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Original research

Magnitude of modulation of gene expression in aneuploid maize depends on the extent of genomic imbalance

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abstract

Aneuploidy has profound effects on an organism, typically more so than polyploidy, and the basis of this contrast is not fully understood. A dosage series of the maize long arm of chromosome 1 (1L) was used to compare relative global gene expression in different types and degrees of aneuploidy to gain insights into how the magnitude of genomic imbalance as well as hypoploidy affects global gene expression. While previously available methods require a selective examination of specific genes, RNA sequencing provides a whole-genome view of gene expression in aneuploids. Most studies of global aneuploidy effects have concentrated on individual types of aneuploids because multiple dose aneuploidies of the same genomic region are difficult to produce in most model genetic organisms. The genetic toolkit of maize allows the examination of multiple ploidies and 1e4 doses of chromosome arms. Thus, a detailed examination of expression changes both on the varied chromosome arms and elsewhere in the genome is possible, in both hypoploids and hyperploids, compared with euploid controls. Previous studies observed the inverse trans effect, in which genes not varied in DNA dosage were expressed in a negative relationship to the varied chromosomal region. This response was also the major type of changes found globally in this study. Many genes varied in dosage showed proportional expression changes, though some were seen to be partly or fully dosage compensated. It was also found that the effects of aneuploidy were progressive, with more severe aneuploids producing effects of greater magnitude.

1. Introduction

Nearly a century ago, Alfred Blakeslee (1921) recovered isolates of the flowering plant Jimson weed (Datura stramonium) that had an extra copy of each of the 12 chromosomes, as well as triploid and tetraploid derivatives of the normal diploid. He noted that each of these trisomic plants had morphological differences from the diploid plants that were more prominent than the differences among the diploid, triploid and tetraploid plants. Furthermore, he mapped a gene with allelic differences for the presence or absence of purple pigment using altered segregation ratios from a trisomy, but noted that every one of the other trisomies modulated the amount of pigment (darker or lighter) when the purple allele was homozygous. In contrast, the pigment phenotypes of diploid, triploid, and tetraploid were similar. Therewith was born the concept of genetic balance that has been recapitulated in many eukaryotic organisms, e.g., Drosophila (Lindsley et al., 1972) and human (Schinzel, 2001). As foreshadowed by the observation of modulation of pigment levels by all trisomies, molecular studies of selected genes found parallels to those classical phenotypic observations (Birchler, 1979; Birchler and Newton, 1981; Rabinow et al., 1991; Guo and Birchler, 1994; Birchler et al., 2001, 2005; Birchler and Veitia, 2007). RNA sequencing studies on gene expression in trisomies of Drosophila and Arabidopsis vs. balanced genotypes illustrate the generalized nature of these alterations on a global scale (Sun et al., 2013a,b; Hou et al., 2018). Here, the question of the impact of increasingly imbalanced genotypes for the same chromosomal segment of the genome is addressed.

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Nondisjunction of the supernumerary B chromosome in Zea mays has proven to be very useful for the study of aneuploidy and dosage effects. Maize B chromosomes are non-vital chromosomes consisting of a centromere with a single long arm; the short arm is vanishingly small (Randolph, 1941). They are selfish elements: in male flowers B chromosomes tend to nondisjoin during the second pollen mitosis (Roman, 1947), and those sperm that have two copies of the B chromosomes tend to fertilize the egg as opposed to the polar nuclei in the process of double fertilization (Roman, 1948). B chromosomes occur naturally in some maize lines, and do not cause any noticeable impairment to the plant unless their number approaches that of the A chromosomes (meaning the regularly numbered chromosomes) (Randolph, 1941). An A chromosome segment can be translocated onto a B centromere (Roman, 1947). Such a B-A chromosome can itself participate in crossing over with homologous A chromosome regions, and researchers have taken advantage of this fact to add marker genes to the B-A chromosome (Birchler and Alfenito, 1993). If a marker for kernel color has been added, for example, this allows easy identification of seeds that have the B-A chromosome. A dominantly marked embryo will have been generated by the joining of the egg and a sperm with two B-A chromosomes; the endosperm resulting from fertilization of the polar nuclei by the counterpart sperm will be missing the dominant marker (Birchler and Alfenito, 1993). A line of maize with B-A chromosomes may be aneuploid for that A chromosome region, which allows for the creation of a dosage series for any chromosome arm so desired. Fifteen unique B-A chromosomes with dominant kernel color markers have been developed and introgressed into the maize W22 line (Birchler and Alfenito, 1993). These stocks can be used to study the effects of both hypoploidy (missing chromosomes) and hyperploidy (extra chromosomes) for most of the maize genome.

The effects of aneuploidy on gene expression levels can be widespread. A B-A chromosome dosage series in maize showed extensive differences in the quantities of selected proteins depending on how many doses of the long arm of chromosome 1 (referred to as 1L) were present (Birchler, 1979; Birchler and Newton, 1981). No detectable effect of B chromosomes alone was found. The gene loci of the affected proteins were not on 1L, but instead were scattered around the genome. This response has come to be defined as a trans-effect, as opposed to a cis-effect that affects genes of the varied region itself. Many of the trans-affected genes showed an inverse relationship to the dosage of 1L (Birchler, 1979; Birchler and Newton, 1981). Given three copies of 1L, affected proteins were found at levels lower than in a normal diploid, and, given one copy, their levels were increased. Another finding of this study was that a protein encoded on the varied portion of 1L was not affected by the dosage of its own locus e it was dosage compensated (Birchler, 1979, 1981; Birchler and Newton, 1981). The results also showed that the inverse effect occurs for most of the other tested chromosomal segments (Birchler and Newton, 1981). This response was initially found with protein levels; mRNA transcripts were later shown to be similarly impacted (Guo and Birchler, 1994). These results suggest that the inverse effect is a general outcome of chromosomal dosage variation, and it has been proposed to be responsible for many aspects of aneuploidy in general (Birchler and Newton, 1981).

In a previous study of all five trisomies of Arabidopsis, which cover the whole genome, as well as a ploidy series of diploid, triploid and tetraploid, trisomy produced much greater modulations of global gene expression than the change of whole-genome copy number (Hou et al., 2018). Although both direct and inverse effects were found in trisomy, the latter was the most prominent. In the present study, ratio distribution plots were used to visualize expression changes at the genome scale, with the ratio of expression (e.g., expression in trisomy compared with balanced diploid) represented on the x-axis and the number of genes in each ratio bin represented on the y-axis. The question of how global patterns of expression are modulated in more extreme imbalanced genotypes that are possible to construct in maize was examined.

Using a set of genetic tools uniquely available in maize, a dosage series for a maize chromosome arm was produced to analyze the effects of the magnitude of genomic imbalance on gene expression. Maize plants were produced that were variable in dosage for chromosome arm 1L. Two separate sets of plants were grown to compare 6 distinct ploidy genotypes: diploids with 1, 2, 3, or 4 copies of 1L and haploids with 1 or 2 copies of 1L. RNA sequencing was used to determine gene expression in mature maize leaf tissue from plants at each dosage level. This approach provided a view of the effects of aneuploidy on the entire transcriptome by comparing different levels of aneuploidy for the same chromosome arm against euploid controls and comparing the same dosage magnitude change for whole-genome ploidy.

2. Results

2.1. Gene expression ratio distributions

The results of RNA sequencing for plants at various chromosomal dosage levels were used to compare the expression of genes in the aneuploid to that in the euploid control. Genes expressed in the euploid but not in the aneuploid were removed from the set; the numbers were variable in the different dosage groups, which resulted in different sizes of compared gene sets. For each gene in the set, mean RNA expression in the dosage-varied plant was divided by that of the control balanced genotype, diploid or haploid, producing a single value for each gene, with a ratio of 1.00 representing no difference between the two sets. These values were plotted in ratio distributions, which has the advantage for examining global trends of modulation, especially close in magnitude to the control, because all genes serve as data points permitting greater statistical power to show narrow range modulations. As shown in Fig. 1 (ratio distribution plots), genes to the left of 1.00 on the x-axis have a decrease of expression compared to normal, and genes to the right have an increase. Data shown in Fig. 1 are comprised of both cis gene ratios (genes located on chromosome arm 1L, as determined by DNA sequencing of a 1L trisomy compared to a diploid) and trans gene ratios (genes located elsewhere in the genome). The 1L and non-1L gene sets show a large difference of characteristic effects.

In all graphs comparing aneuploids to normal (Fig. 1), a large shift of the mode of the distribution from 1.00 or a great deviation of the sample distribution from a normal distribution can be identified. Starting at the low end of the spectrum, some ratio distributions contain a spike in the bin ranging from 0 to 0.05. This bin represents genes that are expressed at the lowest detectable levels in both diploid and aneuploid but show at least a 20-fold decrease of expression in the aneuploid compared to the diploid. Another spike may occur at the high end of the spectrum, indicating a greater-than-6-fold increase in expression in the aneuploid. The genes in these outlying peaks are almost entirely found to have low read counts (less than 0.25 reads per kilobase of transcript per million mapped reads; RPKM), in which case small changes to the numerator or denominator have a larger impact on the ratio. They represent a small fraction of the assayed genes.

A general trend observed in Fig. 1 is that in cis, gene expression changes trend toward correlation with gene dosage. A large peak can be seen near the guide corresponding to dosage increases, 1.50 in trisomy/diploid (Fig. 1A, B and F) and 2.00 in tetrasomy/diploid (Fig. 1C) and disomy/haploid (Fig. 1D). In the monosomy/diploid

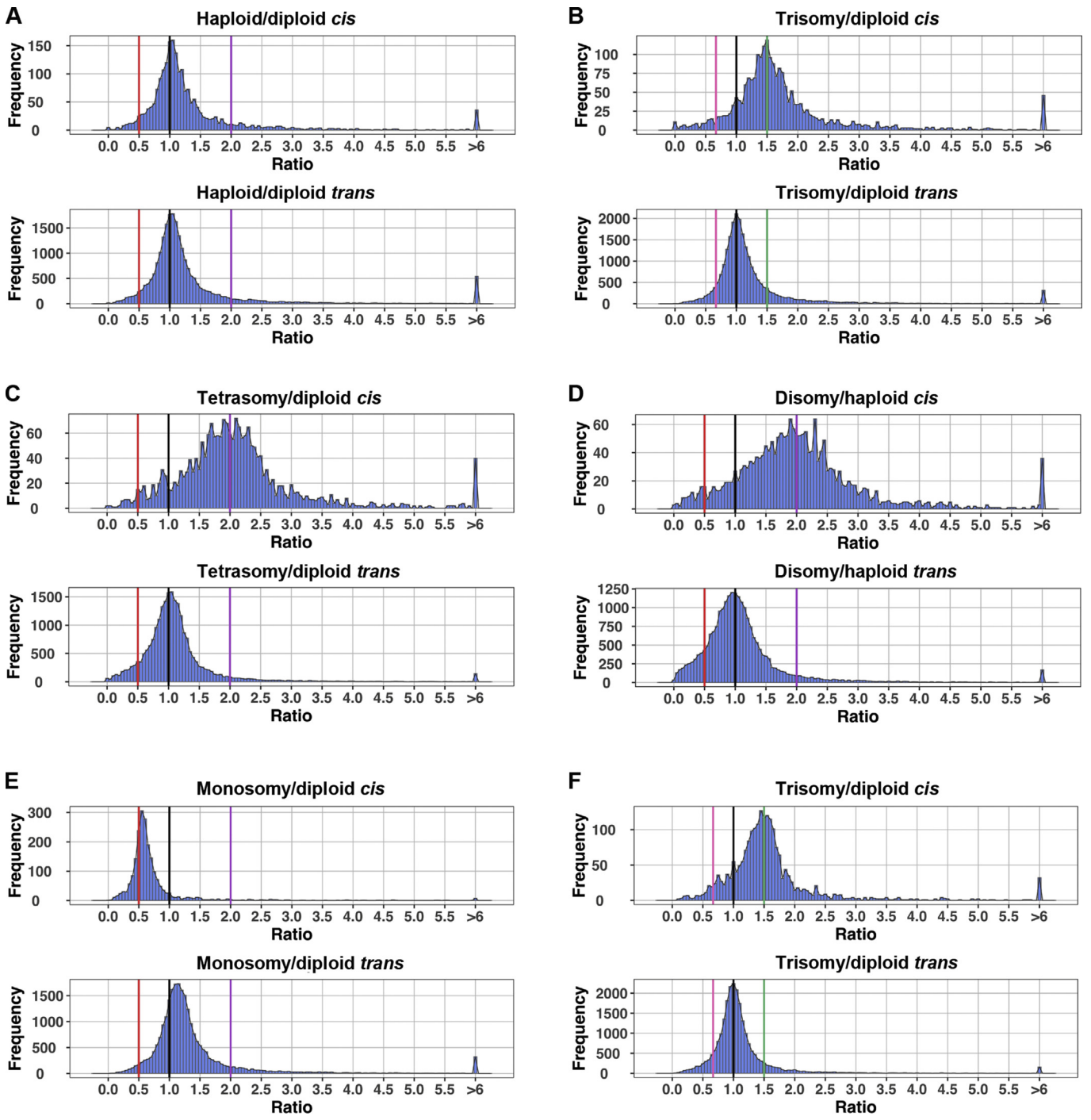

Fig. 1. Ratio distribution plots. For every gene, the ratio of expression in an aneuploid plant compared to a euploid plant was determined. AeD: Ratio distribution plots of the first dosage series. A haploid/diploid comparison was used as a control (A). Trisomy/diploid (B), tetrasomy/diploid (C), and disomy/haploid (D) comparisons were all generated with plants from the same set. E and F: Ratio distribution plots of the second dosage series. Comparison of gene expression in monosomy/diploid (E) and trisomy/diploid (F). The x-axis indicates the expression ratio, and the y-axis indicates the number of genes in each bin. In A and C–E, the three vertical guide bars indicate an inverse relationship to dosage (0.50), a value unchanged from euploid (1.00), and a direct relationship to dosage (2.00), respectively. In B and F, three guide bars indicating an inverse relationship, an unchanged relationship, and a direct relationship to dosage are placed at 0.67, 1.00, and 1.50, respectively. All expressed genes were considered. If a gene has the same average expression in the aneuploid condition as in the euploid condition, its ratio will be 1.00; if expression decreases in an aneuploid, its ratio will fall to the left of 1.00; if expression increases, its ratio will fall to the right of 1.00. For genes with a locus on chromosome 1L, a rightward trend can be observed in hyperploids and a leftward trend in the hypoploid, indicating a direct (but often partial) correlation between gene dosage and RNA expression in cis. For the non-1L genes, a shoulder can be observed on the left side of the central peak in several plots, indicating that some genes show an inverse correlation between 1L gene dosage and non-1L RNA expression.

comparison (Fig. 1E), the peak appears near 0.50, corresponding to a dosage decrease. Though the peaks are broad, more genes tend to fall to the left of the guide in each hyperploid, and to the right in the hypoploid. This indicates that the dosage effect in cis is partly compensated for different genes to different degrees.

Gene expression ratios in trans do not resemble those observed in cis. Instead, in hyperploids smaller peaks appear below 1.00, most prominently in the tetrasomy/diploid (Fig. 1C) and disomy/

haploid (Fig. 1D), towards the inverse guides at 0.50. In the monosomy/diploid (Fig. 1E), the main peak is above 1.00; the peak becomes fairly broad, exceeding 1.00 into the range of increasing gene expression. Although inverse changes in gene expression are distinct to trans, a substantial number of genes are not affected, or are seen to change in the same direction as the varied chromosome. Among genes that are inversely affected, their typical ranges lie between the inverse ratio and 1.00. Table S1 displays the outcome of Kolmogorov-Smirnov (K-S) tests comparing ratio sets of different aneuploid conditions; ratio sets for each dosage are shown to be significantly different from each other. Principal component analysis (PCA) was used to confirm that gene expression levels in biological replicates of the same genotype were similar to each other (Fig. S1).

Then, ratio distributions comparing tetrasomy and trisomy were analyzed (Fig. S2). In the cis comparison, expression is affected up to a 1.33 ratio, consistent with 1L gene dosage; in the trans comparison, most genes are not dramatically different between the two aneuploids, but when they are different, movement trends to the left. This result indicates that some non-1L genes in tetrasomies have lower expression levels than in trisomies. Another conclusion that can be made from this plot is that many of the same genes have altered expression in the two aneuploids in the same direction from the diploid. If a non-1L gene shows a reduced expression in trisomy but not in tetrasomy, the gene would have a high ratio in this comparison. The rarity of this effect compared to low ratios suggests that most genes sensitive to trisomy are also sensitive to tetrasomy. In other words, the ratio distributions show the range of all genes that are modulated even to a slight degree, so the tetrasomy/trisomy distribution illustrates that most genes do not switch direction of modulation compared to the diploid.

2.2. Differential expression

Next, an edgeR test was performed to analyze the significant expression changes in each of the comparisons shown in Fig. 1 (Table 1). The haploid/diploid comparison has no meaningful distinction between cis and trans genes, and both gene sets show similar proportions of affected genes, with some favoring increased expression compared with decreased. For cis genes in all five aneuploid/normal comparisons, a large number show significant expression changes, but the vast majority are directly proportional to gene dosage. In the first dosage series (set 1), the number of significantly affected genes is greater in the tetrasomy/diploid and disomy/haploid than in the trisomy/diploid, correlating with their more imbalanced genotypes. In the same comparisons in trans, the trisomy/diploid distribution has a larger proportion in the decreased-expression category compared to the ploidy distribution of haploid/diploid. In both the tetrasomy/diploid and disomy/haploid comparisons in trans, the number of affected genes is substantially larger than the trisomy/diploid, and the proportion showing decreased expression is greatly increased. In the second dosage series (set 2) in trans, the trisomy/diploid comparison favors decreased expression compared to the haploid/diploid, while the monosomy/diploid comparison shows many more genes with a significant increase than a decrease, in both cases contrasting with their dosage relationship. The results of the edgeR signi fi cance tests were displayed as volcano plots in Fig. 2.

2.3. RT-qPCR verification

In order to validate the results obtained by RNA sequencing, RT-qPCR was performed for a selection of genes, using RNA extracted from materials of the same dosage series. Primers were selected to bind to opposite sides of exon-exon junctions, in order to eliminate the effects of any remaining genomic DNA. External RNA Controls Consortium (ERCC) spike-in was used as an exogenous control, rather than a set of endogenous genes. If overall levels of mRNAs were affected by various aneuploid conditions, housekeeping genes would be subject to changes as well and would not be a reliable control for expression differences in the genome (Birchler, 2014).

Five genes were selected to represent each of five important dosage relationships: direct cis dosage effect, cis dosage compensation, inverse trans effect, direct trans effect, and no trans effect. Relationships between dosage and expression are consistent between RNA sequencing and RT-qPCR, comparing disomy to haploid as well as both trisomy and tetrasomy to diploid (Fig. 3 and Table S2). However, there are two cases of note. One relates to the 1L gene representing dosage compensation; specifically, the compensation is partial in the RT-qPCR results, but complete in the RNA sequencing results. The other is in the non-1L gene representing no change of expression: in the RT-qPCR results, diploid expression was distinctly lower than in the trisomy and tetrasomy. Haploid and disomic haploid for the same genes matched closely. Expression changes were progressively more severe with increasing aneuploidy for the genes representing the direct cis dosage effect and the inverse trans effect. Overall, the two approaches to quantify gene expression support each other.

2.4. Functional group analysis

In a previous study of Arabidopsis trisomies, different functional categories of genes were examined individually with different groups being distinct (Hou et al., 2018). As a means of comparison,

Table 1
Summary of the edgeR significance test results.

Comparison	Total number of genes	Fold change significance below 1	Fold change significance above 1
Cis comparison			
Haploid/diploid (set 1)	2346	18	91
Trisomy/diploid (set 1)	2365	33	179
Tetrasomy/diploid (set 1)	2350	11	1158
Disomy/haploid (set 1)	2342	18	261
Monosomy/diploid (set 2)	2368	380	4
Trisomy/diploid (set 2)	2390	14	353
Trans comparison			
Haploid/diploid (set 1)	26,598	128	1064
Trisomy/diploid (set 1)	26,826	55	308
Tetrasomy/diploid (set 1)	26,371	835	660
Disomy/haploid (set 1)	26,304	526	359
Monosomy/diploid (set 2)	27,417	89	489
Trisomy/diploid (set 2)	26,953	198	445

Summary table of gene expression effects generated by edgeR. Cis (1L) and trans (non-1L) effects are separated. Total number of genes differs due to removal of data points for which the summation of RPKM counts in both conditions is less than 3. Significance is defined by False discovery rate (FDR) less than 0.05.

some functional groups were examined separately for the maize data. Eight functional categories were viewed in terms of both cis and trans impacts of each level of aneuploidy (Figs. 4, 5, and S3–S8). K-S tests for comparing functional groups show distinct patterns among them (Table S3). In cis, chloroplast-targeted genes (Fig. 4) could be distinguished from the set of all 1L genes at all dosage levels and exhibit a stronger inverse trend compared to all genes. The chloroplast-targeted genes also have the most strongly differentiated response in trans. Non-1L mitochondria-targeted and ribosomal genes are also strongly differentiated from the set of all genes in trans (Figs. 5 and S3). Proteasome and transcription factor genes are differentiated to a lesser degree (Figs. S5 and S8), while peroxisome-targeted, signaling-related, and stress-related genes are not consistently different (Figs. S4, S6 and S7).

As with the Arabidopsis study, the functional groups with the strongest inverse effects in trans are chloroplast- and mitochondria-targeted (Figs. 4 and S3). Also, in parallel, the genes encoding the components of the ribosome tend to show a positive effect in trans for trisomies (Fig. 5). In this study, it was possible to examine a tetrasomy for which this trend continues. Interestingly, however, in the disomic haploid distribution, there is a shift downward for ribosomal genes even though other functional classes of genes do not switch direction in this genotype.

3. Discussion

Based upon a comparison of various aneuploid conditions, a central observation from this work is that the effects of dosage appear to be progressive in the sense that increasing levels of aneuploidy correlate with increasing alterations of gene expression. In other words, the greater the genomic imbalance, the greater the modulation of gene expression in both cis and trans. This applies both to the number of genes affected and the typical degree of change. Both disomic haploids and tetrasomic diploids have a greater level of imbalance (the varied chromosome occurs at 2! the copy number of the remainder of the genome) than trisomic diploids (1.5!), and the number of genes with significant expression change is higher in the disomy and tetrasomy than the trisomy. Based on the ratio distribution plots, the range of expression changes in cis moves from near a peak at 1.50 in the trisomy set to near 2.00 in the tetrasomy and disomy sets, proportional with gene dosage. In trans, the broad peak comprising the majority of genes moves further to the left in the tetrasomy and disomy compared with the trisomy, illustrating the greater inverse effect by increasing levels of genomic imbalance. Further, these data suggest that the inverse effect is not a general suppression of transcription due to aneuploidy-induced stress given that the stress-related genes have a distribution similar to all genes. This conclusion is most directly demonstrated by the presence of inverse effects in trans in hypoploid plants, which in that condition equates to an increase in expression. Furthermore, if a general decrease of expression were present, the positively modulated trans effects seen in the trisomic set would be diminished relative to the inverse effect in the more extreme hyperploids. In fact, the number of these positively modulated trans effects also increases with increasing imbalance, implying that increasing disruption of the regulatory network triggers a cascade of more and larger effects on individual genes rather than the entire set of genes as a whole.

At each dosage level, genes with a structural locus on chromosome arm 1L were much more likely to be positively correlated with gene dosage. Nevertheless, the ratio distributions span a range from dosage compensation (no change from diploid) to direct dosage effect (a proportional change matching the gene dosage) with most being intermediate, and there are minor shoulder peaks at compensation and even at the inverse level for cis encoded genes. Few genes are affected beyond the ratio of their dosage change.

A second phenomenon that can be seen in the comparisons of aneuploid to euploid is the prevalence of inverse effects in trans. This directional change of gene expression, opposite to the dosage of the varied chromosome, is in contrast to the haploid/diploid ploidy comparison in which there are few such relative expression changes. In aneuploids, the inverse effect can be seen both holistically (in the ratio distribution plots) and statistically (in the volcano plots). The inverse trans effect is often a partial one, and the positive trans effect also occurs (although it is also typically partial). In most cases, a smaller respective portion of trans genes than cis genes can be statistically confirmed to have altered expression. According to the ratio distribution plots, fully inverse (or direct) effects in trans are relatively rare compared with partial effects. The copy number change of structural genes is likely to have a more intimate impact on expression level than trans-acting effects mediated through multiple steps and interactions. Although molecular kinetics may account for observed effects in both cis and trans, the trans effects are mediated by relationships among genes such as various transcription factors, which have differential rates of synthesis and degradation of both mRNAs and proteins that could affect the magnitude of the regulatory interactions. Expression ratios more extreme than inverse are rare but present.

The modulation of gene expression in aneuploid has been hypothesized to result from an altered stoichiometry of the subunits of regulatory machinery (Birchler and Newton, 1981; Guo and Birchler, 1994; Birchler et al., 2001; Birchler and Veitia, 2012). In this study, it is possible to vary the extent of altered genomic stoichiometry. The modulations in trans in general become more extreme with an increasing imbalance of chromosomes.

In the interesting case of ribosomal genes, trisomies and tetrasomies at the diploid level have a positive trans effect on expression but the disomic haploid flips to an inverse trend. In a theoretical study of how altered regulatory stoichiometry could produce both positive and negative modulations depending on the relative concentrations of interacting regulatory subunits of a multisubunit complex (Birchler et al., 2016), it was illustrated that a positive effect might be conditioned by a low concentration of a critical subunit but at a higher concentration the impact could become dominant negative. The modulation of the ribosomal genes might illustrate such a case.

Previous studies of gene imbalance in maize similarly found inverse trans effects (Guo and Birchler, 1994), although these were conducted with kernel tissue rather than leaf tissue. The similarity of the phenomena observed suggests that despite the developmental differences between the two tissue types and stages, a common mechanism is responsible for the effects seen in both. Phenotypically, aneuploids are affected more strongly than polyploids (Lee et al., 1996a, b; Sheridan and Auger, 2008; Birchler and Veitia, 2012; Brunelle and Sheridan, 2014; Zuo et al., 2016; Bastiaanse et al., 2019), and modulations of gene expression observed here correlate with that observation. These changes in gene expression may be hypothesized to be the cause of the phenotypic effects seen in aneuploidy. However, this relationship is likely to be complicated and might be the result of different sets of genes. For example, 1L monosomies are phenotypically less robust than 1L trisomies, and both are less robust than the diploids, although the trans effects in general are in opposite directions. One possibility to consider is that different effects become rate limiting on the phenotype (Birchler and Newton, 1981). Dosage affected genes in monosomy are reduced to lower levels (0.50), as a general rule, than trans affected genes in trisomy (0.67). The phenotype might be affected by whichever genes become limiting under the respective circumstances. The more severe effects in monosomy

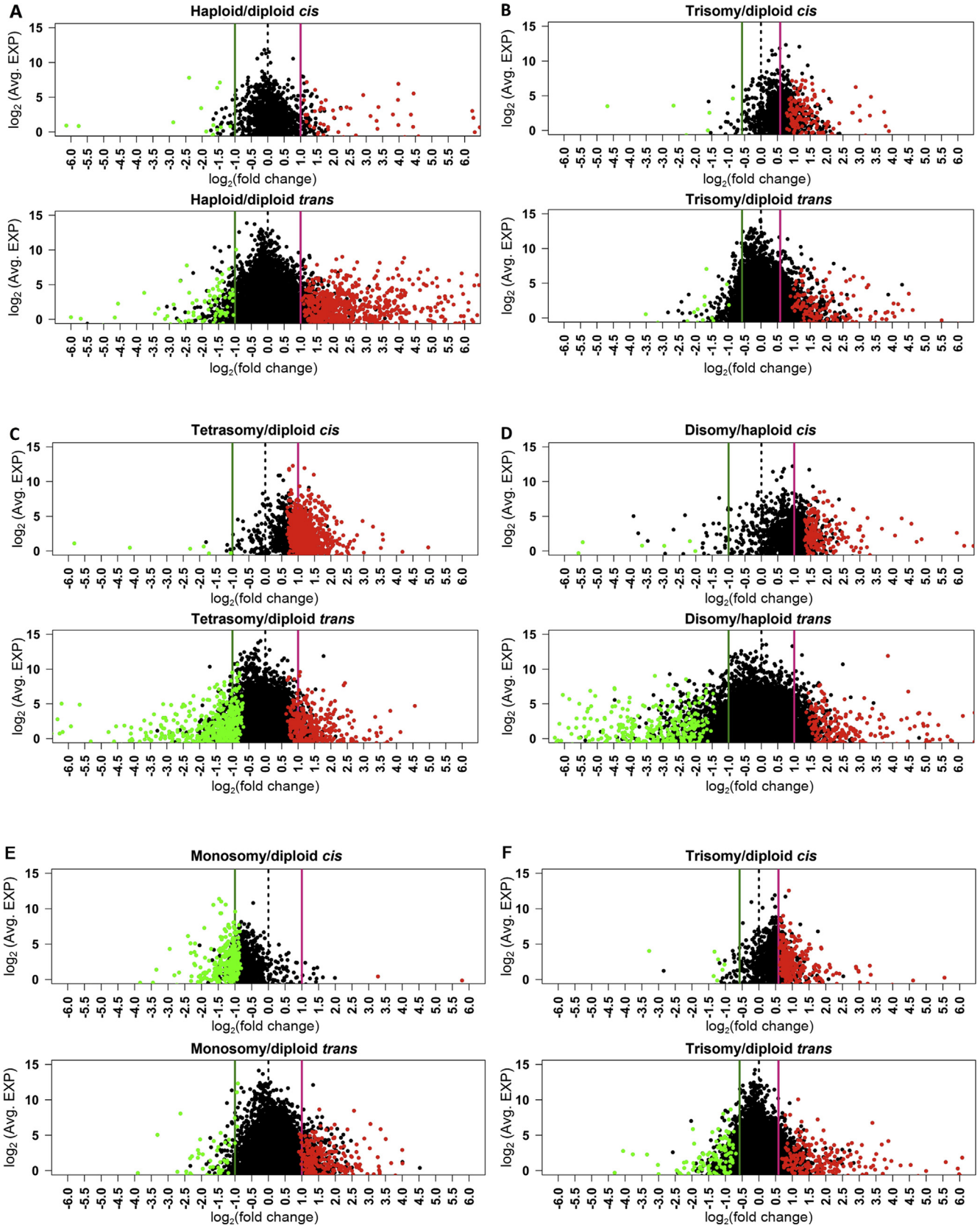

Fig. 2. Volcano plots. AeD: Volcano plots of the first dosage series. The plots are based on the same comparisons of gene expression shown in Fig. 1AeD. E and F: Volcano plots of the second dosage series. The plots are based on the same comparisons of gene expression shown in Fig. 1E and F. The x-axis is fold change, with expression ratio displayed at a log $_2$ scale. The central guide represents log $_2$(fold change) of 0.0, equivalent to an expression ratio of 1.00 (no change). In A and C–E, the guide bars at log $_2$(fold change) of "1.0 and 1.0 represent expression ratios of 0.50 and 2.00, respectively. In B and F, the guide bars represent expression ratios of 0.67 and 1.50, respectively. The y-axis is the mean of RPKM value at a log$_2$ scale in the diploid control. Green points on the left of the centerline represent genes with a statistically significant decrease in gene expression from euploid to aneuploid

than in trisomy correlate with the greatest reductions in gene expression.

In general, the results of this study illustrate that the greater the genomic imbalance, the greater are the modulations of gene expression across the genome. Classical studies at the phenotypic level have shown greater effects with increasing genomic imbalance. Our study illustrates that modulation of gene expression follows the same principle.

4. Materials and methods

4.1. Plant material and tissue collection

To determine RNA levels of all expressed genes, leaf tissue was collected from plants at each dosage level. The first set included diploid plants with 2, 3, and 4 doses of chromosome arm 1L, and haploid plants with 1 and 2 doses. Leaf tissue was harvested at 45e46 days post-germination, so as to avoid differential expression of genes based on varying stages of development. The second set included diploid plants with 1, 2, and 3 doses, and leaf tissue was harvested at 55e57 days post-germination. Plants were greenhouse-grown to minimize environmental variation, and leaf tissue was collected at the same time of day (late mornings) to minimize circadian rhythm variation. The leaf tissue collected was the region approximately 1.5e15 cm above the auricle. Only the leaf blade was kept, and the midrib was discarded. The tissue was collected from the lowest adult-phase leaves, typically leaves 5 and 6 in the W22 inbred line.

The two sets of plants for analysis were phenotyped using different methods, each of which was later verified by checking the relative consistency of cis gene expression in leaf tissue. In the first set, dosage of chromosome 1L was confirmed by FISH karyotyping (Kato et al., 2004; Albert et al., 2010). This dosage series was produced by self- or sibling-crosses of segmental trisomy (hyperploid heterozygotes of TB-1La) parents, which had been phenotyped using the dominant color marker gene Bronze2 on the B-A chromosome (i.e., those kernels with purple embryos and bronze endosperm in a background with R1-scm2 that pigments both the embryo and endosperm). The offspring diploid embryos carried various combinations of 1 or 2 complete copies of chromosome 1, plus 0, 1, or 2 B-1L chromosomes, resulting in euploid, trisomic, or tetrasomic plants (Figs. S9 and S10). No monosomies were recovered from this series. Kernel color in this generation no longer correlates with phenotype, necessitating FISH karyotyping. Haploids and disomic haploids were produced by crossing trisomic females (hyperploid heterozygotes of TB-1La) with males of the haploid inducer RWS (R Gber et al., 2005). The haploid inducer line is homozygous for the dominant inhibitor of color, c1-I, thus maternal haploids have colored embryos and colorless endosperm on ears that are mostly colorless. Haploids and disomic haploids were distinguished by cytological examination of root tip metaphase chromosomes.

In the second set, dosages were limited to 1, 2, and 3 copies of 1L in a diploid background, which allowed for phenotyping of kernels according to the presence of a dominant color gene (Bronze2) carried by the B-1L chromosome in the embryo or endosperm (Birchler and Alfenito, 1993). Crossing a trisomic plant (hyperploid heterozygote, as a male) onto a recessive bz2; R-scm2 tester, if all copies of B-1L were inherited by the embryo (making a trisomy), it had the purple color while the endosperm was bronze. If all copies were inherited by the endosperm (leaving a monosomic embryo), the endosperm carried the purple color while the embryo was bronze. If both embryo and endosperm carried the purple color, normal disjunction had occurred in the preceding second pollen mitosis and the embryo was a balanced diploid.

Though differing methods of dosage verification do not interfere with comparability of results from the two sets, there were some distinctive environmental factors that suggest biological replicates from the two sets are ideally compared within the same set, rather than between the two sets. The age of tissue collection in the second set was adjusted to accommodate the slow growth habit of the monosomies. Regarding adult plant phenotype, diploids were typically taller and more vigorous than trisomies. Tetrasomies were less vigorous than trisomies, and monosomies were the least healthy in a diploid background. Haploids were shorter and less robust than diploids; disomic haploids were severely stunted (Birchler and Veitia, 2012).

Different numbers of biological replicates could be validated for each dosage as outlined above by cis expression. In the first set, four diploid (euploid B-A translocation heterozygotes), five trisomic, and three tetrasomic plants were validated, with the diploid plants serving as the control. Also in the first set were three haploid and two disomic haploid plants, with the haploid plants used as the control. In the second set, four monosomic, three diploid, and three trisomic plants were available, with the diploid plants used as the control.

4.2. RNA and DNA sequencing and data analysis

RNA was extracted using a TRIzol-based protocol (Rio et al., 2010). RNA sequencing was performed by the University of Missouri DNA Core, using Illumina technology (Bentley et al., 2008). TruSeq (Illumina) was used for library preparation, involving poly-A enrichment of mRNA using oligo-dT magnetic beads, first-strand cDNA synthesis using reverse transcriptase, second-strand cDNA synthesis using DNA Polymerase I, ligation of adaptors, and finally PCR amplification. The first set was sequenced on a HiSeq platform with single-end 100 bp reads, and the second set was sequenced on a NextSeq flow cell with single-end 75 bp reads. Data output from Illumina RNA sequencing was processed via RNAMiner pipeline (Li et al., 2015). Specifically, low quality reads were removed, followed by mapping filtered reads to the maize genome (maize W22) using Tophat2 (Trapnell et al., 2009). Uniquely mapped reads were quantified as raw counts and differentially expressed genes were analyzed using R/Bioconductor package edgeR (Robinson et al., 2010). Sequencing and alignment details, including numbers and proportions of uniquely mapped reads, are provided in Table S4. Lists of differentially expressed genes in each comparison are provided in Table S5, including expression data for each gene at each dosage level; Linux command line tools were sufficient to sort cis and trans genes for separate analysis, based on gene locus. The maize W22 reference genome (Springer et al., 2018), including gene locus information, was provided by database MaizeGDB. Statistical analysis, ratio distribution histograms, and volcano plots were produced in R.

DNA sequencing was performed for a trisomy from the second set of plants and a normal diploid (control), using a NextSeq flow cell with single-end 75 bp reads. After mapping to the W22 genome using Bowtie2 (Langmead et al., 2009), gene counts were calculated by customized perl scripts. Then, RPKM-normalized counts were

(FDR < 0.05, $\log_2$(fold change) < 0). Red points on the right represent an increase in gene expression (FDR < 0.05, $\log_2$(fold change) > 0). In cis, the prevalence of direct effects is apparent. In trans, the relationship of dosage to expression is often inverse rather than direct, shown by the relative prevalence of significant down-regulated genes in hyperploid and up-regulated genes in hypoploid.

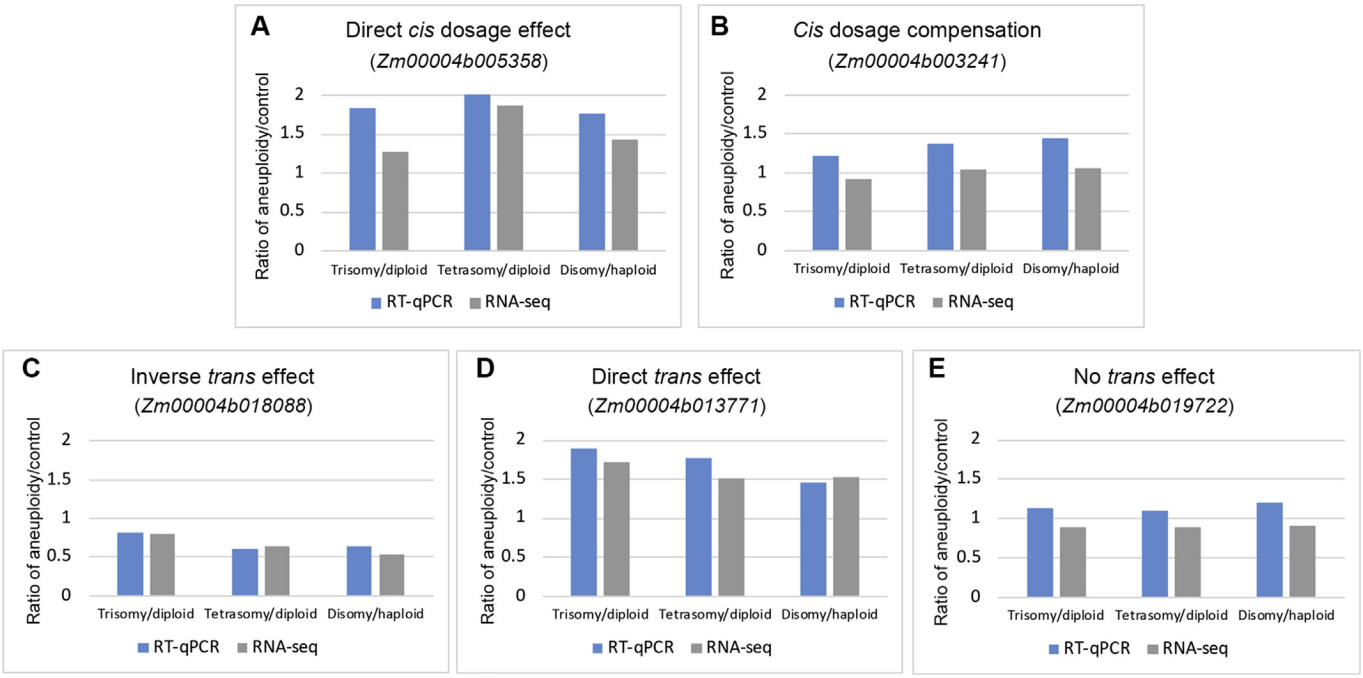

Fig. 3. Comparisons of expression levels of genes representing different dosage reactions by RT-qPCR. Three expression ratios e trisomy/diploid, tetrasomy/diploid, and disomy/haploid e are provided for each representative gene. A: Direct cis dosage effect. B: Cis dosage compensation. C: Inverse trans effect. D: Direct trans effect. E: No trans effect. Expression ratios measured by RT-qPCR generally matched the RNA sequencing results. The progressive impact of aneuploidy on gene expression is apparent in the direct cis dosage effect and inverse trans effect examples.

used to generate gene ratios and to determine directly the dosage of all genes in a 1L trisomy compared with a diploid control; genes in the trisomic portion of chromosome 1 had expression ratios up to 1.50, while all other genes had ratios approaching 1.00. The result suggests a break point at position 164 Mb on chromosome 1, with all genes beyond that position number being part of chromosome arm 1L, defined as cis (between genes Zm00004b002705 and Zm00004b005868). RNA and DNA sequencing data are available in Gene Expression Omnibus (GEO) from NCBI, accession number GSE134936.

Statistical tests were used to determine whether expression of a gene was significantly impacted by 1L aneuploidy (Table 1). Read count data from sequencing experiments were normalized by the RPKM method. Genes with total RPKM less than 3 were excluded from analysis. Then, for each gene, a test for differential expression was performed using edgeR (Robinson et al., 2010) to compare mean gene expression levels of the aneuploid group and the euploid group. False discovery rate (FDR) was computed to adjust multiplicity in hypothesis testing. We chose 0.05 as the cutoff for controlling FDR in testing genome-wide differential expression. In addition, the K-S test was used to determine whether there is a substantial change in expression distribution between two dosages, such as trisomy vs. diploid and tetrasomy vs. diploid (Table S1). Principal Component Analysis (PCA) was used to determine whether biological replicates of the same genotype shared similar expression patterns (Fig. S1). A statistical test based on the PCA was used to identify possible outliers from each genotype group. If any sample was found to be greater than two times the standard deviation from the group mean, it would be considered as an outlier. For all genotypes, values of principal components 1 and 2 (PC1 and PC2) for each biological replicate were within two standard deviations of the group mean indicating that no biological replicate could be considered as an outlier. Differential expression tests, K-S tests, and PCA were performed using R version 3.4.0.

4.3. Generation of gene lists

Gene lists used for the generation of ratio distribution plots were collected from various resources. The list of transcription factors was obtained from PlantTFDB 4.0 (Jin et al., 2017). The lists of chloroplast-, mitochondria-, and peroxisome-targeted genes were generated based on the subcellular localizations predicted or gathered by cropPAL (Hooper et al., 2016). The list of chloroplast-targeted genes contains genes predicted to encode chloroplast-targeted proteins by no less than 5 subcellular localization prediction tools, or those experimentally confirmed to encode chloroplast-located proteins. Mitochondria-targeted genes were defined as genes prediected to encode mitochondria-targeted proteins by no less than 4 prediction tools, or experimentally confirmed to encode mitochondria-targeted proteins. Peroxisome-targeted genes were defined as genes predicted to encode proteins transported to the peroxisome by no less than one prediction tool. All gene model names from the B73 genome were translated to W22 using the Translate Gene Model IDs tool from MaizeGDB (Andorf et al., 2016).

To generate the list of genes related to stress response, signal transduction, and structural components of the ribosome and proteasome, we predicted the function terms of proteins by Gene Ontology (GO) based on protein sequences provided by the maize W22v2 genome annotation (Wang et al., 2013; Springer et al., 2018). In brief, this method integrated profile-sequence alignment, profile-pro file alignment, and domain co-occurrence networks to predict protein function at different levels of complexity (Wang et al., 2013). Genes of interest were selected by their predicted GO terms using customized perl scripts.

4.4. RT-qPCR analysis

Reverse transcription was performed using the RNA-to-cDNA kit (4387406; Applied Biosystems, USA) following the

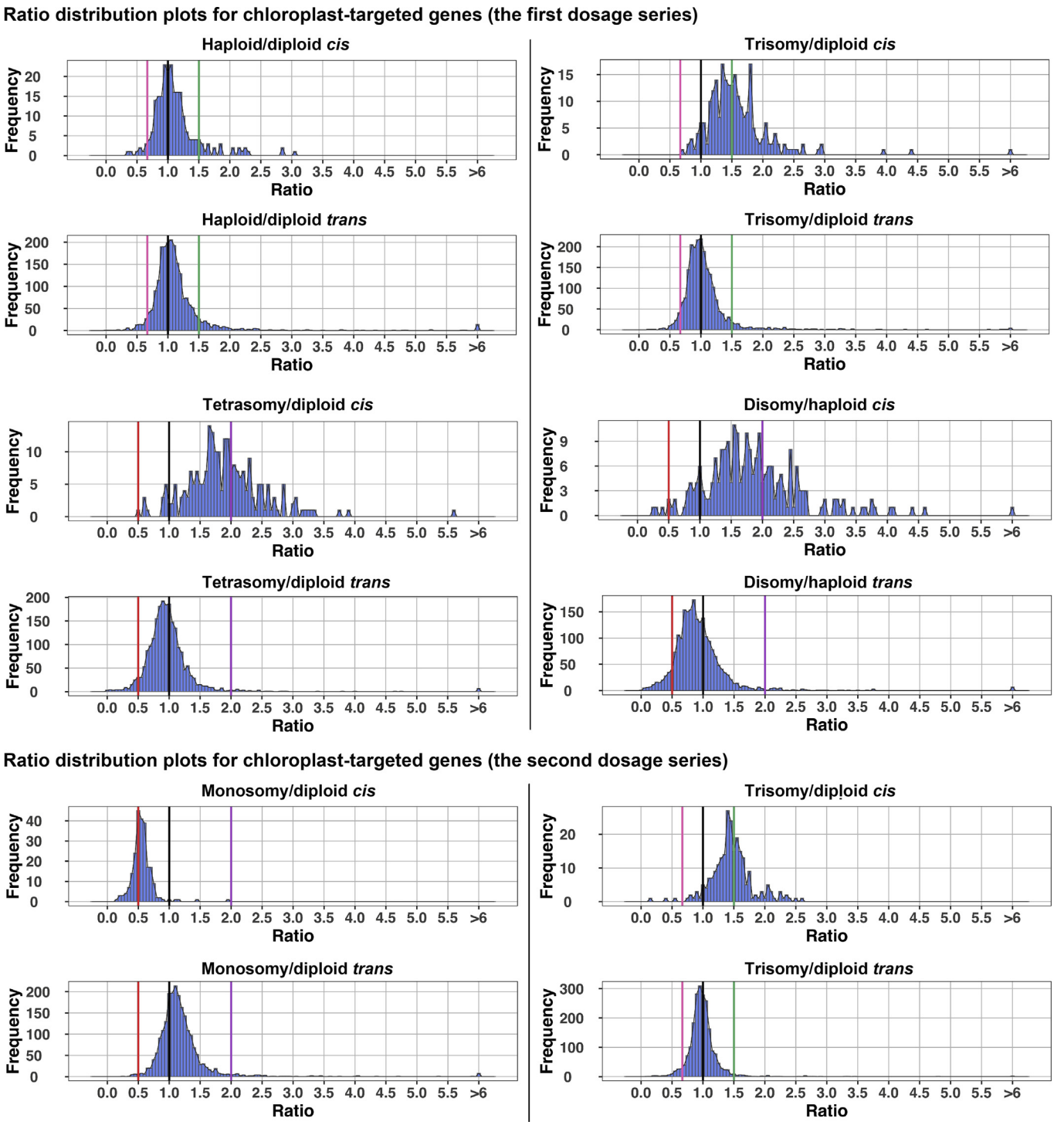

Fig. 4. Ratio distribution plots for chloroplast-targeted genes. Ratio distributions of only chloroplast-targeted genes were prepared. Genotypes, analysis and presentation are as described in Fig. 1.

manufacturer's instructions, using random octamers and oligo dT-16 as primers, 500 ng of total RNA as template, and 1 mL 100! diluted ERCC (External RNA Controls Consortium) RNA Spike-In Mix (4456740; Thermo Fisher Scientific, USA) as an internal control. RT-qPCR was performed using the PowerUp™ SYBR™ Green Master Mix (A25776; Thermo Fisher Scientific) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). PCR conditions were as follows: 50#C for 2 min, 94#C for 2 min, 40 cycles of 94#C for 15 s, 55#C for 15 s, and 72 #C for 1 min, followed by melt curve stage 95 #C for 15 s, and 60 #C for 1 min with ramp temperature 0.3e95 #C. Five different dosage effect categories were identified based on RNA sequencing results, and from each of these, one gene was randomly chosen for RT-qPCR analysis.Zm00004b005358 (direct cis dosage effect) and Zm00004b003241 (cis dosage compensation) are TB-1La cis genes. Zm00004b019722 (no trans effect), Zm00004b018088 (inverse

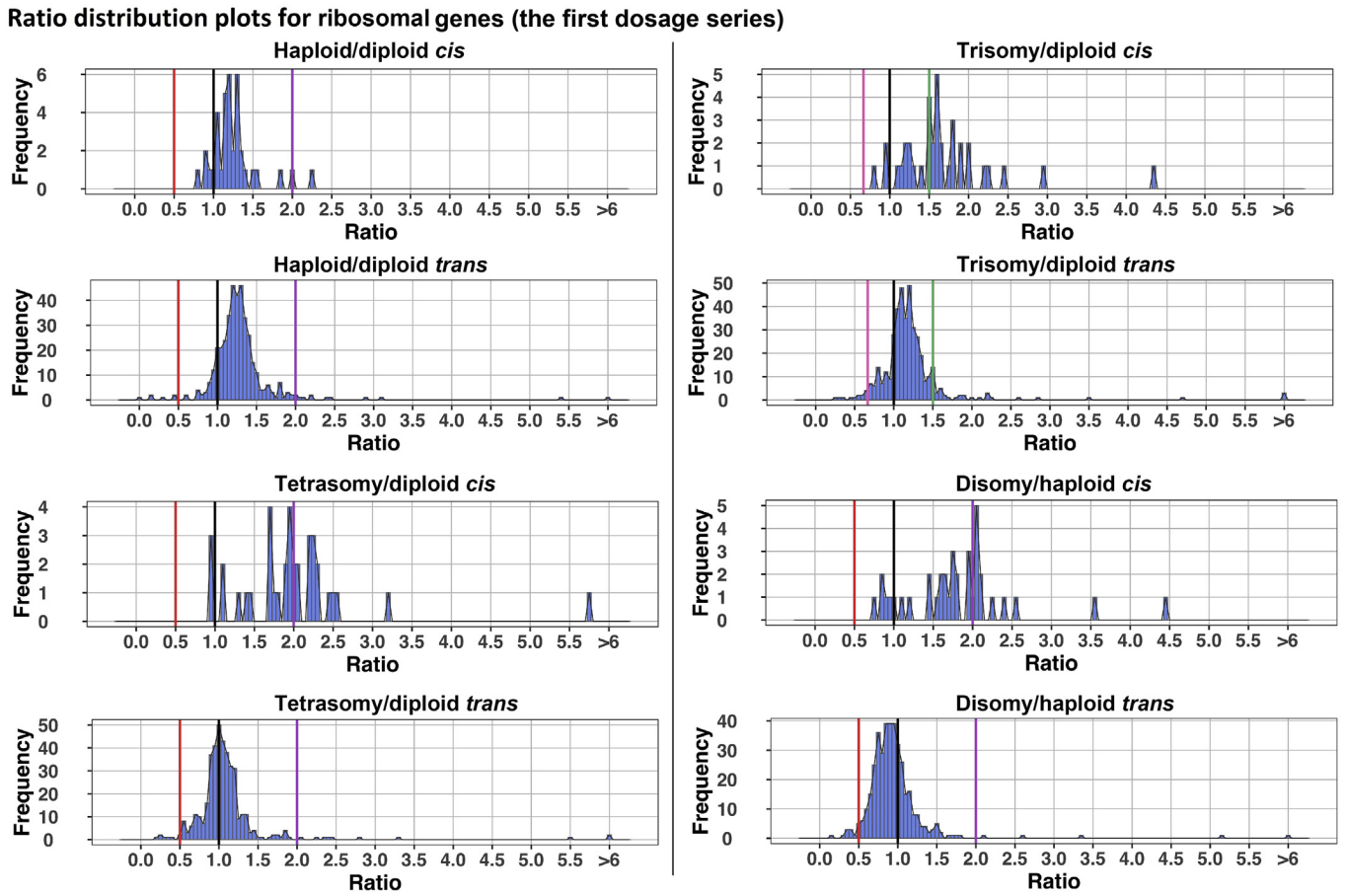

Fig. 5. Ratio distribution plots for ribosomal genes. Ratio distributions of only ribosomal protein encoding genes were prepared. Genotypes, analysis and presentation are as described in Fig. 1.

trans effect), and Zm00004b013771 (direct trans effect) are TB-1La trans genes. ERCC00042 was used as an internal control to calculate relative transcript amount using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Three technical replicates were carried out for each sample.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgg.2020.02.002.

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