Multiple origins, one evolutionary trajectory: gradual evolution characterizes distinct lineages of allotetraploid Brachypodium

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Abstract

The “genomic shock” hypothesis posits that unusual challenges to genome integrity such as whole genome duplication may induce chaotic genomic restructuring. Decades of research on polyploid genomes have revealed that this is often, but not always the case. While some polyploids show major chromosomal rearrangements and derepression of transposable elements in the immediate aftermath of whole genome duplication, others do not. Nonetheless, all polyploids show gradual diploidization over evolutionary time. To evaluate these hypotheses, we produced a chromosome-scale reference genome for the natural allotetraploid grass Brachypodium hybridum, accession “Bhyb26.” We compared 2 independently derived accessions of B. hybridum and their deeply diverged diploid progenitor species Brachypodium stacei and Brachypodium distachyon. The 2 B. hybridum lineages provide a natural timecourse in genome evolution because one formed 1.4 million years ago, and the other formed 140 thousand years ago. The genome of the older lineage reveals signs of gradual post-whole genome duplication genome evolution including minor gene loss and genome rearrangement that are missing from the younger lineage. In neither B. hybridum lineage do we find signs of homeologous recombination or pronounced transposable element activation, though we find evidence supporting steady post-whole genome duplication transposable element activity in the older lineage. Gene loss in the older lineage was slightly biased toward 1 subgenome, but genome dominance was not observed at the transcriptomic level. We propose that relaxed selection, rather than an abrupt genomic shock, drives evolutionary novelty in B. hybridum, and that the progenitor species’ similarity in transposable element load may account for the subtlety of the observed genome dominance.

Keywords: Brachypodium; polyploidy; genomics; fractionation; genome dominance; structural variation; transposable element dynamics; Plant Genetics and Genomics

Introduction

Nearly all plant lineages have experienced at least 1 polyploidy event, or whole genome duplication (WGD), in their recent or ancient past (Clark and Donoghue 2018). Today’s diploids have undergone a process known as genetic diploidization, in which a polyploid loses genomic sequence over evolutionary time and becomes diploid again, though some duplicate genes are retained (Ma and Gustafson 2005). Polyploidy is an important source of genetic novelty and contributes to adaptive evolution (Van de Peer et al. 2017, 2021; Baduel et al. 2018).

In many cases, WGD is accompanied by rapid genome restructuring, in line with the hypothesis that WGD may represent an event that induces a series of rapid genetic and epigenetic modifications as a result of conflicts between parental genomes” (Bird et al. 2018), though in practice “genomic shock” is often used to indicate any sort of dramatic genetic consequence of hybridization or of WGD. The most dramatic examples of genomic shock are chromosomal rearrangements resulting from recombination between homeologous or homologous chromosomes, which may occur in the early generations after WGD (Ramsey and Schemske 2002) in allopolyploids (those whose progenitors are different species) and autopolyploids (those whose progenitors are from the same species), respectively (Grandont et al. 2013). Homeologous rearrangements are common in resynthesized polyploids (Mason and Wendel 2020), and evidence for them has been observed in a number of natural polyploids including Brassica napus.
Some allopolyploids exhibit a dominant subgenome, whose genes are expressed at higher levels than their homeolog(s) on the other subgenome(s) (Alger and Edger 2020). It remains unclear to what extent genome dominance is established instantaneously or gradually. The evidence suggests both: expression bias established in the early generations following WGD may be reinforced over evolutionary time, with the dominant subgenome ultimately contributing more genes to the fully diploidized genome (Flagel et al. 2008; Feldman and Levy 2009; Flagel and Wendel 2010; Woodhouse et al. 2014; Edger et al. 2017).

Transposable element (TE) activation (transcription and/or transposition) can also occur following WGD on short and long timescales. Post-WGD epigenetic changes are not uncommon in polyploids (Ha et al. 2009; Parsons et al. 2009; Kenan-Eichert et al. 2011; Yaakov and Kashkush 2012; Yuan et al. 2020; Jiang et al. 2021). In allopolyploids, a single TE family or several families may be activated immediately following WGD, probably due to epigenetic incompatibilities between subgenomes (Madlung et al. 2005; Parsons et al. 2009; Martienssen 2010; Groszmann et al. 2011; Yaakov and Kashkush 2012; Sarilar et al. 2013; Gantuz et al. 2022). TE movement can also occur in polyploids over longer timescales due to relaxed selection because duplicate genes allow for a greater tolerance for TE insertions (Ágren et al. 2016; Baduel et al. 2019).

While some polyploids show chromosome rearrangements, expression dominance, and TE activation following WGD, these responses are not universal. Many natural allopolyploids show little to no genome restructuring, including Arabidopsis suecica (Burns et al. 2021), Eragrostis tef (VanBuren et al. 2020), Capsella bursa-pastoris (Douglas et al. 2015), and white clover (Griffiths et al. 2013). However, we did find evidence that Bhyb26, unlike the younger lineage, has experienced post-WGD structural change and slight gene loss (Gordon et al. 2015). RNA-Seq

Materials and methods

Sample preparation

For details on the lines used in this study and preparation of high-molecular weight DNA for PacBio sequencing, see Gordon et al. (2020). PacBio sequencing was performed on a PacBio RSII instrument at the HudsonAlpha Institute.

RNA-Seq

To collect leaf tissue, plants were grown in a growth chamber in short-day conditions (26°C 10 h light, 18°C 14 h dark). Leaf tissues were harvested from plants at the 4–5 leaf stage. To collect spikelets, plants were grown in long-day conditions (26°C 16 h light, 18°C 8 h dark). Spikelets were harvested 3 days after inflorescence emergence. For root tissue, plants were grown in plastic sande cups with lids on sterile MS medium, and roots were harvested at 1–2 weeks. Callus tissue was prepared as described (Bragg et al. 2015). RNA was extracted using TRIzol (Invitrogen) and purified with the Purelink RNA Mini Kit (Invitrogen) including DNA extraction from the diploidization experiment.
removal with the Purelink DNAse Set (Invitrogen). Stranded short-read RNA-seq libraries were created using the Illumina TruSeq kit and quantified by qPCR. Sequencing was performed using an Illumina NovaSeq S4 instrument. Stranded long-read RNA-seq libraries were created using the PacBio SMRTbell Template Prep Kit v. 1.0 with or without 2–10-kb size selection using the BluePippin system (Sage Science). Sequencing was performed using a PacBio Sequel II instrument.

Raw RNA-seq reads were filtered and trimmed using BBDuk from the BBtools package (https://sourceforge.net/projects/bbmap). Reads were aligned to the complete reference genome (Bhyb26 v2.1) using BBmap. To increase mapping stringency, given the redundancy of a polyploid genome, reads were required to share 90% sequence identity with the target location, and ambiguous reads were discarded. Gene-level counts were obtained using HTSeq (Anders et al. 2015). Transcripts per million (TPM) values were calculated using a custom Python script (https://github.com/vtartaglio/Scarlett_et_al_2022/blob/master/fig4/countsToTPMbasicNEW.py; last accessed 6-20-2022).

Assembly

Assembly of the Bhyb26 genome was performed with MECAT (Xiao et al. 2017) and polished using ARROW (https://github.com/PacificBiosciences/GCcpp; last accessed 6-20-2022).

Misjoins in the assembly were identified using Hi-C data as part of the JUICER pipeline (Durand, Shamim, et al. 2016). No misjoins were identified in the polished assembly. Scaffolds were then oriented, ordered, and joined together using Hi-C scaffolding. Significant telomeric sequence was properly oriented in the assembly. Hi-C reads were then aligned to the joined release. A contact map was generated using JUICER-pre and visualized using JUICEBOX (Durand, Robinson, et al. 2016) as a quality control check on the order/orientation of contigs in the chromosomes. Care was taken to ensure that telomeres were properly oriented in the chromosomes, and the resulting sequence was screened for retained adapter/vector and/or contaminants.

Adjacent alternative haplotypes were identified on the joined contig set. Adjacent alternative haplotypes were collapsed using the longest common substring between the 2 haplotypes. A total of 22 adjacent alternative haplotypes were collapsed. Chromosomes of the v.2 release were numbered and oriented relative to the previous v.1 release (Gordon et al. 2020). Finally, homozygous SNPs and INDELS were corrected using 40x of Illumina reads.

Annotation

Transcript assemblies were made from Illumina RNA-seq reads using PERTRAN, which conducts genome-guided transcriptome short-read assembly via GSNAP (Wu and Nacu 2010) and builds splice alignment graphs after alignment validation, realignment, and correction. PacBio Iso-Seq circular consensus sequences (CCSs) were corrected and collapsed by a genome guided correction pipeline, which aligns CCS reads to the genome with GMAP (Wu and Nacu 2010) and clusters alignments when all introns are the same or 95% overlap for single exon. Subsequently 625,901 total transcript assemblies were constructed using PASA (Haas et al. 2003) from the Iso-seq transcript assemblies. Loci were determined by transcript assembly alignments and/or EXONERATE (Slater and Birney 2005) alignments of proteins from diverse plant species and Swiss-Prot proteomes to the repeat-soft-masked B. hybridum Bhyb26 genome using RepeatMasker (Smit et al. 2013–2015). Gene models were predicted by homology-based predictors, FGENESH+ (Salamov and Solovyev 2000), FGENESH_EST,

Fig. 1. Both independent allopolyploid lineages reveal remarkable genome stability. a) Cladogram illustrating relationships in the B. hybridum polyploid complex. b) BAC-FISH with probes specific to either the S subgenome (8P20; red fluorescence) or D subgenome (ABR1-63-E6; green fluorescence) indicate the absence of large-scale rearrangement between subgenomes. Left, Bhyb26, right, ABR113. Blue fluorescence, DAPI. Bars, 5 µm. c) Riparian (synteny) plot showing high collinearity between each subgenome and its progenitor, and low collinearity between the polyploid subgenomes, consistent with the high divergence of the progenitor species’ genomes.
and EXONERATE, PASA assembly ORFs (in-house homology constrained ORF finder) and from AUGUSTUS (Stanke et al. 2006) trained by the high confidence PASA assembly ORFs and with intron hints from short-read alignments. The best scored predictions for each locus were selected using multiple positive factors including EST and protein support, and 1 negative factor: overlap with repeats. The selected gene predictions were improved by PASA. Improvement included adding UTRs, splicing correction, and adding alternative transcripts. PASA-improved transcripts were selected based on Cscore, protein coverage, EST coverage, and their CDS overlapping with repeats. Weak gene models, incomplete gene models, gene models whose protein was more than 30% in Pfam TE domains, low homology supported without fully transcriptome supported gene models, and gene models consisting of a short single exon without protein domain nor good expression gene models were manually filtered out.

**BAC-FISH**

BAC-FISH was performed on *B. hybridum* Bhyb26 and ABR113 with *B. distichyon* and *B. stacei*-derived Bacterial Artificial Chromosome clones (BACs): ABR1-63-E6 from *B. distichyon* ABR1 genomic DNA (gDNA) library (Hasterok, Maaeek, et al. 2006) and 08P20 from *B. stacei* gDNA library made by B. Chalhoub. After isolation using the standard alkaline lysis method, BAC DNAs were labeled by nick translation using digoxigenin-11-DUTP (ABR1-63-E6) or tetrathymethylrhodamine-5-DUTP (08P20) as previously described (Jenkins and Hasterok 2007). Chromosome preparations were made using the method of Hasterok, Dulawa, et al. (2006) and Gordon et al. (2020). After germinating seeds in Petri dishes on moist filter paper, seedlings were incubated for 24 h in ice-cold water and fixed in 3:1 methanol-glacial acetic acid. After excision, roots were enzymatically digested for 2 h at 37°C in 1% (w/v) cellulase (Calbiochem), 1% (w/v) cellulase “Ozouka R-10” (Serva), and 8% (v/v) pectinase (Sigma). After removing the root cap and skin, the digested meristematic material was transferred to a slide and squashed in a drop of 45% acetic acid. Coverslips were removed after freezing. Fluorescence in situ hybridization (FISH) followed the method of Jenkins and Hasterok (2007) with previously described modifications (Lusinska et al. 2018; Gordon et al. 2020). The hybridization mixture comprised 40% deionized formamide, 15% (w/v) dextran sulfate, 2× SSC, 0.5% SDS and BAC DNA probes, each at the final concentration of 75–200 ng/slide. The probes in the hybridization mixture were prehydrated at 80°C for 10 min and, after application to the preparations, denatured at 70°C for 4.5 min. Hybridization was carried out at 37°C in a humid chamber for at least 16 h. Posthybridization washes were carried at the equivalent of ~60% stringency, and the digoxigenated probe was immunodetected using relevant (FITC-conjugated anti-digoxigenin, Roche) antibodies. The preparations were counterstained with 2.5 μg/ml DAPI, mounted in Vectashield (Vector Laboratories) and analyzed under Axiosimager Z.2 epifluorescent microscope (Zeiss) coupled with AxioCam Mrm high-sensitivity monochromatic camera (Zeiss).

**Synteny and gene loss**

GENESPACE v.0.9.4 (Lovell et al. 2018, 2022) (https://github.com/jtlovell/Genespace) was run with default parameters to evaluate synteny among *Brachypodium* genomes and rice (*B. distichyon* Bd21 v3.2, proteome id: 556; B. stacei v1.1, proteome id: 316; *B. hybridum* ABR113 v1.1, proteome id: 463; *B. hybridum* Bhyb26 v2.1, proteome id: 693; *Oryza sativa* MSU v0.7, Phytozome, proteome id: 323). All reference genomes were obtained from Phytozome (Goodstein et al. 2012) (https://phytozome-next.jgi.doe.gov/).

Once we had identified the Bhyb26 genomic region potentially containing the missing gene, the region was extracted using bedtools (Quinlan 2014). The diploid peptide was then aligned to that region using the codon- and intron-aware protein2genome model of EXONERATE (Slater and Birney 2005). We found that these EXONERATE alignments were of excellent quality, but EXONERATE codon-aware DNA–DNA alignments were of poor quality, especially on long genes containing frameshifts. Therefore, we next aligned the diploid coding sequence (from Phytozome) to the inferred Bhyb26 coding sequence (from EXONERATE) using MACSE (Ranwez et al. 2011, 2018), and these alignments were used to calculate pairwise nonsynonymous to synonymous substitution rate ratios via the yn00 program from PAML (Yang 2007). The same procedure was applied to fully conserved Bhyb26 genes as a control, with each of 1,000 trials consisting of 224 BhD genes and 240 BhS genes (464 total), since this was the final number of aligned “missing” genes from each subgenome.

### TE annotation and analysis

TE annotation was performed with an in-house pipeline. The pipeline was not designed for external use, but the scripts are available at https://github.com/vtartaglio/Scarlett_et_al_2022/tree/master/fig3, last accessed 6-20-2022.
master/fig5; last accessed 6-202022. First, monocot TEs were pulled from the TREP database to create an initial TE library. To discover TEs from the Brachypodium genomes that are not in public databases, we ran a suite of TE discovery tools. Tools used were LTR-Harvest (Ellinghaus et al. 2008), LTR-retriever (Ou and Jiang 2018), TransposonPSI (http://transposonpsi.sourceforge.net/; last accessed 6-20-2022), MITTE-Tracker (Crescente et al. 2018), and RepeatModeler2 (Flynn et al. 2019). These TEs were added to the library of publicly available repeats and redundancy was removed with CD-HIT (Fu et al. 2012) according to the “80-80-80 rule” (Wicker et al. 2007) (cd-hit-est -c 0.8 -G 0 -as 0.8 -n 5 -T 0.0 -d 0.0 -M 0). Sequences were clustered if they had 80% identity locally, and the alignment had to cover at least 80% of the shorter sequence. Only the longest sequence (the representative sequence) from each cluster was retained. Representative sequences less than 80 bp were discarded. Next, ProtExcluder from the MAKER-P pipeline (Campbell et al. 2014) was used to search the TE library against a plant protein database, and TEs with significant hits to genes were discarded. Only the longest sequence (the representative sequence) from each cluster was retained. Representative sequences less than 80 bp were discarded.

All the genomes listed above were annotated with RepeatMasker (Smit et al. 2013–2015) using the appropriate TE library. Noncontiguous genomic sequences that match the same exemplar were designated fragments of a single TE copy if certain distance and orientation criteria were met, using “one code to find them all” (Bailly-Bechet et al. 2014) with default parameters. A TE family was defined as the set of all TE copies that were hits to a particular exemplar. Subgenome-specific TE families were defined as those that had at least 5 members and that had at least 90% of their copies on one of the 2 subgenomes (this latter criterion comes from Wicker et al. (2018)).

TEMP2 (Yu, Huang, et al. 2021) was used to identify TE polymorphisms relative to the ABR113 reference genome. Library quality was assessed with FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/; last accessed 6-20-2022). Short-read libraries were the same as those used in (Gordon et al. 2020). Only transposon insertion polymorphisms (TIPs) that were supported by reads on both ends ("1p1") and that had a frequency of 20%—that is, at least 20% of sequenced genome supports the insertion—were considered.

To estimate the insertion times of intact LTR-RTs, we largely followed the method of (Wicker et al. 2018), which itself derives from (SanMiguel et al. 1998). The 3’ and 5’ LTRs of individual LTR-RTs were aligned to each other with MAFFT (Katoh et al. 2002) (einsi – adjustdirectionaccurately) and trimmed with trimAl (Capella-Gutiérrez et al. 2009) (trimal -gapthreshold 0.8). Then, EMBOSS dismat (http://emboss.sourceforge.net/; last accessed 6-20-2022) was run on each alignment with the Kimura 2-parameter correction (dismat -nucmethod 2) to obtain the % identity between the LTRs. Insertion time was calculated with the equation: $T = D/2t$, where $T$ is the time elapsed since the insertion, $D$ is the estimated LTR divergence, and $t$ is the substitution rate, for which we used $1.3 \times 10^{-8}$ substitutions per site per year (Ma and Bennetzen 2004).

**Results**

**Assembly and annotation**

We assemled a chromosome-scale reference genome of the naturally inbred allotetraploid B. hybridum accession Bhyb26, which was collected in the wild in Jaen, Spain. In a previous study, we built an illumina-based genome assembly (Bhyb26 v1.1) (Gordon et al. 2020). The new genome assembly (Bhyb26 v2.1) was constructed de novo using PacBio and Hi-C technologies. The main assembly was performed with MECAT (Xiao et al. 2017) using 45× consensus long-read coverage (average read length of 19,692 bp), and the resulting assembly was polished with 40× illumina reads using Arrow (https://github.com/PacificBiosciences/GCpp; last accessed 6-20-2022). Hi-C scaffolding was performed using the Juicer pipeline (Durand, Shamim, et al. 2016). There were 61 contigs with a contig N50 of 16.5 Mb. A total of 51 joins were applied to the broken assembly to form the final assembly consisting of 32 scaffolds. Fifteen of the 32 scaffolds contain 99.69% of the assembled sequence, and these correspond to the 15 chromosomes of B. hybridum (5 BhD and 10 BhS). The remaining 17 scaffolds totaled about 1.6 Mb of sequence. The final genome size is 528.5 Mb and contains less than 0.1% gaps.

Annotation was performed with the JGI pipeline (see Materials and Methods). Transcript assemblies were made from ~290 million 2 x 150 stranded paired-end illumina RNA-seq reads and 23 million PacBio Iso-Seq CCSs, each generated from 4 tissues: leaf, spikelet, root, and callus. The annotation (v.2.1) contains 53,864 primary transcripts with an average of 5.1 exons, a median exon length of 166 bp, and a median intron length of 142 bp. The BUSCO v3.0.2 score on Embryophyta odb9 is 99.7% complete.

**Syntenic and structural variation**

We began our investigation with a survey of Bhyb26 genome structure using molecular cytogenetics. FISH experiments with BACs containing large gDNA inserts as probes (BAC “landing”) (Jenkins and Hasterok 2007) were conducted using 2 clones from previously constructed BAC libraries (Hasterok, Marasek, et al. 2006; Chalhoub B, personal communication) (Fig. 1b). The BAC ABR1-63-E6 containing B. distachyon gDNA was found to reliably hybridize with the entire D subgenome, but it did not hybridize with any chromosomes of the S subgenome. The BAC 8P20 containing B. stacei gDNA hybridized with the entire S subgenome chromosomes, but not with the D subgenome chromosomes. These 2 BACs discriminated between subgenomes in both polymorphisms. Therefore, in both Bhyb26 and ABR113, the subgenomes are readily distinguishable at the level of molecular probes, and no evidence of sequence exchange between subgenomes was observed.

Next, we performed a computational survey of Bhyb26 genome structure. Syntenic blocks between each subgenome and its diploid progenitor species were identified using the GENESPACE pipeline (Lovell et al. 2018, 2022) (Fig. 1c). 97.9% of the Bhyb26 D subgenome was contained within blocks syntenic to B. distachyon (Bd21 v3.2), and 93.4% of the Bhyb26 S subgenome was contained within blocks syntenic to B. stacei (ABR114 v1.1).

There were 41 Bd-BhD syntenic blocks and on average they were 6.6 Mb in length, while the 124 Bs-BhS syntenic blocks were on average 2 Mb in length. This lower concordance between the S subgenome and D subgenome chromosomes, but not with the D subgenome chromosomes. These 2 BACs discriminated between subgenomes in both polymorphisms. Therefore, in both Bhyb26 and ABR113, the subgenomes are readily distinguishable at the level of molecular probes, and no evidence of sequence exchange between subgenomes was observed.
synteny pipeline on each *B. hybridum* ABR113 subgenome against the diploid progenitor species (Fig. 2, d–f). The *B. stacei* reference genome is much lower quality than the *B. distachyon* reference genome, and small inversions were common in the centromeres of the *B. stacei* dot plots (Fig. 2, b and e). The most prominent of these were on BhS3, BhS5, and BhS7. The inversions on BhS3 and BhS7 were not well-supported upon closer inspection of the dot plots (Fig. 2, c and f). The inversion that is apparently private to ABR113 on BhS5 was more clear, that is, all anchor genes in that region supported the inversion. However, given that it is in a centromere that contains small inversions in all 3 dot plots, it is likely that this is an assembly error. Thus, none of the inversions on the ABR113 S subgenome appear to reflect true structural variation.

To ascertain whether inversions or deletions are common between diploid *Brachypodium* accessions, we re-ran our synteny pipeline on 2 more long-read *B. distachyon* genomes, 1 from each of the 3 major populations of *B. distachyon*: Bd21 representing the Turkish + clade, Bd30-1 representing the Spanish + clade, and Bd1-1 representing the extremely delayed flowering + (EDF+) clade (Gordon et al. 2017). We detected no inversions among these genomes. These results indicate that the Bhyb26 genome contains several inversions that are private to that lineage, and such inversions are not common among diverged *B. distachyon* accessions. While it is still possible that these inversions were present in the actual progenitors of Bhyb26 prior to polyploidization, the absence of any similarly dramatic structural variation in the widely sampled natural diversity of *B. distachyon* suggests that these inversions may well have occurred postpolyploidy.

**Gene loss**

In a previous analysis (Gordon et al. 2020), we ascertained that the subgenomes of Bhyb26 were more genetically diverged from the corresponding diploid progenitor species’ reference genomes than were the subgenomes of ABR113, but the low-quality Bhyb26 assembly did not permit in-depth analysis of this variation. We were particularly interested in the degradation or loss of genes, which would be indicative of the early stages of diploidization. However, identifying genes that have been lost in Bhyb26 since WGD is difficult without its true progenitors, since gene presence–absence variation would be common among arbitrary accessions of *B. distachyon* and *B. stacei* (Gordon et al. 2017). We therefore searched for losses of highly conserved genes, reasoning that any gene that is conserved within and beyond the genus *Brachypodium* was probably present in the true progenitors of Bhyb26. Using the synteny and homology-based pipeline GENESPACE (Lovell et al. 2018), we identified 15,217 orthogroups that contained at least 1 orthologous gene in both subgenomes of both polyploids, each diploid genome, and rice (*O. sativa*). In other words, we identified many thousands of genes that are widely conserved across the genus *Brachypodium* and in rice. We then identified orthogroups where all but 1 genome or subgenome was represented (Fig. 3a). Unsurprisingly, orthogroups that had an ortholog in every *Brachypodium* sub/genome but not rice were most common (3,912 orthogroups). More surprisingly, the number of cases where a gene was “missing” from each *B. hybridum* genome was greater than we would expect by summing the progenitors, and this discrepancy was greater for Bhyb26 than for *B. stacei* ABR114.
ABR113. 365 and 299 genes were present in every sub/genome except Bhyb26D and Bhyb26S, respectively; meanwhile, only 143 and 108 genes were present in every sub/genome except ABR113D and ABR113S, respectively. In other words, we identified only 251 “missing” genes in ABR113, but we found 664 in Bhyb26, a significant difference considering the number of genes in each genome (Pearson’s Chi-squared test with Yates’ continuity correction $P = 4.84e^{-70}$). The high number of conspicuously absent genes in Bhyb26 suggests that at least some of these genes may be true pseudogenes or deletions that occurred post-WGD as a consequence of relaxed selection.

We interrogated these “missing genes,” which we dubbed putative pseudogenes, more closely (Fig. 3b). Six hundred sixty-four broadly conserved genes were absent in 1 Bhyb26 subgenome. In 517 of those cases, we were able to definitively identify a region of the Bhyb26 genome where the missing gene “should” be (see Materials and Methods). A total of 464 of those 517 regions contained sequence that could be aligned to the peptide sequence of the corresponding diploid gene (Supplementary Fig. 1). These sequences were scattered throughout the Bhyb26 genome (Supplementary Fig. 2a). As a control, we ran the same procedure on a set of 464 randomly selected Bhyb26 genes that were completely conserved, that is, genes from orthogroups in which all genomes are represented (see Materials and Methods). We repeated the random sampling and the analysis for a total of 1,000 trials. The putative pseudogenes were shorter than expected: for conserved genes, the alignments were 22.7 bp shorter than the peptide on average, while for the putative pseudogenes, they were 224.3 bp shorter (Table 1). In none of the 1,000 samples of conserved genes did this value exceed that of the putative pseudogenes. In addition, 18.8% of the Bhyb26 putative pseudogenes contained a premature stop codon in Bhyb26 relative to the diploid progenitor genome gene. Mean dN/dS (aligned to diploid progenitor genome ortholog) Median transcripts per million (TPM)

Table 1. Characteristics of Bhyb26 putative pseudogenes vs. Bhyb26 annotated, conserved genes.

| Metric                                                                 | Putative pseudogenes | Conserved genes (average of 1,000 trials) |
|------------------------------------------------------------------------|----------------------|------------------------------------------|
| Mean length difference between Bhyb26 alignment and diploid ortholog in progenitor species genome (bp) | 224.3                | 22.7                                     |
| Percentage of alignments that contained a premature stop codon in Bhyb26 relative to diploid progenitor genome gene | 18.8                 | 0.0                                      |
| Mean dN/dS (aligned to diploid progenitor genome ortholog)              | 0.53                 | 0.36                                     |
| Median transcripts per million (TPM)                                   | 0.0                  | 4.14                                     |

Fig. 3. Bhyb26 shows more gene loss than ABR113. a) UpSet plot of orthogroups (groups of orthologous genes) reveals a high number of cases where a single Bhyb26 subgenome lacks an ortholog of an otherwise widely conserved gene. b) Workflow for identifying putative pseudogenes. c) Distribution of dN/dS ratios for Bhyb26 and ABR113 “lost genes,” and Bhyb26 widely conserved genes. All dN/dS values are relative to the corresponding diploid ortholog.
genes among the putative pseudogenes was higher than expected for that tissue (one-sided exact binomial test: \( P = 5.93e^{-78}, P = 1.43e^{-69}, P = 1.72e^{-79}, \) and \( P = 1.40e^{-41}, \) respectively). Finally, a valid dN/dS ratio, that is, the ratio of nonsynonymous to synonymous amino acid substitutions (Yang 2007), could be calculated for 423 of the 464 genes (Fig. 3c). The average dN/dS for the lost genes was 0.53 as opposed to the conserved genes’ 0.36. This is a significant difference (Welch 2 sample t-test \( P = 2.1e^{-9} \)) and is consistent with the hypothesis that the putative pseudogenes are experiencing relaxed selection. Out of 1,000 trials, there were no cases in which the average dN/dS from conserved genes exceeded the average dN/dS from the putative pseudogenes. Interestingly, when we repeated this procedure on the ABR113 “missing” genes, obtaining 220 putative pseudogenes and 106 dN/dS values (many of the alignments had no substitutions in the polyploid), the mean dN/dS for these putative pseudogenes was only 0.324, and their distribution was also similar to that of the conserved genes (Fig. 3c). Thus, only the Bhyb26 genes showed signs of pseudogenization. The putative pseudogenes went unannotated due to lack of homology, incidence of premature stop codons, and weak transcriptome support (Table 1).

We hypothesized that TE insertion into the gene body may have contributed to the inactivation of these putative pseudogenes. A total of 130 of the 464 putative pseudogenes (28%) contained a TE somewhere between the start and end of the alignable region. Meanwhile, in the 1,000 control trials, on average 196 of the 464 randomly selected conserved genes (42.2%) contained a TE (Supplementary Fig. 2c). There were no trials in which the number of putative pseudogenes containing a TE (130) exceeded the number of conserved genes containing a TE; therefore, the \( P \)-value of this one-sided test is zero. This shows that the putative pseudogenes are not more likely to contain a TE than we would expect by random chance, although it is still possible that TE insertions in nearby regulatory regions may have deactivated some of the genes.

Finally, we noticed that both polyploid lineages had apparently lost more genes from the D subgenome than the S subgenome (Fig. 3a). We performed a chi-square test to test whether the biased loss was significantly different from a bias we might expect by chance, based on the total number of genes in each subgenome. In Bhyb26, the difference was significant (Pearson’s Chi-squared test with Yates’ continuity correction \( P = 0.031 \)), but not in ABR113 \( P = 0.34 \). Together, all these results indicate that (1) a significant portion of the “missing” genes in Bhyb26 are of dubious functionality, (2) the gene loss is marked by small-scale substitutions and deletions rather than by rampant TE insertions or by deletion of entire genes, and (3) in Bhyb26, the S subgenome is slightly but significantly dominant in terms of gene retention.

### Gene expression

Using Illumina RNA-seq data, we investigated whether 1 subgenome was systematically more highly expressed than the other in Bhyb26. Two analytical approaches were used: 1 for homeolog expression bias (HEB) and 1 for subgenome expression dominance. Since we did not have biological replicates, we could not conduct a formal HEB analysis, which requires accurate estimation of differential gene expression. Nevertheless, our experiment should be enough to distinguish a genome-wide trend, since the >50,000 genes provide a sort of replication, as do the 4 tissues sampled. We used GENESPACE (Lovell et al. 2018, 2022) to identify 1:1 homeologs between the subgenomes and then filtered out noisy gene pairs (those where both homeologs had a TPM < 1.0), and recorded whether the BhD homeolog or the BhS homeolog had the higher TPM. The chance that the homeolog from a particular subgenome had a higher TPM was near 50/50 in all tissues (Fig. 4a). The most extreme deviation from 50/50 was observed in leaf, in which 49.2% of gene pairs favor the BhD homeolog while 50.8% favor the BhS homeolog. To test whether the deviation from 50/50 was significant in any tissue, we performed an exact binomial test. Leaf was closest to significance \( (P = 0.052, \alpha = 0.0125 \) with Bonferroni correction), but in no case was the pattern of HEB significantly different from what would be expected by random chance.

Finally, we checked for subgenome expression dominance, that is, evidence that the majority of expressed transcripts are coming from one of the 2 subgenomes. For this analysis, we worked with raw read counts rather than TPMs. To control for the fact that 1 subgenome may contribute more reads simply because it has more genes, we summed the lengths of the primary transcripts from all genes in each subgenome and took the total basepairs in each subgenome’s transcriptome to be our null expectation: 50.7% of counts would be expected to originate from BhD and 49.3% from BhS. All 4 tissues were close to this ratio, with floret being the most extreme deviation: 46.41% of counts were from BhD transcripts (Fig. 4b). While there may be some subtle BhS subgenome expression dominance in floret, there is no evidence for overall subgenome expression dominance in Bhyb26. This finding contrasts the above result for subtle BhS genome dominance in terms of gene retention.

### Gradual TE activity post-WGD

We annotated the TEs in Brachypodium genomes to see if disparities in TE content are driving biased genome evolution as has been observed in other polyploids (Woodhouse et al. 2014; Edger et al. 2017). Publicly available repeat sequences and de novo TEs were identified in 5 Brachypodium genomes (see Materials and Methods). The TE content of each polyploid subgenome was examined alone and compared to its progenitor species (Fig. 5a and
The D sub/genomes (BhD of B. hybridum Bhyb26 and ABR113 and B. distachyon Bd21) were slightly more TE rich than the S sub/genomes (BhS of B. hybridum Bhyb26 and ABR113 and B. stacei ABR114). The Bhyb26 D subgenome was most TE rich at 31% TEs, while the B. stacei ABR114 genome was the most TE poor at 20% TEs. The latter figure may be an underestimate because the B. stacei genome is a short-read assembly, however, it is close to the Bhyb26 S TE content of 24%. The Bhyb26 S subgenome was enriched for full-length LTR-retrotransposons (LTR-RTs) relative to the other 2 S sub/genomes (122 vs. 108 and 64), which might be due to the long-read assembly. Gypsy (RLG) and Copia (RLC) elements (Wicker et al. 2007) occupied most of the TE space in all genomes. The ratio of Gypsy to Copia LTR-RT copies ranged from 1.16:1 to 1.30:1 in all genomes, but Gypsy elements were, on average, 1.36–3.01 times longer than Copia elements, so Gypsy elements constituted a much larger portion of the genome space than Copia elements (Fig. 5a and Supplementary File 1). Non-LTR retrotransposons also composed a substantial portion of the TE space, from 2.6 Mb in B. stacei to 5.4 Mb in Bhyb26-D (Supplementary File 1).

We surveyed TE polymorphisms among B. hybridum lines. We used the TE polymorphism detection software TEMP2 (Yu, Huang, et al. 2021) as implemented in the McClintock pipeline (Nelson et al. 2017) to quantify TE polymorphisms in short-read data from 20 B. hybridum lines, using ABR113 as our reference genome (Supplementary Fig. 3a). We focused on TIPs, that is, locations where a TE insertion was present in a resequenced genome but not in the reference. Bhyb26 had by far the greatest number of TIPs relative to ABR113. This increase was not due to sequencing technology because for all samples in this experiment, including Bhyb26, only raw illumina reads were used. TIP number was not correlated with sequencing depth and only loosely correlated with library quality ($r$-squared = 0.34 for total TIPs vs. median per sequence quality score from FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/; last accessed 6-20, 2022)), so observed differences are unlikely to be due to sequencing artifacts. Interestingly, Bhyb118-5 is of the same plastotype as Bhyb26 but does not have nearly as many TIPs (Supplementary Fig. 3a). Previous phylogenetic analysis (Gordon et al. 2020) strongly suggests that Bhyb118-5 and Bhyb26 are of the same origin and neither is admixed with S-plastotype lineages of B. hybridum, so the greater number of polymorphisms in Bhyb26 suggests a possible uptick in TE activity since its divergence from Bhyb118-5. Many TE families contribute to TE diversity in B. hybridum. The 10 TE families that contribute the greatest number of TIPs are responsible for 52% of all TIPs (Supplementary Fig. 3a). The majority of TIPs came from Gypsy and Copia LTR-RTs, the most active classes of TE among B. distachyon accessions as well (Stritt et al. 2018, 2020) (Supplementary Fig. 3b). No single TE family contributed more than 25% of a genome’s total TIPs in any B. hybridum accession (Supplementary Fig. 3a).

Because LTR-RTs are among the most abundant and most active TEs in B. hybridum (Supplementary Table 1 and Supplementary Fig. 3b), we estimated insertion times for intact LTR-RTs in several Brachypodium genomes. Figure 5b shows, for each genome, all LTR-RT families that contain 2 or more intact copies. The number of intact LTR-RTs across our dataset appears
to be a function of genome size and assembly quality. Neither polyploid shows an abundance of TE insertions at or after its WGD (Fig. 5b). All full-length ABR113 LTR-RTs pre-date the WGD. The percentage of full-length LTR-RTs less than 1.4 million years old was similar in ABR113 and Bhyb26: 40% and 44%, respectively. That Bhyb26 has slightly more "young" TEs again hints at the possibility of an uptick in TE activity since the WGD, though the difference is very slight.

We also looked for evidence of overall increased TE activity in and around genes in Bhyb26. We recorded the number of TEs that overlap a gene in each genome, requiring that the TE and gene be on the same strand, and UTR and intronic TEs were included. We found that 43% and 42% of Bhyb26 and ABR113 genes, respectively, were from subgenome-specific TE families. This difference was highly significant \( (P = 2.722 \times 10^{-8}) \). This slight but significant difference suggests that there has been some small-scale transfer of TEs between subgenomes in Bhyb26 post-WGD. Considering that Bhyb26 has a high number of TIPs relative to ABR113, it has a slightly higher proportion of "young" (<1.4 MY) LTR-RTs than ABR113 has, and the Bhyb26 TE fraction is slightly depleted for subgenome-specific TEs relative to that of ABR113, moderate post-WGD TE activity and exchange between subgenomes seems plausible. However, the possibility that the TE landscapes of the true progenitors of Bhyb26 were more similar to each other than those of ABR113 cannot be ruled out.

**Discussion**

Our results indicate that diploidization is progressing slowly in *B. hybridum*, an allotetraploid with multiple origins. In contrast to the more recent *B. hybridum* lineage ABR113, the older Bhyb26 lineage shows several megabase-scale inversions and a greater extent of pseudogenization. In both lineages, gene loss slightly favored retention of genes in the S subgenome, though the difference was significant only in the older line, while in neither line was genome dominance supported by expression data. Finally, we found evidence for gradual rather than instantaneous post-WGD TE activity. We argue that these genomic changes were most likely made possible by relaxed selection post-WGD. The changes are modest overall, consistent with gradual post-WGD evolution.

The chromosomal rearrangements observed in Bhyb26 are not characteristic of homeologous exchange, a classic genomic shock response. Homeologous recombination can lead to duplications, deletions, and translocations (Mason and Wendel 2020). Inversions, on the other hand, more likely result from ectopic recombination or nonhomology directed DNA repair within a single chromosome. The inversions that are unique to Bhyb26 probably did not arise through homeologous exchange, so they could have occurred either pre- or post-WGD. However, we find no megabase-scale inversions between diverse accessions of the well-sampled diplodiploid progenitor species *B. distachyon*. Thus, the available evidence suggests that such large inversions are not typical of intraspecific variation within *Brachypodium* diploids. Neither did we see any definitive evidence of similar inversions in ABR113; higher quality reference genomes for both ABR113 and ABR114, which are in progress, will confirm or refute this finding. It is possible that the true progenitors of Bhyb26 each happened to harbor large inversions relative to all well-characterized modern *B. distachyon* and *B. stacei* lines. However, given the lack of structural variation among diverse diploids, we think a more likely explanation is that the relaxed selection accompanying WGD allowed inversions to persist in the polyploid. Whether these inversions harbor adaptive alleles, as is sometimes the case (Huang and Rieseberg 2020), will be an interesting area for future study.

Some gene loss or gain between lineages, even within the same species, is expected in the normal course of evolution (Gordon et al. 2017). Indeed, we observed that all our *Brachypodium* reference genomes lack at least several dozen genes that are otherwise widely conserved within and beyond the genus. However, such conspicuously absent genes were more common in the polyploids than in the diploid *Brachypodium* genomes, and they were more common in the older polyploid than the younger one. In Bhyb26, the remnants of these genes were shorter, less-expressed, and contained more premature stop codons and nonsynonymous substitutions than would be expected by random chance, suggesting that these were not real genes that were missed due to annotation error. Given that gene loss in ABR113 was greater than the sum of its progenitor species, and gene loss in Bhyb26 was greater than in ABR113, gene loss appears to be progressing gradually with time. One caveat to this analysis is that the greater gene loss in Bhyb26 could be due to demographic factors other than polyploidy. It is worth noting that our current study uncovered more potential pseudogenes in ABR113 than our previous study, likely due to our more sophisticated methods of calling synteny (Gordon et al. 2020).

Bhyb26 shows some evidence of post-WGD TE activity: it is slightly depleted for subgenome-specific TEs, it has more TIPs than its closest relative, and it is slightly more TE-rich than the other *Brachypodium* lineages studied here. These data are reminiscent of the *Capsella bursa-pastoris* case, in which relaxed selection permitted gradual TE proliferation following WGD (Agren et al. 2016). However, we cannot exclude the possibility that these genome features were already present in the true progenitors of Bhyb26; for instance, the progenitors may have shared many TEs already at the time of WGD. Furthermore, it is possible that the inversions, gene losses, and slight TE activation are not really due to buffering by duplicate genes, but due to some demographic factor, such as a smaller population and greater genetic drift in Bhyb26 than in ABR113 for reasons other than polyploidy. Broader sampling of the D-plastotype lineage would allow for greater insight into those polyploids’ demographic histories.

It is not unusual for allopolyploids to preferentially retain genes from 1 dominant subgenome (Garsmeur et al. 2014; Woodhouse et al. 2014; Alger and Edger 2020), and it has been proposed that dominance is established immediately following WGD and increases over time (Edger et al. 2017). *B. hybridum* supports this model in the sense that the biased gene loss does appear to
be stronger in the older lineage. However, given that the RNA-seq data do not reveal any genome dominance in either lineage, which is crucial to the proposed mechanism of genome dominance (Freeling et al. 2012), we cannot conclude that B. hybridum shows subgenome dominance in the classic sense. B. hybridum seems to resemble the paleoallopolyploid Miscanthus sinensis (Mitros et al. 2020) and Cucurbita maxima and Cucurbita moschata (Sun et al. 2017) genomes, as it is an allopolyploid that shows little to no genome dominance. Similar to cotton, our expression data are equivocal, with neither subgenome emerging as dominant across all tissues (Fang et al. 2017).

McClintock’s genome shock question remains a matter of much debate today: is the response to WGD predictable? Today it seems that the answer is yes and no, but our predictions are constantly becoming more sophisticated. For instance, it has been predicted that allopolyploids should show subgenome dominance over the long term (Garsmeur et al., 2014), but Alger and Edger (2020) and Wendel et al. (2018) emphasize that the key predictor of genome dominance is not necessarily progenitor divergence per se, but progenitor divergence in terms of TE load. B. hybridum is in line with this refined prediction, not unlike the cases of Ephedra (Wu et al. 2021) and teff (VanBuren et al. 2020). Genome evolution in B. hybridum is largely subtle and unbiased, even though it formed from a remarkably wide cross (Catalán et al., 2012; Dinh Thi et al., 2016), perhaps because its progenitors bore a similar TE load. Many genetic, epigenetic, and environmental factors contribute to a polyploid’s fate, and there is still much work to be done to determine how these factors work together. B. hybridum has shed some light on this complex process by providing a rare glimpse of diploidization “caught in the act.”

**Data availability**

The Bhyb26 v.2.0 genome and standard annotation files are available on Phytozome. All raw sequence data used in this study, including the Bhyb26 DNA and RNA reads used for genome assembly and annotation, are available on the Joint Genome Institute Genome Portal and The National Center for Biotechnology Information Short Read Archive. Code used in this study is available at https://github.com/vtartaglio/Scarlett_et_al_2022; last accessed 6-20-2022. For accession numbers, download links, and more details on all genomes and sequencing libraries mentioned in this report, see Supplementary File 1. DOIs for the sequences produced by the JGI are: 10.46936/10.25585/60007218; 10.46936/10.25585/60001041; 10.46936/10.25585/60001092; and 10.46936/10.25585/60001143.

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**Conflicts of interest**

None declared.

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