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The fate of 35S rRNA genes in the allotetraploid grass
Brachypodium hybridum

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SUMMARY
Nucleolar dominance (ND) consists of the reversible silencing of 35S/45S rDNA loci inherited from one of the ancestors of an allopolyploid. The molecular mechanisms by which one ancestral rDNA set is selected for silencing remain unclear. We applied a combination of molecular (Southern blot hybridization and reverse-transcription cleaved amplified polymorphic sequence analysis), genomic (analysis of variants) and cytogenetic (fluorescence in situ hybridization) approaches to study the structure, expression and epigenetic landscape of 35S rDNA in an allotetraploid grass that exhibits ND, Brachypodium hybridum (genome composition DDSS), and its putative progenitors, Brachypodium distachyon (DD) and Brachypodium stacei (SS).

In progenitor genomes, B. stacei showed a higher intragenomic heterogeneity of rDNA compared with B. distachyon. In all studied accessions of B. hybridum, there was a reduction in the copy number of S homoeologues, which was accompanied by their inactive transcriptional status. The involvement of DNA methylation in CG and CHG contexts in the silencing of the S-genome rDNA loci was revealed. In the B. hybridum allotetraploid, ND is stabilized towards the D-genome units, irrespective of the polyphyletic origin of the species, and does not seem to be influenced by homoeologous 35S rDNA ratios and developmental stage.

Keywords: 35S rDNA evolution, 35S rRNA gene expression, allopolyploidy, Brachypodium hybridum, nucleolar dominance.

INTRODUCTION
Allopolyploidy consists of interspecific hybridization followed by chromosome doubling, and has long been considered to be a major driving force in evolution (Liu and Wendel, 2003). However, the merger of two or more divergent genomes within one nucleus presents challenges to long-term viability including: deletions, translocations, transposon activation and meiotic irregularities. In addition to these structural changes, the expression of the duplicated genes can result in the silencing of one of the homoeologues (Leitch and Leitch, 2008; Weiss-Schneeweiss et al., 2013; Wendel et al., 2018). One example of an interaction between the ancestral genomes in allopolyploids that results in altered gene expression is nucleolar dominance (ND). ND was discovered in interspecific hybrids of Crepis (Asteraceae), in which the secondary constrictions were only carried by the chromosomes that had been inherited from one of the progenitors (Navashin, 1934). More than 30 years later, it was shown that the tandemly arranged 35S/45S ribosomal DNA (rDNA) units encoding the 18S, 5.8S and 25–28S rRNAs were located within the secondary constrictions (Wallace and Birnstiel, 1966; Phillips et al., 1971). It was found that rDNA arrays may occupy one or more loci in a genome in which individual repeats do not evolve independently but evolve in a concerted manner (Kovarik et al., 2008; Garcia et al., 2010).
2017). Interestingly, the non-coding subregions of the rDNA units, the internal transcribed spacers (ITS1 and ITS2) and the intergenic spacer (IGS) evolve faster than the coding sequences, and therefore are significantly more variable (Eickbush and Eickbush, 2007). Although there are hundreds to thousands of rDNA units per genome, not all rRNA genes undergo transcription. The excess of rDNA units bears epigenetic modifications that are characteristic of heterochromatin and that stay transcriptionally silent (Bockor et al., 2014; Costa-Nunes et al., 2014).

The ND phenomenon consists of the reversible silencing of the 35S/45S rDNA loci that are inherited from one of the ancestral species, and has been described in allopolyploids and hybrids that belong to numerous and diverse plant genera (Pikaard, 2000). Although our understanding of the involvement of epigenetic modifications such as DNA methylation and histone deacetylation in establishing and maintaining the ND has advanced significantly in the last decades (Neves et al., 1995; Chen and Pikaard, 1997a; Lawrence et al., 2004; Earley et al., 2006; Costa-Nunes et al., 2010), the mechanisms by which one ancestral rDNA set is selected for silencing remain elusive (Symonova, 2019). The proposed explanations that the uniparental expression of 35S rDNA corresponds to the physical characteristics of its IGSs (the so-called enhancer imbalance hypothesis, originally proposed for Xenopus hybrids), or to differences in the affinity for transcription factors, are not supported in plants (Chen and Pikaard, 1997b; Frieman et al., 1999). It was also shown that ND in plant allopolyploids is not a maternal effect (Chen and Pikaard, 1997b).

In Arabidopsis thaliana and Hordeum vulgare, the 35S rRNA genes were silenced based on the chromosomal position in which they resided (Nicoloff et al., 1979; Schubert and Künel, 1990; Mohannath et al., 2016), and therefore the influence of the neighbouring DNA sequences on the rRNA gene expression cannot be ruled out.

Most of the research on ND has been performed on dicot species (e.g. Arabidopsis suecica, Brassica and Nicotiana allopolyploids), but rather less is known about this phenomenon in monocots, where the studies have been limited to Triticum aestivum (wheat; Guo and Han, 2014), which harbours very complex genomes with many rDNA loci. To fill a gap in the knowledge about ND in monocots we analysed its molecular basis in a small- and simple-genome allotetraploid grass that exhibits ND, Brachypodium hybridum (2n = 30; genome composition DDSS). Its genomes most likely originated from two diploid progenitors: B. distachyon (2n = 10; DD) and B. stacei (2n = 20; SS). One of the features that made B. hybridum an amenable model in studies of rDNA evolution and expression is the fact that it contains only one rDNA locus per ancestral genome. Moreover, the whole genomic sequence of both diploid progenitors and several B. hybridum genotypes is available for the scientific community (IBI, 2010, https://phytozome.jgi.doe.gov). The preferential silencing of the B. stacei-like rDNA loci was documented in different tissues of B. hybridum, including both meristematic (Idziak and Hasterok, 2008) and differentiated (Borowska-Zuchowska et al., 2016) root cells, prophase-I meiocytes, microspores and different tissues of immature and imbibed embryos (Borowska-Zuchowska et al., 2019). As yet, there have been no reports on the expression status of the S-genome rRNA genes in B. hybridum leaves. We have previously used molecular cytogenetic approaches to study the epigenetic status of 35S rDNA loci in B. hybridum. The actively transcribed D-genome 35S rDNA units exhibited a low DNA methylation level and were enriched in euchromatic marks, such as acetylated histone isoforms (H4K5ac, H4K16ac and H3K9ac) (Borowska-Zuchowska and Hasterok, 2017). In contrast, the B. stacei-like rDNA genes were characterized by a high level of DNA methylation and were enriched with a heterochromatic marker: the dimethylated lysine 9 of histone H3 (H3K9me2). Interestingly, the global cytosine hypomethylation of the B. hybridum genome induced by 5-azacytidine did not lead to a reactivation of the S-genome rDNA loci, which strongly suggests the involvement of other factors that lie behind ND in this species (Borowska-Zuchowska and Hasterok, 2017).

In order to shed more light on the molecular mechanisms that lie behind ND, we applied a combination of molecular and cytogenetic approaches to study the organization, expression and epigenetic landscape of 35S rDNA in 16 B. hybridum genotypes. The following questions were posed: (i) what is the level of the intragenomic homogeneity of the 35S rDNA units in the putative ancestors of B. hybridum; (ii) are the homoeologous 35S rDNA loci in different natural populations of B. hybridum faithfully inherited; (iii) does the expression status of the rRNA genes depend on their dosage in different accessions; and (iv) is DNA methylation involved in the preferential suppression of the S-genome rDNA units?

RESULTS

Assembly of the D- and S-genome 35S rDNA units, their sequence polymorphism and copy number

Although the complete genomic sequence of B. distachyon has been available since 2010 (IBI, 2010), the 35S rDNA unit has not yet been annotated. Based on the conserved nature of the 18S and 25S rRNA genes, we screened the genomic sequence of B. distachyon line Bd21 using the known homoeologous coding sequences of Oryza sativa (rice) as the queries. Two tandemly repeated sequences, located in the nucleolus-organizing region (NOR)-bearing chromosome Bd5, were identified. The 18S and 25S rDNA sequences of B. distachyon showed 98 and 96% nucleotide identity, respectively, with their counterparts from rice. The
sequences of *B. distachyon* ITS1-5.8S rDNA-ITS2 as well as the sequence of the intergenic spacer (Bd IGS) have previously been published (Catalan et al., 2012; Borowska-Zuchowska et al., 2016). These data allowed us to reconstruct the entire 35S rDNA unit that was used as the reference sequence for mapping the raw Illumina reads from two *B. distachyon* genotypes: Bd21 and ABR5. In both cases, the entire 35S rDNA unit, including the non-coding sequences, was covered equally (Figure 1a). The read-mapping details are provided in Table 1. A similar strategy was undertaken to reconstruct the complete 35S rDNA sequence from *B. stacei* by fusing raw genomic reads from the reference genotype ABR114. The entire 35S rDNA reference sequence was covered uniformly (Figure 1c; Table 1). From the mapped reads, we generated 35S rDNA consensus sequences for both diploids that were 8084-bp- and 9215-bp long for *B. distachyon* and *B. stacei*, respectively.

The distribution of the single-nucleotide polymorphisms (SNPs) in the 35S rDNA repeats of both putative ancestral species of *B. hybridum* was determined using the ‘Basic Variant Detector’ tool in the CLC GENOMICS WORKBENCH. We observed a slightly higher number of high-frequency SNPs (≥10%) in the rDNA units of *B. stacei* than in either *B. distachyon* genotype (Figure 1b,d; Tables S1, S3, S5). As expected, the non-coding IGS regions appeared to be more variable than the coding sequences, especially in the *B. stacei* rDNA units (Figure 1d). When the low-frequency SNPs were also considered (decreasing a SNP call threshold to ≥1%), we found a threefold higher intragenomic variability in the *B. distachyon* Bd21 than in ABR5 (Figure 1b; Tables S2 and S4). Most of the SNPs, however, were located in the non-coding regions. Interestingly, we found more than 3000 low-frequency SNPs (Table S6) in *B. stacei* that were uniformly distributed across the entire 35S rDNA unit (Figure 1d) which did not accumulate in specific sub-regions. Substitutions were far more abundant than the InDels (Table S6).

The number of 35S rDNA units in *B. distachyon* and *B. stacei* was estimated based on the slot-blot hybridization with 18S rDNA as a probe (Figure S1). The intensity of the hybridization signals was comparable between the species, thus indicating little variation. Based on radioactivity measurements, Bd21 had the highest number of rDNA units per 1C (approx. 800 copies). The second *B. distachyon* genotype, ABR5, had approximately 700 copies per 1C, while *B. stacei* genotype ABR114 had approximately 650 copies per 1C. The 35S rRNA gene copy number in both diploids and *B. hybridum* ABR113 was also confirmed via the *in silico* analysis of all genomic reads (Table 1). *Brachypodium hybridum* ABR113 had more rDNA copies than any of the progenitor accessions. The values calculated based on the number of mapped reads to the 18S rDNA reference sequences were congruent with those that had been experimentally determined by slot-blot hybridization.

35S rDNA homoeologue ratios among the *B. hybridum* genotypes

In order to determine the ancestral 35S rDNA contributions, Southern blot hybridization with the 25S rDNA probe was performed on genomic DNA (gDNA) from 16 *B. hybridum* genotypes originating from diverse habitats and from two genotypes of each putative ancestral species, *B. distachyon* and *B. stacei* (Table 2). In the 35S rDNA consensus sequences of both diploid species, the two BglII restriction sites were found within the 25S rDNA coding sequence (Figure 2a). As was expected in *B. distachyon*, the short 25S rDNA probe hybridized to a single BglII fragment that was 6.7 kb long (Figure 2b, left panel). The long 25S rDNA probe variant hybridized to the 6.7- and 1.3-kb fragments (Figure 2b, right panel), which was consistent with the restriction map (Figure 2a). Interestingly, in the case of both of the genotypes of *B. stacei*, the 25S rDNA probes hybridized to two and three BglII fragments for the short and long variants of the probe, respectively (Figure 2b). An additional, slow-migrating band was consistent with the full-size rDNA unit of *B. stacei*. Its presence may be explained by a mutation at one of the two BglII restriction sites. Indeed, a site located proximally to the 5.8S rRNA gene (site position: 3850–3855 nt) was less conserved than the distal one (site position: 5185–5190 nt), based on the higher number of SNPs according to the bioinformatic analysis (Table S6). The short 25S rDNA probe that had hybridized to one or two BglII fragments in *B. distachyon* and *B. stacei*, respectively (Figure 2b), was selected for Southern blot hybridization on the gDNA of *B. hybridum*. In all genotypes of the allotetraploid we observed rDNA variants that had derived from both ancestors, however, a reduction of *B. stacei* homoeologues was also denoted in some of the genotypes, e.g. in ABR100 (Figure 2c). To determine the contribution of the D- and S-genome rDNA loci, the radioactivity of the hybridization bands was quantified using a phosphorimager. The D-genome 35S rDNA units were more abundant than the S-genome units for all of the studied genotypes. The contribution of the *B. stacei*-inherited units varied from 7% in ABR100 to 39% in ABR101 (Figure 2d).

Using raw reads from the genome of the *B. hybridum* reference genotype ABR113, we were able to determine *in silico* the ancestral rDNA contributions in this allotetraploid. We selected a 50-bp-long sequence that had derived from a variable part of the ITS1 subregion which had about a 10% divergence between the D- and S-genome rDNA consensus sequences (Figure 3a). The S-genome ITS1 fragment was BLASTed against the *B. hybridum* whole-genome sequence library to identify 3706 sequences, which were then extracted, trimmed, sampled
and aligned, as was described in the Experimental procedures. A neighbour-joining phylogenetic tree was constructed based on the alignment of 222 reads (Figure 3b; Figure S2). The tree was composed of two branches: the first was highly homogenous and consisted of 165 (74%) D-genome sequences and the second was composed of 57 (26%) more divergent S-genome ITS1 sequences. On the Southern blot, phosphorimaging of homoeologous bands revealed 74% of the D-genome rDNA and 26% of the S-genome rDNA in ABR113 rDNA (Figure 2d). Thus, the rDNA ratios determined by in silico calculations were congruent with those of the Southern blot. The haplotypic diversity of S- and D-genome ITS1 homoeologues was determined in S and D genomes of B. hybridum and its putative progenitors (Figure 3c). There was no significant difference (Pearson chi-square test, $P = 0.928$) between both S- and D-genome homoeologous families of B. hybridum ABR113. However, both B. hybridum families and those of B. distachyon had significantly lower ($P < 0.001$) haplotypic diversity than the B. stacei ITS1.

**Number and localization of rDNA loci in the B. hybridum metaphase chromosomes and interphase nuclei**

The number and localization of 35S and 5S rDNA loci were determined on the mitotic metaphase chromosomes from root-tip cells of 14 B. hybridum genotypes using
Table 2 General characteristics of the *Brachypodium* species used in this study

| Species          | Accession number | 2n  | x | Genome designation | Origin; coordinates | Source |
|------------------|------------------|-----|---|--------------------|--------------------|--------|
| *B. distachyon*  | Bd21             | 10  | 5 | DD                 | Iraq               | A      |
|                  | ABR5             | 10  | 5 | DD                 | Spain, Huesca      | B      |
| *B. stacei*      | Bsta5            | 20  | 10| SS                 | Spain, Alicante    | C      |
|                  | ABR114           | 20  | 10| SS                 | Spain, Formentera  | B      |
| *B. hybridum*    | ABR100           | 30  | 5+10| DDSS               | Iran, Khalaf Abad  | B      |
|                  | ABR101           | 30  | 5+10| DDSS               | S. Africa, nr Darling, Cape | B |
|                  | ABR107           | 30  | 5+10| DDSS               | Province, Greece   | B      |
|                  | ABR113           | 30  | 5+10| DDSS               | Portugal, Lisbon   | B      |
|                  | ABR115           | 30  | 5+10| DDSS               | S. Africa          | B      |
|                  | ABR117           | 30  | 5+10| DDSS               | Afghanistan        | B      |
|                  | ABR137           | 30  | 5+10| DDSS               | W. Australia       | B      |
|                  | 3-4-2            | 30  | 5+10| DDSS               | Turkey; 38’17’402"N, 27’24’139"E | D |
|                  | 11-1-1           | 30  | 5+10| DDSS               | Turkey; 36’32’088”N, 29’07’588”E | D |
|                  | 11-9             | 30  | 5+10| DDSS               | Turkey; 36’32’080”N, 29’07’586”E | D |
|                  | 18-8-1           | 30  | 5+10| DDSS               | Turkey; 36’02’662”N, 32’40’504”E | D |
|                  | 18-15            | 30  | 5+10| DDSS               | Turkey; 36’02’662”N, 32’40’504”E | D |
|                  | 18-19            | 30  | 5+10| DDSS               | Turkey; 36’02’662”N, 32’40’504”E | D |
|                  | 19-6-2           | 30  | 5+10| DDSS               | Turkey; 36’10’641”N, 33’26’255”E | D |
|                  | 19-13-2          | 30  | 5+10| DDSS               | Turkey; 36’10’641”N, 33’26’255”E | D |
|                  | 20-15            | 30  | 5+10| DDSS               | Turkey; 36’57’277”N 34’45’042”E | D |

A, US Department of Agriculture, National Plant Germplasm System, Beltsville, MD, USA; B, Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, UK; C, High Polytechnic School of Huesca, University of Zaragoza, Huesca, Spain; D, University of Silesia, Katowice, Poland.

fluorescence in situ hybridization (FISH) data on genotypes ABR113 and ABR117 have already been published; Hasterek et al., 2004; Borowska-Zuchowska et al., 2016). Simultaneous FISH using 25S rDNA and 5S rDNA probes identified two pairs of chromosomes bearing the 35S rDNA loci and another two chromosomal pairs that carried the 5S rDNA loci in all of the studied accessions (Figures 4 and S3–S16). Interestingly, the intensity of 25S rDNA hybridization signals was not uniform across the chromosomes: the signals in the chromosomes that had originated from *B. stacei* were smaller than those inherited from *B. distachyon*, especially in the genotype ABR100 (Figures 4d–f and S4). This accession had also the lowest contribution of S-genome rDNA, revealed by the Southern blot hybridization (Figure 2c,d). We also found that only the terminally located 35S rDNA loci that had originated from the D-genome were able to form secondary constrictions (Figures 4b,e; Figures S3–S16), while the proximally located S-genome rDNA loci always remained condensed (Figures 4b,e and S2–S4). At the level of the interphase nuclei, the FISH signals that corresponded with the 25S rDNA that had been inherited from *B. distachyon* tended to be...
located around the nucleolus (Figures 4c,f and S3–S16), suggesting their transcriptional activity. In contrast, the B. stacei-like 35S rDNA loci were located outside of the nucleolus (Figures 4c,f and S3–S16).

Additional FISH analysis with 25S rDNA as a probe was performed on nuclei isolated from leaves of B. hybridum ABR113 genotype. We found that only D-genome 35S rDNA loci were located adjacent to the nucleolus, while the B. stacei-derived ones were located in the nuclear periphery (Figure S17).

35S rDNA homoeologue expression in B. hybridum

To study the expression of the D- and S-genome 35S rDNA in B. hybridum we analysed the rRNA precursors, taking advantage of the ITS1 sequence divergence. A bioinformatic analysis of the ITS1 sequences from both putative ancestral species revealed the presence of a single restriction site for MluI in the ITS1 of B. distachyon, while there was no site for this enzyme in the corresponding sequence of B. stacei (Figure 5a), which made their restriction
patterns species-specific. Using the reverse-transcription cleaved amplified polymorphic sequence (RT-CAPS) approach, we analysed the expression of rDNA homoeologues in leaf samples from 16 *B. hybridum* genotypes (Figure 5b). We detected both ancestral rDNA variants, evidenced by three bands in the ‘gDNA’ lanes. However, at the level of RNA, we only observed the *B. distachyon*-inherited MluI fragments (two bands for ‘cDNA’ lanes), which implied a strong uniparental expression of the rRNA genes in the leaves of *B. hybridum*

DNA methylation analysis of 35S rDNA homoeologues in *B. hybridum*

DNA methylation patterns of the rDNA units in the 16 genotypes of *B. hybridum* and in both of the putative ancestral species were determined using a Southern blot on gDNAs that had been digested with the methylation-sensitive enzymes *Pst*I and *Xho*I, which recognize the methylated DNA sites in the CHG and CG sequence contexts, respectively. This provided a general outlook on the global differences in the cytosine methylation levels between the rDNA in *B. hybridum* that had derived from both progenitors.

In the 35S rDNA consensus sequence of *B. distachyon*, a single *Pst*I restriction site was present in the ITS2 subregion (Figure 6a). As expected, both the short and long 25S rDNA probes hybridized to a single 8.0-kb *Pst*I fragment, which reflected the full-size non-methylated rDNA unit of the D-genome (Figure 6b). Two restriction sites for *Pst*I located in the ITS2 and IGS subregions were present in the consensus rDNA sequence of the second ancestor (Figure 6a). Both the short and long 25S rDNA probes hybridized to two *Pst*I fragments: the 5.3- and 9.2-kb bands (Figure 6b), thus indicating non-methylated units. The presence of an additional, slow-migrating fragment that reflected a full-size rDNA unit may be explained by either a mutation or the methylation of cytosine in one of the two *Pst*I restriction sites. Interestingly, in the case of the *B. stacei* genotypes, a large amount of high-molecular-weight DNA hybridized to the 25S rDNA (Figure 6b). In all 16 *B. hybridum* accessions, a strong D-genome rDNA band of 8.0 kb was observed, indicating the absence of D-unit methylation at the *Pst*I site. In contrast, with the exception of the ABR117 genotype, no demethylated 5.3-kb band was visualized in *B. hybridum*, while there was a 9.2-kb partially methylated band and a smear of high-molecular-weight DNA.
Figure 4. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of *Brachypodium hybridum*. FISH with 25S rDNA (red fluorescence) and 5S rDNA (green fluorescence) as probes on the mitotic metaphase chromosomes (a,d), prometaphase chromosomes with visible secondary constrictions (b,e) and interphase nuclei (c,f) of the *B. hybridum* genotypes that showed the highest (ABR101, a–c) and the lowest (ABR100, d–f) S-genome 35S rDNA content. The S-genome 35S rDNA loci are indicated by yellow arrows. nu, nucleolus. Scale bars: 5 µm.

Figure 5. Expression analysis of the 35S rRNA genes in the 16 *Brachypodium hybridum* genotypes using the RT-PCR CAPS method.

(a) The *Mlu* restriction profile of the S-genome and D-genome ITS1 amplification products and the expected sizes of the bands after digestion.

(b) The gel restriction profiles of the ITS1 PCR products that were obtained from the leaf cDNA and gDNA from the 16 *B. hybridum* genotypes. Arrowheads denote the bands that corresponded with either S-genome 35S rDNA (S) or D-genome 35S rDNA (D).

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-weight (>10 kb) heavily methylated fragments. Thus the 35S rDNA sequences that originated from the B. stacei parent were almost completely methylated at *PstI* sites in *B. hybridum*. An additional strong band migrating in the 5-kb region (marked as ‘u’, not present in any of the ancestors) was visualized in the 19-6-2 accession (Figure 6c) and may reflect a genetic variation between the progenitors.

The bioinformatic analysis revealed the presence of a single *XhoI* restriction site in the 35S rDNA consensus sequences from the putative progenitor species of *B. hybridum* (Figure 6d). In *B. distachyon* ABR5, the 25S rDNA probe hybridized to two *XhoI* fragments: the fast-migrating band, which was 8-kb long, and a larger fragment that reflected two rDNA units, in one of which a cytosine was methylated (Figure 6e). In the *B. stacei* Bsta5 line, a single *XhoI* fragment (9.2 kb) and a smear of unresolved high-molecular-weight fragments were observed in the upper part of the membrane (Figure 6e). The *XhoI* fragments of *B. distachyon* origin were present in all of the *B. hybridum* accessions, indicating the absence of CG methylation in these homoeologues (Figure 6e). The unmethylated 9.2-kb *XhoI* fragment of *B. stacei* origin was barely visible in the ABR101, ABR113, ABR137, 20–15 and 18–15 *B. hybridum* genotypes. In contrast, a smear of high-molecular-weight methylated fragments inherited from the *B. stacei* parent was clearly visible. These results indicate that the methylation level of *B. distachyon* homoeologues remained unchanged in a hybrid while those of *B. stacei* became hypermethylated.

**DISCUSSION**

**Sequence polymorphism of the 35S rDNA units in *B. distachyon* and *B. stacei***

The genes encoding for 18S-5.8S-25S ribosomal RNAs are known to evolve in a concerted manner. Unequal crossing over and gene conversion are believed to drive the homogenization of the rDNA units, and therefore their sequence homogeneity within an array is higher than would be expected by a random mutation (Szostak and Wu, 1980; Naidoo et al., 2013). Congruent with this hypothesis, we detected a relatively low intragenomic variability of 35S rDNA units, ranging from up to three polymorphic sites in *B. distachyon* to up to 14 polymorphic sites in *B. stacei* (for SNPs frequency ≥10%). As was expected, most of the variation was in the non-coding ITS and IGS subregions, which are under weaker selection constraints than the coding rDNA sequences. Such a distribution pattern of SNPs in the rDNA units has also been observed in other organisms, e.g. *A. thaliana* (Rabanal et al., 2017), *Nicotiana* species (Lunerova et al., 2017), some mosses (Liu et al., 2013; Rosato et al., 2016) and some fungi (Ganley and Kobayashi, 2007). Interestingly, we detected a higher number of SNPs and increased ITS1 diversity in *B. stacei* rDNA than in *B. distachyon* rDNA (both genotypes). It is worth noting that SNPs were uniformly distributed in the coding- and non-coding subregions. Such a mutation pattern could be explained by the presence of putative pseudogenized 35S rDNA copies in the *B. stacei* genome. In support, the mutations affecting the abundant *Cycas revoluta* (gymnosperm) rDNA pseudogenes occurred at nearly the same frequency in the coding and non-coding subregions (Wang et al., 2016). Of note, the pseudogenized rDNA sequences were found in the human genome, mostly in the proximal regions (Robicheau et al., 2017). Similarly, the *B. stacei* rDNA loci are pericentromerically located (Borowska-Zuchowska et al., 2016), suggesting that the propensity to pseudogenization may be increased in these chromosome areas. Certainly, more genomic studies are needed to support the hypothesis.

**Variable inheritance of 35S rDNA homoeologues in the different *B. hybridum* accessions**

At least three different evolutionary scenarios have been observed for the 35S rDNA homoeologues in natural plant allopolyploids to date (Volkov et al., 2007): (i) the presence of rDNA homoeologues from both parents without any visible changes (Volkov et al., 1993); (ii) a uniparental rDNA inheritance as a result of the elimination/conversion of the rDNA units that had been inherited from the second parent (Wendel et al., 1995; Kotseruba et al., 2003; Dadejova et al., 2007; Lim et al., 2007; Volkov et al., 2017; Kolano et al., 2019); or (iii) a uniparental rDNA inheritance that is followed by rearrangements and the appearance of new rDNA classes (Clarkson et al., 2005; Kovarik et al., 2008). As is shown in the present work, the first scenario, i.e. genetic additivity, does not apply to the allotetraploid *B. hybridum*. Although the 35S rDNA units that had derived from both progenitors were present in all of the studied accessions of *B. hybridum*, a marked reduction in copy number of the S-homoeologues occurred during their evolution (Figure 2). This trend was also confirmed in the reference genotype ABR113 using the available genomic approaches. The S-genome rDNA contributions to total rDNA varied from 7 to 39% between different *B. hybridum* genotypes. This variation in the rDNA ratios most likely is related to the polyphyletic origin of *B. hybridum*. Recent dating analysis revealed that different *B. hybridum* lines arose from multiple crosses between *B. distachyon* and *B. stacei*-like ancestral species during the Quaternary, ca. 0.05–1 Ma (Catalan et al., 2012; Diaz-Perez et al., 2018), thus the elimination of the S-genome rDNA may still be in progress. Interestingly, the haplotypic diversity of S-genome units was markedly lower in *B. hybridum* compared to parental *B. stacei*, suggesting that the divergent and perhaps pseudogenized units were preferentially eliminated from the *B. hybridum* genome. Alternatively, rDNA homoeologues could still homogenize despite their inactivity.
Considering the relatively ancient origin of *B. hybridum*, the homogenization of 35S rDNA proceeds relatively slower than in other allopolyploid systems of a comparable age (Dadejova et al., 2007; Kolano et al., 2019). Interestingly, there were no intergenomic translocations between the D and S genomes of the *B. hybridum* allotetraploid (Lusinska et al., 2018). It is therefore possible that the relatively slow tempo of rDNA homogenization may reflect an overall stasis of the *B. hybridum* subgenomes.

As previously shown for many allopolyploids, one parental set of rDNA can be overwritten by the rDNA units derived from the second parental species. Such a process of sequence conversion may encompass the whole parental rDNA set (e.g. in *Nicotiana arenstii*) or just a portion of the rDNA units that had derived from one progenitor (e.g. *Nicotiana rustica*, *Nicotiana tabacum*, *Brassica napus* and *Thinopyrum intermedium*; Kovarik et al., 2008; Mahelka et al., 2013; Sochorova et al., 2017). The complete homogenization towards the D-genome rDNA units in *B. hybridum* may be excluded (at least in the accessions analysed by us), as we detected S-rDNA variants in all of the accessions studied by Southern blot hybridization.
highly stringent FISH experiments, only the proximally located S-genome rDNA loci were detected in the metaphase chromosomes of the B. hybridum ABR113 using a B. stacei-specific IGS probe (Borowska-Zuchowska et al., 2016). Although the partial homogenization of the S-genome rDNA units cannot be ruled out, the size of the FISH hybridization signals that corresponded with 25S rDNA in the B. stacei-inherited chromosomes seems to corroborate a reduction of the S-genome rDNA units. For instance, in the ABR100 accession in which the contribution of the S-units was 7% of the total 35S rDNA, we observed significantly weaker rDNA FISH signals in the B. distachyon-like chromosomes than in the B. distachyon ones.

Interestingly, the S-genome 35S rDNA units were present in the ABR117 genotype of B. hybridum. The contribution of S-genome 35S rDNA units to the total number of rDNA units was low (8%) in this accession, however, only one of the two S-genome rDNA families that were visible after BgIII restriction was detected in this genotype. Our previous analysis using FISH with 25S rDNA as a probe on the mitotic metaphase chromosomes of ABR117 revealed only the presence of terminally located D-genome rDNA units, which suggests that the S-genome repeats were completely eliminated (Hasterok et al., 2004; Borowska-Zuchowska et al., 2016). It seems that both the reduction of the number of S units and the elimination of one S-genome rDNA family prevented the visualization of these loci in situ.

In the case of the D-genome 35S rDNA loci, length polymorphism was detected among different B. hybridum genotypes. In the ABR101, ABR137 and 20–15 lines, slightly shorter restriction fragments were detected than in the parental B. distachyon rDNA. Such a polymorphism is most likely caused by the length variability in the intergenic spacers of 35S rRNA genes, as shown in other plant species, e.g. Atropa, Nicotiana and Prunus (Volkov et al., 1993; Borisjuk et al., 1997; Volkov et al., 2017). Since B. hybridum is a polyphyletic species we cannot exclude the possibility that these IGS polymorphisms were already present among the populations of progenitor species that donated their rDNAs to the allotetraploid.

**Nucleolar dominance of the D-genome rDNA in B. hybridum leaves and root tips**

As has been shown in many allopolyploids, because the repressed rDNA loci may be reactivated at certain developmental stages, ND seems to be a reversible, developmentally modulated phenomenon (Volkov et al., 2007). For instance, in synthetic Solanum allopolyploids, the reactivation of the silenced 35S rRNA genes was observed in the callus and anthers (Komarova et al., 2004). Similarly, in the allotetraploid Brassica napus, Brassica oleracea-derived rRNA genes were activated after the floral meristem transition. Interestingly, transcripts of Brassica oleracea origin were found in all floral organs of Brassica napus, i.e. the floral buds, petals, sepals, anthers and siliques (Chen and Pikaard, 1997b). However, the studies of Sochorova et al. (2017) on different Brassica napus cultivars showed only a weak expression of Brassica oleracea-inherited rDNA in the flower buds of only a few of accessions that were studied, thus the developmental regulation of ND may even be genotype specific. This fact is further supported by the Brassica napus cultivar ‘Norin 9’, in which a co-dominance of Brassica rapa- and Brassica oleracea-inherited 35S rDNA was present in the leaves, roots and flower buds (Sochorova et al., 2017). A co-dominant expression profile of the parental rDNA in Brassica napus was also observed in the meristematic cells of 2- and 3-day-old roots (Hasterok and Maluszynska, 2000). In Arabidopsis suecica, which is another dicot allotetraploid that exhibits ND, a gradual silencing of the A. thaliana-derived 35S rDNA loci occurred during early postembryonic development (Pontes et al., 2007). In contrast, in wheat–rye hybrids, it was found that the ND may be fully established as early as 4–5 days after fertilization (Castilho et al., 1995), thus ND regulation may vary significantly between allopolyploid species.

To date, D- and S-genome rDNA activity was studied mainly in the reference genotype of B. hybridum, ABR113. It was shown that the B. distachyon-inherited 35S rRNA genes are dominant at all developmental stages studied, including vegetative (Borowska-Zuchowska et al., 2016; Borowska-Zuchowska and Hasterok, 2017) and generative tissues (Borowska-Zuchowska et al., 2019). Unlike in diploid allopolyploids, e.g. Brassica napus (Chen and Pikaard, 1997b), in B. hybridum the repressed S-genome rRNA genes are not reactivated in generative tissues. Moreover, it was found that in contrast to developmentally unstable ND in Brassicaceae polyploids, ND is abolished neither in different tissues of immature embryos nor during early postembryonic development in B. hybridum (Borowska-Zuchowska et al., 2019). In this work, we found that only D-genome 35S rDNA loci are transcriptionally active in the leaf tissue of B. hybridum. We also revealed that the S-genome loci were unable to form nucleoli in the interphase and correspondingly were not located within the secondary constrictions of the chromosomes in the root-tip meristem of any of the 16 accessions studied. Since the ability of the 35S rDNA loci to form a nucleolus is an indirect measure of their transcriptional activity (Shaw, 2013), we conclude that a strong uniparental expression of the D-genome genes is present in both the leaf and root-tip cells of all of the studied B. hybridum genotypes. Based on the data presented here, the S-genome rDNA repression is not dependent on the ancestral rDNA ratios in the studied B. hybridum accessions.
Involvement of DNA methylation in the suppression of S-genome rDNA

Using two different enzymes that recognize methylated cytosines in the CHG and CG contexts, we found a differential DNA methylation status of the 35S rDNA homoeologues in B. hybridum (Figure 6). A large portion of the D-genome 35S rDNA units was not methylated at the sites studied. The S-genome bands that reflected the unmethylated rDNA loci from the second ancestral species, however, were either absent or significantly weaker than the D-genome ones (Figure 6). These data are in agreement with studies on numerous plant hybrids and allopolyploids in which the involvement of DNA methylation in establishing and maintaining the ND was shown (Vieira et al., 1990; Houchins et al., 1997; Komarova et al., 2004; Lawrence et al., 2004; Costa-Nunes et al., 2010; Dobesova et al., 2015). In the allotetraploid A. suecica, the small interfering RNA (siRNA)-directed DNA methylation pathway is required to inactivate the 35S rDNA of an A. thaliana origin (Preuss et al., 2008). According to the proposed model of rRNA gene silencing, the siRNA molecules direct de novo DNA methyltransferase DRM2 activity to both the promoter and regulatory sequences that are present within the IGS of the targeted 35S rDNA unit. CHH methylation is then recognized by the methylcytosine-binding protein MBD6, which is thought to react with other co-repressors (Preuss et al., 2008; Costa-Nunes et al., 2010). This crosstalk may mediate methylation in both the CHG and CG sequence contexts in the targeted rDNA units (Guo and Han, 2014). Thus, significant differences in the DNA methylation levels between the transcriptionally active and repressed rDNA units are observed in different plant species that exhibit ND (Neves et al., 1995; Houchins et al., 1997; Chen and Pikaard, 1997a; Komarova et al., 2004; Guo and Han, 2014).

Similar differences in the DNA methylation patterns between the 35S rDNA loci from the D and S genomes in B. hybridum were revealed using the molecular cytogenetic approaches (Borowska-Zuchowska and Hasterok, 2017). The repressed S-genome units were characterized by a significantly higher DNA methylation level than the D-genome units, which is in agreement with the Southern blot analysis that is presented here (Figure 6). Interestingly, it was also revealed that the S-genome rDNA units were not reactivated after the global hypomethylation of the B. hybridum genome induced by 5-azacytidine (Borowska-Zuchowska and Hasterok, 2017), which strongly suggests that DNA methylation alone is not sufficient to establish and maintain the ND in the studied allotetraploid. We speculate that the non-methylated B. stacei units were methylated de novo in the allotetraploid as a result of epigenetic silencing and were shifted to the heterochromatic fraction.

To date, neither reverse dominance, i.e. the silencing of the D-genome rDNA loci, nor co-dominance of the D- and S-genome rDNA have been reported in any tissue of B. hybridum (Idziak and Hasterok, 2008; Borowska-Zuchowska et al., 2016; Borowska-Zuchowska et al., 2019), which suggests that the S-genome rDNA units may be irreversibly inactivated. However, investigations of the activity of the 35S rDNA homoeologues during early stages of embryo development in B. hybridum, as well as further analyses of the rDNA expression in different B. hybridum genotypes that have a more balanced ratio of parental rDNA copies, need to be performed in order to confirm our hypothesis. It is possible that strong silencing and methylation may be related to the proximal, pericentromeric position of the B. stacei NORs. In support of this, the ‘always’ dominant D-genome NORs are located at the chromosome termini. There is some evidence that the chromosomal position of the rDNA locus may influence its activity (Schubert and Künzel, 1990; Neves et al., 2005; Báez et al., 2019). For instance, the 5S rDNA arrays that are located proximally to the centromeres are more methylated than the units that are located in distal positions in A. thaliana (Mathieu et al., 2002). The relationship between the chromosomal position of NOR, array homogeneity, chromatin condensation, epigenetic state and rDNA behaviour in allopolyploids requires further investigation.

EXPERIMENTAL PROCEDURES

Plant material

Twenty genotypes of three Brachypodium species, B. hybridum, B. distachyon and B. stacei, were used in this study. Information on their origins and basic cytogenetic characteristics are listed in Table 2. Nine B. hybridum genotypes were derived from the T1 generation of the germplasm that we had recently collected in climatically diverse habitats in Turkey (Table 2). The seeds were sown in pots with soil mixed with vermiculite (3:1, w/w) and grown at 22°C and a 16-h light/8-h dark photoperiod in a greenhouse.

For the cytogenetic analyses, the seeds were grown on a filter paper moistened with tap water for 3 days at 20–22°C in the dark. Whole seedlings with approximately 2-cm-long roots were immersed in ice-cold water for 24 h, fixed in 3:1 (v/v) methanol: glacial acetic acid at 4°C overnight and stored at –20°C.

In silico reconstruction of the B. distachyon and B. stacei 35S rDNA units, SNP calling and copy-number estimation

To annotate the B. distachyon and B. stacei 35S rDNA units, the following sequences were used as queries in the Bd21 and ABR114 genomic sequence screening:

i 18S rDNA sequence from rice (GenBank ID: X00755.1) (Takaïwa et al., 1984);
ii 25S rDNA sequence from rice (GenBank ID: M11585.1) (Takaïwa et al., 1985);
iii B. distachyon and B. stacei ITS1-5.8S-ITS2 sequences (GenBank IDs: JN187608.1 and JN187611) (Catalan et al., 2012); and
iv B. distachyon and B. stacei IGSs (GenBank IDs: KX263276 and KX263278) (Borowska-Zuchowska et al., 2016).
The sequences were ordered into 18S-ITS1-5.8S-ITS2-25S-IGS rDNA units using CLC GENOMICS WORKBENCH (Qiagen, https://www.qiagen.com). The complete units were used as the reference sequences in the B. distachyon and B. stacei raw Illumina reads mapping using the 'Map Read Reference' tool. The following genomic libraries were used: B. distachyon Bd21 (SRP081388), B. distachyon ABR5 (SRP001538) and B. stacei ABR114 (SRP025051). All of the reads that contained any ambiguous nucleotides; were shorter than 100 bp (in the case of the Bd21 and ABR5 libraries) or were shorter than 150 bp (in the case of the ABR114 library); and/or failed to pass a quality score limit of P ≥ 0.05 were removed using the 'TRIM' command in CLC GENOMICS WORKBENCH. The mapping parameters were as follows: mismatch cost, 2; insertion cost, 3; deletion cost, 3; with the length fraction set at 0.5 and the similarity fraction set at 0.8. The consensus 35S rDNA sequences were then extracted from the mapped reads.

For SNP calling, the ‘Basic Variant Detector’ tool in CLC GENOMICS WORKBENCH was used. The parameters were as follows: minimum read coverage, 300; minimum read count, 30; and minimum frequency (the ratio of ‘the number of countable reads supporting the allele’ to ‘the number of countable reads that covered the position of the variant’); ≥10% for high-frequency SNPs or ≥1% for low-frequency SNPs.

The copy number of the 35S rDNA units was calculated from the NGS read count using the following scheme: (i) the genome proportion (GP) was calculated from the number of mapped reads to the 18S rDNA consensus sequences divided by the total number of reads in percentages; (ii) the genome space (GS) of 35S rDNA was determined using the formula GP x size of the genome in Mb (309, 276 and 620 Mb for B. distachyon, B. stacei and B. hybrیدum, respectively); and (iii) the copy number of 35S rDNA was calculated as the GS value divided by the size of a 18S rDNA in Mb (0.00181 Mb).

In silico rDNA haplotypic analysis of the B. hybrídum reference genotype

A short 50-bp-long ITS1 fragment that showed a 10% sequence variation between the D- and S-genome 35S rDNA consensus sequences was selected for the haplotype analysis. The B. stacei-like ITS1 fragment was BLASTed against a local whole-genomic B. hybrídum database (genotype ABR113, accession no. SRP025053). The hit sequences were trimmed for lengths (≥50 nt) and quality (no ambiguous nt were allowed; quality score limit P ≥ 0.05), and then sampled for approximately 220 reads. The sampled reads were aligned using the ‘Multiple Alignment’ tool in CLC GENOMICS WORKBENCH with the following parameters: gap open cost, 10; gap extension cost, 1; end gap cost, as any other; and alignment mode, very accurate. A neighbour-joining phylogenetic tree was constructed in CLC GENOMICS WORKBENCH using the Jukes-Cantor distance algorithm and a bootstrap of 100 replicates.

Intragenomic diversity was estimated by calculating the number of haplotypes in the aligned sequences. Each alignment contained 400 reads of each B. stacei, B. distachyon and B. hybrídum ABR113, D-genome ITS1, and 326 reads from B. hybrídum ABR113, S-genome ITS1. All sequences were of the same length (50 bp). DNA polymorphisms were calculated using a stand-alone DNA SP6 program (Rozas et al., 2017). Haplotype diversity was calculated as the number of haplotypes divided by number of sequences.

DNA isolation and Southern blot hybridization

Total genomic DNA (gDNA) from the leaves from 1-month-old plants of diploid and allotetraploid Brachypodium species (Table 2) was isolated using a cetyltrimethylammonium bromide (CTAB) buffer as described by Doyle (1991). The gDNA was treated with 50 µg RNase for 1 h at 65°C. The samples were extracted with chloroform/isooamyl alcohol (24:1) and precipitated with isopropanol. The purified gDNAs (0.5–1 µg per sample) were digested with the restriction enzymes BglII, PsI or XhoI, separated by gel electrophoresis on 1% (w/v) agarose gel and alkali-blotted onto nylon membranes (Hybond XL; GE Healthcare Life Sciences, now Cytiva, https://www.cytiva.com). The membranes were hybridized with a 32P-labelled DNA probes (DekaLabel kit; ThermoFisher Scientific, https://www.thermofisher.com) using either the approximately 220-bp PCR product derived from the 3’ end of the 25S rDNA of Nicotiana tabacum (tobacco); short probe; red rectangle in Figure 2a; forward primer, 5'-GAATTCCACCAAGTGTGGAT-3'; reverse primer, 5'-AGGGCTTACGTTCAATAC-3') or a long 25S rDNA probe of approximately 2470 bp (long probe; green rectangle in Figure 2a) that contained an EcoRI-EcoRI fragment of the 25S rRNA gene from Solanum lycopersicum (tomato) (Kiss et al., 1988). The Southern hybridization followed the procedure described in Kovarik et al. (2005). The hybridization bands were visualized with a phosphorimager (Typhoon 9410; GE Healthcare, https://www.gehealthcare.com) and the radioactivity of the bands was quantified with IMAGEQUANT (GE Healthcare).

FISH on squashed mitotic preparations, image acquisition and analysis

The chromosomes were prepared as described in Jenkins and Hasterok (2007) from meristematic root-tip cells of 14 B. hybrídum genotypes. For the preparation of the slides with nuclei isolated from leaf tissue of B. hybrídum ABR113, fresh, young leaves were washed briefly in PBS buffer, then washed in TRIS buffer (10 mM TRIS–HCl, pH 7.5, 10 mM Na2-EDTA, 100 mM NaCl) for 20 min and chopped with a razor blade in LB01 buffer (15 mM TRIS–HCl, pH 7.5, 2 mM Na2-EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1% Triton X-100) on ice. The suspension of nuclei was filtered through a mesh filter (pore size of 30 µm), centrifuged (700 g, 4°C, 2 x 3 min) in 0.1% Triton X-100 in 1 x PBS to remove the chloroplasts and dropped onto microscopic slides that had been cooled to about 0°C. The slides were air-dried and stored at –20°C until use.

Due to the highly conserved nature of the rDNA coding sequences, a 2.3-kb ClaI subclone of the 25S rDNA of A. thaliana (Unfried and Gruendler, 1990) and the clone pTa794, which contained the SS rRNA gene from wheat (Gerlach and Dyer, 1980), were used as the probes to detect the 35S and SS rRNA gene clusters. The clones were labelled by nick translation with either tetramethylrhodamine-5-dUTP (Roche, https://www.roche.com) or digoxigenin-11-dUTP (Roche). Fluorescence in situ hybridization
(FISH) was performed according to the protocol described by Idziak et al. (2011). The precipitated probes were dissolved directly in a hybridization mixture containing 50% deionized formamide and 20% dextran sulphate in 2 × SSC, denatured at 75°C for 10 min and applied to the slides. The preparations were allowed to hybridize at 37°C for 16–20 h. The hybridization signals were detected using green fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibodies (Roche) or, in the case of the probes that had been labelled with tetramethylrhodamine-5-dUTP, directly visualized. The chromosomes were counterstained with 2.5 μg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, https://vectorlabs.com). Images from slides with root-tip cells were acquired using a Zeiss Axioscop 2 Plus microscope equipped with an AxioCam HRm monochromatic camera, while images of the interphase nuclei isolated from B. hybridum leaves were acquired using an Olympus BX 51 confocal system (Olympus, https://www.olympus-global.com). All images were processed using IMAGEJ (https://imagej.nih.gov/ij/).

35S rDNA expression analysis

The expression levels were analysed using the RT-CAPS method according to the protocol of Sochorova et al. (2017). In brief, total RNA was isolated from fresh leaves that had been collected from 1-month-old plants using an RNeasy Plant Mini Kit (Qiagen). Contaminating DNA was removed from the samples using TURBO DNA-free (Ambion, now ThermoFisher Scientific). The reverse transcription reactions (20 μl) typically contained 1 μg of total RNA and 200 units of PrimeScript reverse transcriptase (Takara, https://www.takarabio.com), and were performed using the conditions recommended by the supplier. For ITS1 region amplification using primer pairs ITS1f (Forster et al., 2005) were used. The ITS1 PCR products were digested with a MluI restriction enzyme in an amplification mixture. For each, a control PCR, with gDNA as the template, followed by the MluI restriction of the ITS1 PCR product was performed.

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AUTHOR CONTRIBUTIONS

All of the authors contributed their intellectual input and assistance to this study. NB-Z, AK and RH conceived of and designed the study, NB-Z, AK and ER performed the experiments. SG and JV provided the NGS data. NB-Z, AK, ER and RH analysed the data. MT, GST and RH collected the plant material from Turkey. NB-Z, RH, AK and JV acquired the financial support. NB-Z, ER and RH wrote the article, which was edited and approved by all of the authors.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the article and its supporting materials.

SUPPORTING INFORMATION

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

Figure S1. The quantity of 35S rDNA in the genomes of Brachypodium distachyon and Brachypodium stacei that were analysed by slot-blot hybridization.

Figure S2. Alignment of 222 50-bp-long ITS1 variants from Brachypodium hybridum ABR113, based upon which the neighbour-joining phylogenetic tree in Figure 3(b) was constructed.

Figure S3. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum ABR101 genotype.

Figure S4. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum ABR110 genotype.

Figure S5. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum ABR115 genotype.

Figure S6. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum ABR116 genotype.

Figure S7. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum ABR137 genotype.

Figure S8. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 20-15 genotype.

Figure S9. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 19-13-2 genotype.

Figure S10. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 19-6 genotype.

Figure S11. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 18-17 genotype.

Figure S12. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 18-15 genotype.

Figure S13. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 18-13-2 genotype.

Figure S14. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 11-8 genotype.

Figure S15. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 11-1-1 genotype.

Figure S16. The distribution of the 35S and 5S rDNA loci in the mitotic metaphase chromosomes and interphase nuclei of the Brachypodium hybridum 11-1-1 genotype.

Figure S17. The distribution of the 35S rDNA loci in the nuclei isolated from leaves of the Brachypodium hybridum ABR113 genotype.
Table S1. The distribution of high-frequency SNPs (≥10%) in the 35S rDNA sequences of the Brachypodium distachyon genotype ABR5.

Table S2. The distribution of low-frequency SNPs (≥1%) in the 35S rDNA sequences of the Brachypodium distachyon genotype ABR5.

Table S3. The distribution of high-frequency SNPs (≥10%) in the 35S rDNA sequences of the Brachypodium distachyon genotype Bd21.

Table S4. The distribution of low-frequency SNPs (≥1%) in the 35S rDNA sequences of the Brachypodium distachyon genotype Bd21.

Table S5. The distribution of high-frequency SNPs (≥10%) in the 35S rDNA sequences of the Brachypodium stacei genotype ABR114.

Table S6. The distribution of low-frequency SNPs (≥1%) in the 35S rDNA sequences of the Brachypodium stacei genotype ABR114.

REFERENCES

Baer, M., Souza, G. and Guerra, M. (2019) Does the chromosomal position of 35S rDNA sites influence their transcription? A survey on Nothocar- dium species (Amaryllidaceae). Genet. Mol. Biol. https://doi.org/10.1590/ 1678-6665-GMB-2018-0194.

Becker, V.V., Barisic, D., Horvat, T., Maglica, Z., Vojta, A. and Zoldos, V. (2014) Inhibition of DNA methylation alters chromatin organization, nuclear positioning and activity of 45S rDNA loci in cycling cells of Q. robur. PLoS One, 9, e103954.

Borjus, R.V., Davidjuk, Y.M., Kostishin, S.S., Miroshnichenko, G.P., Velasco, R., Hemieben, V. and Volkov, R.A. (1997) Structural analysis of rDNA in the genus Nicotiana. Plant Mol. Biol. 35, 655-660.

Borowska-Zuchowska, N. and Hasterok, R. (2017) Epigenetics of the prefer- ential silencing of Brachypodium stacei-oligomeric 35S rDNA loci in the allotetraploid grass Brachypodium hybridum. Sci. Rep. 7, 5260.

Borowska-Zuchowska, N., Kwasiwinski, M. and Hasterok, R. (2016) Cyto- molecular analysis of ribosomal DNA evolution in a natural allotetraploid Brachypodium hybridum and its putative ancestors - dissecting complex repetitive structure of intergenic spacers. Front. Plant Sci. 7, 1499.

Borowska-Zuchowska, N., Robaszkiewicz, E., Wolny, E., Betekhtin, A. and Hasterok, R. (2019) Ribosomal DNA loci derived from Brachypodium stacei are switched off for major parts of the life cycle of Brachypodium hybridum. J. Exp. Bot. 70, 805-815.

Castilho, A., Queiroz, A., Neves, N., Barao, A., Silva, M. and Viegas, W. (1995) The developmental stage of inactivation of rye origin rRNA genes in the embryo and endosperm of wheat x rye F1 hybrids. Chromosome Res. 3, 169-174.

Catalan, P., Muller, J., Hasterok, R., Jenkins, G., Mur, L.A., Langdon, T., Betekhtin, A., Siwinska, D., Pimentel, M. and Lopez-Alvarez, D. (2012) Evolution and taxonomic split of the model grass Brachypodium dis- tachyon. Ann. Bot. 109, 385-405.

Chen, Z.J. and Pikaard, C.S. (2003) Epigenetic silencing of RNA polyme- rase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. Gene Dev. 17, 2124-2136.

Chen, Z.J. and Pikaard, C.S. (1997b) Transcriptional analysis of nucleolar dominance in polyploid plants: biased expression/silencing of progenitor rRNA genes is developmentally regulated in Brassica. Proc. Natl. Acad. Sci. USA, 94, 3442-3447.

Clarkson, J.J., Lim, K.Y., Kovarik, A., Chase, M.W., Knapp, S. and Leitch, A.R. (2005) Long-term genomic diploidization in allopolyploid Nicotiana section Repandae (Solanaceae). New Phytol. 168, 241-252.

Costa-Nunes, P., Kim, J.Y., Hong, E. and Pontes, O. (2014) The cytological and molecular role of DOMAINS REARRANGED METHYLTRANSFER- ASE3 in RNA-dependent DNA methylation of Arabidopsis thaliana. BMC Res. Notes, 7, 721.

Costa-Nunes, P., Pontes, O., Preuss, S.B. and Pikaard, C.S. (2010) Extra- views on RNA-dependent DNA methylation and MBD2-dependent heterochromatin formation in nucleolar dominance. Nucleus, 1, 254-259.

Dadejova, M., Lim, K.Y., Soucova-Skalicka, K., Matyasek, R., Grandbastien, M.A., Leitch, A. and Kovarik, A. (2007) Transcription activity of rRNA genes correlates with a tendency towards intergenic homogenization in Nicotiana allotetraploids. New Phytol. 174, 658-668.

Diaz-Perez, A., Lopez-Alvarez, D., Sancho, R. and Catalan, P. (2018) Recon- structing the origins and the biogeography of species' genomes in the highly reticate allopolyploid-rich model grass genus Brachypodium using minimum evolution, coalescence and sequence likelihood approaches. Mol. Phylogenet. Evol. 127, 256-271.

Dobesova, E., Malinska, H., Matyasek, R., Leitch, A.R., Solits, D.E., Solits, P.S. and Kovarik, A. (2015) Silenced RNA genes are activated and substitute for partially eliminated active homeologs in the recently formed allotetraploid, Tragopogon mirus (Asteraceae). Heredity, 114, 356-365.

Dolezel, J., Bartos, J., Voglmayr, H. and Greilhuber, J. (2003) Nuclear DNA content and genome size of trit and human. Cytometry A 51, 127-128.

Doyle, J. (1991) DNA protocols for plants. In Molecular Techniques in Tax- onomy (Hewitt, G.M., Johnston, A.W.B. and Young, J.P.W., eds), Berlin, Heidelberg: Springer, Berlin Heidelberg, pp. 283-293.

Earley, K., Lawrence, R.J., Pontes, O., Reuther, R., Enciso, A.J., Silva, M., Neves, N., Gross, M., Viegas, W. and Pikaard, C.S. (2006) Erasure of his- tone acetylation by Arabidopsis HDA6 mediates large-scale gene silenc- ing in nucleolar dominance. Gene Dev. 20, 1283-1293.

Eickbush, T.H. and Eickbush, D.G. (2007) Finitely orchestrated movements: evolution of the ribosomal RNA genes. Genetics, 175, 477-485.

Frierman, M., Chen, Z.J., Saez-Vasquez, J., Shen, L.A. and Pikaard, C.S. (1999) RNA polymerase I transcription in a Brassica interspecific hybrid and its progenitors: tests of transcription factor involvement in nuclear dominance. Genetics, 152, 451-460.

Ganley, A.R.D. and Kobayashi, T. (2007) Highly efficient concerted evolution in the ribosomal DNA repeats: total rDNA repeat variation revealed by whole-genome shotgun sequence data. Genome Res. 17, 184-191.

Garcia, S., Kovarik, A., Leitch, A.R. and Garnatje, T. (2017) Cyto genetic fea- tures of rRNA genes across land plants: analysis of the Plant rDNA data- base. Plant J. 89, 1020-1030.

Gerlach, W.L. and Dyer, T.A. (1980) Sequence organization of the repeating units in the nucleus of wheat which contains 5S ribosomal RNA genes. Nucleic Acids Res. 8, 4851-4865.

Guo, X. and Han, F. (2014) Asymmetric epigenetic modification and elimina- tion of rDNA sequences by polyploidization in wheat. Plant Cell, 26, 4311-4327.

Hasterok, R., Draper, J. and Jenkins, G. (2004) Laying the cytotoxic foundations of a new model grass, Brachypodium distachyon (L.) Beauv. Chromosom Res. 12, 397-403.

Hasterok, R. and Maluszynska, J. (2000) Nucleolar dominance does not occur in root tip cells of allotetraploid Brassica species. Genome, 43, 574-579.

Houchins, K., O’Dell, M., Flavell, R.B. and Gustafson, J.P. (1997) Cytosine methylilation and nucleolar dominance in cereal hybrids. Mol. Gen. Genet. 255, 294-301.

IBI (2010) Genome sequencing and analysis of the model grass Brachy- podium distachyon. Nature, 463, 763-768.

Idziak, D. and Pikaard, C.S. (1997b) Transcriptional analysis of nucleolar dominance in polyploid plants: biased expression/silencing of progenitor rRNA genes is developmentally regulated in Brassica. Proc. Natl Acad. Sci. USA, 94, 3442-3447.

Kotseruba, V., Gernand, D., Meister, A. and Houben, A.

Kovarik, A., Dadejova, M., Lim, K.Y., Chase, M.W., Clarkson, J.J., Knapp, S. and Leitch, A.R. (2008) Evolution of rDNA in Nicotiana allopolyploids: a
potential link between rDNA homogenization and epigenetics. Ann. Bot. 101, 816–823.

Kovarik, A., Pires, J.C., Leitch, A.R., Lim, K.Y., Sherwood, A.M., Matysyak, R., Rocca, J., Soitits, D.E. and Soitis, P.S. (2005) Rapid concerted evolution of nuclear ribosomal DNA in two Tragopogon allopolyploids of recent and recurrent origin. Genetica, 169, 931–944.

Lawrence, R.J., Earley, K., Pontes, O., Silva, M., Chen, Z.J., Neves, N., Viegas, W. and Pikaard, C.S. (2004) A concerted DNA methylation/histone methylation switch regulates rDNA gene dosage control and nucleolar dominance. Mol. Cell, 13, 599–609.

Leitch, A.R. and Leitch, I.J. (2008) Genomic plasticity and the diversity of polyploid plants. Science, 320, 481–483.

Lim, K.Y., Matysyak, R., Kovarik, and A. Leitch, (2007) Parental origin and genome evolution in the allopolyploid Iris versicolor. Ann. Bot. 100, 219–224.

Liu, B. and Wendel, J.F. (2003) Epigenetic phenomena and the evolution of plant allopolyploids. Mol. Phylogenet. Evol. 29, 365–379.

Liu, Y., Forrest, L.L., Bainard, J.D., Budke, J.M. and Goffinet, B. (2013) Organellar genome, nuclear ribosomal DNA repeat unit, and microsatellites isolated from a small-scale of 454 GS FLX sequencing on two mosses. Mol. Phylogenet. Evol. 66, 1089–1094.

Lunero, J., Renny-Byfield, S., Matyasek, R., Leitch, A. and Kovarik, A. (2017) Concerted evolution rapidly eliminates sequence variation in rDNA coding regions but not in intragenic spacers in Nicotiana tabacum allotetraploid. Plant Syst. Evol. 303, 1043–1060.

Lusinska, J., Majka, J., Betekhtin, A., Susek, K., Wolny, E. and Hasterok, R. (2018) Chromosome identification and reconstruction of evolutionary rearrangements in Brachypodium distachyon. B. stacei and B. hybridum. Ann. Bot. 122, 445–459.

Mahelka, V., Kopecky, D. and Baum, B.R. (2013) Contrasting patterns of evolution of 45S and 4S rDNA families uncover new aspects in the genome constitution of the agronomically important grass Thinopyrum intermedium (Triticeae). Mol. Biol. Evol. 30, 2065–2086.

Mathieu, O., Picard, G. and Tournement, S. (2002) Methylation of a euchromatin-heterochromatin transition region in Arabidopsis thaliana chromosome 5 left arm. Cell 108, 455–466.

Mohannath, G., Pontvianne, F. and Pikaard, C.S. (2016) Selective nucleolar organizer inactivation in Arabidopsis is a chromosomal position-effect phenomenon. Proc. Natl. Acad. Sci. USA, 113, 13426–13431.

Naidoo, K., Steenkamp, E.T., Coetzee, M.P., Wingfield, M.J. and Wingfield, B.D. (2013) Concerted evolution in the ribosomal RNA cistron. PLoS One, 8, e69355.

Navashin, M. (1934) Chromosomal alterations caused by hybridization and their bearing upon certain general genetic problems. Cytologia, 5, 169–203.

Neves, N., Delgado, M., Silva, M., Caperta, A., Morais-Cecilio, L. and Viegas-Wang, W., Ma, L., Becher, H., Garcia, S., Kovarikova, A., Leitch, I.J., Leitch, A.R. and Kovarik, A. (2016) Astonishing 35S rDNA diversity in the gymnosperm species Cypres revoluta Thunb. Chromosoma, 125, 883-699.

Phillips, R.L., Kleese, R.A. and Wang, S.S. (1977) Nucleolar organizer region of maize (Zea mays L.) - chromosomal site of DNA complementary to ribosomal RNA. Chromosoma 36, 79–88.

Pikaard, C.S. (2000) Nucleolar dominance: uniparental gene silencing on a multi-megabase scale in genetic hybrids. Plant Mol. Biol. 43, 163–177.

Pontes, O., Lawrence, R.J., Silva, M., Preuss, S., Costa-Nunes, P., Earley, K., Neves, N., Viegas, W. and Pikaard, C.S. (2007) Postembryonic establishment of megabase-scale gene silencing in nucleolar dominance. PLoS One, 2, e1157.

Preuss, S.B., Costa-Nunes, P., Tucker, S. et al. (2008) Multimegalbase silencing in nucleolar dominance involves siRNA-directed DNA methylation and specific methylocysine-binding proteins. Mol. Cell, 32, 673–684.

Rabanal, F.A., Mandakova, T., Soto-Jimez, L.M. et al. (2017) Epistatic and allelic interactions control expression of ribosomal RNA gene clusters in Arabidopsis thaliana. Genome Biol. 18, 75.

Robichue, B.M., Susko, E., Harrigan, A.M. and Snyder, M. (2017) Ribosomal RNA genes contribute to the formation of pseudogenes and junk DNA in the human genome. Genome Biol. Evol. 9, 380–397.

Rosato, M., Kovarik, A., Garilleti, R. and Rossello, J.A. (2016) Conserved organisation of 45S rDNA sites and rDNA gene copy number among major clades of early land plants. PLoS One, 11, e0162544.

Rozas, J., Ferrer-Mata, A., Sanchez-Delbarrio, J.C., Guiro-Rico, S., Librado, P., Ramos-Onsins, S.E. and Sanchez-Gracia, A. (2017) DnaSP 6: DNA sequence polymorphism analysis of large data sets. Mol. Biol. Evol. 34, 3299–3302.

Schubert, I. and Kunzel, G. (1990) Position-dependent NOR activity in barley. Chromosoma, 99, 352–359.

Shaw, P. (2013) The plant nucleus. In Plant Genome Diversity, vol 2, (Leitch, I.J., Greilhuber, J., Doelezel, J. and Wendel, J.F., eds). Wien: Springer-Verlag, pp. 65–76.

Sochorova, J., Coriton, O., Kuderova, A., Lunero, J., Chevre, A.M. and Kovarik, A. (2017) Gene conversion events and variable degree of homogenization of rDNA loci in cultivars of Brassica napus. Ann. Bot. 119, 13–26.

Ssymonova, R. (2019) Integrative rDNAomics-Importance of the oldest repetitive fraction of the eukaryote genome. Genes, 10(5), 345.

Szostak, J.W. and Wu, R. (1980) Unequal crossing over in the ribosomal DNA of Saccharomyces cerevisiae. Nature, 284, 426–430.

Takaiwa, F., Oono, K., Iida, Y. and Sugiiura, M. (1985) The complete nucleotide sequence of a rice 25S rRNA gene. Gene, 37, 255–259.

Takaiwa, F., Oono, K. and Sugiiura, M. (1984) The complete nucleotide sequence of a rice 17S rRNA gene. Nucleic Acids Res. 12, 5441–5448.

Unfried, I. and Gruenderl, P. (1990) Nucleotide sequence of the 5.8S and 25S rRNA genes and of the internal transcribed spacers from Arabidopsis thaliana. Nucleic Acids Res. 18, 4011.

Vieira, R., Mellosampayo, T. and Viegas, W.S. (1990) 1R chromosome nucleolar organizer region activation by 5-azacytidine in wheat x rye hybrids. Genome, 33, 707–712.

Volkov, R.A., Komarova, N.V. and Hemleben, V. (2007) Ribosomal DNA in plant hybrids: inheritance, rearrangement, expression. Syst. Biodivers. 5, 261–276.

Volkov, R.A., Kostishin, S.S. and Panchuk, I.I. (1993) rDNA organization in species from subfamily Prunoideae. Mol. Biol. 27, 1356–1367.

Wendel, J.F., Lisch, D., Hu, G. and Mason, A.S. (2010) The long and short of doubling down: polyploidy, epigenetics, and the temporal dynamics of genome fractionation. Curr. Opin. Genet. Dev. 49, 1–7.

Wendel, J.F., Schnabel, A. and Seelanan, T. (1995) Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (Gossypium). Proc. Natl. Acad. Sci. USA, 92, 280–284.

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