Trends in Plant Science

CellPress OPEN ACCESS

Review

Switch them off or not: selective rRNA gene repression in grasses

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Nucleolar dominance (ND) is selective epigenetic silencing of 35-48S rDNA loci. In allopolyploids, it is frequently manifested at the cytogenetic level by the inactivation of nucleolar organiser region(s) (NORs) inherited from one or several evolutionary ancestors. Grasses are ecologically and economically one of the most important land plant groups, which have frequently evolved through hybridisation and polyploidisation events. Here we review common and unique features of ND phenomena in this monocot family from cytogenetic, molecular, and genomic perspectives. We highlight recent advances achieved by using an allotetraploid model grass, *Brachypodium hybridum,* where ND commonly occurs at a population level, and we cover modern genomic approaches that decipher structural features of core arrays of NORs.

Highlights

Selective silencing of 35S rRNA genes (35S rDNA) via nucleolar dominance (ND) is accompanied by epigenetic repatterning, including extensive DNA methylation of promoter sequences through RNA-directed DNA methylation and histone deacetylation.

Although ND was first observed nearly a century ago, the molecular mechanisms behind when and how ancestral rDNA sets are chosen for silencing are still unknown.

Recent advances in technologies such as single-molecule real-time and nanopore sequencing and optical mapping allow long (>10 kb) DNA segments, including whole rDNA units, to be analysed, which improves characterisation of 35S rDNA loci in plants.

Brachypodium hybridum is a unique model species for studying the mechanisms of ND in grasses due to its small nuclear genome size and the presence of only one 35S rDNA locus per subgenome.

Nucleolar dominance: 90 years ago to present

A **nucleolus** (see Glossary) is formed in the vicinity of the chromosomal locus whose tandemly repeated **35-48S rDNA** is transcribed (Figure 1) [1,2]. 35-48S rRNA genes encode 18S, 5.8S, and 25-28S rRNA molecules, which constitute essential components of ribosomes and which are vital for the viability of the cell. In many **allopolyploids** and interspecific hybrids, nucleoli are formed through the transcription and processing of 35-48S rRNA genes from one of two or more evolutionary ancestors. This phenomenon of selective repression, called **Nucleolar dominance (ND)**, is a manifestation of subgenome dominance in these organisms (Figure 1) [3–10] and was observed in the representatives of both plant and animal subkingdoms [1].

ND was first demonstrated in the first half of the past century by Navashin [11], who observed the loss of **secondary constrictions** on chromosomes that had been inherited from one particular parent in several hybrids of *Crepis* (Asteraceae). Interestingly, in backcrosses of the hybrids that exhibit this phenomenon to their underdominant parents, secondary constrictions were restored, showing that ND is a reversible process [11]. On the basis of Navashin's results and studies on maize (*Zea mays*) translocation lines, McClintock [12] suggested that particular species differ in their ability to form a nucleolus, implying that a hierarchy of ND exists. For example, if species A dominates species B, and species B is dominant to species C, then species A should always be dominant over species C. Later molecular studies revealed that ND results from expressing only one parental set of rRNA genes in *Xenopus* (frog) hybrids [13]. Transcriptionally active 35S rRNA genes within secondary constrictions are responsible for nucleolus assembly. By contrast, their inactive counterparts are located in heterochromatic knobs [14].

Since these pioneering studies, much attention has been given to determine whether ND is common or exceptional in plant hybrids and allopolyploids across different genera. Another aim was to discover the molecular mechanisms of ND to answer the question how parental 35S rDNA loci are selected for silencing. Although plant and animal hybrids have been studied for a considerable

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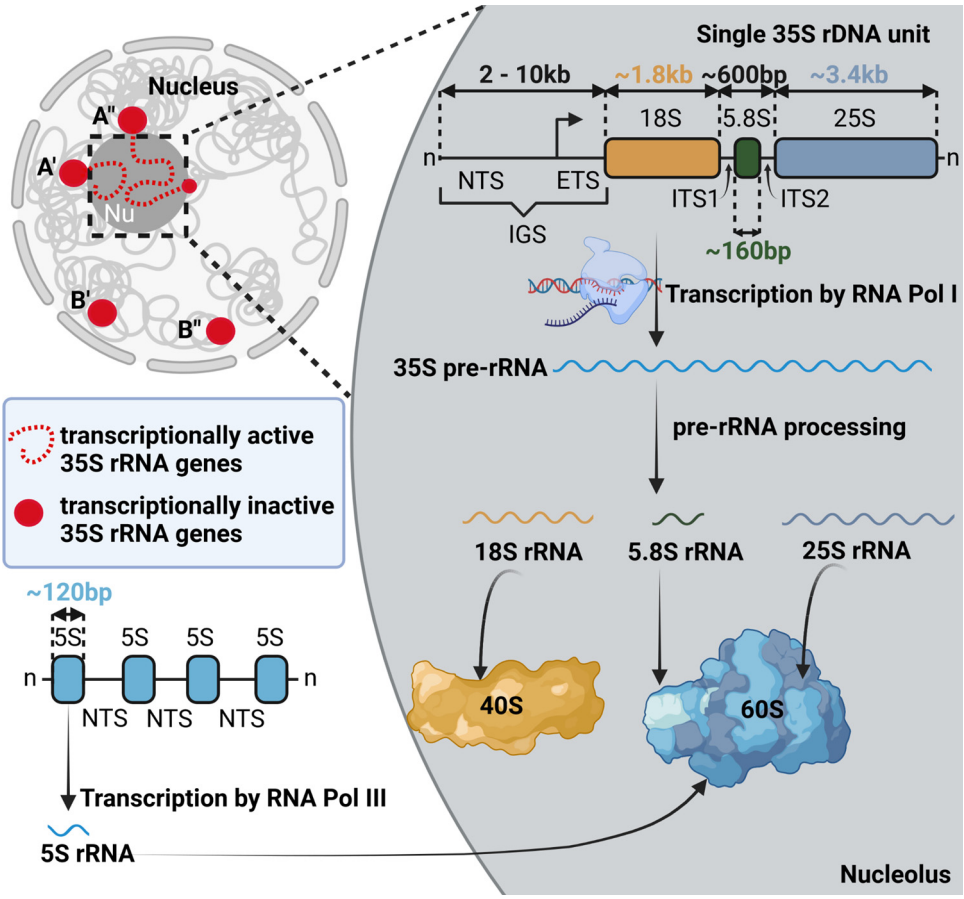

Figure 1. Schematic of ribosome subunit biogenesis with a special emphasis on rDNA contribution and nucleolar dominance (ND). Top left panel: Interphase nucleus of a typical allotetraploid species (subgenome composition AABB) exhibiting ND. Only the 35S rDNA loci from the A subgenome (homologs are marked as A′ and A″) can form a nucleolus (Nu), whereas those of the B-subgenome (marked as B′ and B″) are silenced via ND and located at the nuclear periphery. Right panel: In the nucleolus, RNA polymerase I (Pol I), together with a set of transcription factors, transcribes hundreds of A-subgenome 35S rRNA genes to generate 35S pre-rRNA, which is further cotranscriptionally processed to form mature 18S, 5.8S, and 25S rRNAs. The processing involves multiple endonucleolytic and exonucleolytic cleavages and the modification of rRNA residues (mainly by 2′-O-ribose-methylation and pseudouridylation; reviewed in [91]). The assembly of the ribosomal subunits occurs cotranscriptionally and requires the so-called ribosome biogenesis factors (RBFs). The nascent 35S pre-RNAs interact with RBFs that are necessary for pre-rRNA processing and ribosomal subunit assembly (reviewed in [92]). The 18S rRNAs assemble with ribosomal proteins of the small subunit (RPSs) to form 40S ribosome subunits. By contrast, 5.8S and 25S assemble with ribosomal proteins of the large subunit (RPLs) and 5S rRNA (transcribed outside a nucleolus, processed and imported into the nucleolus; bottom left panel) to form 60S ribosome subunits. The figure was created with BioRender.com. Abbreviations: ETS, external transcribed spacer; IGS, intergenic spacer; ITS, internal transcribed spacer; NTS, nontranscribed spacer.

time, little is known about the exact mechanism by which ND is established and maintained. Here, we review the current knowledge on ND in plants, focusing primarily on the economically and ecologically important grass family. We highlight recent methodological advances that enable studies of individual 35S rDNA loci in complex genomes of allopolyploids. We also outline the importance of a new flagship allopolyploid model grass, *Brachypodium hybridum* (2n = 30, DDSS), which emerges as a model for studying ND in grasses. Improving our understanding of ND is a key to decipher polyploidy, one of the major forces governing seed plant evolution [6,15,16].

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A brief overview of the early cytological observations of ND in cereals

ND is widespread among grasses, such as allohexaploid wheat [17], triticale [18], barley [19], oats and oatlike grasses [20,21], maize [22], and many others (Table 1). For many years, its occurrence was determined on the basis of cytological observations, such as the presence or absence of secondary constrictions and the number and size of nucleoli per nucleus [23–25]. The absence of secondary constrictions on mitotic metaphase chromosomes implied that specific regulatory proteins and their complexes did not associate with inactive rRNA genes. The corollary was that silenced rRNA genes reside in a different chromatin configuration from their active counterparts [17]. This assumption has been tested directly by assessing the activity of ancestral rDNA sets using silver staining that highlights NOR regions [26]. Some proteins remain associated with NORs during mitosis; thus, only 35S rDNA loci that were transcribed during the previous interphase are stained by silver [27]. Numerous studies have used silver staining to examine rDNA activity in grass hybrids and allopolyploids, particularly those of the Triticeae tribe. For example, in the artificial intergeneric triticale [18,28], derived from a cross between wheat (tetra- or hexaploid; subgenome composition AABB and AABBDD, respectively) and rye (*Secale cereale*; RR), it was shown that only the 35S rDNA loci of the wheat progenitors were expressed, with the selective silencing of the loci of the rye subgenome [18,29]. Triticale containing two (ABRR) or three (ABRRR) rye genomes did not change the inactive transcriptional state of the 35S rRNA genes of the rye subgenomes. This indicates that, at least in triticale [29] and wheat [30], ND is not a simple dosage-dependent phenomenon, because it may be influenced by the unit structure and chromosome features. In wheat-rye hybrids, in which the 35S rDNA derived from rye is also suppressed, underdominant rRNA genes are highly condensed and less dispersed in interphase nuclei than the nucleoli-associated wheat loci, thereby showing a distinctly different organisation of active compared with inactive rRNA gene loci [28]. Further studies on wheat-rye hybrids and hexaploid triticale revealed transcriptional reactivation and decondensation of the rDNA of the R-subgenome after treatment with the DNA hypomethylating agent, 5-azacytidine. This suggests that epigenetic mechanisms may be important in maintaining ND in grasses [9,31].

Cytological analyses still have much to offer in the study of ND. The use of the silver staining, together with the flagship cytomolecular method, **fluorescence *in situ* hybridisation (FISH)**, and its modification, **genomic *in situ* hybridisation (GISH),** not only discriminate between active and inactive 35S rDNA loci but also identify the subgenome origin of particular loci, also in interphase nuclei [32–34].

ND in monocots versus dicots

Even though ND is prevalent among grasses, most of the information about this phenomenon has come from research on dicot plants from various genera, such as *Arabidopsis* [5,35], *Brassica* [3,36], *Nicotiana* [37], *Solanum* [38], *Tragopogon* [39,40], and *Anemone* [41]. A holistic understanding of molecular mechanisms that shape ND, however, requires verification as to whether they are the same or not in dicot and monocot representatives.

In both groups of angiosperms, ND is driven and maintained through epigenetic mechanisms [5,7–9,42]. Early experiments using cytosine methyltransferase (5-azacytidine; 5-azadeoxycytidine) and histone deacetylase (sodium butyrate; trichostatin A) inhibitors reactivated underdominant 35S rRNA genes in some dicots [5,10,43] and monocots [9,31,44]. Indeed, the differential DNA methylation status of active and silenced ancestral rDNA sets has been documented in many allopolyploids, including the allotetraploid grass *B. hybridum*, in which the silenced 35S rDNA loci of the S-subgenome have significantly higher DNA methylation levels than those of the D-subgenome (Figure 2) [45]. The underdominant rRNA genes of rapeseed (dicot allotetraploid)

Glossary

35-48S rDNA: also known as 35-48S rRNA genes, encodes rRNAs, a vital component of ribosomes. It is essential for the metabolism of every cell and belongs to the so-called group of housekeeping genes. 35-48S rDNA is organised as chromosomal locus consisting of long arrays of tandemly repeated units. Each unit comprises 18S, 5.8S, and 25S (or 28S in mammals) rRNA genes, two internal transcribed spacers (ITS1 and ITS2), and an intergenic spacer (IGS) that contains a nontranscribed spacer (NTS) and an external transcribed spacer (ETS; Figure 1). A single rDNA transcription unit is known as 35S-45S rDNA in plants (depending on the size of 5′-ETS), 48S rDNA in mammals, and 35S rDNA in yeast. The genic regions are highly conserved even between distantly related species, whereas the intergenic regions are highly variable and lend themselves frequently therefore to phylogenetic studies.
Allopolyploid: an individual with at least two complete disomic chromosome sets (subgenomes) derived from hybridisation between different taxonomic species followed by the doubling of their chromosome number in order to restore sexual fertility (e.g., AA × BB → AB → AABB).
Fluorescence *in situ* hybridisation (FISH): a cytomolecular method to visualise a specific DNA sequence on a substrate (e.g., mitotic or meiotic chromosomes, interphase nuclei, extended chromatin fibres). FISH relies on kinetically controlled annealing of fluorescent or immunolabelled DNA probes with complementary substrates in a cytological preparation.
Genomic *in situ* hybridisation (GISH): a modification of FISH using whole genomic DNA probes rather than specific DNA sequences.
Long-read sequencing: also known as third-generation sequencing, a modern DNA sequencing approach that helps to close gaps in a genome, with particular utility in mapping repetitive sequences. Depending on the technology used, read length may vary from tens of kilobases (single-molecule real-time sequencing) to hundreds of kilobases (nanopore sequencing).
Nucleolar dominance (ND): a common epigenetic phenomenon describing the selective transcription of the 35S rRNA genes of one or more of

and bread wheat (monocot allohexaploid) are extensively methylated within the promoters recognised by **RNA polymerase I (Pol I)** [7,46]. *Arabidopsis suecica* is a dicot allotetraploid in which the rRNA genes derived from *Arabidopsis arenosa* are dominant over those from the *Arabidopsis thaliana*-like subgenome. Reverse genetic approaches in this species identified the proteins that are involved in rRNA gene silencing. These include histone deacetylase 6 (HDA6), domains rearranged methyltransferase 2 (DRM2), methylcytosine binding domain protein 6 (MBD6), histone H3K9 methyltransferase (SUVR4), RNA-dependent RNA polymerase 2 (RDR2), and dicer-like 3 protein (DCL3) [4,5,47,48]. These proteins are involved in the RNA-directed DNA methylation (RdDM) pathway, where siRNAs of 24 nt in length elicit the transcriptional silencing of a gene by directing repressive epigenetic modifications such as DNA and histone methylation to the genomic regions with which they share homology [49,50]. Interestingly, preferential silencing of *A. thaliana*-derived rDNA of *A. suecica* is correlated with both the occurrence of 24-nt-long siRNAs that match the intergenic spacer (IGS) region and rRNA gene promoter and the *de novo* cytosine methylation by DRM2, implicating the involvement of the RdDM pathway in ND [4,47].

Because the inactive state of underdominant rDNA loci is maintained via repressive epigenetic mechanisms, ND in both groups of angiosperms seems to be a fully reversible, developmentally regulated phenomenon, which has to be restored in each generation. One example of this is the progressive inactivation of *A. thaliana*-derived rRNA genes during early postembryonic development of *A. suecica* [51]. Similar ND establishment was also observed in rapeseed, in which both ancestral 35S rDNA sets are active in 2–3-day-old seedlings [52], but stable ND towards the *Brassica rapa*-derived rRNA genes in the leaves of mature plants exists [3,36]. Moreover, at least in some rapeseed genotypes [3] and artificial *Solanum* allopolyploids [38], a transcriptional reactivation of the underdominant rRNA genes was observed after the transition from the vegetative to the generative phase. The establishment of ND in monocots seems to occur early in development. For example, rDNA loci of rye origin in wheat-rye hybrids are repressed as early as 4–5 days after fertilisation in both embryo and endosperm [53].

Resynthesised forms of allopolyploids are often used in studies of the genetic and epigenetic dynamics of rDNA loci of the first generations following hybridisation. Analyses of the resynthesised rapeseed revealed that the silencing of the *Brassica oleracea*-originated rRNA genes may be initiated as early as in the F1 hybrids and is correlated with the progressive hypermethylation of the underdominant C-subgenome rRNA genes [46]. The rDNA expression patterns in the resynthesised rapeseed mirrored those of its natural forms [36]. Thus, at least in the case of *Brassica* allopolyploids, it seems that interspecific hybridisation itself is sufficient to induce epigenetic repatterning of the C-subgenome rDNA set and, consequently, triggers uniparental silencing [46]. In newly formed *A. suecica* F1 hybrids, the silencing of *A. thaliana*-originated rDNA was variable, with two generations needed to establish stable ND in some lines [43]. By contrast, in resynthesised wheat, allopolyploidisation constitutes a critical step for the silencing trigger [7]. As in *Arabidopsis*, in wheat, the repression of the A-subgenome rDNA is accompanied by increased CHG and CHH (H = A, C, T) DNA methylation on rRNA gene promoters, followed by recruitment of repressive histone modifications, that is, dimethylation of lysine 9 of histone H3 (H3K9me2) and trimethylation of lysine 27 of histone H3 (H3K27me3) [7]. In summary, ND establishment may be triggered by interspecies hybridisation only or may require subsequent whole-genome duplication followed by meiotic cycles.

ND is one specific aspect of rRNA gene dosage control [54]. It is an open question whether the repressive mechanisms underlying rRNA gene dosage control are the same or different in diploid and allopolyploid organisms. On the basis of studies in genera differing in ploidy levels [8,10,38,45,47,55], it is likely that the maintenance of ND relies mostly on DNA methylation and

the constituent subgenomes in interspecific hybrids and allopolyploids. Only transcriptionally active rRNA genes form a nucleolus, hence the name given for this phenomenon.

Nucleolus: a nuclear domain whose primary function is the biogenesis of ribosomal subunits. This process involves the transcription of 35S rRNA genes by RNA polymerase I (Pol I), followed by primary 35S rRNA (pre-rRNA) transcript processing. Small and large ribosomal subunits are assembled from individual, mature rRNA molecules together with ribosomal proteins.

Optical map: a physical map visualising hundreds to thousands of kilobase stretches of DNA in a nanochannel array using short-sequence motifs/labels.

RNA polymerase I (PolI): a highly specific eukaryotic nuclear DNA-dependent RNA polymerase catalysing transcription of 35-48S rRNA genes in the nucleolus. RNA polymerase I is a complex enzyme made up of 12 or more subunits.

Secondary constriction: a chromosomal region that morphologically marks the site of 35S rRNA genes expressed during the previous interphase. It can be visualised at the microscopic level.

Table 1. Occurrence of nucleolar dominance in selected grass representatives

Taxon	Short characteristics	Dominant	Underdominant	Method	Refs
Aegilops cylindrica	Tetraploid $C^cC^cD^cD^c$ (*Aegilops caudata* × *Aegilops tauschii*)	C^cC^c	D^cD^c	Silver staining, FISH	[33]
Avena barbata	Tetraploid AABB	AA	BB	Silver staining, FISH	[20,93]
Brachiaria decumbens	Tetraploid cv. Basilisk, $B^1B^1B^2B^2$	Unknown	Unknown	Silver staining, FISH	[94,95]
Brachypodium hybridum	Tetraploid DDSS (*B. distachyon* × *B. stacei*)	DD	SS	Sequential silver staining and FISH	[34,45,88]
Elymus tetraploids	Tetraploid SSHH	SS	HH	Silver staining	[96]
Hordeum vulgare	Translocation lines T505, T506, T248, T571, and T2052 combining NOR6 and NOR7 on one chromosome	NOR6	NOR7	Silver staining, FISH	[19,64]
Lolium multiflorum	cv. Barjumbo (4x) and cv. ABARP (3x)	Unknown	Unknown	Silver staining, FISH	[97]
Triticale	cv. Cachirulo, Senatore Capelli, and Bidi 17 (hexaploid AABBRR)	AABB	RR	Silver staining	[18,98]
Triticum aestivum	Hexaploid cv. Chinese Spring (AABBDD)	1B and 6B	5D and 1A	Silver staining RNA-seq, RT-qPCR	[25,29,30,56]
T. aestivum × *Secale cereale*	cv. Chinese Spring × cv. Centeio do Alto (F1 hybrid, ABDR)	1B and 6B	*1R*	Silver staining	[9]
Triticum spelta	Hexaploid AABBDD	1B and 6B	5D and 1A	Silver staining, ISH	[29,99]
Triticum turgidum durum	Tetraploid AABB. cv. Enano de Andújar, cv. Nordum, and cv. Calvin	1B and 6B	1A	Silver staining, FISH, ISH, N-banding	[18,29,99,100]
Z. mays inbred line	Intraspecific F1 hybrid Sx19 (♀ B73 × ♂ Mo17)	B73-derived rDNA	Mo17- derived rDNA	Silver staining, RNA slot blot	[22,101]
Z. mays inbred line	Intraspecific F1 hybrid Sx19 (♀ Mo17 × ♂ B73)	Mo17- derived rDNA	B73- derived rDNA	Silver staining, RNA slot blot	[22,101]

histone modifications. Thus, the repressive epigenetic marks maintaining ND are conserved and somewhat similar between diploids and allopolyploids, in dicots and monocots. However, the enzymatic machinery involved in the 35S rRNA gene dosage control may differ between diploid and allopolyploid plants, as shown in *A. thaliana* and *A. suecica*, where distinct H3K9 methyltransferases are required for rDNA variant-specific silencing and ND, respectively [48].

The mechanisms determining which ancestral rDNA set is destined for silencing remain elusive. Because ND seems to occur independently of paternal or maternal effects [3,11] and is established either during embryogenesis [53] or early after embryogenesis [51], ancestral rDNA imprinting in the gametes does not appear to be involved. In both dicots and monocots, it is usually the same parental rDNA set chosen for inactivation, implying that ND is not random yet may be dosage dependent, as was shown in *Arabidopsis* allopolyploids [43]. Also, there seems to be no simple relationship between the number of rDNA units within a locus and the direction of ND, because NORs with fewer genes can be dominant over those with more rRNA genes, as was shown in bread wheat [30,56] and *Brassica* allopolyploids [3]. Certainly, loci with a low number of genes are usually inactive in plants with multiple 35S rDNA sites [36,57], suggesting that there might be a minimum number of functional genes constituting a dominant NOR.

Several hypotheses have been proposed to explain molecular mechanisms that shape ND. One of them posits that the rapid evolution of rRNA gene promoters should be accompanied by coevolution of transcription factors (TFs) that recognise the promoter and interact with Pol I [58,59]. Thus, the lack of species-specific TFs in the hybrid or allopolyploid should result in ND enforcement. Indeed, this hypothesis appears accurate for distantly related species that cannot

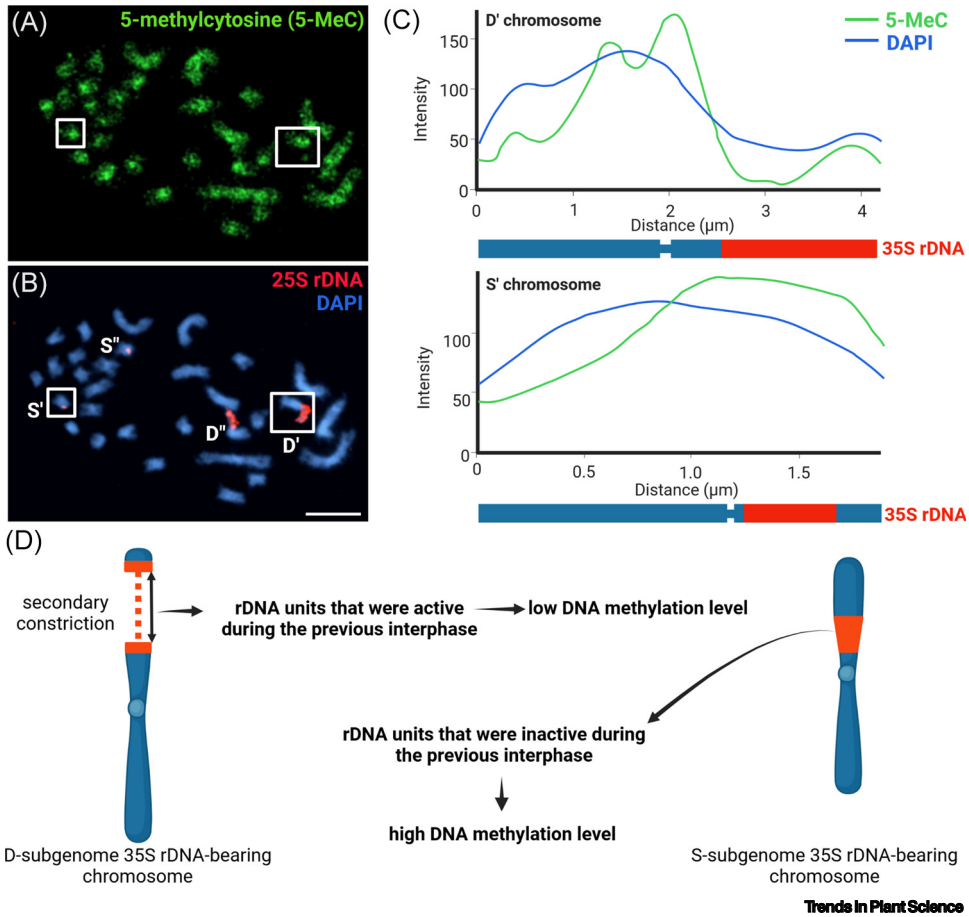

Figure 2. DNA methylation status and chromosomal organisation of 35S rDNA loci in *Brachypodium hybridum*. (A) 5-Methylcytosine immunopatterns (green). (B) Fluorescence *in situ* hybridisation with the 25S rDNA probe (red) in the chromosome complement shown on (A). Bar, 5 μm. (C) 5-Methylcytosine foci distribution along the longitudinal axes of chromosomes D′ (the top profile) and S′ (the bottom profile), which are surrounded by white rectangles on (A,B). The length of the chromosomes in micrometres is shown on the *x*-axis. Chromosomes are oriented from their long arm to the short. The fluorescence intensity on the *y*-axis is presented in arbitrary units. The blue curves denote the fluorescence intensity of the counterstain [4′,6-diamidino-2-phenylindole (DAPI); blue], whereas the green curves indicate the distribution of DNA methylation foci. The localisation of 35S rDNA loci is presented below the profiles on the chromosome diagrams. (D) Schematic representation of D- and S-subgenome 35S rDNA loci, which are characterised by differential DNA methylation levels in relation to their activity. Only rDNA loci from the D-subgenome are transcriptionally active. Based on [45]. The figure was created with BioRender.com.

be crossed. However, it fails to explain ND in plant hybrids in which the TFs of one progenitor could efficiently interact with the promoter of the second parental species [60]. Another hypothesis is based on the structural features of IGSs, whose repetitive regions are the most rapidly evolving of rDNA units. It proposes that the 35S rDNA derived from the progenitor with longer IGSs containing more subrepeats upstream of the transcription initiation site is dominant over those derived from the progenitor(s) with shorter IGSs [61]. Such a correlation was first found in *Xenopus* frog hybrids, where upstream subrepeats act as transcription enhancers (so-called enhancer imbalance hypothesis) [62,63]. However, 35S rDNA sets with longer IGSs in *Brassica*, *Arabidopsis*, and maize were underdominant [3,22,43], so this hypothesis needs further scrutiny in plants.

Interestingly, some studies on the behaviour of rDNA loci in diploid plants suggest a chromosomal role in the context of ND. For example, rRNA gene activity may depend on the position on the chromosome in a barley translocation line, in which both 35S rDNA loci are present on the opposite arms of one chromosome [64]. In wild-type barley, 35S rDNA loci are on different chromosomes and are transcriptionally active. In the translocation line, however, one locus is repressed, implying interference between the two loci that impacts rRNA gene expression. A similar conclusion was reached in *A. thaliana* mutant deficient for histone H3 lysine 27 monomethylase activity, where normally silent rDNA units from NOR2 are translocated to NOR4 [65]. At the new chromosomal position, the NOR2 rRNA genes are activated, revealing a potential role of the neighbouring pericentromeric sequences in the selective silencing of rRNA genes in NOR2. Handa *et al.* [66] suggested that the highly methylated, transposon-rich regions adjacent to the major wheat NORs on chromosomes 1B and 6B not only may regulate the expression of neighbouring NORs but also may influence minor unlinked rDNA loci. Thus, the impact of the chromosomal position still needs to be further examined in both dicot and monocot allopolyploid systems to explain ND. Despite some reports demonstrating the effect of supernumerary B chromosomes [67] and intergenomic non-NOR chromosome substitutions [68] on 35S rDNA expression patterns in hybrids and allopolyploids, a mechanistic explanation of chromosome effects on ND remains elusive.

Modern methodological approaches provide new insights into 35S rDNA genomic organisation, epigenetic modification, and ND in plants

Studies on the molecular basis of ND for many years were limited through a lack of methodological approaches enabling more detailed scrutiny of the sequence organisation and transcriptional activity of rRNA genes from individual loci. Repetitive rDNA arrays spanning millions of base pairs were refractory to mapping and assembling, leading to significant gaps in sequenced plant genomes. The onset of the **long-read sequencing** technologies with ultralong reads, such as nanopore sequencing and single-molecule real-time sequencing, has opened a new chapter in studies of tandem repeats, including 35S rDNA, in both the sequence and DNA methylation contexts [69–71]. Recently, a combination of long- and short-read sequencing of bacterial artificial chromosomes containing rDNA has assessed higher-order organisation of the NOR2 locus of *A. thaliana* [72]. This study revealed not only heterogeneity of rDNA units but also, and even more interestingly, tissue-specific patterns of their expression. These observations are consistent with the existence of tissue-specific ribosome populations, which are characterised by different rRNA variants, an exciting new aspect to be considered in future ND studies. Considering that previous findings [55,73] on *A. thaliana* clearly showed significant differences in the expression and copy number of rDNA clusters among different accessions and the regulation of rRNA gene expression via complex epistatic and allelic interactions between rDNA cluster haplotypes, future studies should address ND at the population level in order to characterise intraspecific variability as well. Burns *et al.* [35] showed wide variation in both 35S rDNA cluster size and expression among different accessions of *A. suecica*, adding support to the notion that ND may be partly explained by variation in its constituent ancestral genomes.

Another intriguing question is whether a significant reduction in dominant rDNA copy number in allopolyploid species may change the direction of ND. In the recently emerged allotetraploid, *Tragopogon mirus* (DDPP), 35S rDNA of the D-subgenome, accounting for ~25% of the total rDNA in this species, is dominant over the P-subgenome [39]. Individuals of *T. mirus* carrying a homozygous macrodeletion, which reduced the number of D-subgenome units to ~4% of total rDNA, had no ND in flowers, roots, and calli but not in leaves. Thus, repressed rDNA homoeologues can be activated to alleviate the mutational damage [39]. Also, a significant decrease in the 35S rDNA copy number in diploid plants may affect selective variant-specific

silencing [74–76]. For example, in *A. thaliana* mutant deficient for the main subunits of chromatin assembly factor 1 (CAF1): FACIATA 1 (FAS1) and FAS2, the number of rRNA genes was decreased to ~40% and ~10–15% of the original number in the wild type by the second and fifth generations of plants, respectively [74]. The transcriptionally active rDNA variants located in the nucleolus were preferentially lost in mutants, and therefore the inactive copies (including the typically silent variant 1) were activated [76]. Except for the late (fifth to ninth) mutant generations, the *fas* mutants maintained levels of rRNA transcripts similar to those of the wild-type plants. More recent studies on *A. thaliana* employed Cas9-mediated genome editing to decrease the 35S rDNA copy number by up to 93% [77]. The mutants possess as few as ~25–30 rDNA copies per NOR (~50–60 copies/genome), which was still sufficient to sustain their viability, although dramatic changes in their transcriptomes were observed. These studies supported the previous notion that plants harbour more rDNA copies than are needed for their developmental programs, a redundancy that is difficult to explain at present. Also, the developmentally silenced NOR2 in wild-type *A. thaliana* accessions [65,78] becomes activated in lines with decreased rDNA copy number and is accompanied by loss of the heterochromatic mark H3K9me2 [77]. There is no doubt that CRISPR-Cas-mediated targeted mutagenesis to study rRNA gene dosage promises new possibilities for understanding ND in more complex allopolyploid genomes.

Differentiation between individual rDNA loci in allopolyploids with ND is a challenge worthy of mention, especially in species where there is more than one rDNA locus per subgenome. Bread wheat (*Triticum aestivum*; AABBDD) is a good example, with its large and complex genome of over 16.5 Gbp [79] and at least four 35S rDNA loci distributed on chromosomes 1A, 1B, 6B, and 5D. Recently, combining **optical maps** of flow-sorted chromosomes with short-read sequencing data has enabled the reconstruction of individual rDNA loci in wheat for the first time [56]. In the Chinese Spring variety, major and minor loci from the B-subgenome and the D-subgenome, respectively, have high intra-array homogeneity. By contrast, the A-subgenome locus has an irregular structure containing incomplete units, indicating that this locus is in a phase of disintegration and pseudogenisation. DNA methylation (bisulphite sequencing) and expression (RNA-seq) analyses have been used to investigate the epigenetic status of these wheat loci. Such a multiomic approach revealed various mechanisms of rRNA dosage control, such as stable silencing of the pseudogenised A-subgenome locus, complete silencing of the minor D-subgenome locus, and the developmental regulation of the 6B locus. The locus on the 1B chromosome is stably expressed throughout development and contributes most of the 35S rRNA transcripts [56].

B. hybridum as a model in ND studies in grasses

Functional studies of ND in large complex cereal genomes may be difficult to interpret due to epistatic interactions of numerous rDNA loci. It was therefore desirable to find a more tractable model. One of the most promising current candidates for this role is a small-genome annual allotetraploid grass, *B. hybridum* (~0.630 pg/1C) (e.g., [80–82]). This species is closely related to the economically important temperate cereals, such as bread wheat, rye, barley, and oat [83]. Moreover, it is the only polyploid within the *Brachypodium* genus, whose evolutionary ancestors (*Brachypodium distachyon*, 2n = 10; DD and *Brachypodium stacei*, 2n = 20; SS) have been unambiguously identified [83–85]. The durable stasis of the subgenomes of *B. hybridum* has been demonstrated using both genomic [86] and cytomolecular [87] approaches. However, 35S rDNA in this species does not seem to follow this rule and appears to be a more dynamic part of the *B. hybridum* genome [32,80]. In 2008, ND favouring the D-subgenome rDNA loci was observed for the first time in root-tip cells of several *B. hybridum* genotypes [34]. The difference in chromosomal position of the two loci, together with the inheritance of only one rDNA locus from each progenitor, is a handy feature for studying ND in this allotetraploid, because these

features significantly simplify cytogenetic analyses. As in other allopolyploids that exhibit ND, the underdominant S-subgenome 35S rDNA loci of *B. hybridum* exhibit high levels of repressive epigenetic modifications, that is, methylation of cytosine residues (Figure 2) and H3K9me2 [45]. Hence, ND in this species has an epigenetic origin. Interestingly, the developmental regulation of ND was shown to be dependent on a particular *B. hybridum* genotype. Whilst ND was present in all tissues studied so far in the ABR113 genotype, including immature and imbibed embryos, primary and adventitious roots, leaves, spikes, and meiocytes [81,88], the 3-7-2 genotype reactivated the S-subgenome loci in root tissue only (Figure 3) [81]. ND is already detectable in immature embryos [88] and during the early postembryonic development of *B. hybridum* [34,80,81]. Similarly, a complete suppression of the D-subgenome locus in wheat (Chinese Spring variety) occurs in young embryos and cotyledons [56]. These studies indicate that the establishment of ND in monocots (specifically in grasses) may occur earlier in development than in the dicots [51,52].

It has been shown that the underdominant S-subgenome loci in *B. hybridum* are gradually eliminated during evolution [80], as in rapeseed [36]. The contribution of the underdominant loci to the total rDNA in this grass varied between genotypes from ~7% up to 39% [80,81], whereas the relationship between copy number and ND is currently unclear. This phenomenon is probably correlated with the polyphyletic origin of *B. hybridum* [83,89]. The most recent dating analysis

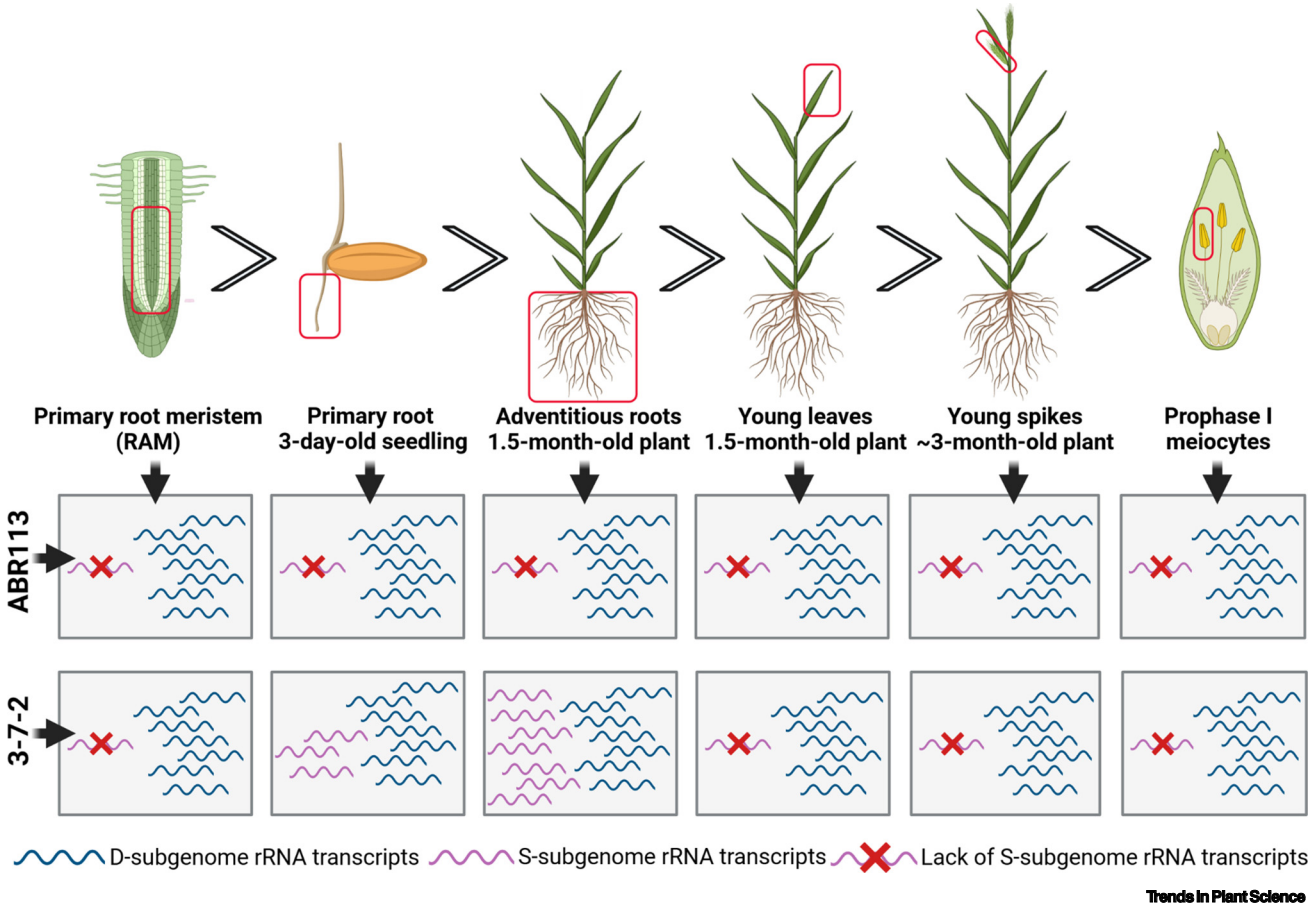

Figure 3. Genotype-specific developmental regulation of nucleolar dominance in *Brachypodium hybridum*. Based on [81,88]. The figure was created with BioRender.com.

shows that its different lineages arose from multiple crosses between the D- and S-subgenome donors during the Quaternary period, ~1.4 and ~0.14 million years ago [86].

Due to its small and relatively simple genome, and a wide range of available genetic resources and sequencing data (including whole-genome sequences for a range of accessions and two of its putative evolutionary ancestors) [86,90], *B. hybridum* has the potential to become a flagship model for ND studies in monocots or grasses as a whole.

Concluding remarks and future perspectives

Despite nearly a century of studies on ND in a variety of dicot and monocot allopolyploids and hybrids, the factors determining which rDNA loci are chosen to be repressed and the biological significance of this selective rDNA inactivation are still enigmatic. The advent of new sequencing techniques offers a much closer look at the structure, epigenetic pattern, and expression of individual rRNA gene loci, so far intractable. The increasing interest in *B. hybridum* as a model representative of the *Brachypodium* genus, with its superlative combination of plant resources and cutting-edge methods, bodes well for our gaining new insights into the mechanisms that shape this intriguing phenomenon. This should improve our comprehension of allopolyploidy, one of the major evolutionary forces in angiosperms (see Outstanding questions).

Outstanding questions

Are the molecular mechanisms that shape ND universal among monocot and dicot allopolyploids and hybrids?

Is a tissue-specific expression of ancestral rRNA genes in allopolyploids linked with the needs of the cell for a particular ribosome subpopulation?

Is there any correlation between ND in plants and their adaptation to environmental stresses?

Is there a relationship between the position of the NOR locus along the chromosome and its predisposition to silencing in hybrids and allopolyploids?

Acknowledgments

R.H. recognises the support of the National Science Centre Poland (2018/31/B/NZ3/01761) and the Research Excellence Initiative of the University of Silesia in Katowice. R.W. thanks the Medical Research Agency (2019/ABM/01/00047-00). A.K. recognises the support of the Czech Science Foundation (22-16826S). We thank Emeritus Professor Glyn Jenkins (Aberystwyth University, Aberystwyth, UK) for all his critical reading and valuable comments on the manuscript.

Declaration of interests

The authors declare no conflict of interest.

References

1. Pikaard, C.S. (2000) Nucleolar dominance: uniparental gene silencing on a multi-megabase scale in genetic hybrids. *Plant Mol. Biol.* 43, 163–177
2. Viegas, W. *et al.* (2002) Nucleolar dominance: a 'David and Goliath' chromatin imprinting process. *Curr. Genomics* 3, 563–576
3. Chen, Z.J. and Pikaard, C.S. (1997) Transcriptional analysis of nucleolar dominance in polyploid plants: biased expression/silencing of progenitor rRNA genes is developmentally regulated in *Brassica*. *Proc. Natl. Acad. Sci. U. S. A.* 94, 3442–3447
4. Costa-Nunes, P. *et al.* (2010) Extra views on RNA-dependent DNA methylation and MBD6-dependent heterochromatin formation in nucleolar dominance. *Nucleus* 1, 254–259
5. Earley, K. *et al.* (2006) Erasure of histone acetylation by *Arabidopsis* HDA6 mediates large-scale gene silencing in nucleolar dominance. *Genes Dev.* 20, 1283–1293
6. Glombik, M. *et al.* (2020) Competition of parental genomes in plant hybrids. *Front. Plant Sci.* 11, 200
7. Guo, X. and Han, F. (2014) Asymmetric epigenetic modification and elimination of rDNA sequences by polyploidization in wheat. *Plant Cell* 26, 4311–4327
8. Lawrence, R.J. *et al.* (2004) A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. *Mol. Cell* 13, 599–609
9. Vieira, R. *et al.* (1990) 1R chromosome nucleolus organizer region activation by 5-azacytidine in wheat × rye hybrids. *Genome* 33, 707–712
10. Chen, Z.J. and Pikaard, C.S. (1997) Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. *Genes Dev.* 11, 2124–2136
11. Navashin, M. (1934) Chromosome alterations caused by hybridization and their bearing upon certain general genetic problems. *Cytologia* 5, 169–203
12. McClintock, B. (1934) The relationship of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Zeit. Zellforsch. Mik. Anat.* 21, 294–328
13. Honjo, T. and Reeder, R.H. (1973) Preferential transcription of *Xenopus laevis* ribosomal RNA in interspecies hybrids between *Xenopus laevis* and *Xenopus mulleri*. *J. Mol. Biol.* 80, 217–228
14. Wallace, H. and Langridge, W.H.R. (1971) Differential amphiplasty and the control of ribosomal RNA synthesis. *Heredity* 27, 1–13
15. Borowska-Zuchowska, N. *et al.* (2022) Tracing the evolution of the angiosperm genome from the cytogenetic point of view. *Plants* 11, 784
16. Soltis, P.S. *et al.* (2015) Polyploidy and genome evolution in plants. *Curr. Opin. Genet. Dev.* 35, 119–125
17. Flavell, R.B. *et al.* (1986) The differential expression of ribosomal RNA genes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 314, 385–397
18. Lacadena, J.R. *et al.* (1984) Evidence for wheat-rye nucleolar competition (amphiplasty) in triticale by silver-staining procedure. *Theor. Appl. Genet.* 67, 207–213
19. Schubert, I. and Künzel, G. (1990) Position-dependent NOR activity in barley. *Chromosoma* 99, 352–359
20. Fominaya, A. *et al.* (1988) C-banding and nucleolar activity of tetraploid *Avena* species. *Genome* 30, 633–638
21. Winterfeld, G. *et al.* (2012) Origin of highly polyploid species: different pathways of auto- and allopolyploidy in 12–18x species of *Avenula* (Poaceae). *Int. J. Plant Sci.* 173, 399–411
22. Jupe, E.R. and Zimmer, E.A. (1993) DNaseI-sensitive and undermethylated rDNA is preferentially expressed in a maize hybrid. *Plant Mol. Biol.* 21, 805–821
23. Crosby, A.R. (1957) Nucleolar activity of lagging chromosomes in wheat. *Am. J. Bot.* 44, 813–822

24. Darvey, N.L. and Driscoll, C.J. (1972) Nucleolar behaviour in *Triticum*. *Chromosoma* 36, 131–139
25. Flavell, R.B. and O'Dell, M. (1979) The genetic control of nucleolus formation in wheat. *Chromosoma* 71, 135–152
26. Hizume, M. *et al.* (1980) A highly reproducible method of nucleolus organizing regions staining in plants. *Stain. Technol.* 55, 87–90
27. Sirri, V. *et al.* (2000) The AgNOR proteins: qualitative and quantitative changes during the cell cycle. *Micron* 31, 121–126
28. Appels, R. *et al.* (1986) The structure of DNA from the rye (*Secale cereale*) NOR R1 locus and its behaviour in wheat backgrounds. *Can. J. Genet. Cytol.* 28, 673–685
29. Cermeño, M.C. *et al.* (1984) Nucleolar organizer activity in wheat, rye and derivatives analyzed by a silver-staining procedure. *Chromosoma* 89, 370–376
30. Flavell, R.B. and O'Dell, M. (1976) Ribosomal RNA genes on homoeologous chromosomes of groups 5 and 6 in hexaploid wheat. *Heredity* 37, 377–385
31. Amado, L. *et al.* (1997) Development-dependent inheritance of 5-azacytidine-induced epimutations in triticale: analysis of rDNA expression patterns. *Chromosom. Res.* 5, 445–450
32. Borowska-Zuchowska, N. *et al.* (2016) Cytomolecular 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
33. Mirzaghaderi, G. *et al.* (2017) Dynamic nucleolar activity in wheat × *Aegilops* hybrids: evidence of C-genome dominance. *Plant Cell Rep.* 36, 1277–1285
34. Idziak, D. and Hasterok, R. (2008) Cytogenetic evidence of nucleolar dominance in allotetraploid species of *Brachypodium*. *Genome* 51, 387–391
35. Burns, R. *et al.* (2021) Gradual evolution of allopolyploidy in *Arabidopsis suecica*. *Nat. Ecol. Evol.* 5, 1367–1381
36. Sochorova, J. *et al.* (2017) Gene conversion events and variable degree of homogenization of rDNA loci in cultivars of *Brassica napus*. *Ann. Bot.* 119, 13–26
37. Kovarik, A. *et al.* (2008) Evolution of rDNA in *Nicotiana* allopolyploids: a potential link between rDNA homogenization and epigenetics. *Ann. Bot.* 101, 815–823
38. Komarova, N.Y. *et al.* (2004) Organization, differential expression and methylation of rDNA in artificial *Solanum* allopolyploids. *Plant Mol. Biol.* 56, 439–463
39. Dobešová, E. *et al.* (2015) Silenced rRNA genes are activated and substitute for partially eliminated active homeologs in the recently formed allotetraploid, *Tragopogon mirus* (Asteraceae). *Heredity* 114, 356–365
40. Matyasek, R. *et al.* (2007) Concerted evolution of rDNA in recently formed *Tragopogon* allotetraploids is typically associated with an inverse correlation between gene copy number and expression. *Genetics* 176, 2509–2519
41. Mlinarec, J. *et al.* (2022) Structure and methylation of 35S rDNA in allopolyploids *Anemone multifida* (2n = 4x = 32, BBDD) and *Anemone baldensis* (2n = 6x = 48, AABBDD) and their parental species show evidence of nucleolar dominance. *Front. Plant Sci.* 13, 908218
42. Pikaard, C.S. (2000) The epigenetics of nucleolar dominance. *Trends Genet.* 16, 495–500
43. Chen, Z.J. *et al.* (1998) Gene dosage and stochastic effects determine the severity and direction of uniparental ribosomal RNA gene silencing (nucleolar dominance) in *Arabidopsis* allopolyploids. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14891–14896
44. Neves, N. *et al.* (1995) rRNA gene activity and control of expression mediated by methylation and imprinting during embryo development in wheat x rye hybrids. *Theor. Appl. Genet.* 91, 529–533
45. Borowska-Zuchowska, N. and Hasterok, R. (2017) Epigenetics of the preferential silencing of *Brachypodium stacei*-originated 35S rDNA loci in the allotetraploid grass *Brachypodium hybridum*. *Sci. Rep.* 7, 5260
46. Książczyk, T. *et al.* (2011) Immediate unidirectional epigenetic reprogramming of NORs occurs independently of rDNA rearrangements in synthetic and natural forms of a polyploid species *Brassica napus*. *Chromosoma* 120, 557–571
47. Preuss, S.B. *et al.* (2008) Multimegabase silencing in nucleolar dominance involves siRNA-directed DNA methylation and specific methylcytosine-binding proteins. *Mol. Cell* 32, 673–684
48. Pontvianne, F. *et al.* (2012) Histone methyltransferases regulating rRNA gene dose and dosage control in *Arabidopsis*. *Genes Dev.* 26, 945–957
49. Matzke, M.A. and Mosher, R.A. (2014) RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nat. Rev. Genet.* 15, 394–408
50. Singh, J. *et al.* (2019) Reaction mechanisms of Pol IV, RDR2, and DCL3 drive RNA channeling in the siRNA-directed DNA methylation pathway. *Mol. Cell* 75, 576–589.e575
51. Pontes, O. *et al.* (2007) Postembryonic establishment of megabase-scale gene silencing in nucleolar dominance. *PLoS One* 2, e1157
52. Hasterok, R. and Maluszynska, J. (2000) Nucleolar dominance does not occur in root tip cells of allotetraploid *Brassica* species. *Genome* 43, 574–579
53. Castilho, A. *et al.* (1995) The developmental stage of inactivation of rye origin rRNA genes in the embryo and endosperm of wheat × rye F1 hybrids. *Chromosom. Res.* 3, 169–174
54. Grummt, I. and Pikaard, C.S. (2003) Epigenetic silencing of RNA polymerase I transcription. *Nat. Rev. Mol. Cell Biol.* 4, 641–649
55. Rabanal, F.A. *et al.* (2017) Epistatic and allelic interactions control expression of ribosomal RNA gene clusters in *Arabidopsis thaliana*. *Genome Biol.* 18, 75
56. Tulpová, Z. *et al.* (2022) Fine structure and transcription dynamics of bread wheat ribosomal DNA loci deciphered by a multi-omics approach. *Plant Genome* 15, e20191
57. Hasterok, R. *et al.* (2001) Ribosomal DNA is an effective marker of *Brassica* chromosomes. *Theor. Appl. Genet.* 103, 486–490
58. Grummt, I. *et al.* (1982) Ribosomal RNA transcription *in vitro* is species specific. *Nature* 296, 173–174
59. Miesfeld, R. and Arnheim, N. (1984) Species-specific rDNA transcription is due to promoter-specific binding factors. *Mol. Cell. Biol.* 4, 221–227
60. Frieman, M. *et al.* (1999) RNA polymerase I transcription in a *Brassica* interspecific hybrid and its progenitors: tests of transcription factor involvement in nucleolar dominance. *Genetics* 152, 451–460
61. Houchins, K. *et al.* (1997) Cytosine methylation and nucleolar dominance in cereal hybrids. *Mol. Gen. Genet.* 255, 294–301
62. Caudy, A.A. and Pikaard, C.S. (2002) *Xenopus* ribosomal RNA gene intergenic spacer elements conferring transcriptional enhancement and nucleolar dominance-like competition in oocytes. *J. Biol. Chem.* 277, 31577–31584
63. Pikaard, C.S. and Reeder, R.H. (1988) Sequence elements essential for function of the *Xenopus laevis* ribosomal DNA enhancers. *Mol. Cell. Biol.* 8, 4282–4288
64. Nicoloff, H. *et al.* (1979) 'Nucleolar dominance' as observed in barley translocation lines with specifically reconstructed SAT chromosomes. *Theor. Appl. Genet.* 55, 247–251
65. Mohannath, G. *et al.* (2016) Selective nucleolus organizer inactivation in *Arabidopsis* is a chromosome position-effect phenomenon. *Proc. Natl. Acad. Sci. U. S. A.* 113, 13426–13431
66. Handa, H. *et al.* (2018) Structural features of two major nucleolar organizer regions (NORs), Nor-B1 and Nor-B2, and chromosome-specific rRNA gene expression in wheat. *Plant J.* 96, 1148–1159
67. Morais-Cecílio, L. *et al.* (2000) Modification of wheat rDNA loci by rye B chromosomes: a chromatin organization model. *Chromosom. Res.* 8, 341–351
68. Neves, N. *et al.* (1997) Nucleolar dominance in triticales: control by unlinked genes. *Chromosom. Res.* 5, 125–131
69. McKinlay, A. *et al.* (2021) Targeted enrichment of rRNA gene tandem arrays for ultra-long sequencing by selective restriction endonuclease digestion. *Front. Plant Sci.* 12
70. Rhoads, A. and Au, K.F. (2015) PacBio sequencing and its applications. *Genom. Proteom. Bioinform.* 13, 278–289
71. Gouil, Q. and Keniry, A. (2019) Latest techniques to study DNA methylation. *Essays Biochem.* 63, 639–648
72. Sims, J. *et al.* (2021) Sequencing of the *Arabidopsis* NOR2 reveals its distinct organization and tissue-specific rRNA ribosomal variants. *Nat. Commun.* 12, 387
73. Riddle, N.C. and Richards, E.J. (2002) The control of natural variation in cytosine methylation in *Arabidopsis*. *Genetics* 162, 355–363

74. Mozgová, I. *et al.* (2010) Dysfunction of chromatin assembly factor 1 induces shortening of telomeres and loss of 45S rDNA in *Arabidopsis thaliana*. *Plant Cell* 22, 2768–2780
75. Pavlištová, V. *et al.* (2016) Phenotypic reversion in fas mutants of *Arabidopsis thaliana* by reintroduction of FAS genes: variable recovery of telomeres with major spatial rearrangements and transcriptional reprogramming of 45S rDNA genes. *Plant J.* 88, 411–424
76. Pontvianne, F. *et al.* (2013) Subnuclear partitioning of rRNA genes between the nucleolus and nucleoplasm reflects alternative epiallelic states. *Genes Dev.* 27, 1545–1550
77. Lopez, F.B. *et al.* (2021) Gene dosage compensation of rRNA transcript levels in *Arabidopsis thaliana* lines with reduced ribosomal gene copy number. *Plant Cell* 33, 1135–1150
78. Chandrasekhara, C. *et al.* (2016) Chromosome-specific NOR inactivation explains selective rRNA gene silencing and dosage control in *Arabidopsis*. *Genes Dev.* 30, 177–190
79. Doležel, J. *et al.* (2018) One major challenge of sequencing large plant genomes is to know how big they really are. *Int. J. Mol. Sci.* 19, 3554
80. Borowska-Zuchowska, N. *et al.* (2020) The fate of 35S rRNA genes in the allotetraploid grass *Brachypodium hybridum*. *Plant J.* 103, 1810–1825
81. Borowska-Zuchowska, N. *et al.* (2021) To be or not to be expressed: the first evidence of a nucleolar dominance tissue-specificity in *Brachypodium hybridum*. *Front. Plant Sci.* 12, 768347
82. Hasterok, R. *et al.* (2022) *Brachypodium*: 20 years as a grass biology model system; the way forward? *Trends Plant Sci.* 27, 1002–1016
83. Catalan, P. *et al.* (2012) Evolution and taxonomic split of the model grass *Brachypodium distachyon*. *Ann. Bot.* 109, 385–405
84. Hasterok, R. *et al.* (2004) Laying the cytotaxonomic foundations of a new model grass, *Brachypodium distachyon* (L.) Beauv. *Chromosom. Res.* 12, 397–403
85. Hasterok, R. *et al.* (2006) Alignment of the genomes of *Brachypodium distachyon* and temperate cereals and grasses using bacterial artificial chromosome landing with fluorescence *in situ* hybridization. *Genetics* 173, 349–362
86. Gordon, S.P. *et al.* (2020) Gradual polyploid genome evolution revealed by pan-genomic analysis of *Brachypodium hybridum* and its diploid progenitors. *Nat. Commun.* 11, 3670
87. Lusinska, J. *et al.* (2018) Chromosome identification and reconstruction of evolutionary rearrangements in *Brachypodium distachyon*, *B. stacei* and *B. hybridum*. *Ann. Bot.* 122, 445–459
88. Borowska-Zuchowska, N. *et al.* (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
89. Díaz-Pérez, A. *et al.* (2018) Reconstructing the origins and the biogeography of species' genomes in the highly reticulate allopolyploid-rich model grass genus *Brachypodium* using minimum evolution, coalescence and maximum likelihood approaches. *Mol. Phyl. Evol.* 127, 256–271
90. International Brachypodium Initiative (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 463, 763–768
91. Streit, D. and Schleiff, E. (2021) The Arabidopsis 2'-O-ribose-methylation and pseudouridylation landscape of rRNA in comparison to human and yeast. *Front. Plant Sci.* 12, 684626
92. Sáez-Vásquez, J. and Delseny, M. (2019) Ribosome biogenesis in plants: from functional 45S ribosomal DNA organization to ribosome assembly factors. *Plant Cell* 31, 1945–1967
93. Irigoyen, M.L. *et al.* (2001) Discrimination of the closely related A and B genomes in AABB tetraploid species of *Avena*. *Theor. Appl. Genet.* 103, 1160–1166
94. de Paula, C.M. *et al.* (2016) Chromosomal distribution of H3K4me2, H3K9me2 and 5-methylcytosine: variations associated with polyploidy and hybridization in *Brachiaria* (Poaceae). *Plant Cell Rep.* 35, 1359–1369
95. Nani, T.F. *et al.* (2016) Physical map of repetitive DNA sites in *Brachiaria* spp.: intravarietal and interspecific polymorphisms. *Crop Sci.* 56, 1769–1783
96. Dubcovsky, J. *et al.* (1992) Variation in the restriction fragments of 18S–26S rRNA loci in South American *Elymus* (Triticeae). *Genome* 35, 881–885
97. Bustamante, F.O. *et al.* (2014) Distribution of rDNA in diploid and polyploid *Lolium multiflorum* Lam. and fragile sites in 45S rDNA regions. *Crop Sci.* 54, 617–625
98. Orellana, J. *et al.* (1984) Nucleolar competition analysis in *Aegilops ventricosa* and its amphiploids with tetraploid wheats and diploid rye by the silver-staining procedure. *Can. J. Genet. Cytol.* 26, 34–39
99. Hutchinson, J. and Miller, T.E. (1982) The nucleolar organisers of tetraploid and hexaploid wheats revealed by *in situ* hybridisation. *Theor. Appl. Genet.* 61, 285–288
100. Armstrong, K.C. *et al.* (1991) Expression of *Thinopyrum distichum* NORs in wheat×*Thinopyrum* amphiploids and their backcross generations. *Theor. Appl. Genet.* 81, 363–368
101. McMurphy, L.M. and Rayburn, A.L. (1994) Cytological evidence for nucleolar competition in a maize hybrid. *J. Hered.* 85, 407–410