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Persistence of sub-genomes in paleopolyploid cotton after 60 million years of evolution

Simon Renny-Byfield1§, Lei Gong1, Joseph P. Gallagher1, Jonathan F. Wendel1\*

1Department of Ecology, Evolution and Organismal Biology, Iowa State University, Ames, IA 50014, USA.

§current address: Department of Plant Sciences, University of California, Davis. One Shields Avenue, Davis, CA, 95616, USA

\*Corresponding author

email: jfw@iastate.edu

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The importance of whole genome multiplication (WGM) in plant evolution has long been recognized. Indeed, in flowering plants WGM is both ubiquitous and in many lineages cyclical, each round followed by substantial and in some cases massive gene loss. This fractionation process may be biased with respect to duplicated donor chromosomes (homoeologs), often with over-expression of genes in less fractionated (LF) relative to more fractionated (MF) regions. This bias is hypothesized to arise through down-regulation of genes via silencing of local transposable elements (TEs). We report that genomic signatures of biased fractionation remain evident in the paleopolyploid genome of modern diploid cotton even after 60 million years of post-polyploid evolution. Biased fractionation is associated with over-expression of genes in LF regions whereas MF regions exhibit elevated GC content, higher TE density and preferential mapping of siRNAs. Contrary to previous observations, we show that gene expression is not influenced by TE proximity, nor siRNA targeting, both of which are therefore unlikely to be primary drivers of biased fractionation. We propose that repeat content *per se* and differential recombination rates may drive biased fractionation following WGM.

Whole genome multiplication (WGM or polyploidy) is ubiquitous and cyclical in flowering plants and is thought to have played important roles in angiosperm diversification and the success of crop plants 1-9. The realization that all flowering plants are paleopolyploid indicates that, over time, a process of diploidization operates to return polyploids to a diploid-like condition 10-13. This transition involves the large-scale loss of duplicate genes 14-19. This process may lead to differential loss of duplicated genes from homoeologous genomic regions, a phenomenon termed biased fractionation 15,20.

Recent analyses in maize 16 and *Brassica*21 indicate that homoeologous regions experiencing greater gene loss tend to have lower levels of gene expression, such that for any given syntenic paralog pair, the gene with the highest expression is likely to reside on the genomic segment that has experienced less gene loss. Similarly, more recent polyploids also experience nonequivalence of gene expression between sub-genomes, e.g., cotton 22,23, coffee 24 and *Tragopogon* 25. These observations have led to the hypothesis that following WGM, differences in gene expression between duplicated regions drive differential gene loss 26. This hypothesis is attractive in that nascent or evolutionarily young polyploids often exhibit biased homoeolog expression 27 suggesting that WGM, especially in allopolyploids 14, may establish the initial conditions that set in motion the mechanistic underpinnings of biased fractionation. This process is suggested to be accompanied by selection, where under-expressed genes contribute less to fitness than do their over-expressed homoeologous counterparts, and therefore are more likely to become dispensable 26.

Additional support for this hypothesis was generated by recent work involving *Brassica*; homoeologous regions experiencing greater gene loss were enriched for mapping of 24 nt siRNAs to transposable elements (TEs) surrounding resident genes 18. As shown in *Arabidopsis*, gene expression may be down-regulated by positional effects associated with local TEs, which are silenced by the 24-nt siRNA machinery 28. These observations of TE proximity, siRNA frequency, and relative gene expression of homoeologs in *Brassica* led to the suggestion 18 that positional-effect, down-regulation by local TEs drives differences in gene expression between duplicate regions, thus providing a potential explanation for biased fractionation.

Here we extend the temporal window for studying biased fractionation and demonstrate that the genomic footprints of the process persist following ancient (~ 60 mya) WGM29 in the modern genome of diploid cotton (*Gossypium* *raimondii* L.). We assess differences in gene expression between duplicated regions and demonstrate that the rate of fractionation is negatively correlated with gene expression. We examine recent hypotheses regarding the source of fractionation bias and show that TE-mediated, positional down-regulation is absent in the modern cotton genome, seemingly excluding this phenomenon as the primary driver of fractionation bias. We present evidence of other genomic features that distinguish the most fractionated and least fractionated components of the genome and suggest how these characteristics might drive differential gene loss. Our observations indicate that the impact of biased fractionation extends well beyond the time-scale over which it was originally identified ( ~10 mya in maize16,30 and 20 mya in *Brassica* 17,21,31).

Results

*Chromosome reconstruction, biased fractionation and gene expression*

Using the SynMap tool of CoGe (https://genomevolution.org/CoGe; last accessed 7/7/14), we identified blocks of genes in synteny between *Gossypium raimondii* (diploid cotton) and its relative *Theobroma cacao* (cacao; SynMap output in File S1). Importantly, comparative genome sequence data indicate that relative to cacao, the lineage that gave rise to modern diploid cotton experienced a 5-6 fold ploidy increase approximately 60 mya29. Accordingly, we identified duplicate regions in the cotton genome resulting from the cotton-specific WGM (Fig. S1 and S2). The relative antiquity of WGM in the cotton genome, however, makes identification of duplicate regions challenging compared to efforts in paleopolyploids where WGM, although still ancient, has occurred much more recently. Indeed, the genome of cotton is substantially re-arranged relative to cacao (Fig. S1 and S2), and in some regions five or six duplicate segments per haploid cacao genome are evident in cotton. It was not possible to re-construct all sets of chromosomes, as has been possible in maize 16 and *Brassica* 17*.* We were, however, able to reconstruct a subset of the chromosomes, in the same manner as in Schnable et al. 16; we restricted further analyses to those genes and genomic regions covered by the whole chromosome reconstructions (highlighted green in Fig. S1).

In total, we were able to reconstruct at least two ancestral cotton chromosomes for six of the ten pre-duplicated cacao chromosomes (Chromosomes 2, 6, 7, 8, 9, 10; Table 1), allowing for comparisons of gene loss between homoeologous regions in the cotton genome (Table 1, Fig. S1). Assuming that each duplicate chromosome has the same number of genes at the time of duplication, we estimated gene loss by assessing the number of genes remaining in each reconstruction, in a manner similar to that used earlier 17. The hypothesis of gene retention equivalence among homoeologs was evaluated using Pearson’s chi-squared test. Importantly, all tests indicate statistically significant deviation from random (equivalent) gene loss, providing strong evidence for biased gene fractionation between duplicate regions in the cotton genome. Thus we are able to detect signatures of chromosome-wide biased fractionation following WGM on time-scales of ~60 mya, far in excess of those previously reported in *Brassica*17,18,21 and maize 16.

*Fractionation and expression of duplicate genes*

We binned chromosome reconstructions such that, for each pre-duplicated ancestral chromosome, the least fractionated of the post-duplicated (cotton) reconstructions was distinguished LF and all others were grouped together and designated as most fractionated (MF). These classifications were used to group retained paralogous as belonging to LF or MF fractions of the genome. We then examined gene expression levels in three tissues and compared expression of syntenic paralogs residing in LF and MF fractions. In this case we took the most conservative approach and discarded any genes that lacked a suitable paralog for comparison, or genes that did not have a corresponding syntenic ortholog in the chocolate genome. Despite such a restrictive set of comparisons, this analysis revealed that genes on LF chromosomes are generally more highly expressed than those on MF (Fig. 1 A); of the 1064 paralogous gene pairs compared, LF genes were more highly expressed in 559-589 cases, depending on the tissue. To test for statistically significant deviation from a random number of up-regulated genes in each group (LF of MF) we used a cumulative binomial distribution with the probability of LF being more highly expressed at 0.5. This result was statistically significant for expression data for both leaf and seed tissue, but not for petals (Fig. 1A). To examine the robustness of the bias we restricted our analysis to those syntenic paralogs that varied in expression by greater than two-fold (Fig. S3). In this case, the bias was greater and statistically significant in all tissues examined. Furthermore, bias remained evident even when considering only syntenic paralogs exhibiting differential gene expression and fold-change greater than two, confirming previous results (Fig. S3). Thus, the data indicate that genes on LF chromosomes are typically expressed at higher levels than their MF counterparts.

*Local transposable element density*

Using transposable element (TE) annotations from the cotton reference genome 29, we identified TEs near each gene and used sliding windows to calculate the average TE density in regions surrounding genes (Fig. 2). Unsurprisingly, TE density is low close to transcription start/stop sites and increases further from genes. Importantly, a comparison of the local TE density for genes on LF and MF chromosomes revealed that TE density is higher in MF chromosomes (Wilcoxon’s paired sum rank test, V=502503, p=<0.0001; Fig. 2). In addition, genes residing on MF chromosomes generally have more internal TE insertions (Fig. S4) than do genes on LF fragments. Using the same sliding windows, we observed that GC content in both LF and MF fractions steadily decreases toward transcription start/stop sites and gradually increases following transcription start/stop sites (Fig. S5) and the GC content of LF chromosomes is significantly higher than that of MF 5,000 bp either side of genes models (Wilcoxon’s paired sum rank test, V =0, p = <0.0001;Fig. 3).

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*Enrichment of mapped siRNAs on MF chromosomes*

The higher density of TEs surrounding MF genes prompted us to ask whether those same regions upstream and downstream of genes on MF chromosomes are enriched for TE-derived, 24-nt siRNAs. We mapped 24 nt siRNAs from leaves of *G. raimondii* to the cotton reference genome, averaging the number of uniquely mapped reads along sliding windows near genes of interest. Surrounding regions of MF genes have higher proportions of TE-derived siRNAs (Wilcoxon’s paired sum rank test, V =327477, p = <0.0001;Fig. 3). In addition to enrichment of siRNAs, the MF fraction of the genome also exhibits a sharp increase in siRNAs mapping ~200 bp upstream and ~800 bp downstream of the transcription start/stop site, a pattern absent in the LF fraction of the genome (Fig. 3).

*TE proximity and gene expression*

All 37,000 cotton gene models were binned according the distance to the nearest TE, taking into account both up and downstream insertions. Using RNA seq data from petal, leaf and seed of diploid cotton 8, we examined the relationship between TE proximity and gene expression. Importantly, expression was relatively uniform across all bins and across all tissues (Fig. 4 A). Indeed, correlation analysis revealed a statistically significant negative correlation between expression and TE proximity (Pearson’s correlation coefficient = -0.03, df = 111667, t = -10.166, p = <0.001). Although statistically significant, the effect is minimal and in the opposite direction of expectation, indicating that TE proximity is likely to have little impact on levels of gene expression. Furthermore, linear modeling revealed an R-squared value of <0.001, indicating that TE proximity explains only a very small fraction of variation in gene expression. We broadened our analysis to consider local TE density, rather than the nearest TE insertion (Fig. S6). Interestingly, in this case, local TE density seems to be more strongly negatively correlated with expression of nearby genes (Pearson0.125, df = 116637, t = -42.003, p = < 0.001); nevertheless, and similarly to TE proximity, only a very weak correlation was observed.

We separately binned genes into two pools based on the presence or absence of local siRNA-inducing TEs and compared the impact of TE proximity on gene expression in the two groups (Fig. 4 B). Analyses using linear modeling and ANOVA indicate that presence of siRNAs has little impact on the expression of nearby genes (Table S1).

Discussion

The extensive occurrence of episodically recurrent WGM in the history of flowering plants has only recently become evident 1-9. This legacy of genome multiplicity is exemplified by the genome of modern cotton, which in addition to experiencing more ancient WGM events, underwent a 5-6 fold ploidy increase near the start of the Tertiary, well after its divergence from cacao 29. A universal attribute of WGM is the loss of duplicated genes 15, with many returning to single copy status via deletional processes, be they biased or unbiased with respect to homoeologous chromosomes. Examples of the former are phylogenetically widespread among angiosperms, as documented in recent analyses of maize, *Brassica*, *Arabidopsis* and poplar 14,16,17,19,32,33.

By reconstructing ancestral chromosomes of cotton, often in multiple copies, we demonstrate that this fractionation process has acted differentially on what initially were homoeologous chromosomes. Furthermore, this bias has occurred at the whole-chromosome level, and is evident in every chromosomal comparison we performed (p <0.0001; Table 1). Despite the antiquity of the genome multiplication in the cotton lineage, and the extensive genome rearrangement that has occurred between the genomes of modern cotton and cacao, we show here that fractionation in the cotton lineage remains evident after ~ 60 million years of evolution (Fig. S1 and Fig. S2; Table 1). This result extends our understanding of the scale, scope, and temporal depth of the diploidization processes and reveals the signatures of biased fractionation are evident far beyond the time-frame reported for other genomes; around 20 mya in *Brassica*17, 10 mya post WGM in maize16 and a few million years in *Arabidopsis* 20. Considering that most paleopolyploid genomes appear chromosomally and functionally diploid it is noteworthy that such signatures, as well as differentiation of genomic content, remain evident after ~60 mya. By extension, we deduce that the process of biased fractionation has a long-lasting legacy, raising a myriad questions regarding the functional and adaptive implications of biased fractionation over vast evolutionary timescales.

One of the primary correlates of biased fractionation in plants is differential expression of the duplicated genes that are retained on homoeologous segments. In maize and *Brassica,* biased fractionation is associated with over-expression of genes on the homoeologs experiencing less gene loss 16-18. We report similar results here, i.e., that genes on LF chromosomes generally are more highly expressed than their counterparts on MF chromosomes (Fig. 1 and Fig. S3), demonstrating the same correlation between fractionation and gene expression in the cotton lineage as in other recent examples from plants. Remarkably, this quantitative difference in homoeolog expression levels remains detectable despite many tens of millions of years of evolution since WGM (Fig. 1).

The correlation between biased fractionation and gene expression has led to a novel hypothesis regarding causation, i.e., that selection has favored retention of the homoeolog with higher expression 16. Under this hypothesis, conditions established at the time of genome merger (hybridization) and doubling (polyploidization), or those derived following autopolyploidization, generate widespread differences among homoeolog expression levels. These differences are commonly observed in cotton diploid hybrids and neo-allopolyploids 23,34-36, showing that initial conditions may be sufficient to set in motion subsequent selective forces operating on gene expression. Moreover, in modern allopolyploid cotton, which traces to a hybridization event 1-2 mya 37, gene expression levels among linked genes on homoeologous segments may be correlated 38. Collectively, these data indicate that homoeolog expression level differences may be established on temporal scales ranging from immediate, accompanying genome merger, to evolutionary timescales encompassing millions of years.

The question arises as to the proximate driver of expression level differences between MF and LF regions. An important insight in this respect stems from the work of Freeling and colleagues 18, who noted that in *Brassica*, regions experiencing greater gene loss were enriched for mapping of 24 nt siRNAs to TEs near genes. This observation, in conjunction with the earlier demonstration 28 that gene expression may be down-regulated by positional effects associated with TEs, led to the suggestion that positional-effect down-regulation drives differences in gene expression between duplicate regions, thus providing a potential explanation for biased fractionation.

Here we also show that 24 nt siRNAs are enriched in MF compared to LF regions (Fig. 3), with patterns similar to those recently described in *Brassica* 18. Thus, one might expect that genes residing near TEs are generally expressed at lower levels, particularly when close to TEs subject to siRNA silencing. Our data, however, show only a weak relationship between TE proximity and gene expression in cotton (Fig. 4 A). Furthermore, there is a slight negative correlation between gene expression and TE proximity, a trend that is opposite to expectations. Furthermore, the presence of local TEs that are silenced via siRNA pathways has no apparent impact on local gene expression levels (Fig. 4 B). Yet, the correlation remains between homoeolog expression levels and fractionation bias (Fig. 1 and Table 1, respectively). Thus, siRNA-mediated, positional down-regulation may not be the primary driver of biased gene loss, at least in cotton.

The foregoing observations raise the question as to whether homoeolog expression level differences are a consequence, rather than a driver, of biased fractionation. Others have previously predicted that differential TE load between sub-genomes might be a determinant of LF and MF; that is, the sub-genome with the lowest TE density would be less fractionated and over expressed 18. Our data generally support this notion, in that several genomic characteristics differentiate LF and MF genome fractions: local TE density is higher in MF than LF chromosomes (Fig. 2), whereas GC content is lower (Fig. S5). Given that ancient TEs are difficult to identify, we cannot exclude the possibility that differences in apparent TE content among homoeologous regions may be due to invasion of TE sequences post paleopolyploidy and are not reflective of conditions at the time of WGM. With this caveat in mind, our observations, coupled with data showing that TE proximity appears to be de-coupled with expression levels (Fig. 4), suggest that some other genomic feature associated with increased TE density content between sub-genomes might drive differentiation in rates of gene loss following WGM.

In the present context, irrespective of the deletional mechanism, we forward the hypothesis that elevated TE density may have triggered a greater fixation of gene losses in MF chromosomes via indirect effects on recombination rate. Such a hypothesis may be testable given appropriate genetic maps, but currently suitable data are not available. Nevertheless, recombination rate has been shown to be negatively correlated with TE prevalence 39,40. Similarly, GC content can be positively correlated with recombination rate 41. Therefore, higher TE density and lower GC content in MF suggest a history of reduced recombination relative to LF. This is important as the impact of selection is weaker in regions of low recombination 42, leading to the possibility that mildly deleterious gene deletions in LF fragments, where selection can operate strongly, are less likely to be fixed compared to similar deletions in MF fractions, where selection is weaker. Moreover, at the time of WGM, effective population size is likely to be very small, in which case, in the absence of effective selection on MF fragments, deletions could be fixed via drift. Although speculative, it seems to us possible that TE load (or perhaps some other determinant of recombination rate) may be a proximate evolutionary force responsible for the genesis of LF and MF genomic compartments.

Methods

*Biased fractionation*

We examined gene fractionation following the cotton-specific WGM using the reference genome sequences for *Gossypium raimondii* 29 and its close relative, *Theobroma cacao* L.43. The SynMap function of the online tool CoGe (https://genomevolution.org/CoGe) was used to identify blocks of syntenic orthologs using BlastN, relative gene order and the following parameters: -D 50, -A 10, the Quota Align function to merge syntenic blocks, -Dm 80 and a ratio of syntenic depth of 6:1 (*Gossypium raimondii*: *Theobroma cacao*).

We further reconstructed ancestral chromosomes (Fig. 1), according to the logic of Schnable et al. 16. Briefly, re-arrangements on the same chromosome are presumed to be more frequent than exchanges between different chromosomes. Thus, segments of the cotton genome that reside on the same chromosome and are orthologous to the same chromosome of the cacao genome are assumed to originate from an ancestral chromosome in the common ancestor of the two species. Furthermore, under the assumption that gene loss and chromosomal re-arrangements are more likely after than before a WGM event 44, we took gene content and gene order in the outgroup *Theobroma cacao* to be representative of the ancestral model of thepre-duplicatedcotton genome, as has been done previously 16. We selected those reconstructions for which we had the greatest confidence of a full-length reconstruction, provided there was a homoeologous reconstruction of comparable quality with which to compare. We subsequently limited our analyses of fractionation to these selected regions (highlighted green in Supplementary Fig. 1). Assuming that the number of genes on duplicated chromosomes is initially equal following duplication, we assessed differences in the numbers of remaining genes and used a Pearson’s chi-squared test to investigate biased fractionation between re-constructed cotton chromosomes as in 17. We subsequently binned chromosome reconstructions such that, for each ancestral chromosome, the least fractionated homoeolog was designated as LF and all other reconstructions were designated as most fractionated (MF).

*Gene Expression in LF and MF*

We determined the overlap in gene content between reconstructions and compared the relative expression of syntenic paralogs on LF and MF fractions. In detail we restricted our expression analysis to those genes with paralogs on both MF and LF fractions. We further restricted those genes under consideration by using only syntenic paralog pairs that had a corresponding syntenic ortholog in the unduplicated (relative to cotton) chocolate genome. We used gene expression data from petal, leaf and seed, previously published in 8. Briefly, RNA-seq reads were filtered for quality using the program sickle (https://github.com/najoshi/sickle; last accessed 7/7/14), and mapped to the cotton reference genome 29 using GSNAP (http://research-pub.gene.com/gmap/; last accessed 7/7/14). Mapped reads were sorted and indexed using SAMtools (http://samtools.sourceforge.net; last accessed 7/7/14) and coverage of each gene annotation was calculated using custom perl scripts and normalized using reads per kilobase per million (RPKM). We compared paralogs on LF and MF reconstructions using methods similar to Schnable et al. 16 and Woodhouse et al. 18, where for each comparable paralog pair the gene with the highest expression was declared the winner. In cases where there were three reconstructions (Table 1) we compared expression of LF genes with their syntenic paralogs on either, or both of the MF homoeologs, depending on whether a syntenic paralog was present on each MF reconstruction. We tested for differential gene expression of syntenic paralogs using a student’s t-test, correcting p values for a false discovery rate of 0.01 using the method of 45. Using a cumulative binomial distribution function we assessed the chances of observing the number of wins in LF, given a random chance of LF being more highly expressed for each gene (i.e. the probability of LF being more highly expressed for a given comparison is 0.5).

*Mapping of siRNAs*

Small RNA libraries, previously published in 46, and produced from seedling leaves of *Gossypium raimondii* analysed (deposited in NCBI SRA database SRP017133). From the dataset we extracted 24-nt siRNAs and mapped these to the cotton reference genome using bowtie 0.12.7 47. In order to determine the precise TE from which a given siRNA derived we restricted mapping to those reads to those with perfect and unique alignments. All 24-nt siRNAs were mapped to TEs with all non-TE derived nucleotides masked. For each gene, sliding windows of 100 bp in size (moving by increments of ten) where used to characterize the siRNA distribution 5000 bp up- and downstream of the transcription start/stop sites. Coverage of siRNAs was characterized by counting the number of mapped reads inside each window.

*Local GC content and local TE density*

For each genewe assessedthe local GC content 5000 bp either side of the transcription start/stop site in sliding windows of 100 bp, moving by increments of ten, using *BEDtools* (http://bedtools.readthedocs.org/en/latest/; last accessed 7/7/14). We then grouped genes according to their status with respect to MF and LF and separately plotted average GC content over each window. We examined if mean GC content in LF and MF groups was different using a Wilcoxon’s sum rank test. Similarly, for each protein gene, the TE density (the proportion of TE derived bp) within each sliding window was estimated using *BEDtools*. We examined differences in TE density between LF and MF groups using Wilcoxon’s sum rank test.

*TE density, proximity and gene expression*

Repetitive DNA annotations from 29 were used to identify TEs. Using the ‘closest’ functionality of *BEDtools* we ascertained the nearest TE to each gene, allowing for TEs inside gene models. Using the same expression data as above, each of the global set of ~37,200 gene annotations was assessed for expression level using three biological replicates. Genes were subsequently binned according to distance to nearest TE and expression was compared between bins using boxplots. The statistical relationship between TE proximity and the log of expression was examined using Pearson’s correlation coefficient and linear modeling. All statistical analyses were performed in the statistical package R.

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Figure Legends

Figure 1. Gene expression in LF and MF fractions of the genome. Syntenic paralogs were binned according to residency on least fractionated (LF) or most fractionated (MF) chromosome reconstructions, and compared for gene expression levels in three tissues, petal, leaf and seed. Three biological replicates were used in each tissue comparison. (A) The number of genes more highly expressed in each category is given; significance deviation from an equal number of genes being more highly expressed in each category was examined using a cumulative binomial distribution with the odds of an LF gene being more highly expressed at 0.5. (B) Density plots comparing gene expression values for syntenic paralogs on LF and MF fragments of the genome. The white line in each plot indicates a 1:1 relationship and equal expression of syntenic paralogs.

Figure 2. Transposable element (TE) density flanking genes in LF and MF fractions of the genome. Annotations from the cotton reference genome were used to identify TEs, and the proportion of base pairs annotated as TE-derived was calculated over sliding windows of 5000 bp (10 bp increments), either side of transcription start/stop sites. Mean density for LF and MF genes are displayed separately. The dashed line indicates the start/stop site and the intervening genic region is excluded from the plot.

Figure 3. GC content in LF and MF fractions of the genome. Shown are the proportions of GC nucleotides in regions surrounding LF and MF genes 5000 bp either side of gene transcription start/stop sites. The vertical dashed line indicates the start/stop site and the intervening genic region is excluded from the plot.

Figure 4. Enrichment of 24-nt siRNAs mapped to TEs that flank MF genes. All genes in LF and MF categories were assessed for uniquely mapped siRNAs, allowing no mismatches, 1,250 bp either side of transcription start/stop sites. I n order to limit mapping of TE-derived siRNA non-TE derived genomic sequences were masked. The vertical dashed line indicates the start/stop site and the intervening genic region is excluded from the plot.

Figure 5. TE proximity, siRNAs and gene expression. (A) Boxplot of a transcriptomic analysis of 37,200 cotton genes examined for expression level and binned according the proximity to the nearest TE. Gene expression levels are given for each of three tissues. Boxes indicate 95% confidence intervals for median gene expression (solid black lines), mean expression is indicated by solid diamonds, and outliers are indicated with solid circles. (B) Expression of genes grouped into those with local siRNA producing TEs (siRNA+) and genes lacking local siRNA-inducing TEs (siRNA-). Linear modeling and ANOVA revealed that the presence or absence of siRNAs mapping to nearby TEs had no statistically significant effect on gene expression (Table S1).

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