expansion of the map compared to the
 17 reference sequence (Fig. 1B). To eliminate this inaccuracy, coefficient of 0.92 was used to
 18 correct the rDNA unit size estimate from the OM data.

19 *Quantification of units in rDNA arrays*

20 An algorithm included in the IrysView 2.0 software package (Bionano Genomics) was used to
 21 identify labelled tandemly organized repeats in size-filtered (>150 kb) raw (single-molecule)
 22 data. Regular arrays of six and more repeat units were considered in the analysis. Repeat
 23 stretch tolerance of 0.1 was applied for 5DS (one DLE1 site per unit) while more relaxed 0.19
 24 tolerance was used for 1BS and 6BS data generated with Nt.BspQI enzyme, because of two
 25 merging recognition sites in the unit. The detected repeats were quantified and their unit size

1 and frequency in the dataset were plotted in a histogram for visual analysis. The fractions of
2 telocentric chromosomes purified by flow cytometric sorting were contaminated by a mixture
3 of other chromosomes/arms, totalling 9.6-19.7% of the sorted particles (Table 1). This was
4 considered in recalculating the proportion of rDNA units per pure chromosomal fraction.
5 Since the 1BS, 6BS and 5DS arms differ significantly in size (Table 1), their mutual
6 contamination was unlikely and was not considered in the recalculation.

7 *Reconstruction of wheat rDNA units*

8 A wheat consensus and three chromosome-specific 45S rDNA units were reconstructed from
9 published short-read data using RepeatExplorer pipeline (Novák *et al.*, 2013), as previously
10 described in Kapustová *et al.* (2019). The consensus sequence was generated from whole-
11 genome Illumina data of wheat Synthetic W7984 (SRP037990, Chapman *et al.*, 2015) while
12 the chromosome-specific units were reconstructed from Illumina reads for CS 1BS
13 (ERX250504, IWGSC 2014) and Roche/454 reads for CS 6BS (DRX007672, Tanaka *et al.*,
14 2014) and 5DS arms (ERA296180, Akpinar *et al.*, 2015). For the unit reconstructions, a
15 random data set of 4,471,956 filtered reads, corresponding to 0.13x genome coverage, was
16 used for the wheat rDNA consensus, and reads corresponding to 0.15-0.77x coverages of
17 particular chromosome arms were applied for the chromosomal consensus. In case a
18 chromosomal rDNA unit was not reconstructed to a contiguous sequence after one round of a
19 graph-based clustering, the available contigs were used to extract all putative reads
20 homologous to 45S rDNA using BLASTn. A second round of clustering was done on a data
21 set enriched for putative rDNA reads and lacking other abundant repeat types. Finally,
22 particular components of the 45S rDNA unit (genes and spacers) were ordered and annotated
23 according to a rye 45S rDNA (JF489233.1, Fluch *et al.*, 2012), except transcription start site,
24 which was determined according to Vincentz and Flavell (1989).

1 *Identification and validation of diagnostic SNPs*

2 Chromosome-arm-specific SNPs in coding regions were identified after aligning consensual
3 rDNA sequences of 1BS, 6BS and 5DS in Geneious v7.1.2 (<http://www.geneious.com>).
4 Diagnostic value of all putative SNPs was tested by cross-mapping of available telosome-
5 derived Illumina reads (IWGSC, 2014) from 1AS (ERX250501), 1BS (ERX250504), 6BS
6 (DRR008486) and 5DS (ERX250533) to individual chromosomal rDNA consensus.
7 Representation of particular SNPs in three *T. aestivum* ‘Chinese Spring’ assemblies
8 (SRX2994097, Zimin *et al.*, 2017; ERX1700146, Clavijo *et al.*, 2017; SRX3059308, IWGSC,
9 2018) was assayed by mapping whole-genome Illumina reads generated in particular projects
10 to the wheat consensual rDNA unit. The reads were filtered for quality and length and aligned
11 to wheat rDNA consensus sequence using Bowtie2 aligner with default parameters
12 (Langmead and Salzberg, 2012). The resulting SAM files were converted to BAM files,
13 sorted by SAMtools package version 1.6 and 0.1.18, respectively and viewed in Integrative
14 Genomics Viewer v2.7.2, which was also used to quantify particular SNP variants. To verify
15 the proposed locus-specific haplotypes, we exploited merged 2 x 250 bp (PE450) reads from
16 whole-genome libraries generated for the IWGSC RefSeq v1.0 genome (SRP114784;
17 IWGSC, 2018) and aligned them to the wheat rDNA consensus. Reads overlapping all three
18 diagnostic SNPs were extracted by SAMtools and checked for haplotype constitution using
19 Jvarkit Biostar214299 utility (Lindenbaum, 2015).

20 *RNA-seq*

21 Five plant tissues - embryo, coleoptile (including plumule), root tips, primary leaf and mature
22 leaf – each in two biological replicates were used for transcriptome analysis. Seeds were
23 incubated for 5 days at 4°C in Petri dishes on wet cellulose covered by filter paper, followed
24 by incubation at 26°C in the dark for 4-6 hours for embryo and 24 hours for coleoptile and

1 root tips. After 24-hour incubation at 26°C, five germinating seeds were planted in garden soil
2 and maintained in a growth chamber under long-day (16 hours' day light) conditions and
3 20°/16°C for five days or until the flag leaf was visible (Zadoks' stage 37) to collect the
4 primary and the mature leaf, respectively. Three embryos, 2-5 coleoptiles, 10 root tips about
5 2mm in length and up to 100 mg of the primary leaf tissue and the mature leaf blade,
6 respectively, were collected per sample and flash-frozen in liquid nitrogen. Total RNA was
7 isolated using RNEasy Plant Mini Kit (Qiagen, Germany) and RNA quality was checked on
8 Bioanalyzer using Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, USA).
9 Sequencing libraries were prepared by NEBNext® Ultra™ II Directional RNA Library Prep
10 Kit for Illumina (New England Biolabs, Ipswich, USA) and pair-end sequenced on the
11 Illumina NextSeq550 System. Summary of the sequencing output is provided in Table S2
12 **[Supplementary Information]**.

13 *Calling of transcript variants*

14 Raw RNA-seq data were quality controlled using fastqc, trimmed with trimalore/0.6.2 using
15 default settings and rid of chloroplast and mitochondrial DNA by mapping to the respective
16 references (accession number KC912694.1, Middleton *et al.*, 2014; accession number
17 MH051716.1, IWGSC, 2018) using HiSat2 v2.1.0 (Table S2)**[Supplementary Information]**.
18 Two million (one million of pairs) of clean reads were used as an input for SNV calling. Out
19 of these 90-96 % (Table S2) **[Supplementary Information]** were mapped to the wheat rDNA
20 consensus unit, which included the external transcribed spacer (ETS)-18S-ITS1-5.8S-ITS2-
21 26S subregions (Fig. S1A) **[Supplementary Information]**. Mapping was carried out using
22 commands in the CLC genomics workbench (Qiagen, Germany) with the following
23 parameters: Match score – 1, mismatch cost – 2, insertion cost – 3, deletion cost – 3, length
24 fraction – 0.5, similarity fraction – 0.8. Read tracks were visually checked in the program
25 window and coverage graphs were constructed. Variants were called via the 'Probabilistic

1 Variant Detection' function tool in CLC using default settings. SNPs were filtered as follows:
 2 minimum read coverage – 400, count (the number of countable reads supporting the allele) -
 3 40, frequency (the ratio of “the number of 'countable' reads supporting the allele” to “the
 4 number of 'countable' reads covering the position of the variant”): $\geq 10\%$ (high-frequency
 5 SNPs). Frequency of rRNA variants across five tissues were evaluated by a Pearson's chi-squared test.

6 *Mapping of Iso-Seq data*

7 To identify possible rare transcripts originating from 5DS and other minor rDNA loci,
 8 available wheat CS Iso-Seq data from PRJEB15048 (Clavijo *et al.*, 2017) were mapped to the
 9 wheat rDNA consensus and 5DS rDNA unit, respectively. A total of 817,892 high-quality
 10 (CCS) Iso-Seq reads, divided into six sets corresponding to six plant developmental stages -
 11 leaf, root, seed, seedling, stem and spike - were mapped using minimap2 software with
 12 default parameters. Resulting SAM files were converted into BAM files and sorted using
 13 SAMtools. The data were visualised and variant proportion was estimated using Integrative
 14 Genomics Viewer (IGV).

15 *Quantification of rRNA*

16 A hybrid reference wheat genome was generated by concatenating repeat- and rDNA-masked
 17 IWGSC RefSeq v1.0 genome sequence, the wheat consensus 45S rDNA unit, and the wheat
 18 chloroplast (KC912694.1) and mitochondrial (MH051716.1) genomes. Trimmed RNA-seq
 19 data were mapped to this reference using HiSat2 v2.1.0 (-k 1), followed by running the
 20 featureCounts function from the package Subread v1.5.2 in a paired-end mode using
 21 'iwgsc_refseqv1.0_HighConf_wheat_rDNA.gtf' annotation file (IWGSC, 2018). Per-genome
 22 counts were obtained using counts and lengths of corresponding wheat nuclear (18S, 26S),
 23 chloroplast (16S, 23S) and mitochondrial (18S, 26S) rRNA genes. For mitochondria,
 24 comprising three and two non-clustered copies of 18S and 26S rRNA genes, respectively,

counts for each of the genes were summed. Other rRNA types were not considered. For relative quantification of the nuclear/chloroplast/mitochondrial 18/16S and 26/23S rRNA gene transcripts, TPM (Transcripts Per Kilobase Million) values were calculated that normalize for both gene length and sequencing depth.

Methylation of rDNA

To evaluate the level of DNA methylation in particular rDNA loci, an artificial reference sequence was compiled of the chromosomal rDNA units specific for 1BS, 6BS and 5DS (Dataset S1) [Supplementary Information], respectively, which were tailed with the initial 1000 bp of the IGS to mimic concatenation. The reference was used to map three replicas of published wheat bisulphite-sequencing (BS-seq) datasets (SRR6792673, SRR6792679, SRR6792686, SRR6792688, SRR6792689, SRR6792681, SRR6792684, SRR6792687, SRR6792678, IWGSC 2018), obtained from two-week-old CS leaf tissue. The raw sequences were trimmed from the adaptors and low-quality reads were removed using Trim Galore! v0.6.2 (trim_galore -j 4 --paired --trim1). Subsequent analysis was run in Bismark-0.22.1 program (Krueger and Andrews, 2011), making use of SNPs specific for particular chromosomal loci (Dataset S2) [Supplementary Information]. Mapping by Bismark results in the best unique alignment by default and was run with additional parameters '-I 0 -X 1000 -non_directional'. The mapping was followed by deduplicate_bismark and bismark_methylation_extractor '--paired-end --comprehensive --no-overlap --CX --bedGraph --counts'. Methylation level in CpG, CHG and CHH contexts was calculated as a percentage of methylated cytosines (mC) out of total cytosines (C+mC) per region - 5'ETS or selected part of the 26S rRNA gene) - using SeqMonk. Differential methylation between the three chromosomal units was statistically evaluated using ANOVA test and using two-tailed t-test for all pairs of chromosomal units and methylation contexts.

1 RESULTS

2 *Positioning and characterization of rDNA arrays*

3 Using bread wheat whole-genome Illumina reads and RepeatExplorer pipeline, we
 4 reconstructed a wheat consensus 45S rDNA unit with the length of 8193 bp (Dataset S1)
 5 **[Supplementary Information]**. We searched the unit for GCTCTTC and CTTAAG motifs,
 6 which are the recognition sites of Nt.BspQI and DLE-1 enzymes, respectively, used to label
 7 DNA molecules on optical mapping platforms of Bionano Genomics. The rDNA unit
 8 comprises one CTTAAG and two GCTCTTC sites, all of them located in the 26S rRNA gene.
 9 Since the two GCTCTTC motifs were positioned just 1126 bp apart, they could not be
 10 spatially discriminated and were expected to be recorded as a single label. Thus, each of the
 11 enzymes was predicted to introduce one label per unit and an array of tandemly organized
 12 rDNA units was expected to generate a regular labelling pattern with a spacing of 8-9 kb.

13 To verify this prediction and to visualize particular rDNA arrays in their genomic context, we
 14 constructed chromosome-arm-specific Bionano optical maps for wheat 1BS, 6BS, 5DS and
 15 1AS chromosome arms, respectively, which bear rRNA multigene loci. Parameters of the
 16 chromosomal maps are given in Table S1 **[Supplementary Information]**. The optical map
 17 contigs were aligned to the IWGSC RefSeq v1.0. Among 369 OM contigs from the 1BS arm,
 18 we found several comprising a regular label pattern with the spacing of 8.8-10 kb. Only 1BS
 19 contig 447, having a longer segment with an irregular pattern, could be hereby aligned to the
 20 1BS pseudomolecule at ~80.7 Mb position (Fig. 1B). A non-aligned 200-kb part of this contig
 21 had a regular pattern with 9.4-kb label spacing that overhung towards the telomere, indicating
 22 a presence of an rDNA array at this position. This presumption was supported by the presence
 23 of a scaffold boundary at 80.65 Mb and of a cluster of incomplete rDNA units between 80.65-
 24 80.88 Mb. Another cluster of rDNA fragments was found at 111.1-111.2 Mb, but this region

1 was spanned by an OM contig without a regular pattern, excluding the presence of a regular
2 rDNA array at this position.

3 We analogously assigned rDNA arrays to positions 111.58 Mb and 19.01 Mb of 6BS and 5DS
4 pseudomolecules, respectively. In both the 6BS and the 5DS, we found rDNA-comprising
5 OM contigs at both the distal and the proximal sides of the arrays (Fig. 1B). The 5DS optical
6 map indicated that 5DS scaffold 34514 (5D:19005535..19736721 bp), flanking the rDNA
7 array at the proximal side, was misoriented in the RefSeqv1.0 pseudomolecule, artificially
8 breaking the rDNA array into two parts. Clusters of incomplete rDNA units flanking the
9 rRNA multigene loci indicated that 1BS and 6BS rRNA genes were oriented from the
10 centromere to the telomere while the 5DS rRNA locus had the opposite orientation. The
11 rDNA-bearing OM contigs showed a highly regular pattern in 1BS and 5DS but the regularity
12 has been disrupted in several positions by label-free gaps of tens of kilobases in the 6BS,
13 suggesting that the rDNA array has been invaded by larger blocks of transposable elements or
14 interspersed by shorter arrays of unlabelled tandem repeats.

15 We also searched for the characteristic pattern in OM from the 1AS arm but failed in finding
16 a single OM contig with a clear ~9-kb pattern. Only 1AS OM contig 42 comprised segments
17 resembling the rDNA pattern with more condensed spacing (around 8.6 kb) and some
18 irregularities (Fig. 1B). This contig aligned with high confidence to the start of the 1A
19 pseudomolecule, thus confirming the correctness of the sequence assembly in this region. In
20 the sequence, we found 29 units comprising all of 18S, 5.8S and 26S rRNA genes, featured by
21 high sequence and length diversity and variable orientation, and numerous rDNA fragments
22 (Fig. 2A, C). This degenerated rDNA array spanned over 430 kb. The rDNA units were
23 interspersed by telomeric repeats (Fig. 2B), indicating a dynamic character of the region and a
24 loss of function. Majority of molecules involved in the assembly of the distal part of the OM

1 contig 42 terminated with label-free overhangs, suggesting the possible presence of a regular
2 telomeric sequence in the immediate vicinity of the rDNA array.

3 *Quantification of rDNA units in arrays*

4 The optical map contigs comprised segments with rDNA-specific patterns spanning over 200-
5 370 kb, but it was obvious that these lengths did not correspond to the full size of the rDNA
6 arrays, but rather reflected the sizes of DNA molecules used for optical map assembly, which
7 did not exceed 500 kb. Still, an approximate quantification could be done by the IrysView
8 software, which includes algorithms that calculate the proportion of nucleotides comprised in
9 tandemly organised repeats of a particular size in OM raw data. Results of this analysis
10 revealed repeats of 8.5-10 kb as the major labelled tandem repeats in chromosome arms 1BS,
11 6BS and 5DS (Fig. S2) [**Supplementary Information**]. We quantified tandem-organized
12 rDNA units in each rDNA locus, employing information on the percentage of nucleotides in
13 repeats, sizes of the telosomic chromosomes and purities of particular sorted fractions (Table
14 1). This analysis indicated that the largest array of rDNA units was located in the 6BS
15 chromosome arm (2813 units, 26.87 Mb), followed by arrays in 1BS (1376 units, 12.96 Mb),
16 5DS (170 units, 1.5 Mb) and 1AS (29 complete units, 0.43 Mb), being the smallest locus. The
17 total of 4388 units occupied approximately 42 Mb of the wheat CS genome space. Relative
18 quantification of the units assigned 31.4 and 64.1% of them to the major NORs in 1BS and
19 6BS, respectively. The remaining 3.9 and 0.7% belong to the minor loci in 5DS and 1AS,
20 respectively.

21 *Chromosome-specific rDNA units and diagnostic SNPs*

22 Chromosome-specific consensual units were reconstructed for 1BS, 6BS and 5DS loci,
23 featured by regular rDNA arrays (Dataset S1) [**Supplementary Information**]. Mutual
24 comparison of the chromosomal consensual sequences (Dataset S2) [**Supplementary**

1 **Information]** revealed a high sequence diversity in IGS, including 5'ETS, and several SNPs
2 in both ITS1 and ITS2. While the 18S and 5.8S rRNA gene sequences were identical among
3 the three chromosomal consensus, a total of eight putative arm-specific SNPs were
4 identified in the 26S rRNA gene: two 6BS-specific, two 1BS-specific and four 5DS-specific.
5 Diagnostic value of all these SNPs was tested by cross-mapping Illumina reads from 1AS,
6 1BS, 6BS and 5DS to the three consensus. This assay led to the selection of two diagnostic
7 SNPs for each rRNA multigene locus. The 5DS-2 SNP variant was also found in 1A and other
8 minor rDNA clusters outside the B genome (Table S3) **[Supplementary Information]**, thus
9 we called it “non-B”. Further, we analysed the frequency of allelic variants of the proposed
10 diagnostic SNPs in the genome of CS wheat by investigating raw data that had been used for
11 RefSeq v1.0 assembly (IWGSC, 2018), and also Illumina reads used for two additional CS
12 genome assemblies (Triticum 3.1, Zimin *et al.*, 2017; TGACv1, Clavijo *et al.*, 2017) (Table
13 S4) **[Supplementary Information]**. Pairs of SNPs featuring 1BS and 6BS, respectively, had
14 highly consistent representations in IWGSC RefSeq v1.0 and Triticum 3.1 data, while
15 showing slight inconsistencies in TGACv1, possibly due to a small number of available reads
16 or difference in CS accessions used in particular projects. The RefSeq v1.0 analysis showed
17 that the 1BS, 6BS and 5DS repeat unit variants represented 36.2, 56.6 and 4.2% of the whole-
18 genome data, respectively. Similar representation of particular chromosomal loci has been
19 found in raw data of the other CS projects.

20 Based on this analysis, we proposed locus-specific haplotypes and verified them using merged
21 450-bp Illumina pair-end reads, applied for the IWGSC RefSeq v1.0 assembly. Alignment of
22 the reads to the wheat consensus rDNA (Fig. S3) **[Supplementary Information]** showed
23 6240 of them covering a triplet of chromosome-specific SNPs, 5DS-1, 6BS-2 and 1BS-1, that
24 enables discrimination of haplotypes proposed for 1BS, 6BS and 5DS loci, respectively. Out
25 of the 6240 reads, 2177 (34.9%) corresponded to the predicted allelic constitution of 1BS,

1 3667 (58.8%) to 6BS and 240 (3.8%) to 5DS. Another haplotype, observed in 135 (2.2%)
 2 reads was found in most units of the 1AS array and in small clusters of degenerated rDNA
 3 units in 7DS and other arms, which besides that comprised units with the 1BS haplotype.
 4 Only 21 (0.3%) 450-bp reads had a different combination of SNPs (Table 1). The results of
 5 the haplotype analysis thus supported validity of the proposed diagnostic SNPs.

6 *Locus- and tissue-specific transcription of 26S rDNA*

7 To assess locus-specific transcription, we used short Illumina RNA-seq and long Iso-Seq
 8 (PacBio) reads of high and low sequence coverage, respectively. A typical profile of RNA-seq
 9 read coverage along the rDNA unit is shown in Fig. S1B [Supplementary Information]. As
 10 expected, the coverage was high in genic regions and low in the non-coding regions.
 11 Depending on the tissue, only 0.006-0.122% reads mapping to wheat consensus rDNA unit
 12 belonged to the 5'ETS, that represents 16.3% of the primary 45S rDNA transcript length. This
 13 implies that just a fraction of a percent of sequenced rRNAs had full-length 5'ETS, indicating
 14 that a minute amount of 45S pre-rRNA was present in the sequenced RNA samples. The
 15 highest and the lowest 5'ETS coverage was observed in the embryo and the primary leaf,
 16 respectively.

17 The analysis of high-frequency (>10%) variants in the RNA-seq data obtained from five
 18 tissues confirmed the presence of significant transcript variants at the diagnostic SNPs in both
 19 major rDNA loci in the B genome, namely 6BS-1, 6BS-2, 1BS-1 and 1BS-2. In all tissues, the
 20 1BS rRNA variants predominated those of the 6BS in a ratio roughly 2:1, which slightly
 21 varied among tissues (Fig. 3A; Table S5) [Supplementary Information]. The highest
 22 frequency of 6BS-specific variants was in the coleoptile (35 and 37.6% in 6BS-1 and 6BS-2,
 23 respectively) while the highest frequency of the 1BS-specific variants was in the mature leaf
 24 (71.9 and 75.1% in 1BS-1 and 1BS-2, respectively).

1 Our failure to identify allelic variants in 5DS-1 and 5DS-2 SNP positions suggested the
2 absence of transcription outside the major loci. To validate this finding, we inspected
3 publically available Iso-Seq data of CS wheat with reads of several kilobases in length,
4 promising to reliably identify possible 5DS transcripts. The majority of transcripts in all
5 datasets started at position 2351 of the rDNA consensus, which is the start of the 18S rRNA
6 gene, and did not extend beyond position 8154 (Fig. S4A, B) **[Supplementary Information]**,
7 preceding a polypyridine CCCTCCCC tract. The Iso-Seq datasets for particular
8 developmental stages (leaf, root, seed, seedling, stem and spike) differed in read length and
9 amount of Iso-Seq data mapped to the 45S rDNA unit. The most beneficial showed the
10 dataset of “Seed” comprising 1951 reads mapping to the 5DS rDNA unit, which was used to
11 search for transcripts of 5DS and possibly other minor rRNA loci. In five out of 1079 „Seed“
12 Iso-Seq reads mapping to the position of 5DS-1 in the 5DS rDNA unit, we could
13 unambiguously recognize the 5DS-specific haplotype based on the 5DS-1, several 5DS-
14 specific SNPs in ITS2 (Dataset S2) **[Supplementary Information]** and, in two cases, also
15 5DS-2 and 1BS-2 SNPs (Fig. S4C) **[Supplementary Information]**. Additional two reads out
16 of 425 mapping to the terminal part of the 26S rRNA gene, covering 5DS-2 and 1BS-2 but
17 not 5DS-1, suggested a non-B origin of the transcripts, and another three reads, covering the
18 entire 26S rRNA gene, had a non-B non-5DS combination of SNP alleles, indicating possible
19 1A origin. To summarize, we observed among the “Seed” transcripts of CS wheat possible
20 non-B variants of 26S rRNA with the frequency of around one percent.

21 *Unequal cytosine methylation of rDNA loci*

22 To clarify mechanisms controlling the expression of particular rRNA multigene loci, we
23 carried out a comparative analysis of cytosine methylation assigned to particular
24 chromosome-specific rDNA sequences. The methylation was evaluated after mapping BS-seq
25 reads to chromosomal consensual units of 1BS, 6BS and 5DS, respectively. This approach

1 allowed comparing methylation ratios in non-repetitive segments of the rDNA units that
2 showed polymorphisms between chromosomal consensual sequences. The reads covered well
3 5'ETSs, both ITSs and the polymorphic parts of the 26S rRNA genes. The 5'ETS region of
4 1141bp length contained 13 evenly distributed SNPs discriminating 1BS- and 6BS-specific
5 reads; the ETS of the 5DS unit comprised 39 SNPs that distinguished it from those of the B-
6 genome loci (Dataset S2) **[Supplementary Information]**. The extent of cytosine methylation
7 was quantified for the 5'ETS and the part of the 26S rRNA gene with validated SNPs (Fig. 4,
8 Fig. S5) **[Supplementary Information]**. In the 5'ETS, the comparative analysis revealed
9 generally high levels of DNA methylation in the CpG contexts, reaching 92.05, 94.77 and
10 94.47% for 1BS, 6BS and 5DS unit, respectively. The difference between 1BS and 6BS
11 (2.72%) was highly significant ($p < 0.001$). On the contrary, the frequency of cytosine
12 methylation in the CHG and CHH contexts was markedly lower (23.28, 22.97 and 43.55% for
13 CHG, and 1.4, 1.49 and 2.31% for CHH in 1BS, 6BS and 5DS unit, respectively) and
14 significantly ($p < 0.001$) higher in 5DS compared to 1BS and 6BS loci. The difference
15 between 1BS and 6BS in the CHG and CHH contexts was non-significant. Pattern of cytosine
16 methylation in the analysed part of the 26S rRNA gene was highly similar to that in the 5'ETS
17 (Fig. S5) **[Supplementary Information]**, except for a noticeable increase in methylation in
18 the CHG contexts in both 1BS (32.2 vs. 23.3%) and 6BS (30.7 vs. 23%) units.

19 *Dynamic expression of organellar rDNA*

20 Relative quantification of nuclear, chloroplast and mitochondrial ribosomal RNAs across five
21 tissues was based on TPM estimates for the large rRNAs (16/18S and 23/26S). The relative
22 amount of rRNA in transcripts culminated in tissues with high proportion of rapidly dividing
23 meristematic cells (embryo, coleoptile, root tip), likely reflecting a high demand on ribosome
24 biogenesis and proteosynthesis in these tissues. As expected, production of plastid rRNA was
25 minute in embryo, coleoptile and root tip but the proportion of chloroplast rRNA increased

1 dramatically in the leaf samples, in line with the proliferation of chloroplasts and high
2 photosynthetic activity. Alongside this chloroplast rRNA increase, the proportion of nuclear
3 rRNA dropped to about one half in the leaf samples. The relative contribution of
4 mitoribosomal RNA to overall rRNA production was negligible in all tissues (Fig. 3B; Table
5 S6) [Supplementary Information].

6 **DISCUSSION**

7 *Ribosomal DNA quantity and distribution*

8 Instability in rDNA copy number has been well documented in both plants and animals at the
9 inter-population and even inter-individual levels (Rogers and Bendich, 1987; Rabanal *et al.*,
10 2017, Nelson *et al.*, 2019). In our study, we used a new approach to quantify 45S rDNA in
11 the genome, which was based on calculating tandemly organized repeats of the corresponding
12 size in optical map raw data. Our total estimate - 4388 rDNA units in arrays - was remarkably
13 similar to that of Flavell and O'Dell (1976) who, based on classical filter hybridization
14 techniques, estimated 4575 copies per 1C genome of CS wheat. The accordance between both
15 studies suggests robustness of both quantification approaches and points to relative stability of
16 rDNA copy number, even between standard karyotype of CS wheat and derived telosomic
17 lines used in our study, which have undergone multiple meiotic cycles since their generation
18 in 1970's (Sears and Sears, 1978). Nevertheless, a recent study of Handa *et al.* (2018)
19 reported 11,160 rDNA units in the CS genome, which is roughly 2.5 higher estimate than that
20 of Flavell and O'Dell (1976) and ours. The incongruence between the studies can be assigned
21 to different approaches used to quantify the rDNA units rather than to inter-individual
22 variability. The hybridization techniques used by Flavell and O'Dell (1976) are likely to pick
23 up only longer DNA sequences while qPCR used by Handa and co-workers can target also
24 short rDNA-unit fragments of a few hundred base pairs, which are abundant in marginal parts

1 of rDNA arrays and are also dispersed across the genome (Fig. 1). The optical-mapping
2 approach applied in the current study considered only units organized in arrays, which are
3 likely to be functional, while qPCR cannot discriminate the arrayed genes from the dispersed
4 ones that are frequently truncated or recombined and likely pseudogenized. Of note, BLAST
5 search for the consensual rDNA unit in the IWGSC RefSeq v1.0 genome, carried out in
6 Ensembl Plants (https://plants.ensembl.org/Triticum_aestivum/Tools/Blast), provided 1431
7 hits of high confidence ($E\text{-value} < e^{-100}$), distributed on all pseudomolecules (743 hits) and in
8 unassigned scaffolds (688 hits). Majority of them were located outside regular arrays and
9 represented rDNA fragments. Taking this into account, we consider our approach more
10 relevant for assessing sequences with potential to contribute to rRNA production.
11 Nevertheless, we cannot exclude that our outcomes slightly underestimate the number of
12 rDNA units due to false negative/positive labels occurring occasionally in the OM raw data
13 and possibly disrupting the regularity of the label pattern.

14 The relative proportion of particular rDNA loci was determined both from the optical map
15 data and from the representation of locus-specific haplotypes in whole-genome data (Table 1).
16 The results obtained by both approaches were concordant, except for a slight shift (34.9 vs.
17 31.4%) in favour of 1B in the haplotype data. This may reflect the fact that the haplotype
18 analysis does not exclude the non-arrayed units and larger unit fragments dispersed across the
19 genome where the 1BS haplotype predominates, probably due to the higher transcription
20 activity. The 1BS : 6BS ratio of 31.4 : 64.1 is in a good agreement with 30/30.5 : 60/60.9
21 obtained in previous studies (Flavell and O'Dell, 1976; Handa *et al.*, 2018) but our OM data
22 ascribe a smaller percentage to other chromosomes (4.6 vs. 10/8.6%), for the reasons
23 explained above. The occurrence of haplotypes in 26S rRNA gene with high locus specificity
24 suggests limited interchromosomal conversion events between particular arrays, which is
25 supported by observations of Lassner *et al.* (1987) who did not find evidence of

1 homogenization between B- and D-genome rDNA based on comparing IGS sequences from
2 the 6B and 5D loci. On the other hand, Handa *et al.* (2018), analysing 3'ETS region in 45S
3 rDNA, identified two rDNA subtypes that were shared between 1B and 6B chromosomes,
4 suggesting sequence interchange between the two chromosomes.

5 *Nucleolar dominance of B-genome NORs*

6 To assess the transcription of particular chromosomal rDNA loci, we analysed data obtained
7 by high-throughput sequencing of total RNA, making use of validated diagnostic SNPs
8 located in 26S rRNA gene, which was well covered by transcripts. The results for 1B- and
9 6B-specific transcription supported findings of Martini and Flavell (1985) who measured
10 volumes of nucleoli belonging to *NorB1* and *NorB2*, respectively, and predicted 2:1
11 transcription ratio for the 1B and 6B loci. Fractional transcription from the 5DS locus detected
12 in our study is in agreement with observations of Mukai *et al.* (1991) who found 5DS-
13 associated micronucleoli in ~3% of CS nuclei. Several studies (Cormeño *et al.*, 1974; Martini
14 and Flavell, 1985; Handa *et al.*, 2018) observed activation of minor rDNA loci, mainly *Nor*-
15 *D3* in 5D, after removal of one of the major NORs. This is consistent with our finding that the
16 5DS locus is represented by a regular array of compactly organized rDNA units, suggesting
17 their full functionality. The silenced state of the 5DS locus is likely maintained by epigenetic
18 mechanisms, among which cytosine methylation in CHG and possibly CHH contexts appears
19 to play a significant role, as suggested by our comparative analysis of DNA methylation
20 levels in 1BS, 6BS and 5DS-specific 45S rDNA units (Fig. 4, Fig. S5) **[Supplementary**
21 **Information]**. Similarly, in a study of Guo and Han (2014), promoter methylation in CHG
22 and CHH rather than CpG contexts was involved in silencing of A-genome rDNA loci in
23 synthetic allotetraploid wheats. Given that the CHG methylation is ultimately associated with
24 heterochromatic H3K9m2 histone mark (Lindroth *et al.*, 2004), it is likely that the 5D NOR is
25 in phase of heterochromatization and permanent inactivation.

1 We failed to find clear evidence for 26S rDNA expression from the 1AS locus that is featured
 2 by large scale rearrangements and truncated copies, indicating extensive pseudogenization,
 3 which suggests that silencing of this locus is most probably terminal (mutation-directed), in
 4 contrast to that of 5DS. Still, several non-truncated units seem to be present in 1AS,
 5 harbouring shorter IGS than in other loci. Given that lengths of the IGS were correlated with
 6 transcription activity in wheat (Sardana *et al.*, 1993), it is likely that the 1AS array became
 7 underdominant early after hybridization of the A and B genomes, which gave rise to tetraploid
 8 AABB wheat, and that the rearrangements arrived later in the evolution. This is supported by
 9 the observation of Guo and Han (2014) that the A genome loci were silenced in the synthetic
 10 lines of wheat and eventually lost in subsequent generations. That study also demonstrated
 11 nucleolar dominance of the D over the A and the B over the D genome in newly formed
 12 allotetraploids. Interestingly, our evidence of an irregular rDNA array in 1A and a potentially
 13 functional array in 5D, whose suppression was reverted in several studies, indicates that the
 14 process of elimination of the subdominant rDNA loci has been much slower in natural bread
 15 wheat than in the synthetic polyploids reported by Guo and Han (2014). Since the D genome
 16 has been a relatively recent addition to the hexaploid bread wheat genome (IWGSC, 2014),
 17 the difference in the extent of suppression between the two minor loci may reflect the length
 18 of co-existence of the A and the D with the dominant B genome. Alternatively, the lower
 19 suppression of the D genome might be due to higher competitiveness of its rDNA, possibly
 20 due to longer IGS compared to the A genome.

21 *Uneven transcription of the major B-genome rDNA loci*

22 In contrast to the B genome nucleolar dominance, explored in several studies, the partial
 23 predominance of 1B over 6B rDNA transcription gained less attention and its mechanism has
 24 not been clarified yet. Handa *et al.* (2018) suggested a regulation mechanism based on rDNA
 25 sequence difference because their predominantly expressed subtype of 3'ETS sequence was

1 found on both 1B and 6B chromosomes. In our study, and also in that of Martini and Flavell
2 (1985), the higher expression was associated with the NOR in 1B chromosome. Similarly, the
3 transcription activity was found associated with a particular chromosome in a diploid species
4 *Arabidopsis thaliana*, (Chandrasekhara *et al.*, 2016). Since the active and the developmentally
5 silenced NORs, located in *A. thaliana* chromosomes 4 and 2, respectively, are comparable in
6 size, unit sequence and position on the chromosome in the Col-0 ecotype, the authors
7 proposed a critical role of a *NOR2*-adjacent region in initiating the selective silencing of the
8 locus. However, the mechanism of spreading heterochromatization appears unlikely in the
9 partial silencing of the 6B locus since wheat NORs were found to form nucleoli with
10 expressed (decondensed) and unexpressed (condensed) rDNA segments interspersed, in
11 contrast to other species (Leitch *et al.*, 1992).

12 Obviously, the expression dominance of the 1B NOR is rather labile and could be genotype-
13 specific and unit-structure-dependent. For example, another wheat variety, *T. aestivum*
14 ‘Bezostaya’, having larger 6B rDNA units, expressed the 6B NOR more efficiently than that
15 in 1B, as concluded from cytogenetic observations (Sardana *et al.*, 1993). The authors
16 suggested that the IGS size, specifically the number of 135-bp repeats therein, and
17 methylation status of a specific CCGG site downstream of the repeats might be decisive for
18 transcription activity of particular rDNA loci in wheat. They assumed for 1B units in CS a
19 larger IGS size compared to those in 6B and 5D, as demonstrated in several studies (Appels
20 and Dvořák, 1982; Lassner *et al.* 1987; Flavell *et al.* 1988). Our optical map data (Table 1)
21 confirm a larger rDNA unit size for 1B vs. 5D but not 6B locus, whose unit mean size appears
22 ~130 bp larger than that of the 1B locus. Nevertheless, the analysis of OM single-molecule
23 data revealed for each chromosomal locus a broader range of unit sizes (Fig. S2)
24 **[Supplementary Information]**, suggesting that rDNA arrays in particular chromosomal loci
25 are not completely homogenous, at least in terms of the unit size. Consistent with this, Flavell

1 *et al.* (1988) found the longer spacers only in a small fraction of rRNA genes from the 1B
2 NOR of CS. Such heterogeneity might eventually result into a variability in the proportions of
3 longer (possibly highly active) and shorter (moderately active or inactive) units, which would
4 explain observation of Mukai *et al.* (1991) who found the two major loci equally active in CS,
5 based on cytogenetic analyses.

6 *Developmental control of rRNA dosage*

7 We carried out transcriptomic analysis across five wheat tissues aiming to identify differential
8 transcription of 45S rDNA, as observed in both diploid and allopolyploid species of other
9 genera (Chen and Pikaard, 1997; Komarova *et al.*, 2004; Pontvianne *et al.*, 2010; Dobešová *et*
10 *al.*, 2015). Here we observed moderate developmental silencing of the 6B rDNA locus, which
11 was most apparent in the adult leaf tissue. The higher expression of the 6B-specific rDNA in
12 early-stage tissues with rapidly dividing cells (embryo, coleoptile, root tip), might be
13 conducted by their overall high demands on nuclear rRNA production, as suggested by our
14 transcript analysis across tissues (Fig. 3B). Studies in *A. thaliana* ascribed the major role in
15 the developmental regulation of minor variants in both 45S and 5S rDNA to chromatin state
16 of the loci, related to the proportion of meristematic cells in particular tissues (Mathieu *et al.*,
17 2003; Pontvianne *et al.*, 2010). The expression of a developmentally silenced 45S rDNA
18 variant *VARI*, specific for the *NOR2*, was found associated with a lower degree of chromatin
19 condensation in the early stage of seedling development and also with decreased cytosine
20 methylation in 5'ETS of 45S rDNA. The *VARI* expression was apparent up to the second day
21 of seed germination and in later stages only weakly in root and floral tissues, while
22 completely disappearing from leaves (Pontvianne *et al.*, 2010). This expression pattern is
23 consistent with the trend observed for the 6B locus in our transcriptomic data (Fig. 3A), thus
24 we propose an analogous mechanism to control the developmental dynamics of rRNA
25 transcription in wheat. Nevertheless, the extent of 6B-NOR silencing detected in our study

1 was less significant, perhaps due to small differences in IGS sizes between 1B and 6B loci,
 2 which appear determining for the unit methylation status and transcription activity of rRNA
 3 genes in wheat and its relatives (Flavell *et al.*, 1988; Sardana *et al.*, 1993). We observed
 4 small, but highly significant difference in the level of CpG methylation between 1B and 6B
 5 units and no difference in CHG methylation (Fig. 4, Table S7) [**Supplementary**
 6 **Information**]. This subtle variance might reflect the fact that only a minor part of rDNA units
 7 in both 1B and 6B NORs appear active (Mukai *et al.*, 1991), thus diminishing potential
 8 differences in global epigenetic patterns between the two loci.

9 *Conclusions*

10 Our study provided comprehensive information on fine structure of major and minor rDNA
 11 loci and their activity and evolution in bread wheat genome (summarised in Fig. 5). We
 12 documented various mechanisms of rRNA dosage control, including gene elimination and
 13 stable inactivation related to nucleolar subdominance of the A and D-genome loci, and partial,
 14 developmentally regulated silencing of one of the major B-genome loci. To fully understand
 15 the latter mechanism, further studies across developmental stages will be needed to assess
 16 involvement of epigenetics and chromatin arrangement as well as the role of intergenic
 17 spacers and their transcripts.

18 **Supplementary Data**

19 Table S1. Optical map statistics

20 Table S2. RNA-seq data

21 Table S3. Frequency of allelic variants at diagnostic SNPs in Illumina reads of flow-sorted
 22 chromosome arms

- 1 Table S4. Frequency of allelic variants at diagnostic SNPs in Illumina reads from three *T.*
- 2 *aestivum* 'Chinese Spring' genome projects
- 3 Table S5. Variant analysis of 26S rRNA in different tissues - statistics
- 4 Table S6. Transcripts per kilobase million (TPM) values for nuclear, chloroplast and
- 5 mitochondrial 16S/18S and 23/26S rRNAs across five tissues
- 6 Table S7. Cytosine methylation in 5'ETS of 45S rDNA units - statistics
- 7 Fig. S1. Wheat consensus 45S rDNA unit and its coverage by RNA-seq reads.
- 8 Fig. S2. Quantification of 45S rDNA units in arrays from optical map data.
- 9 Fig. S3. Analysis of chromosome-specific haplotypes in 45S rDNA.
- 10 Fig. S4. Mapping of Iso-Seq reads to wheat consensus and 5DS rDNA units.
- 11 Fig. S5. Cytosine methylation in 5'ETS and 26S rRNA gene from 1B, 6B and 5D loci
- 12 Dataset S1. Sequences of wheat 45S rDNA consensus and chromosome-specific consensual
- 13 rDNA units.
- 14 Dataset S2. Multiple alignment of wheat 45S rDNA consensus and chromosome-specific
- 15 consensual rDNA units.

16 **Funding**

17 This work was supported by the Czech Science Foundation [grant numbers 17-17564S, 19-
18 03442S] and by the European Regional Development Fund project "Plants as a tool for
19 sustainable global development" [No. CZ.02.1.01/0.0/0.0/16_019/0000827].

20

1 **Acknowledgements**

2 We thank to Zdeňka Dubská, Jitka Weiserová and Eva Jahnová for excellent technical
3 assistance. We are grateful to M. Mascher for sharing merged PE450 Illumina reads of wheat.
4 Computational resources were supplied by the project "e-Infrastruktura CZ" (e-INFRA
5 LM2018140) provided within the program Projects of Large Research, Development and
6 Innovations Infrastructures, and by the ELIXIR-CZ project (LM2018131), part of the
7 international ELIXIR infrastructure. Author contribution: HŠ planned and designed the
8 research. AK and ZT conducted transcriptome analysis, ZT and HT carried out optical
9 mapping, including data analysis. PN performed rRNA quantification and cytosine
10 methylation analysis. VK validated diagnostic SNPs and together with JM a EH reconstructed
11 consensual rDNA units. JV flow sorted chromosomes for optical mapping. HŠ, AK and JD
12 were involved in writing the manuscript and acquisition of funding for the study.

13 **Data Availability**

14 Raw RNA-seq data for five tissues generated for this study are openly available in the NCBI
15 Short Read Archive under BioProject PRJNA657991. Consensual sequences of rDNA units
16 and their alignments are available within Supplementary Data published online. Optical maps
17 of 1AS, 1BS, 6BS and 5DS wheat chromosome arms (bnx and cmap files) are available from
18 the corresponding author, Hana Šimková, upon request.

19 **Literature Cited**

20 **Akpınar BA, Yuce M, Lucas S, *et al.* 2015.** Molecular organization and comparative analysis
21 of chromosome 5B of the wild wheat ancestor *Triticum dicoccoides*. *Scientific Reports*
22 **5**:10763. doi: 10.1038/srep10763

- 1 **Appels R., Dvořák J. 1982.** The wheat ribosomal DNA spacer: its structure and variation in
2 populations and among species. *Theoretical and Applied Genetics* **63**: 337-348.
3 doi.org/10.1007/BF00303905
- 4 **Cermeño MC, Orellana J, Santos JL, Lacadena JR. 1974.** Nucleolar organizer activity in
5 wheat, rye and derivatives analyzed by a silver-staining procedure. *Chromosoma* **89**(5): 370-
6 376. doi: 10.1007/BF00331254
- 7 **Chandrasekhara C, Mohannath G, Blevins T, Pontvianne F, Pikaard CS. 2016.**
8 Chromosome-specific NOR inactivation explains selective rRNA gene silencing and dosage
9 control in Arabidopsis. *Genes and Development* **30**(2):177-190. doi: 10.1101/gad.273755.115
- 10 **Chapman JA, Mascher M, Buluc A, et al. 2015.** A whole-genome shotgun approach for
11 assembling and anchoring the hexaploid bread wheat genome. *Genome Biology* **16**(26). doi:
12 10.1186/s13059-015-0582-8
- 13 **Chen ZJ, Pikaard CS. 1997.** Transcriptional analysis of nucleolar dominance in polyploid
14 plants: biased expression/silencing of progenitor rRNA genes is developmentally regulated in
15 Brassica. *Proceedings of the National Academy of Science of the United States of America*
16 **94**(7): 3442-3447. doi: 10.1073/pnas.94.7.3442
- 17 **Clavijo BJ, Venturini L, Schudoma C, et al. 2017.** An improved assembly and annotation of
18 the allohexaploid wheat genome identifies complete families of agronomic genes and
19 provides genomic evidence for chromosomal translocations. *Genome Research* **27**(5): 885–
20 896. doi: 10.1101/gr.217117.116
- 21 **Crosby AR. 1957.** Nucleolar activity of lagging chromosomes in wheat. *American Journal of*
22 *Botany* **44**(10): 813–822. doi: 10.1002/j.1537-2197.1957.tb08268.x

- 1 **Darvey NL, Driscoll CJ. 1972.** Nucleolar behavior in *Triticum*. *Chromosoma* **36**: 131-139.
- 2 doi: 10.1007/bf00285208
- 3 **Dobešová E, Malinská H, Matyášek R, et al. 2015.** Silenced rRNA genes are activated and
- 4 substitute for partially eliminated active homeologs in the recently formed allotetraploid,
- 5 *Tragopogon mirus* (Asteraceae). *Heredity* **114**(3): 356-365. doi: 10.1038/hdy.2014.111
- 6 **Doležel J, Čížková J, Šimková H, Bartoš J. 2018.** One major challenge of sequencing large
- 7 plant genomes is to know how big they really are. *International Journal of Molecular*
- 8 *Sciences* **19**(11): 3554. doi: 10.3390/ijms19113554
- 9 **Flavell RB, O'Dell M. 1976.** Ribosomal RNA genes on homoeologous chromosomes of
- 10 groups 5 and 6 in hexaploid wheat. *Heredity* **37**: 377–385. doi: 10.1038/hdy.1976.102
- 11 **Flavell RB, O'Dell M, Thompson WF. 1988.** Regulation of cytosine methylation in
- 12 ribosomal DNA and nucleolus organizer expression in wheat. *Journal of Molecular Biology*
- 13 **204**(3): 523-534. doi.org/10.1016/0022-2836(88)90352-X
- 14 **Fluch S, Kopecky D, Burg K, et al. 2012.** Sequence composition and gene content of the
- 15 short arm of rye (*Secale cereale*) chromosome 1. *PloS ONE*, **7**(2): E30784. doi:
- 16 10.1371/journal.pone.0030784
- 17 **Gerlach WL, Bedbrook JR. 1979.** Cloning and characterization of ribosomal RNA genes
- 18 from wheat and barley. *Nucleic Acids Research* **7**(7): 1869–1885. doi: 10.1093/nar/7.7.1869
- 19 **Guo X, Han F. 2014.** Asymmetric epigenetic modification and elimination of rDNA
- 20 sequences by polyploidization in wheat. *Plant Cell* **26**(11): 4311-4327. doi:
- 21 10.1105/tpc.114.129841

1 **Handa H, Kanamori H, Tanaka T, et al. 2018.** Structural features of two major nucleolar
2 organizer regions (NORs), Nor-B1 and Nor-B2, and chromosome-specific rRNA gene
3 expression in wheat. *Plant Journal* **96**(6): 1148–1159. doi: 10.1111/tpj.14094

4 **International Wheat Genome Sequencing Consortium (IWGSC). 2014.** A chromosome-
5 based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science*
6 **345**(6194), 1251788. doi: 10.1126/science.1251788

7 **International Wheat Genome Sequencing Consortium (IWGSC). 2018.** Shifting the limits
8 in wheat research and breeding using a fully annotated reference genome. *Science* **361**(6403):
9 eaar7191. doi: 10.1126/science.aar7191

10 **Kapustová V, Tulpová Z, Toegelová H, et al. 2019.** The Dark Matter of Large Cereal
11 Genomes: Long Tandem Repeats. *International Journal of Molecular Sciences* **20**(10): 2483.
12 doi: 10.3390/ijms20102483

13 **Komarova NY, Grabe T, Huigen DJ, Hemleben V, Volkov RA. 2004.** Organization,
14 differential expression and methylation of rDNA in artificial *Solanum* allopolyploids. *Plant*
15 *Molecular Biology* **56**(3): 439-463. doi: 10.1007/s11103-004-4678-x

16 **Krueger F, Andrews SR. 2011.** Bismark: a flexible aligner and methylation caller for
17 Bisulfite-Seq applications. *Bioinformatics* **27**(11): 1571-2. doi: 10.1093/bioinformatics/btr167

18 **Kubaláková M, Vrána J, Čihalíková J, Šimková H, Doležel J. 2002.** Flow karyotyping and
19 chromosome sorting in bread wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics*
20 **104**(8): 1362–1372. doi: 10.1007/s00122-002-0888-2

21 **Lam ET, Hastie A, Lin C, et al. 2012.** Genome mapping on nanochannel arrays for structural
22 variation analysis and sequence assembly. *Nature Biotechnology* **30**(8): 771–776. doi:
23 10.1038/nbt.2303

1 **Langmead B, Salzberg SL. 2012.** Fast gapped-read alignment with Bowtie 2. *Nature*
2 *Methods* **9**(4): 357–359. doi: 10.1038/nmeth.1923

3 **Lassner M, Anderson O, Dvořák J. 1987.** Hypervariation associated with a 12-nucleotide
4 direct repeat and inferences on intergenomic homogenization of ribosomal-RNA gene spacers
5 based on the DNA-sequence of a clone from the wheat NOR-D3 locus. *Genome* **29**(5): 770-
6 781. doi: 10.1139/g87-130

7 **Leitch AR, Mosgoller W, Shi M, Heslop-Harrison JS. 1992.** Different patterns of rDNA
8 organization at interphase in nuclei of wheat and rye. *Journal of Cell Science* **101**: 751-757.

9 **Lindenbaum P. 2015.** Jvarkit: java-based utilities for Bioinformatics. figshare. *Journal*
10 *contribution*.

11 **Lindroth AM, Shultis D, Jasencakova Z, et al. 2004.** Dual histone H3 methylation marks at
12 lysines 9 and 27 required for interaction with CHROMOMETHYLASE3. *EMBO Journal*
13 **23**(21): 4286-96. doi: 10.1038/sj.emboj.7600430

14 **Mathieu O, Jasencakova Z, Vaillant I, et al. 2003.** Changes in 5S rDNA Chromatin
15 Organization and Transcription during Heterochromatin Establishment in Arabidopsis. *Plant*
16 *Cell* **15**(12): 2929-2939. doi: 10.1105/tpc.017467

17 **Martini G, Flavell R. 1985.** The control of nucleolus volume in wheat, a genetic study at
18 three developmental stages. *Heredity* **54**: 111–120. doi: 10.1038/hdy.1985.15

19 **Middleton CP, Senerchia N, Stein N, et al. 2014.** Sequencing of chloroplast genomes from
20 wheat, barley, rye and their relatives provides a detailed insight into the evolution of the
21 Triticeae tribe. *PLoS ONE* **9**(3): e85761. doi: 10.1371/journal.pone.0085761

- 1 **Mukai Y, Endo TR, Gill BS. 1991.** Physical mapping of the 18S.26S rRNA multigene family
2 in common wheat: Identification of a new locus. *Chromosoma* **100**(71–78). doi:
3 10.1007/bf00418239

- 4 **Nelson JO, Watase GJ, Warsinger-Pepe N, Yamashita YM. 2019.** Mechanisms of rDNA
5 copy number maintenance. *Trends in Genetics* **32**(10): 734-742. doi:
6 10.1016/j.tig.2019.07.006

- 7 **Novák P, Neumann P, Pech J, Steinhaisl J, Macas J. 2013.** RepeatExplorer: a Galaxy-based
8 web server for genome-wide characterization of eukaryotic repetitive elements from next-
9 generation sequence reads. *Bioinformatics* **29**(6): 792–793. doi:
10 10.1093/bioinformatics/btt054

- 11 **Pontvianne F, Abou-Ellail M, Douet J, et al. 2010.** Nucleolin is required for DNA
12 methylation state and the expression of rRNA gene variants in *Arabidopsis thaliana*. *PLoS*
13 *Genetics* **6**(11): e1001225. doi: 10.1371/journal.pgen.1001225

- 14 **Pruitt RE, Meyerowitz EM. 1986.** Characterization of the genome of *Arabidopsis thaliana*.
15 *Journal of Molecular Biology* **187**(2): 169-183. doi: 10.1016/0022-2836(86)90226-3

- 16 **Rabanal FA, Nizhynska V, Mandaková T, et al. 2017.** Unstable inheritance of 45S rRNA
17 genes in *Arabidopsis thaliana*. *G3-Genes Genome Genetics* **7**(4): 1201-1209. doi:
18 10.1534/g3.117.040204

- 19 **Rogers SO, Bendich AJ. 1987.** Heritability and variability in ribosomal RNA genes of *Vicia*
20 *faba*. *Genetics* **117**(2): 285-295.

- 21 **Šafář J, Šimková H, Kubaláková M, et al. 2010.** Development of chromosome-specific
22 BAC resources for genomics of bread wheat. *Cytogenetic and Genome Research* **129**(1-3):
23 211-223. doi: 10.1159/00031

- 1 **Sardana R, O'Dell M, Flavell R. 1993.** Correlation between the size of the intergenic
2 regulatory region, the status of cytosine methylation of rRNA genes and nucleolar expression
3 in wheat. *Molecular and General Genetics* **236**(2-3): 155-162. doi: 10.1007/BF00277107
- 4 **Sears ER. 1954.** The aneuploids of common wheat. *Missouri Agricultural Experiment Station*
5 *Research Bulletin* **572**: 1–59.
- 6 **Sears ER, Sears LMS. 1978.** The Telocentric Chromosomes of Common Wheat.
7 *Proceedings of the 5th International Wheat Genetics Symposium*, New Delhi, 23-28, 389-407.
- 8 **Sims J, Sestini G, Elgert C, von Haeseler A, Schlögelhofer P. 2021.** Sequencing of the
9 Arabidopsis NOR2 reveals its distinct organization and tissue-specific rRNA ribosomal
10 variants. *Nature Communications* **12**(1): 387. doi: 10.1038/s41467-020-20728-6.
- 11 **Staňková H, Hastie AR, Chan S, et al. 2016.** BioNano genome mapping of individual
12 chromosomes supports physical mapping and sequence assembly in complex plant genomes.
13 *Plant Biotechnology Journal* **14**(7): 1523-1531. doi: 10.1111/pbi.12513
- 14 **Symonová R, Ocalewicz K, Kirtiklis L, et al. 2017.** Higher-order organisation of extremely
15 amplified, potentially functional and massively methylated 5S rDNA in European pikes (*Esox*
16 sp.). *BMC Genomics* **18**(1): 391. doi: 10.1186/s12864-017-3774-7
- 17 **Tanaka T, Kobayashi F, Joshi GP, et al. 2014.** Next-generation survey sequencing and the
18 molecular organization of wheat chromosome 6B. *DNA Research* **21**(2): 103-114. doi:
19 10.1093/dnares/dst041
- 20 **Vincentz M, Flavell RB. 1989.** Mapping of ribosomal RNA transcripts in wheat. *Plant Cell*
21 **1**(6): 579-589. doi: 10.1105/tpc.1.6.579
- 22 **Zimin AV, Puiu D, Hall R, Kingan S, Clavijo BJ, Salzberg SL. 2017.** The first near-
23 complete assembly of the hexaploid bread wheat genome, *Triticum aestivum*. *GigaScience*
24 **6**(11): 1-7. doi: 10.1093/gigascience/gix097

1 **Legends to Figures**

2 **Fig. 1.** 45S rDNA loci in bread wheat genome. (A) Graphical outcome of a BLAST search in
3 Ensembl Plants/Triticum aestivum database for sequences homologous to wheat consensus
4 rDNA unit in the IWGSC RefSeq v1.0 genome. The search was done with default settings.
5 Vast majority of hits correspond to rDNA fragments; positions of larger rDNA clusters are
6 marked by rectangles, with green colour pointing to rDNA loci analysed in the study. About
7 30% hits fall into unassigned scaffolds (Un). (B) Positioning of 45S rDNA arrays by optical
8 mapping. Optical maps (blue bars) generated from rDNA-bearing chromosomes 1AS, 1BS,
9 6BS and 5DS were aligned to corresponding pseudomolecules of IWGSC RefSeq v1.0 wheat
10 genome (green bars), digested *in silico* with enzymes used to construct the optical map. The
11 vertical lines represent BspQI (red) and DLE-1 (blue) recognition sites labelled in particular
12 optical map. Regular pattern with 8.4-9.6-kb spacing (highlighted by a yellow line) indicates
13 presence of a 45S rDNA array. Only the irregular rDNA array in 1AS is present in the
14 sequence while the regular arrays in the other chromosomes are missing in the
15 pseudomolecules. The relevance of the arrays for the rDNA is supported by the presence of
16 rDNA fragments found in the adjacent genomic regions (highlighted by violet lines).
17 Sequence scaffold 34514, flanking the 5DS rDNA array at the proximal side, is misoriented in
18 the IWGSC RefSeq v1.0 genome. The red arrows indicate scaffold boundaries in the
19 reference genome.

21 **Fig. 2.** Degenerated 45S rDNA array in wheat chromosome arm 1AS. (A) The 1AS rDNA
22 array is composed of both complete (comprising all of 18S, 5.8S and 26S rRNA genes; blue
23 boxes), and incomplete units. Separated 18S and 26S rRNA genes and gene fragments are
24 marked purple and yellow, respectively. (B) Ribosomal DNA units are interspersed by
25 telomeric repeats (red triangles) of various orientation. (C) Comparison of genic parts of 29

1 complete 45S rDNA units building the 1AS array shows a high level of diversity. Grey bars
2 represent individual rDNA units, disagreements are indicated by black lines.

3

4 **Fig. 3.** Tissue-specific contribution of particular loci to rRNA production. (A) Representation
5 of 6BS- and 1BS-specific SNP variants in 26S rDNA, as estimated from raw Illumina data
6 used to generate IWGSC RefSeq v1.0 (*genome*), and in 26S rRNA from five tissues (*embryo*,
7 *coleoptile*, *root tip*, *primary leaf*, *mature leaf*). (B) Relative contribution of nuclear,
8 mitochondrial and chloroplast genomes to overall rRNA production across five tissues,
9 expressed as TPM (Transcripts Per Kilobase Million) values for each of the 16S/18S and
10 23/26S rRNA types. The graphs show mean values of two replicas.

11

12 **Fig. 4.** Cytosine methylation in 5'ETS of 45S rDNA unit from 1B, 6B and 5D loci. The
13 methylation was evaluated after mapping three replicas of publically available bisulfite-
14 sequencing reads (IWGSC, 2018) to consensual units of 1BS, 6BS and 5DS, respectively. The
15 methylation was quantified in 5'ETS of each unit as percentage of methylated cytosine in
16 CpG, CHG and CHH contexts, respectively. Asterisks indicate levels of statistical
17 significance (** $p < 0.01$; *** $p < 0.001$) of differences between methylation in 1BS, 6BS and
18 5DS loci. Absence of asterisks indicates non-significant difference ($p > 0.05$).

19 **Fig. 5.** A scheme for the NOR evolution and transcription regulation in bread wheat genome

20

21

22

23

24

25

1 Tables

Table 1. Quantification of 45S rDNA units

Quantification of arrayed 45S rDNA units based on optical map raw data

Chromosome arm	1BS	6BS	5DS	1AS ²	Total
Arm size (Mb) ¹	314	415	258	275	
% contaminating chromosomes	15	19.7	15		
% nucleotides in repeats	3.51	5.2	0.54		
Mb in repeats - contaminated sample	11.2	21.58	1.28		
Mb in repeats - pure sample	12.96	26.87	1.5	0.43	41.76
45S rDNA unit size (kb) ³	9.42	9.55	8.8	8.6	
Number of 45S rDNA units in arrays	1376	2813	170	29	4388
% total number units in arrays⁴	31.4	64.1	3.9	0.7	

¹ according to Šafář *et al.*, 2010

² 1AS rDNA was quantified based on IWGSC Ref Seq v1.0

³ estimated from the optical map; the size of the 5DS unit has been corrected for concordance with the sequence

⁴ calculated as the number of 45S rDNA units divided by 4388*100

Quantification of 26S rDNA haplotypes across 5DS-1, 6BS-2 and 1BS-1 SNPs

Chromosome arm	1BS	6BS	5DS	1AS + others	Non-specified Other combinations
Haplotype	CGT	CTC	TGC	CGC	
No. haplotypes	2177	3667	240	135	21
% haplotypes	34.9	58.8	3.8	2.2	0.3

Haplotypes were analysed in 6240 PE-450 reads (IWGSC, 2018) spanning over SNP positions 5300 bp, 5348 bp and 5533 bp of wheat 45S rDNA consensus sequence, which discriminate the chromosomal haplotypes.

2

3 Appendix

4 Abbreviations

5 BS-seq – bisulphite sequencing

6 CS – *T. aestivum* cv. Chinese Spring

7 ETS – external transcribed spacer

8 IGS – intergenic spacer

9 ITS – internal transcribed spacer

10 IWGSC – International Wheat Genome Sequencing Consortium

- 1 NOR – nucleolus organizer region
- 2 OM – optical map
- 3 TPM – Transcripts Per Kilobase Million
- 4

A

B

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5