Gene balance in allopolyploids: Homoeologous exchanges show signs of dosage constraint and dosage constraint of biased homoeologs differs between subgenomes.

Short title: Gene dosage constraints in allopolyploids

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Abstract

Allopolyploidy involves the hybridization of two evolutionary diverged species and the doubling of genomic material. Allopolyploids often exhibit homoeologous exchanges (HEs) that recombine, duplicate, or delete homoeologous regions. These changes to gene dosage are hypothesized to be constrained by selection to maintain balanced gene dosage. However, the dynamics of this dosage constraint in response to HEs or in the context of biased subgenome expression is poorly understood. We used genomic and transcriptomic data for six independently resynthesized, isogenic *Brassica napus* lines in the first, fifth, and tenth generation to identify HEs. We modified a recently developed method (polyploid response variance, PRV) to analyze HE events, which we call homoeologous exchange response variance (HERV), and tested HEs for gene expression dynamics reflective of gene dosage constraint. Results from the HERV analyses showed that dosage-sensitive groups of genes (GO terms) had a less variable expression response to HEs than dosage-insensitive genes; thus, HEs showed a sign of selective constraint for balanced gene dosage. We also discovered this dosage constraint is present for homoeologous pairs where expression is biased toward the dominant BnC subgenome, but not the BnA subgenome. A PRV analysis of the expression response to polyploidy and an integrated PRV/HERV analysis confirmed this pattern of dosage constraint. These findings expand our knowledge of the prevalence of dosage constraint and the interplay of dosage constraints and biased subgenome expression. We speculate how these results connect evolutionary patterns in polyploids over time; from homoeolog expression bias to biased fractionation and reciprocal retention.

Introduction

Changes in gene dosage are known to be a powerful and important driver of gene expression abundance, quantitative trait variation, and the evolution of genomes (Birchler and Veitia 2007, 2010, 2012). The observation that imbalanced gene dosage changes can have a large phenotypic impact and can be highly deleterious for certain classes of genes, especially those involved in highly connected regulatory networks and multimeric protein complexes led to the formulation of Gene Balance Hypothesis (Birchler and Newton, 1981; Birchler et al., 2001; Makino and McLysaght, 2010; Birchler and Veitia, 2012). The core of the Gene Balance Hypothesis argues that changing the stoichiometry of members of networks and protein complexes involved in multicomponent interactions affects their kinetics, assembly, and function of the whole, which causes negative fitness consequences (Birchler et al., 2005; Birchler and Veitia, 2007, 2010, 2012). The need to maintain the stoichiometric balance of gene products in the face of changes in gene dosage from both small-scale and whole-genome duplication influences genome evolution in important and predictable ways. Comparative genomic studies have supported predictions from the Gene Balance Hypothesis, showing that for certain classes of genes, the retention of duplicate genes shows biased patterns depending on whether a gene is duplicated by whole-genome duplication or by small-scale duplications. In particular, duplicate copies from many transcription factor families, genes involved in signaling pathways and multimeric protein complexes, and others tend to be retained more than expected after whole-genome duplication, and duplicates from small-scale duplications tend to be retained less than expected (Blanc and Wolfe, 2004; Maere, 2005; Paterson et al. 2006; Thomas and Freeling, 2006; Freeling, 2009; Edger and Pires, 2009; De Smet et al., 2013; Conant et al., 2014; Li et al., 2016; Tasdighian et al., 2018). This pattern of preferential retention from whole-genome duplication and loss from small-scale duplication has been called “reciprocal retention” (Freeling, 2009; Tasdighian et al., 2018).

Many of these studies have focused on meso- or paleopolyploids, where genomes have returned to a diploid-like state, leaving the immediate transcriptional impact of large-scale gene dosage changes less well understood. However, several authors have recently investigated the expression responses caused by aneuploidy and polyploidy (Coate et al. 2016; Hou et al. 2018; Song et al. 2020; Shi et al. 2021; Yang et al. 2021). Coate et al. (2016) and Song et al. (2020),

in particular, attempt to connect observed patterns of long-term duplicate gene retention to short-term duplicate gene expression responses. They use tenets of the Gene Balance Hypothesis to predict two patterns in short-term expression response. First, genes that are reciprocally retained after whole-genome duplication (e.g. those that are highly connected in gene networks, involved in multicomponent protein complexes, etc.) should experience a change in gene expression in response to genome duplication. Second, these changes should be less variable for all genes in the network, what they call a “coordinated response”. Coate et al. (2016) address this question using natural soybean (*Glycine* L.) allopolyploids with an origin ~500,000 years ago and known diploid progenitors, while Song et al. (2020) use three *Arabidopsis thaliana* autopolyploid/diploid pairs. Both studies determined that highly reciprocally retained genes showed a less variable gene expression response to polyploidy (Coate et al. 2016; Song et al. 2020). However, whether this observation holds true in recent allopolyploids remains unexplored.

Early studies in resynthesized polyploids showed extensive genetic changes in a short period of time (Song et al. 1995). Subsequent investigations showed major genome structural changes from the first meiosis after polyploid formation, primarily in the form of homoeologous exchanges which can result in partial or complete deletion and duplication of chromosomal segments (Sharpe et al. 2005; Osborn et al. 2003; Jenczewski et al. 2003; Pires et al. 2004; Gaeta et al. 2007; Nicolas et al. 2007; 2012; Szadowski et al. 2010; Xiong et al. 2011; Chalhoub et al. 2014; He et al. 2017; Rousseau-Geutin et al. 2017; Samans et al. 2017; Stein et al. 2017; Hurgobin et al. 2018; Lloyd et al. 2018; Pele et al. 2018; Mason and Wendel 2020; Bayer et al. 2021; Chawla et al. 2021; Ferreira de Carvalho et al. 2021; Higgins et al. 2021; Xiong et al. 2021; Orantes-Bonilla et al. 2022). These rearrangements continue to accumulate over time, generating genomic diversity in early polyploids (Gaeta and Pires 2010; Xiong et al. 2011; Mason and Wendel, 2020; Bird et al. 2021). Homoeologous exchanges are often destructive to the organism and meiotic stability is more frequently observed in natural polyploids compared to resynthesized and it is likely that meiotic stability is under strong selection in natural polyploid populations (Gaeta and Pires, 2010; Rousseau-Gueutin et al. 2017; Pele et al. 2018; Xiong et al. 2020; Gonzalo et al. 2019; Gaebelein et al. 2019; Ferreira de Carvalho et al. 2021). At the same time, homoeologous exchanges generate phenotypic novelty in resynthesized polyploids (Pires et al. 2004; Gaeta et al. 2007; Wu et al. 2021) and are frequently observed in natural polyploids (Chalhoub et al. 2014; Lloyd et al. 2018; Edger et al. 2019; He et al. 2017, Chawla et

al. 2021). Additionally, homoeologous exchanges may be under genetic control (e.g., Jenczewski et al. 2003; Higgins et al. 2021), may affect meiotic stability (Xiong et al. 2021), may underlie gene presence-absence variation and agronomically valuable quantitative trait loci in *Brassica napus* (Samans et al. 2017; Stein et al. 2017; Hurgobin et al. 2018; Bayer et al. 2021), and may generate novel, chimeric transcripts as recently observed in several polyploid species including wheat, *Brassica napus, Arabidopsis suecica*, banana, peanut, and synthetic tetraploid rice (Zhang et al, 2020).

Unlike aneuploidy and polyploidy, the impact of gene dosage constraint on gene expression changes from homoeologous exchanges is largely unexplored. There are reasons to believe homoeologous exchange can alter the dosage balance of gene products. Lloyd et al. (2018) investigated the effect of homoeologous exchanges on expression in natural *Brassica napus* and found that homoeologous exchanges (HEs) alter expression in a dosage-dependent manner that greatly resembles the gene dosage effects seen in aneuploid and polyploid organisms (Birchler and Newton, 1981). Furthermore, a key result from Lloyd et al. 2018 relates to unequal expression of homoeologous copies in polyploid genomes. Polyploid genomes also must accommodate inherited and novel expression differences in homoeologous genes which often results in subgenome dominance, where expression is biased in favor of homoeologs from one progenitor genome over others. (Alger et al. 2021; Bird et al. 2018,2021; Wendel et al. 2018). This effect is driven by the merger of evolutionarily diverged genomes, which frequently results in remodeling of epigenetic markers (Madlung et al., 2001; Edger et al., 2017; Bird et al., 2021), alterations in gene regulation (Chen, 2007), and activation of transposable elements (Vicient and Casacuberta, 2012). Importantly, there is also a continuum of polyploidy as parental genomes within and among species can vary in evolutionary distance and subsequent genome evolution blurs the distinction between autopolyploidy and allopolyploidy (Stebbins 1947; Leal-Bertioli et al. 2018; Mason and Wendel 2020; Blischak et al. 2022; Bomblies 2022). Subgenome expression dominance has been defined in terms of a subgenome possessing a greater amount of dominantly expressed homoeologs and has been identified in many allopolyploid species, including maize (Schnable et al. 2011) *Mimulus peregrinus* (Edger et al. 2017), garden strawberry (Edger et al. 2019), *Brassica rapa* (Cheng et al. 2012; Cheng et al. 2016), and a population of resynthesized *Brassica napus* (Bird et al. 2021). Lloyd et al. (2018) found when homoeologous gene pairs have unequal expression, altering the ratio of homoeologous copies does not accurately compensate to maintain the same level of combined

homoeolog expression. Similar results have since been observed in tetraploid wheat lines (Zhang et al. 2022). Therefore, in the presence of unequal homoeolog expression, HEs will alter the stoichiometry of gene products for a homoeologous pair and would be predicted by the Gene Balance Hypothesis to be under selective constraint to maintain gene dosage balance. Whether the perturbation to dosage is large enough to be constrained for gene balance and if this predicted pattern is observed has not yet been explored.

We analyzed paired WGS and RNASeq data for six independently resynthesized and isogenic *Brassica napus* (CCAA) lines, which are known to accumulate large amounts of genomic rearrangement (Xiong et al. 2011), at three generations to determine if the immediate gene expression responses to allopolyploidy are consistent with the Gene Balance Hypothesis. We further identified homoeologous exchange events to test if changes in gene expression from homoeologous exchanges exhibit signs of dosage constraint consistent with the Gene Balance Hypothesis. Based on previous results indicating subgenome dominance in this population of resynthesized lines favoring the BnC subgenome (Bird et al. 2021), we then tested the expression response to homoeologous exchange and whole-genome duplication to see signs of dosage constraint differed based on which homoeolog is more highly expressed. Additionally, plants from the first, fifth, and tenth generations are used to see if expression responses change over time. Our findings provide novel insights into the alteration of global expression by homoeologous exchanges and extend our understanding of how the drive for balanced gene products constrains gene expression and genome evolution across various modes of gene dosage changes.

Methods

Sequencing data

We downloaded the data and files for previously identified genomic rearrangements and transcript quantification from leaf samples from Bird et al. (2021) at the associated Data Dryad repository https://doi.org/10.5061/dryad.h18931zjr. These previous analyses identified 26,111 syntenic ortholog pairs between the progenitor genomes, treated as homoeologous pairs from here on. We used the dosage assignments from Bird et al. (2021). Briefly, read depth ratio for homoeologous pairs were calculated over a 50 gene sliding window with stepsize of one.

Homoeologous pairs were assigned 0:4, 1:3, 2:2 3:1, 4:0 based on distorted ratios of reads mapping to one homoeolog over the other along a sliding window of 170 genes with a stepsize of one gene. To account for uncertainty in alignment and potential cross-mapping, the read depth was split into equal sized quintiles to assign a dosage (0-20% as 0:4, 20-40% as 1:3, etc). Additionally, a region was only assigned as a homoeologous exchange if 10 or more consecutive genes had read depths within a preselected range. Read count files for these samples had previously been filtered to remove lowly expressed pairs by removing gene pairs with summed TPM < 10, allowing for the potential that one copy is truly silenced. The number of homoeologous gene pairs with expression quantification in these samples ranged from 11,355 to 12,939, while the number of gene pairs affected by putative homoeologous exchanges with expression quantification ranged from 148 for one plant in the first generation to 4606 for a plant in the tenth generation.

Dosage sensitivity assignment

To leverage the well-curated gene annotations of *Arabidopsis thaliana*, and the close phylogenetic relationship between *A. thaliana* and the *Brassica* genus, we assigned our *Brassica* gene pairs to the GO category of their *A. thaliana* ortholog. Orthologs between *A. thaliana* and *Brassica oleracea* were identified with Synmap (Lyons et al. 2008) on CoGe (Lyons and Freeling, 2008) and the *A. thaliana* GO annotations were directly assigned to the *B. oleracea* orthologs and from *B. oleracea* to the *B. rapa* syntelogs. Next, we used the GO term dosage response assignments (dosage-insensitive and dosage-sensitive) from Song et al.'s (2020) analysis of gene retention patterns of *A. thaliana* genes to classify our syntenic homoeologs as belonging to dosage-sensitive and dosage-insensitive GO terms. Arabidopsis genes, their associated GO terms and classification from Song et al. (2022), and the identified *Brassica oleracea* orthologs can be found in the supplementary dataset.

Homoeologous exchange response variance

We included only syntenic homoeolog pairs that diverged from 2:2 dosage ratio (e.g. gene pairs with read-depth ratio less than 0.4 or greater than 0.6), as identified by Bird et al. (2021), to investigate the effects of gene dosage changes. Previous cytogenetic analysis of these lines revealed substantial aneuploidy and partial chromosomal duplication/deletion,

especially among the most syntenic chromosome pairs like A1/C1, A2/C2, and C9/A10 (Xiong et al. 2011). To eliminate confounding effects of these kinds of rearrangements, we checked our lines for regions of skewed read depth that covered the majority or entirety of a chromosome. We fully excluded chromosomes where the majority of plants showed these large regions of skewed read-depth ratios and individually removed cases where large skewed ratios were seen for chromosomes in one sample. Plots of read depth ratios along the genome for each line and generation are shown in Figs S1-S6. This resulted in the removal of syntenic homoeologs from chromosomes A1/C1, A2/C2, and C9/A10 from all lines, and chromosome C4 only for line EL-1100 at generation 10. Parental expression was taken from Bird et al. (2021), where RNAseq from independent libraries of B. rapa acc. IMB218DH and B. oleracea acc. TO1000DH, were each aligned separately to the "in silico polyploid" concatenated reference genome comprised of the B. rapa acc. R500 genome (Lou et al. 2020) with SNP correction using IMB218DH resequencing data and the B. oleracea TO1000 reference genome (Parkin et al. 2014). This was the same concatenated reference genome used to align the RNAseq data from the resynthesized lines.

We defined the expression response to homoeologous exchange as the fold change of the summed homoeolog pair expression ($Exp_{BnC} + Exp_{BnA}$) and the midparent expression of the parents, accounting for dosage, ($\frac{X*Exp_{B.\,oleracea} + Y*Exp_{B.\,rapa}}{2}$) where X and Y represent the appropriate dosage of homoeologs for a particular class of homoeologous exchanges. For example for a pair assigned a dosage ratio of 3:1, X=3 and Y=1. This calculation represents the fold change of the summed expression for a homoeologous pair in the polyploids and the hypothetical expression of the progenitor orthologs when mapped to the *in silico* polyploid genome. We modify the approach of Coate et al. (2016) and Song et al. (2020) and calculated the coefficient of variation of this expression response ($\frac{\sigma_{exp}}{\mu_{exp}}$) and termed it the homoeologous exchange response variance (HERV). Statistical analysis was done with a Kruskal-Wallis test applied by the function stat_compare_means() in the R package ggpubr v.0.04.0 (R core team, 2020; Kassambara, 2020). We calculated HERV only for GO terms that contained more than 20 genes. When analyzing the response to polyploidy among different homoeolog expression biases, the expression bias of progenitor orthologs was used. Previous analysis showed that for over 70% of homoeologs, all six resynthesized *B. napus* lines shared the same homoeolog expression bias as the parents (Bird et al. 2021).

Dosage response to polyploidy

When investigating the dosage response to polyploidy, we limited our analysis to the syntenic homoeologous genes identified as being in a 2:2 dosage ratio. We created our dataset by combining data across individuals, selecting gene pairs in 2:2 for a particular individual sample. We did not require that a gene pair was in 2:2 dosage in every line. We calculated expression response to polyploidy for each gene pair, defined as the fold change of polyploid expression for a 2:2 syntenic homoeolog pair and the mid-parent expression of the progenitor ortholog pair ($\frac{Exp_{B.\,oleracea} + Exp_{B.\,rapa}}{2}$). We used the same parental expression data from Bird et al. 2021 as the HERV analysis. We applied the same approach as Coate et al. (2016) and Song et al. (2020) and focused on the coefficient of variation of expression response ($\frac{\sigma_{exp}}{\mu_{exp}}$), which we similarly termed the polyploid response variance (PRV). The Kruskal-Wallis implementation from ggpubr (Kassambara, 2020) was used again for statistical analysis. As for the previous analysis, we only included GO terms with 20 or more genes and defined homoeolog expression bias in terms of expression bias in parental orthologs.

Data availability:

Raw data from this project are available on the NCBI Sequence Read Archive (SRA) Project PRJNA577908. Intermediate files can be found at https://doi.org/10.5061/dryad.h18931zjr and code to recreate main figures and results can be found at https://github.com/KevinABird/Bird_GenomeInFlux_BNapus

Results

Homoeologous exchange expression response (HERV) shows dosage constraint

To assess how the dosage sensitivity of genes affects their expression response to gene dosage changes from allopolyploidy, we used the dosage-sensitivity gene class assignments for *Arabidopsis thaliana* from Song et al. (2020). As per Song et al. (2020), Class I Gene Ontology

(GO) categories are putatively dosage-insensitive and Class II are putatively dosage-sensitive and these classes are based on the observed reciprocal retention (over-retention after whole-genome duplication, and under-retention after small-scale duplication) of genes from the investigated GO categories following the At-alpha duplication event in the Brassicaceae. Similar patterns of reciprocal retention have been identified across angiosperms. To leverage the superior annotation quality of *A. thaliana*, *B. rapa* and *B. oleracea* orthologs were assigned to dosage-sensitivity GO classes based on their ortholog in *Arabidopsis*. These dosage-sensitivity assignments were used to assess how expression response differs between classes in the resynthesized allopolyploids. GO categories were then filtered so that only those with 20 or more genes in our dataset were included in the analysis

The extensive genomic rearrangements observed in this population of resynthesized lines (Xiong et al. 2011; Bird et al. 2021) provide an opportunity to test for the first time whether gene expression changes from homoeologous exchange events experience dosage balance constraints as predicted by the gene balance hypothesis. Using the published results from Bird et al. (2021), we focused on genomic regions identified as not being in 2:2 dosage, representing homoeologous exchanges with 0:4, 1:3, 3:1, and 4:0 dosage ratios (BnC:BnA). To avoid the inclusion of likely aneuploidy events, genes on chromosomes that frequently showed dosage changes for the entirety or majority of the chromosome were excluded. This affected chromosome pairs 1A/1C, 2A/2C, 10A/9C (FigS1-S6). With this dataset of gene pairs affected by putative homoeologous exchange events, we compared their expression to the hypothetical expression in parents with appropriate dosage. It should be noted, this approach did not normalize RNA with exogenous spike-in as other studies have, meaning values reported are relative gene expression levels rather than the absolute expression response. Although we can not assign expression responses to categories like dosage-dependent or compensation as previous studies have (Song et al. 2020; Shi et a. 2020; Hou et al. 2018), we can investigate the relative change in expression and test to see if it matches predictions laid out by the Gene Balance Hypothesis. This type of analysis should be robust to the issues caused by the lack of an exogenous spike-in and has been previously employed in expression comparisons of natural allopolyploid and diploid species, without the use of spike-ins (Coate et al. 2016).

We investigated the extent that expression responses from homoeologous exchanges differ among the identified dosage-sensitive and dosage-insensitive GO terms (Fig 1). We

looked at the expression response of gene pairs in a given GO term, only for those gene pairs affected by a homoeologous exchange event. We used the coefficient of variation of this expression response, which we call the Homoeologous Exchange Response Variance (HERV), to assess how variable the expression response was for genes from dosage-sensitive and insensitive GO categories. After filtering GO terms with fewer than 20 genes represented in our dataset, we had 299 GO terms total, with 138 dosage-insensitive and 161 dosage-sensitive). Across all lines, genes belonging to putatively dosage-sensitive GO terms showed significantly lower HERV, indicating a less variable expression response than genes from putatively dosage-insensitive GO terms (Fig 1a, Kruskal-Wallis test, p=0.0087).

It is possible that the higher variation in expression of dosage-insensitive GO terms may be may an artifact of this difference in expression. To rule this out we compared average TPM of homoeolog pairs for GO terms assigned as dosage insensitive (Class I) and dosage sensitive (Class II) with a Kruskal-Wallis test to make sure dosage sensitive GO terms did not have significantly higher expression compared to dosage sensitive GO terms. For this dataset our results showed that dosage-sensitive (Class II) GO terms had significantly lower expression on average compared to dosage-insensitive (Class I; p=0.033; Fig S7). These results are similar to what Song et al. (2020) found for their Arabidopsis polyploids and should provide strong support that our results are not an artifact of higher expression among dosage-sensitive genes.

Using an allopolyploid gave us the opportunity to observe if dosage constraint varies based on homoeolog expression bias. Previous transcriptomic analysis of these resynthesized lines from Bird et al. (2021) identified significantly more homoeolog pairs with expression biased toward the BnC subgenome, which was dubbed the dominant subgenome (Bird et al. 2021). We compared the dosage-sensitive and dosage-insensitive GO terms, this time only including gene pairs with particular homoeolog expression bias in GO terms. This resulted in three datasets: expression response of GO terms only considering expression data for gene pairs with expression biased toward the BnC subgenome, pairs only with expression biased toward the BnA subgenome, and pairs with no expression bias. Expression bias of the gene pair was based on the expression relationship of the parental orthologs. Previous analyses by Bird et al. (2021) found these parental expression differences to match homoeolog expression bias in all six lines for over 70% of homoeologous gene pairs (Bird et al. 2021).

When broken down by direction of homoeolog expression bias there were 53 GO terms for pairs biased toward the non-dominant BnA subgenome (29 dosage-insensitive and 24 dosage-sensitive), 105 GO terms for pairs biased toward the dominant BnC subgenome (50 dosage-insensitive and 55 dosage-sensitive), and 234 for gene pairs without expression bias (101 dosage-insensitive and 133 dosage-insensitive). We found that homoeologous gene pairs with expression biased toward the dominant BnC subgenome (Kruskal-Wallis test, p=0.022) show significantly lower HERV in dosage-sensitive GO terms than dosage-insensitive GO terms while pairs with expression biased toward the BnA subgenome and unbiased gene pairs did not (Fig 1b; Kruskal-Wallis test,p=0.68;p=0.053) compared to dosage-insensitive GO terms. This result suggests that dosage constraint on the expression response differs depending on which homoeolog is more highly expressed.

When analyzing expression response by generation, there were 79 GO terms (34 dosage-insensitive and 45 dosage-sensitive) that passed filtering for generation one, 139 (60 dosage-insensitive and 79 dosage-sensitive) for generation five, and 186 (87 dosage-insensitive and 99 dosage-insensitive) for generation 10. We found that there was not a significant difference in HERV between dosage-sensitive and dosage-insensitive GO terms at the first or tenth generation (Fig 1c, Kruskal-Wallis test, p=0.332, p=0.288), but dosage-sensitive and insensitive GO terms did show different HERV at the fifth (Fig 2c, Kruskal-Wallis test, p=0.022). We also found that homoeologous exchange response variance increased over time with dosage-sensitive and dosage-insensitive GO terms showing mean HERV of 0.661 and 0.502, respectively, in generation one and increasing to 0.828 and 0.697, respectively, in generation ten.

Figure 1. Expression changes from homoeologous exchange reflect predictions from the gene balance hypothesis

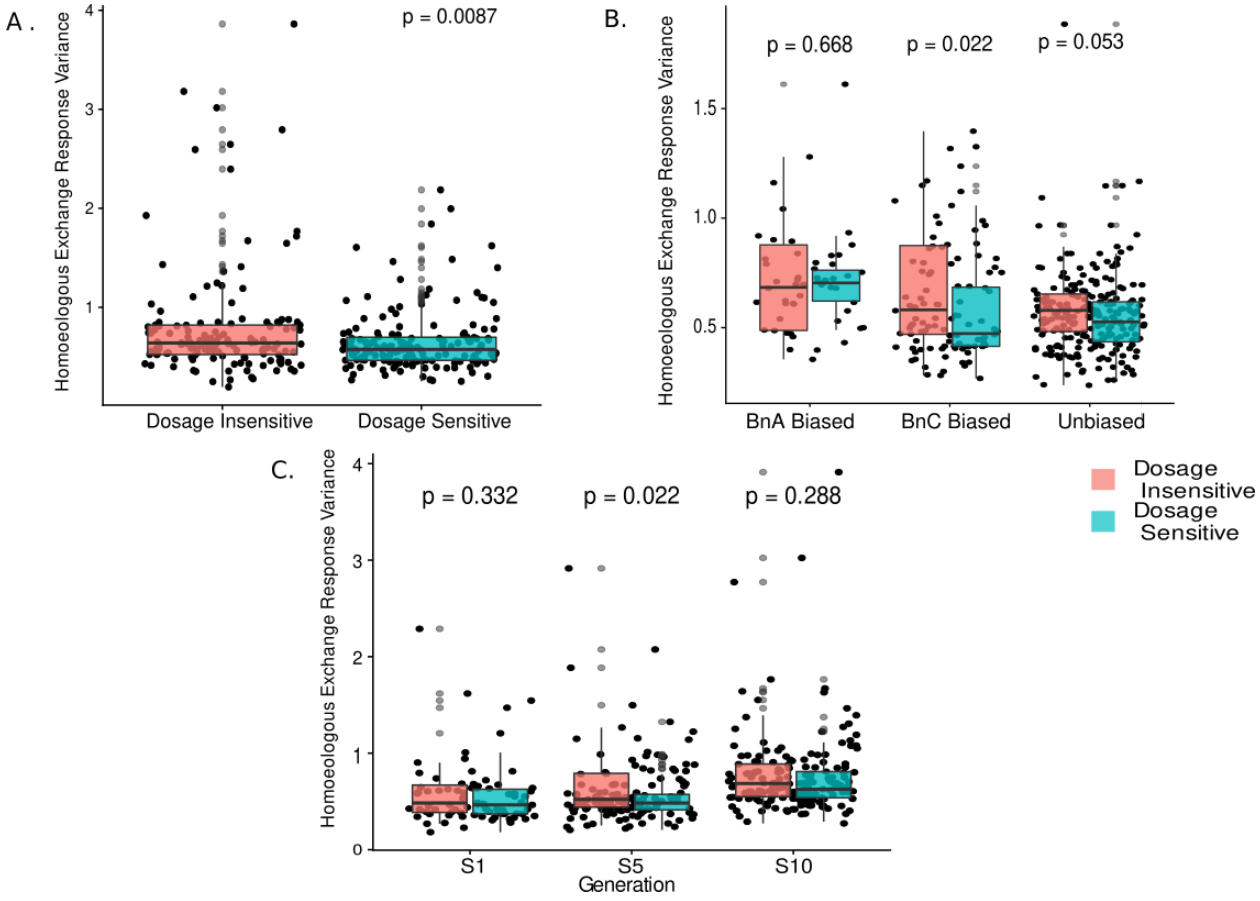

Fig 1. Homoeologous Exchange response variance (coefficient of variation of dosage response from homoeologous exchange) for all dosage imbalanced homoeologs in all 16 isogenic polyploid plants broken down by **A)** only putatively dosage-insensitive (Class I) and dosage-sensitive (Class II) GO categories from Song et al. 2020, **B)** GO Dosage categories and subgenome dominance relationship in parental lines, **C)** GO Dosage categories and generation. P-values represent results of Kruskal-Wallis test of polyploid response variance between Class I vs Class II dosage categories. In all plots, individual dots represent a GO term, restricted only to GO terms that were represented by 20 or more genes in our dataset.

Global allopolyploidy expression response (PRV) shows signs of dosage constraint

We further investigated the relative gene expression change for individual homoeologous gene pairs in 2:2 dosage by taking the fold change of the summed transcript count for homoeologous gene pairs in the allopolyploid individuals and mid-parent value of the progenitors. We used the polyploid response variance (PRV) measure from Song et al. (2020) and Coate et al. (2016), defined as the coefficient of variation of the relative expression

response, to assess how variable the expression response to polyploidy is in the different gene groups. These analyses allowed us to establish the expression response to polyploidy in a newly formed allopolyploid and further explore and validate the findings about homoeolog expression bias in the HERV analysis.

Analyzing data across all lines and filtering out GO terms with fewer than 20 genes, we had a final count of 376 GO terms of which 181 were classified dosage-insensitive and 195 were dosage-sensitive. As observed previously in resynthesized autopolyploids and natural *Glycine* allopolyploids, the polyploid response variance was significantly lower (i.e. the expression response was less variable) in genes from GO categories in the dosage-sensitive class compared to the dosage-insensitive class (Kruskal-Wallis test, p=0.0024; Fig 1; Fig 1a). We again checked for expression differences between dosage-sensitive and dosage-insensitive groups of genes. For this dataset of homoeologous pairs at 2:2 dosage, our results showed that dosage sensitive (Class II) GO terms had significantly lower expression on average compared to dosage insensitive (Class I; p=0.0085; Fig S8). This again supports that our results are not due to differences in expression between genes in the Class I and Class II GO terms.

Fig 2. Expression changes from allopolyploidy reflect predictions from the gene balance hypothesis

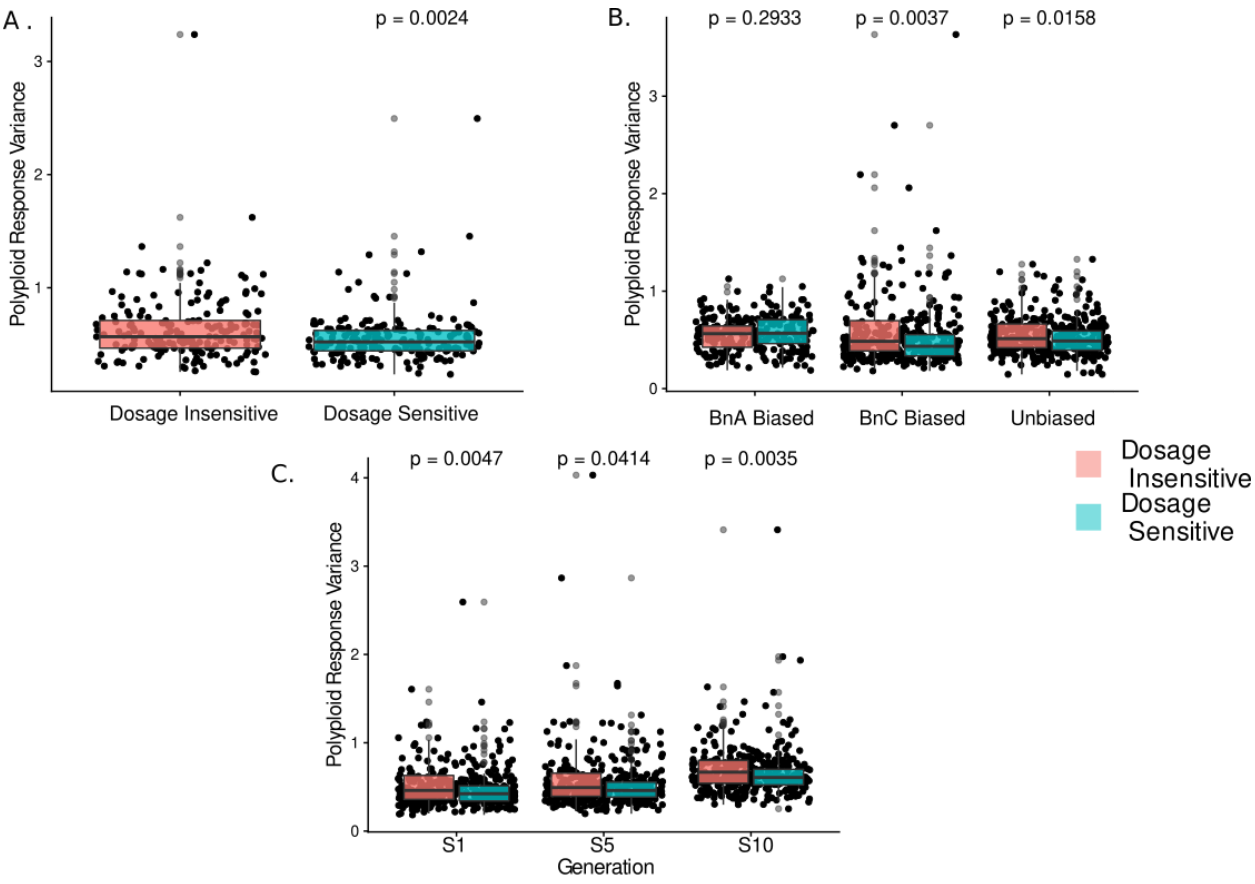

Fig 2. Polyploid response variance (coefficient of variation of dosage response) for all 2:2 balanced homoeologs in all 16 isogenic polyploid plants broken by **A)** only putatively dosage-insensitive (Class I) and dosage-sensitive (Class II) GO categories from Song et al. 2020, **B)** GO Dosage categories and subgenome dominance relationship in parental lines, **C)** GO Dosage categories and generation. P-values represent results of Kruskal-Wallis test of polyploid response variance between Class I vs Class II dosage categories. In all plots, individual dots represent a GO term, restricted only to GO terms that were represented by 20 or more genes in our dataset.

We next sought to replicate the above results showing signs of dosage constraint differing based on which homoeologous pair was more highly expressed, this time using PRV. After filtering out GO terms with fewer than 20 genes, there were 274 GO terms (113 dosage-insensitive, 124 dosage-sensitive) for gene pairs biased toward the non-dominant BnA subgenome, 330 GO terms (156 dosage-insensitive and 174 dosage-sensitive) for pairs biased toward the dominant BnC subgenome, and 374 GO terms (179 dosage-insensitive and 195

dosage-sensitive) for genes not biased toward either subgenome. We found that pairs with expression biased toward the *B. napus* C subgenome (BnC) biased show a significant difference between PRV of dosage-sensitive and dosage-insensitive GO categories as above (Kruskal-Wallis test, p=0.0037; p=0.0158, respectively). As before, gene pairs biased toward the *B. napus* A subgenome (BnA) showed no significant difference in PRV between dosage-sensitive and insensitive GO classes (Kruskal-Wallis test, p=0.2933; Fig 1b). However, unlike for HERV we found that pairs with unbiased expression dosage-sensitive GO terms had a significantly lower PRV than dosage-insensitive GO terms. These results provide further support that constraint on the gene dosage response manifests differently depending on homoeolog expression bias.

When broken down by generation, there were 375 GO terms (180 dosage-insensitive and 195 dosage-sensitive) that passed filtering for generation one, 368 GO terms (174 dosage-insensitive and 194 dosage-sensitive) for generation five, and 362 GO terms (172 dosage-insensitive and 190 dosage-sensitive) for generation ten. In all three generations, dosage-sensitive GO terms have significantly lower PRV than dosage-insensitive GO terms (Fig 1c; Gen 1 p=0.0047, Gen 5 p=0.0414, Gen 10 p=0.0035). We observed an increase in the coefficient of variation over time, with both dosage-sensitive and dosage-insensitive showing higher PRV in generation ten than in the first generation (Fig 1c). Notably, in generation ten the dosage-sensitive GO categories show higher mean polyploidy response variance than dosage-insensitive GO categories in the first generation.

Homoeologous exchange (HERV) and global polyploid (PRV) expression responses are, at least partially, distinct

While our findings suggest that the expression response to dosage changes caused by homoeologous exchanges is less variable for dosage-sensitive genes, in line with predictions from the Gene Balance Hypothesis, it is possible these results are an artifact of our analysis also picking up the effects of dosage changes caused by allopolyploidy or trans dosage effects of aneuploidy. To determine if the results obtained for homoeologous exchanges are distinct from the effect of polyploidy, we compared the coefficient of variation for the expression response of GO terms common to the polyploid and homoeologous exchange datasets (Fig 3).

First, we compared the proportion of gene pairs belonging to dosage-sensitive and dosage-insensitive GO terms in all 16 individuals for the polyploidy and homoeologous exchange analysis. For the polyploid analysis, the mean proportion of genes belonging to dosage-insensitive GO terms is 0.554, while it is 0.544 for the homoeologous exchange analysis. This difference in proportion of Class I and Class II GO terms between the PRV and HERV analysis was not statistically significant (^{2}-test, p=0.983). Even considering the direction of difference in proportion of dosage-insensitive genes, a greater proportion of gene pairs having dosage-insensitive GO terms would be predicted to result in a higher coefficient of variation. Instead, we found a significantly higher coefficient of variation from homoeologous exchanges (Fig 3a, Kruskal-Wallis test, p=0.0015), which had a lower proportion of genes belonging to dosage-insensitive GO categories. Both allopolyploidy and homoeologous exchange dosage changes produced significantly different expression responses from genes belonging to dosage-sensitive and insensitive GO categories (Fig 3b), and we determined that the coefficient of variation was significantly different between polyploidy and homoeologous exchange dosage changes for gene pairs from dosage-sensitive (Kruskal-Wallis test, p = 0.0052) but not for dosage insensitive (Kruskal-Wallis test, p=0.1) GO categories.

Likewise, for both homoeologous exchange and polyploidy-induced dosage changes, the difference in expression response between genes belonging to dosage-sensitive and insensitive GO terms was significantly different for BnC biased (HERV p-value = 0.22; PRV p-value=0.11), but not for BnA biased pairs (HERV p-value=0.67; PRV p-value=0.45) (Fig 3c). While unbiased gene pair results are qualitatively similar, the PRV for dosage-sensitive and dosage-insensitive GO terms are significantly different (p-value=0.0048) but the HERV are not (p=0.053). Our results also showed that the coefficient of variation from homoeologous exchange-induced dosage changes was not significantly different than for polyploidy-induced dosage changes for gene pairs belonging to both dosage-sensitive and insensitive for all homoeolog expression bias relationships (Table 1).

Figure 3. Expression responses from allopolyploidy and homoeologous exchange appear to be distinct

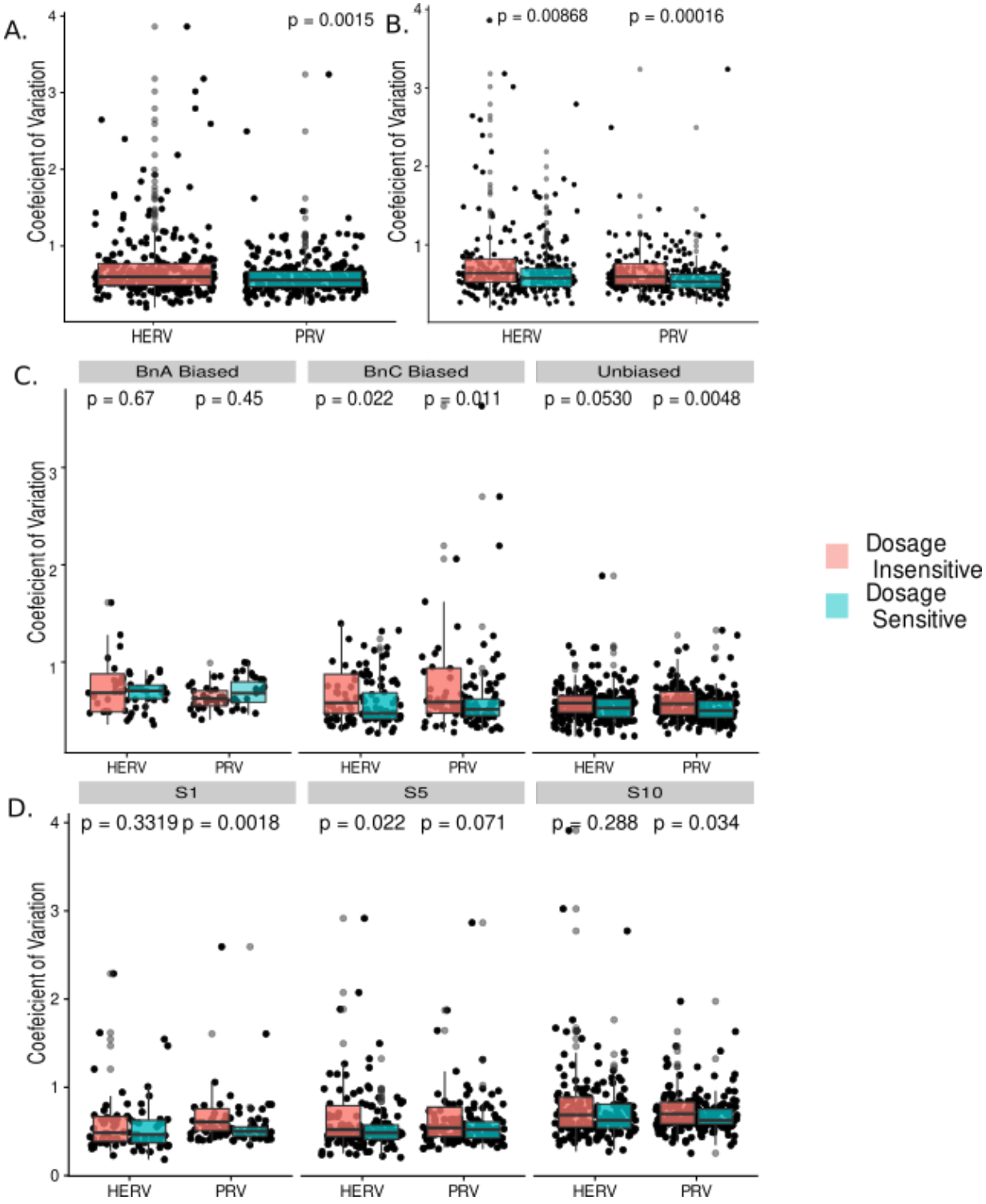

Fig 3. Comparison of expression response variance for homoeologous exchanges (HERV) and allopolyploidy (PRV) for **A)** all lines and gene groups combined, **B)** all lines grouped by dosage class from Song et al. 2020, **C)** GO

Dosage categories and subgenome dominance relationship in parental lines and **D)** GO Dosage categories and generation. For **A)** and **B)** P-values represent results of Kruskal-Wallis test of expression response variance between HERV and PRV and for **C)** and **D)** P-values represent results of Kruskal-Wallis test of expression response variance for Class I vs Class II dosage categories. In all plots, individual dots represent a GO term, restricted only to GO terms that were represented by 20 or more genes in our dataset and shared between the two datasets. Note that p-values may differ from Figs 1 and 3 because only common GO terms between two datasets are analyzed.

In generational comparisons, homoeologous exchange and polyploidy-induced dosage changes diverged. In generation one, the coefficient of variation did not significantly differ by dosage sensitivity for homoeologous exchange-induced dosage changes but it did for polyploid-induced dosage changes(Fig 3d; Kruskal-Wallis test; PRV p-value=0.0018; HERV p-value =0.3319). In generation five, HERV was significantly different between dosage-sensitive and dosage-insensitive GO terms (Kruskal-Wallis test; p=0.022) but PRV was not (Kruskal-Wallis test; p= 0.071). In generation ten we found again that the coefficient of variation from homoeologous exchange dosage changes did not differ significantly between dosage-sensitive and dosage-insensitive GO terms (Kruskal-Wallis test; p=0.288) but they did for the coefficient of variation from polyploid-induced dosage changes (Kruskal-Wallis test p=0.034). We also found that the coefficient of variation for homoeologous exchange-induced dosage changes was not significantly different for both dosage-sensitive and insensitive GO terms for any comparison (Table 2).

That the expression response to homoeologous exchanges and polyploidy-induced dosage changes are significantly different overall, and among a subset of comparisons, is strong evidence that the patterns observed for homoeologous exchange-induced dosage changes are, at least partially, distinct from the effects of polyploidy-induced dosage change. Furthermore, it is likely that dosage constraint is weaker for dosage changes from homoeologous exchanges, leading to a more variable expression response compared to polyploidy. This is because the coefficient of variation for the expression response to homoeologous exchange was higher than that for polyploidy-induced dosage changes for both dosage-sensitive and dosage-insensitive GO terms.

Table 1: Kruskal-Wallis test exploring the difference in expression coefficient of variation from homoeologous exchange and allopolyploidy-induced dosage changes broken down by dosage sensitivity and subgenome bias.

GO Class	Subgenome Bias	HERV mean (SD)	PRV mean (SD)	X2	df	p-value
Dosage Insensitive	BnC Biased	0.677 (0.279)	0.792 (0.575)	0.335	1	0.5625
Dosage Insensitive	BnA Biased	0.722 (0.290)	0.656 (0.143)	0.196	1	0.6576
Dosage Insensitive	Unbiased	0.575 (0.157)	0.587 (0.176)	0.004	1	0.9472
Dosage Sensitive	BnC Biased	0.565 (0.227)	0.569 (0.331)	0.354	1	0.5520
Dosage Sensitive	BnA Biased	0.698 (0.109)	0.681 (0.142)	0.072	1	0.7887
Dosage Sensitive	Unbiased	0.556 (0.204)	0.524 (0.150)	1.177	1	0.2781

Table 2: Kruskal-Wallis test exploring the difference in expression coefficient of variation from homoeologous exchange and allopolyploidy-induced dosage changes broken down by dosage sensitivity and generation.

GO Class	Generation	HERV mean (SD)	PRV mean (SD)	X2	df	p-value
Dosage Insensitive	S1	0.661 (0.456)	0.647 (0.233)	3.206	1	0.073
Dosage Insensitive	S5	0.692 (0.466)	0.635 (0.288)	0.0186	1	0.8915
Dosage Insensitive	S10	0.828 (0.557)	0.745 (0.245)	0.1496	1	0.6989
Dosage Sensitive	S1	0.502 (0.179)	0.551 (0.322)	1.009	1	0.3151
Dosage Sensitive	S5	0.522 (0.208)	0.559 (0.306)	1.769	1	0.1835
Dosage Sensitive	S10	0.697 (0.241)	0.668 (0.199)	0.0047	1	0.9456

Discussion

Two recent investigations have helped demonstrate the connection between gene expression responses to dosage changes and dosage sensitivity (Coate et al. 2016; Song et al.

2020). These authors showed in resynthesized *Arabidopsis* autopolyploids and natural *Glycine* allopolyploids that the expression response to whole-genome duplication in dosage-sensitive genes was less variable than for dosage-insensitive genes. They concluded that dosage constraints produce a less variable expression for dosage-sensitive genes and that this provides a proximal mechanism by which dosage constraint can impact long-term gene retention. We extended this analytical approach to analyze expression responses to homoeologous exchange to test if the dosage-dependent expression changes and alterations to total expression of a gene pair from unequal homoeolog expression (Lloyd et al. 2018) show signs of dosage constraint. By leveraging our population of resynthesized allopolyploid *B. napus* lines, this study could investigate the immediate response to homoeologous exchange and whole-genome duplication in allopolyploids. The unique aspects of *B. napus* also allowed for a novel investigation of how biased subgenome expression interacts with dosage balance constraints.

Dosage constraint and evolution of homoeologous exchanges

Homoeologous exchanges have long been recognized as an engine of phenotypic diversity and novelty in newly formed polyploids (Pires et al. 2004; Gaeta et al. 2007; Xiong et al. 2011; Rousseau-Guetin et al. 2017; Stein et al. 2017; Leal-Bertioli et al. 2018; Lloyd et al. 2018; Mason and Wendel 2020; Ferreira de Carvalho et al 2021, Wu et al. 2021; Bomblies 2022). Our analysis of genomic rearrangements and homoeologous exchanges in resynthesized *B. napus* confirmed at higher resolution the extensive rearrangements in these lines (Gaeta et al. 2007; Xiong et al. 2011). Investigations of genome imbalance and dosage sensitivity have predominantly focused on polyploidy and aneuploidy as the sources of gene dosage alteration (Hou et al. 2018; Yang et al. 2021; Shi et al. 2021). However, homoeologous exchanges, which alter the ratio of parental chromosomes, have also been shown to produce dosage-dependent expression changes and perturb the total expression of a gene pair (Lloyd et al. 2018). These dosage changes from homoeologous exchanges have not been investigated for dosage constraints or more general patterns of expression response expected from the gene balance hypothesis.

We found that expression changes resulting from homoeologous exchanges show the same signs of dosage constraint as those found in response to whole-genome duplication (Fig 1a) (Coate et al. 2016; Song et al. 2020). Gene expression responses from dosage sensitive

GO terms are less variable than those from dosage-insensitive GO terms, a prediction entailed by the Gene Balance Hypothesis. We also saw similar patterns of lower expression coordination in later generations and a lack of differences in expression coordination from homoeolog pairs biased toward the non-dominant subgenome that we observed when investigating expression response to polyploidy. Such results have not been reported before, to our knowledge, and suggest that homoeologous exchanges also experience selective constraint to maintain balance of gene dosage, just like polyploidy or aneuploidy.

If homoeologous exchanges evolve in ways predicted by the gene balance hypothesis then we might expect selection to disfavor homoeologous exchanges containing dosage-sensitive genes, producing biases in gene functions surviving homoeologous exchanges that are similar to those for small-scale duplications. Following these predictions, Hurgobin et al. (2018) and Bayer et al. (2021) identified a significant degree of gene presence-absence variation in *B. napus* arising from homoeologous exchanges, and these genes were associated with membership in the protein-protein interaction network (Bayer et al. 2021) and GO terms related to plant defense and stress pathways (Hurgobin et al. 2018). They also observed several homoeologous exchanges generating presence-absence variation in paralogs of the large gene family *FLC,* which regulates flowering time. Analysis of expression dynamics of *FLC* paralogs in *B. napus* showed that while FLC paralogs are dosage-sensitive, dosage constraints act on overall *FLC* gene family expression allowing compensatory drift (Thompson et al. 2016) and expression divergence (Calderwood et al. 2020). This *FLC* example shows that the interplay of homoeologous exchange and dosage constraint may be highly dynamic depending on the gene family in question. The dosage constraint observed here may also help understand the mechanisms by which Lloyd et al. (2018) observed a tendency of transcriptional compensation of older HE events in natural *B. napus*. Finally, Dosage constraint may also drive subgenome biases in the direction of homoeologous exchange. For example, Edger et al. (2019) proposed that constraints on stoichiometric balance and altered gene dosage explained the overwhelming bias in direction of homoeologous exchange, favoring the dominant subgenome, in the octoploid strawberry genome. These results might help explain why natural *B. napus* lines tend to show bias in favor HE events decreasing copies of C subgenome regions (Nicolas et al. 2012; Chalhoub et al. 2014), as perturbing dosage among only a fraction of the members of interacting units is expected to incur a fitness cost similar to aneuploidy. Our results showing signs of dosage constraint on homoeologous exchanges

differing for gene pairs depending on which homoeolog is more highly expressed lends further credence to this idea.

Dosage constraint on homoeologous pairs appears to differ depending on which subgenome is more highly expressed

In both our HERV and PRV analyses, we observed dosage constraint on pairs where the BnC homoeolog is more highly expressed while not in cases where the BnA homoeolog is more highly expressed. In the HERV analysis (Fig 1b), unbiased homoeolog pairs do not show signs of dosage constraint either while they do in the PRV analysis (Fig 2b, Fig 3c). This may suggest that dosage constraint for homoeologous exchange is driven by some unique aspect of BnC biased homoeologs. Previous analysis of these resynthesized lines showed that homoeologous pairs biased toward the BnC subgenome, which was the maternal contributor and called the dominant subgenome, were highly connected in a protein-protein interaction network, while pairs with expression biased toward the paternal BnA, and non-dominant, subgenome showed no such enrichment for connectivity (Bird et al. 2021). This lack of connectivity may explain why putatively dosage-sensitive genes with biased expression toward the non-dominant subgenome do not show less variable expression; without high connectivity in gene networks, they do not experience strong dosage constraints. Bird et al. (2021) speculated that this enrichment was driven by interactions between the nuclear and organellar genomes, given the functional enrichment for mitochondria, chloroplast, and cytoplasm among the PPI network, however, some recent work casts doubt on the impact of cyto-nuclear incompatibilities on this kind of response to allopolyploidy (Ferreira de Carvalho et la. 2019; Sharborough et al. 2022). Assessing the generality of these subgenome differences in network connectivity and their relation to cyto-nuclear interaction will be a promising avenue for future research in this area.

The fact that this difference in dosage constraint between biased homoeolog pairs is also present in the PRV analysis also suggests that the interaction of homoeolog expression bias dosage constraint may have implications for long-term duplicate gene retention patterns. For example, over the long term, subgenome variation in dosage constraint might be predicted to preserve more dosage-sensitive genes from the dominant subgenome than the non-dominant. In line with this, Schnable et al. (2012) observed that biased retention of dosage

sensitive genes broke down over time, with only 50% of genes retained from one genome duplication event being retained in duplicate after a subsequent duplication event. They further observed that the lower expressed copy was more likely to be lost and proposed the lower expressed copies contribute less to overall gene product dosage, and so experience less purifying selection and weaker dosage constraint (Schnable et al. 2012). Similarly, when subgenome dominance was first described in *Arabidopsis*, the dominant subgenome was also associated with the production of clusters of dosage-sensitive genes across the genome (Thomas et al. 2006).

Implications for long-term duplicate gene evolution and the interplay of biased fractionation and reciprocal retention of duplicate genes

We propose a unified model for short-term and long-term interactions of subgenome dominance and gene dosage constraint. This model involves: (1) greater retention of dosage-sensitive gene pairs that are biased toward the dominant subgenome due to dosage constraint and (2) the eventual divergence of duplicates over long evolutionary time and loss of non-dominant homoeologs due to biased fractionation. Following duplication, gene pairs biased toward the dominant subgenome in these synthetic *B. napus* show higher connectivity in protein-protein interaction networks and functional enrichment. Since dosage-sensitivity is a spectrum, largely positively correlated with connectivity of the gene product in a network or macromolecular complex (Veitia and Birchler, 2012). Dosage constraint is predictive of which genes will be retained long-term to maintain proper stoichiometry. Thus, the more dominantly expressed copy will be expected to persist longer in the genome given that their loss is more likely to perturb the relative balance of interacting gene products.

Dosage constraint is not permanent and can subside over long evolutionary time (Schnable et al. 2012; Conant et al. 2014), additionally the relative dosage of interacting proteins is what is truly under selective constraint and the expression of paralogs can diverge so long as relative dosage is largely left intact, a phenomenon called compensatory drift (Thompson et al. 2016). In the case of subgenome dominance, one copy is contributing a greater fraction of the overall dosage of that gene product. As dosage constraint weakens, this more highly expressed copy will cause a greater disturbance to relative dosage if deleted. As

an extreme example, if one copy contributes 90% to total expression and the other 10%, a greater dosage imbalance would be observed with interactors when losing the dominant (90%) copy. As such, the dominant copy will be under stronger purifying selection. Additionally, under compensatory drift it is easier for the dominant copy to change expression enough to account for the all or most of the relative dosage of a pair, thus reducing purifying selection on the non-dominant copy which now contributes little-to-no expression for the overall relative dosage.

This difference in purifying selection reduces the likelihood that the dominant copy is fractionated by the short-deletion mechanism postulated to drive genome fractionation in plant genomes (Woodhouse et al. 2010). Ultimately, genes on the non-dominant subgenome will be preferentially lost and the dominant subgenome will maintain higher gene content and more enrichment for dosage sensitive genes - even through successive polyploid events (Woodhouse et al., 2014 PNAS).

Future Directions

Several findings may warrant follow-up or more targeted investigation.Our comparison of homoeologous exchange and polyploidy response variance showed that overall gene expression was more variable in response to homoeologous exchange compared to polyploidy. This may mean that genes affected by homoeologous exchange experience weaker dosage constraints, although it may also simply be due to high levels of inter-individual variation among lines. Additionally, this study only looks at expression in leaf tissue. Expression differences and differences in homoeolog expression bias are likely to exist across tissues. Investigation in more tissue types and looking for variation by tissue is a promising avenue of research. Future work would also benefit from approaches using spike-ins and those which can isolate homoeologous exchange from other trans-effects on expression, both from hybridization and aneuploidy that could not be controlled for when assessing expression changes in this study. Such work would provide more precise estimates of the magnitude of the dosage constraint effect. This improved experimental design would also help make sense of the finding that PRV and HERV for both dosage-sensitive and -insensitive GO terms is interesting (Fig 1c, Fig 2c, Fig 3d). Currently, it is not possible to distinguish whether this result is from a loosening of dosage constraint or an accumulation of inter-individual variation from trans dosage effects in the genomic background. Disentangling these two explanations will reveal novel insights into the dynamics of expression

changes and dosage constraint over short evolution time scales. One particularly interesting possibility would be exploring ways to generate or introduce homoeologous exchanges of a specific dosage in a controlled genetic background, allowing a more precise investigation of the effect of these dosage changes.

Conclusion

This study provides new insight into the role of dosage constraint on gene balance in affecting gene expression changes from genomic rearrangements. These findings may help fuel more integrative genetic and evolution investigations of homoeologous exchange, subgenome expression dominance, and duplicate gene evolution that can leverage the vast new output of genomes with ancestral and recent polyploidy and explicit evolutionary models of ancestral subenomes (Emery et al. 2018; Hao et al. 2021, 2022; Parey et al. 2022). This new avenue of investigation may help further examine evolution and epistasis as well as selection and divergence among paralogs (Qi et al. 2021; Conover and Wendel, 2022; Kwon et al. 2022), and spur further integration of methods and data across phylogenomics, comparative and population genomics, and network biology (Renny-Byfield et al. 2017; Blischak et al. 2018). Such work can enhance plant breeding efforts by providing a strengthened evolutionary understanding of the consequences of gene duplications, gene balance, and subgenome dominance (Rodriguez-Leal et al. 2017; Bird et al. 2018; Salman-Minkov et al. 2016; Turner-Hissong et al. 2020; Bayer et al. 2021; Bomblies 2022).

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Author Contributions:

KAB contributed to conceptualization, formal analysis, investigation, methodology, validation, visualization, writing—original draft, and writing—review editing; JCP. contributed to resources, and writing—review editing; RV. contributed to supervision, methodology, and writing—review editing; ZX. contributed resources, experimental design, and writing—review editing; and PPE. contributed supervision, methodology, and writing – review editing.

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