Unraveling *cis* and *trans* regulatory evolution during cotton domestication

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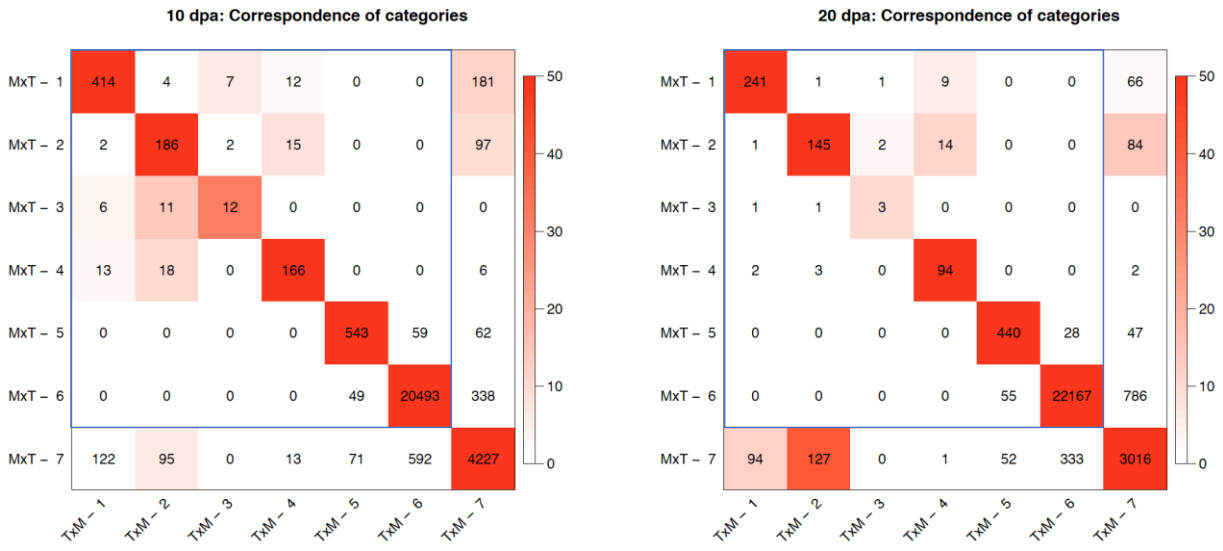
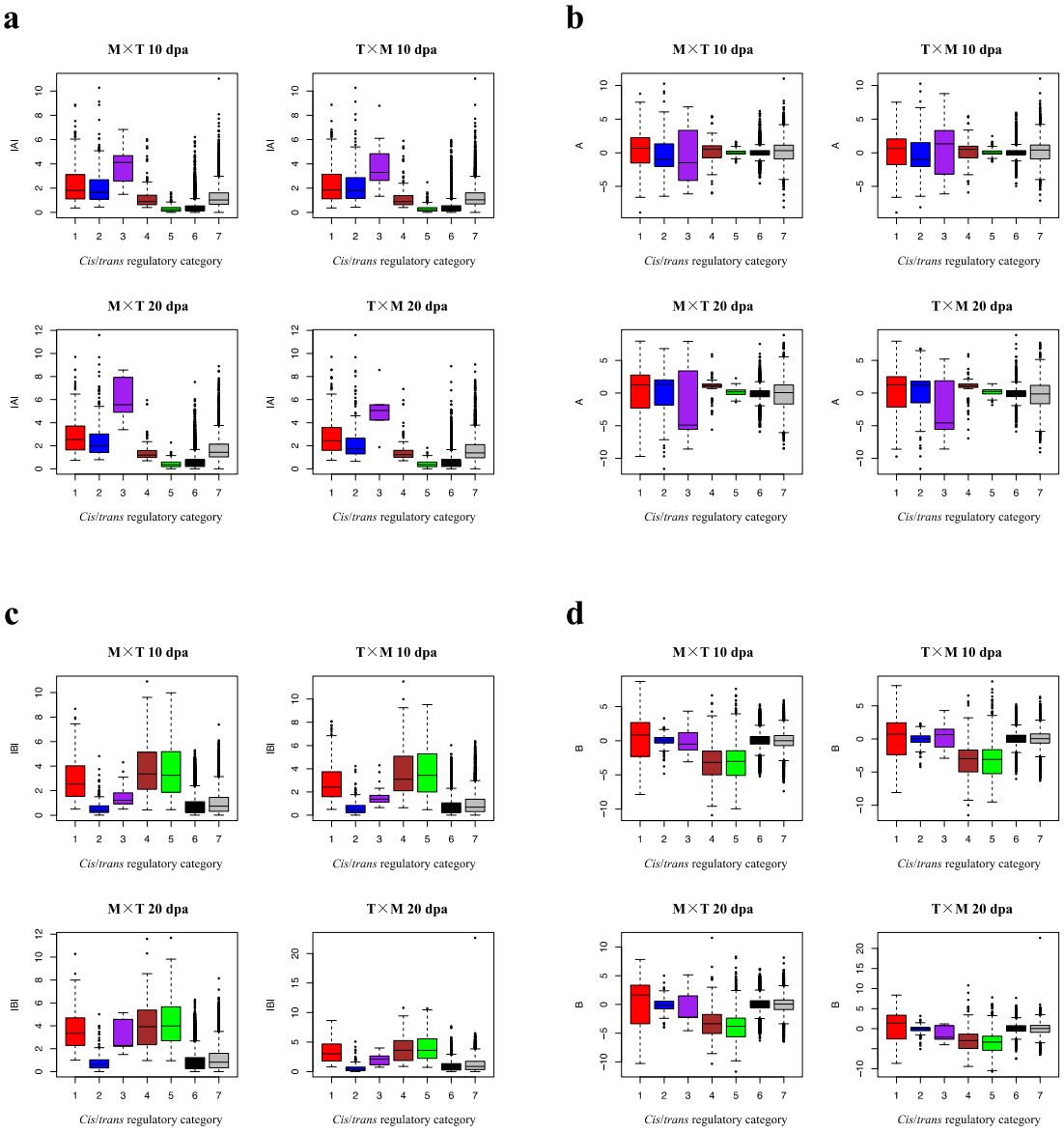
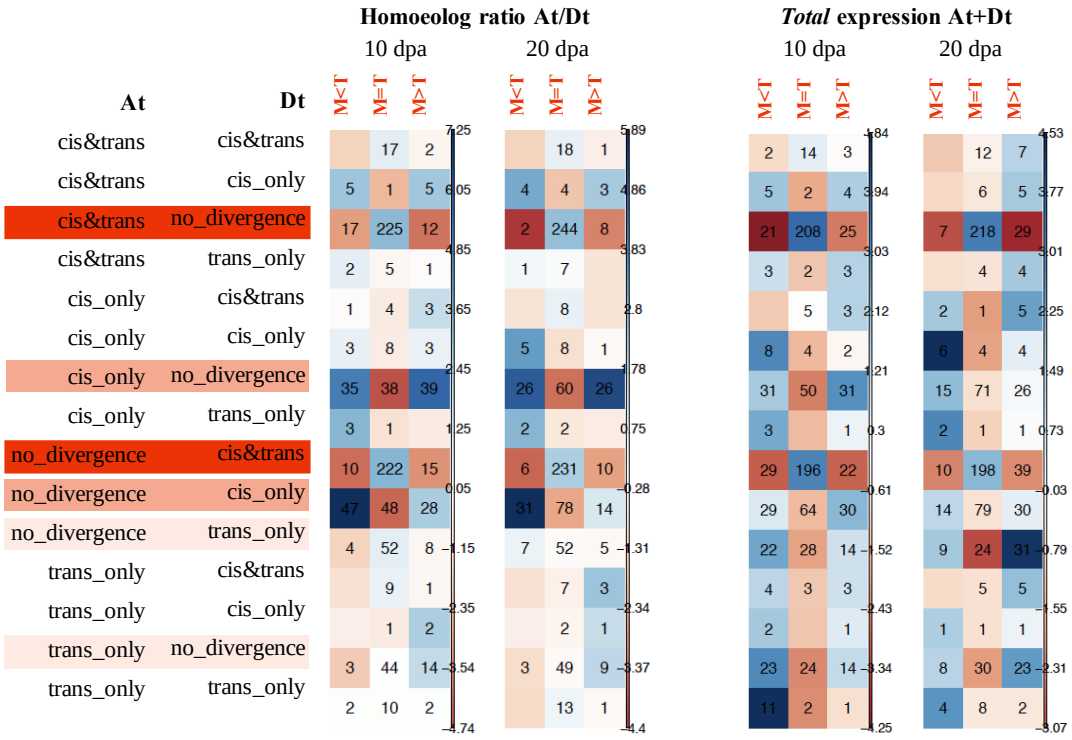


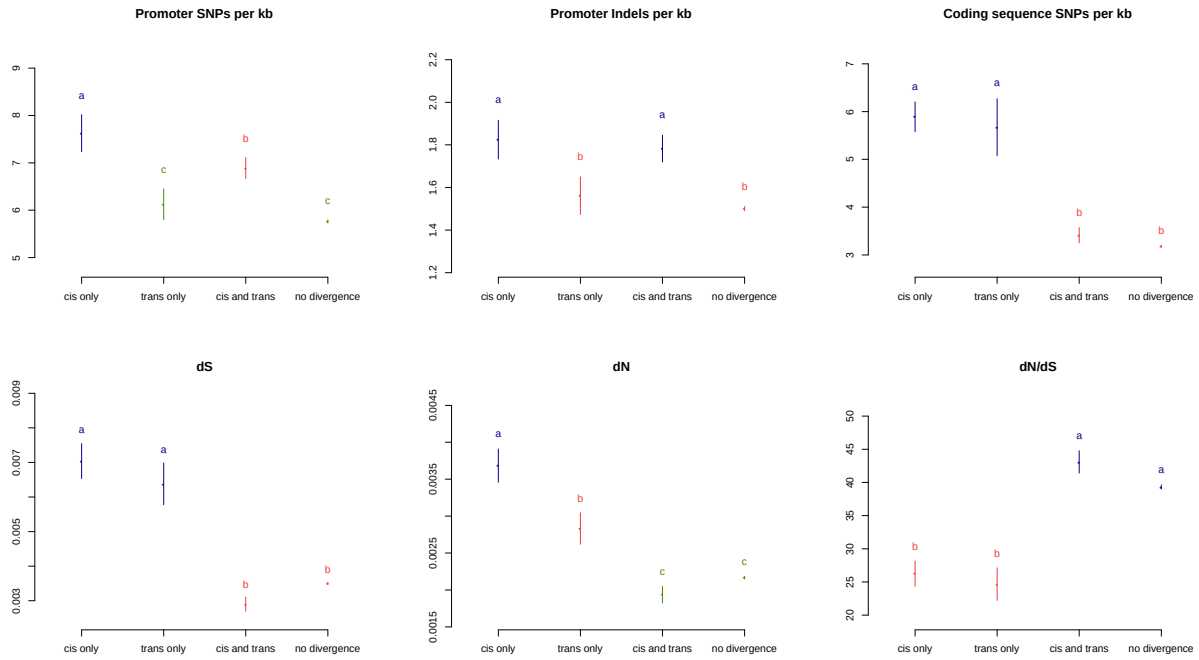
Supplementary Figure 1. Correspondence of *cis* and *trans* regulatory categorization between M×T and T×M. Cross-tabulation of gene numbers is shown at 10 and 20 dpa. Pearson's Chi-square test of independence revealed significant association between M×T and T×M for both contingency tables ($P < 0.05$). Based on Fisher's exact test, statistical significance of enrichment is shown by the color of each cell. Darkest red cells indicate the most significantly enriched overlap between their corresponding row (M×T) and column (T×M) categories. Inside the boxes excluding ambiguous genes, over 99% of genes are located in the diagonal cells.



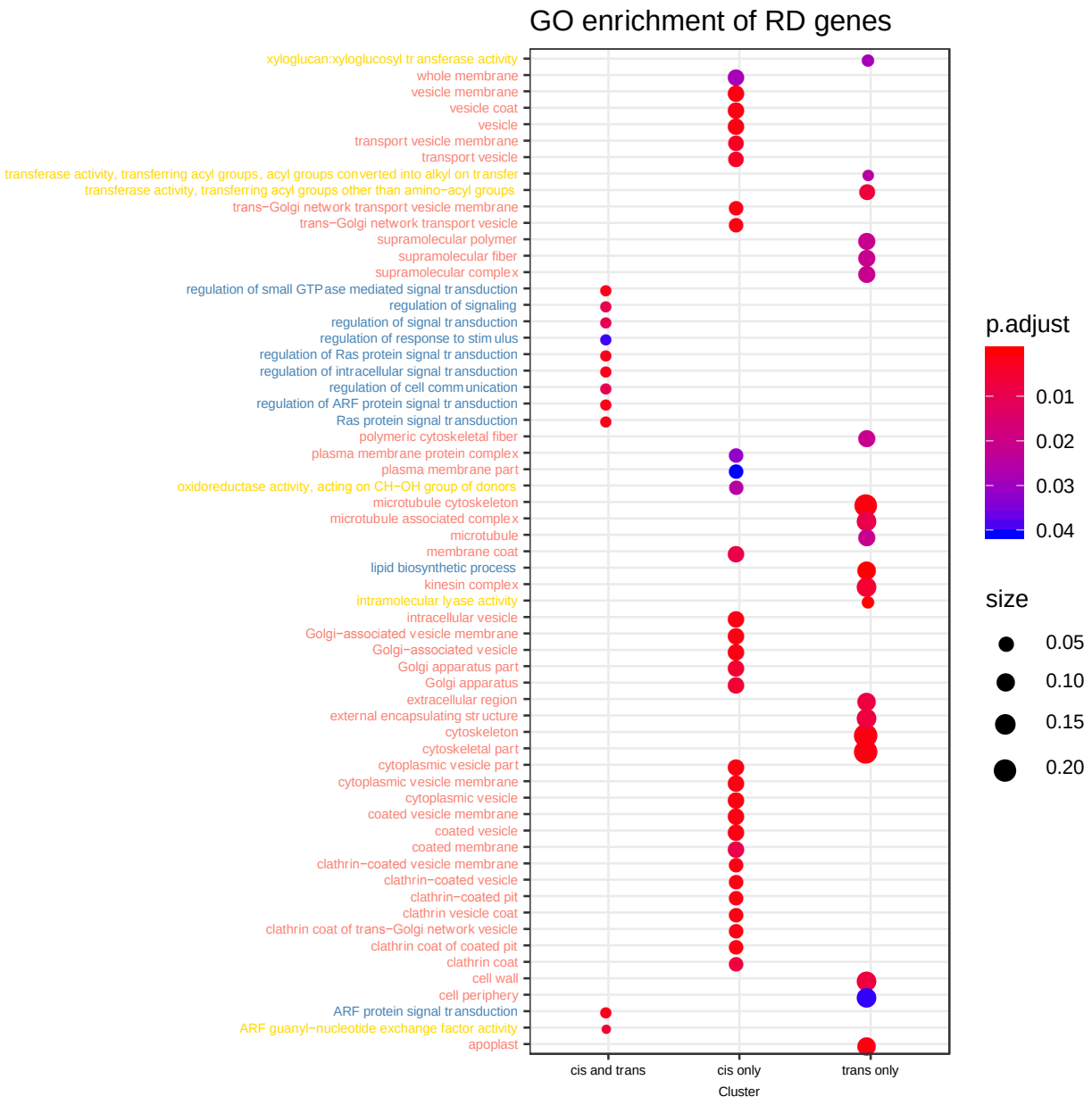
Supplementary Figure 2. Boxplots of seven *cis* and *trans* regulatory categories corresponding to parental expression divergence and *cis* regulatory divergence. As in Figure 2c-f, the magnitude and direction of parental expression divergence (**a** - |***A***| and **b** - ***A***) and *cis* regulatory divergence (**c** - |***B***| and **d** - ***B***) were plotted for four F_1 hybrid samples within each panel. M×T, Maxxa × TX2094; T×M, TX2094 × Maxxa. Boxplot elements: center line - median; box limits - upper (Q3) and lower (Q1) quartiles; whiskers - smallest and largest non-outlier; points - outliers. Source data are provided as a Source Data file.



Supplementary Figure 3. Homoeolog expression patterns altered by At and Dt regulatory variation. On the left, 15 combinatorial patterns of At and Dt regulatory variations are listed, with the most overrepresented patterns as colored in Figure 4c. Corresponding to each combinatorial pattern, expression changes between Maxxa and TX2094 in At/Dt ratio and At+Dt *total* expression are tabulated at 10 and 20 dpa. The color of each cell shows the magnitude of gene enrichment (blue) and depletion (red) based on residuals of Pearson's Chi-square test of independence (blue indicates a positive residual, i.e., more genes observed than expected, and red indicates fewer genes than expected).



Supplementary Figure 4. Relationships between sequence divergence and regulatory divergence. Measures of DNA sequence divergence, including promoter SNPs and indels, coding region SNPs, synonymous rate (*dS*), nonsynonymous rate (*dN*) and *dN*/*dS*, were contrasted between genes of different regulatory patterns using Duncan's multiple range test, with error bars depicting standard errors. The different letters (e.g., a, b, c) denote significant differences ($P < 0.05$). Source data are provided as a Source Data file.



Supplementary Figure 5. GO enrichment of regulatory divergent genes. Enriched GO terms are compared for three different categories of regulatory divergent (RD) genes on the x-axis, and the corresponding ontology of molecular function (MF - gold), biological process (BP – steel blue), and cellular component (CC - salmon) are colored on the y-axis. Dot size and dot color represent gene ratio and adjusted p-value, respectively.

Supplementary Table 1. Summary of fiber RNA-seq samples included in this study.

SRA	accession	dpa	rep	Total reads number	Filtered/mapped reads number	%	note
Datasets generated in this work							
SRR8797010	M×T	10	1	10,756,588	6,570,501	61.08%	
SRR8797009	M×T	20	1	8,985,380	6,105,101	67.94%	
SRR8797008	M×T	10	2	10,110,567	6,009,681	59.44%	
SRR8797007	M×T	20	2	11,494,379	6,918,189	60.19%	
SRR8797006	M×T	10	3	10,740,520	6,061,867	56.44%	
SRR8797005	M×T	20	3	10,778,185	6,747,724	62.61%	
SRR8797004	T×M	10	1	10,026,565	5,977,375	59.62%	
SRR8797003	T×M	20	1	9,685,175	5,926,936	61.20%	
SRR8797012	T×M	10	2	10,000,290	5,774,898	57.75%	
SRR8797011	T×M	20	2	10,178,768	6,186,586	60.78%	
SRR8797028	T×M	10	3	10,815,961	6,520,713	60.29%	
SRR8797027	T×M	20	3	10,780,275	6,519,842	60.48%	
SRR8797030	Maxxa	10	1	9,839,562	5,491,071	55.81%	
SRR8797029	Maxxa	20	1	9,986,304	5,731,427	57.39%	
SRR8797024	Maxxa	10	2	11,374,202	5,972,051	52.51%	
SRR8797023	Maxxa	20	2	10,779,389	5,977,830	55.46%	&
SRR8797026	Maxxa	10	3	10,593,243	5,845,661	55.18%	
SRR8797025	Maxxa	20	3	10,045,980	6,351,115	63.22%	
SRR8797022	Maxxa	10	4	10,773,224	6,202,329	57.57%	
SRR8797021	Maxxa	20	4	9,646,587	5,389,200	55.87%	
SRR8797013	TX2094	10	1	11,309,911	6,303,933	55.74%	
SRR8797014	TX2094	20	1	10,770,070	4,334,720	40.25%	*
SRR8797015	TX2094	10	2	9,971,759	6,100,029	61.17%	
SRR8797016	TX2094	20	2	11,483,824	3,929,115	34.21%	*&
SRR8797017	TX2094	10	3	11,206,757	5,701,339	50.87%	
SRR8797018	TX2094	20	3	10,740,479	4,136,004	38.51%	*
SRR8797019	TX2094	20	4	27,700,290	14,260,153	51.48%	
Downloaded datasets							
SRR8797020	TX2094	10	JJ	21,052,971	12,383,164	58.82%	
SRR203242	Maxxa	10	BYU	13,555,520	10,240,026	75.54%	
SRR203244	TX2094	10	BYU	12,954,154	8,960,954	69.17%	
SRR203247	Maxxa	20	BYU	11,862,948	7,340,486	61.88%	
SRR611435	TX2094	20	MJY	23,925,569	10,409,586	43.51%	
SRR611436	Maxxa	10	MJY	20,872,445	10,005,262	47.94%	
SRR611443	Maxxa	20	MJY	19,740,447	5,714,567	28.95%	
SRR611445	TX2094	10	MJY	16,282,320	8,718,768	53.55%	
SRR8837952	Maxxa	5	SRB	44,718,091	32,057,851	71.69%	
SRR8837953	Maxxa	10	SRB	29,367,962	19,629,588	66.84%	
SRR8837954	Maxxa	15	SRB	31,686,165	14,551,393	45.92%	
SRR8837955	Maxxa	20	SRB	2,769,552	1,622,905	58.60%	
SRR8837969	TX2094	5	SRB	27,776,650	15,530,616	55.91%	
SRR8837971	TX2094	15	SRB	26,232,665	18,863,550	71.91%	
SRR8837970	TX2094	20	SRB	38162310	4,768,072	12.49%	*

* Lower mapping rates due to higher sequence duplication levels, indicating some kind of enrichment bias in generating RNA-seq libraries for TX2094 20dpa samples.
& Included for SNP detection but excluded from other analyses (including DE, cis/trans and network analysis) due to incorrect labeling, as both 20 dpa samples were clustered with corresponding 10 dpa samples.

Supplementary Table 2. Summary of F_1 allelic read assignment.

	Maxxa		TX2094		Unassigned		
Sample	number	%	number	%	number	%	Total
M×T.10dpa.1	965,758	7.3%	1,011,273	7.7%	11,163,977	85.0%	13,141,008
M×T.10dpa.2	885,338	7.4%	927,945	7.7%	10,206,087	84.9%	12,019,370
M×T.10dpa.3	901,602	7.4%	931,438	7.7%	10,290,728	84.9%	12,123,768
T×M.10dpa.1	871,128	7.3%	904,285	7.6%	10,179,339	85.1%	11,954,752
T×M.10dpa.2	851,544	7.4%	878,906	7.6%	9,819,356	85.0%	11,549,806
T×M.10dpa.3	959,844	7.4%	998,359	7.7%	11,083,233	85.0%	13,041,436
M×T.20dpa.1	983,266	8.1%	966,027	7.9%	10,260,917	84.0%	12,210,210
M×T.20dpa.2	1,037,788	7.5%	1,069,653	7.7%	11,728,953	84.8%	13,836,394
M×T.20dpa.3	1,047,691	7.8%	1,070,737	7.9%	11,377,028	84.3%	13,495,456
T×M.20dpa.1	883,080	7.4%	913,776	7.7%	10,057,022	84.8%	11,853,878
T×M.20dpa.2	930,008	7.5%	952,974	7.7%	10,490,198	84.8%	12,373,180
T×M.20dpa.3	1,015,130	7.8%	1,032,388	7.9%	10,992,174	84.3%	13,039,692
All F_1s	**11,332,177**	**7.5%**	**11,657,761**	**7.7%**	**127,649,012**	**84.7%**	**150,638,950**

Supplementary Table 3. Differential expression analyses of 28,716 genes containing allelic SNPs.

Analysis	Sample 1	Sample 2	1≠2	1>2	1<2	Chi-squared test *P*-value
Between parents (*A*)	Maxxa.10dpa	TX2094.10dpa	5168	2715	2453	0.0003
	Maxxa.20dpa	TX2094.20dpa	3528	1916	1612	0.0000
Between alleles in M×T (*B*)	Maxxa.M×T.10dpa	TX2094.M×T.10dpa	1965	652	1313	0.0000
	Maxxa.M×T.20dpa	TX2094.M×T.20dpa	1190	320	870	0.0000
Between alleles in T×M (*B*)	Maxxa.T×M.10dpa	TX2094.T×M.10dpa	1827	563	1264	0.0000
	Maxxa.T×M.20dpa	TX2094.T×M.20dpa	1378	405	973	0.0000
Between alleles in F_1	Maxxa.F1.10dpa	TX2094.F_1.10dpa	3800	1583	2217	0.0000
	Maxxa.F1.20dpa	TX2094.F_1.20dpa	2888	1158	1730	0.0000
Between reciprocal F_1s	M×T.10dpa	T×M.10dpa	824	239	585	0.0000
	M×T.20dpa	T×M.20dpa	1	0	1	0.3173
Maxxa vs M×T	Maxxa.10dpa	M×T.10dpa	3580	1739	1841	0.0882
	Maxxa.20dpa	M×T.20dpa	520	200	320	0.0000
Maxxa vs T×M	Maxxa.10dpa	T×M.10dpa	2536	1114	1422	0.0000
	Maxxa.20dpa	T×M.20dpa	1586	762	824	0.1195
TX2094 vs M×T	TX2094.10dpa	M×T.10dpa	3895	1697	2198	0.0000
	TX2094.20dpa	M×T.20dpa	1290	564	726	0.0000
TX2094 vs T×M	TX2094.10dpa	T×M.10dpa	2129	1018	1111	0.0438
	TX2094.20dpa	T×M.20dpa	5091	2323	2768	0.0000
Maxxa vs F_1	Maxxa.10dpa	F_1.10dpa	4210	1995	2215	0.0007
	Maxxa.20dpa	F_1.20dpa	1312	538	774	0.0000
TX2094 vs F_1	TX2094.10dpa	F_1.10dpa	3372	1354	2018	0.0000
	TX2094.20dpa	F_1.20dpa	4661	1822	2839	0.0000
Maxxa allele expression between reciprocal F_1s	Maxxa.M×T.10dpa	Maxxa.T×M.10dpa	85	15	70	0.0000
	Maxxa.M×T.20dpa	Maxxa.T×M.20dpa	2	2	0	0.1573
TX2094 allele expression between reciprocal F_1s	TX2094.M×T.10dpa	TX2094.T×M.10dpa	74	17	57	0.0000
	TX2094.M×T.20dpa	TX2094.T×M.20dpa	0	0	0	n/a

Supplementary Table 4. Weighted co-expression gene network and subnetwork density.

Network	Gene number	TX2094	Maxxa
Whole	63665	0.00912485	0.00608408
Non-RD	26161	0.0087424	0.00595536
RD	1655	0.01897275	0.01897275
*Cis*_only	513	0.01898896	0.00820015
Cis&trans	841	0.01807372	0.01146843
*Trans*_only	301	0.03144944	0.01262072

Supplementary Note 1. Sequence variation in association with regulatory evolution.

To understand the genetic basis of *cis* divergence, we utilized genomic sequences for the two parental lines, Maxxa and TX2094 (SRA accession number SRR617482 and SRR3560138-3560140), to characterize genetic variants within a 2-kb promoter region upstream of the transcription start site. We asked whether *cis* regulatory divergence is mirrored in sequence variation in the gene promoter regions. A total of 340,642 SNPs (in 53,252 genes) and 88,137 indels (in 40,218 genes) were identified. As shown in Supplementary Figure 4, RD genes with *cis* divergence contain more SNPs and indels than genes without *cis* regulatory divergence (*cis*-only or *cis*+*trans* > *trans*-only; Duncan's multiple range test $P < 0.05$), and *trans*-only RD genes showed no significant difference from non-RD genes.

We next examined whether regulatory divergence was also associated with evolutionary rates (dN and dS) in coding regions. Because of the small values of dN (peak at 0.0011) and dS (peak at 0.0026), the calculation of dN/dS is subject to stochastic variance of ratios of small numbers, and was omitted in the following analysis. Both the distributions of dN and dS were significantly different between RD and non-RD genes (Kolmogorov–Smirnov test $P < 0.01$). *Cis*-only and *trans*-only genes tend to display higher substitution rate (dS = 0.0063-0.0070 and dN = 0.0028-0.0037) than those exhibiting *cis*+*trans* divergence and non-RD genes (dS = 0.0029-0.0035 and dN = 0.0019-0.0021; Duncan's multiple range test $P < 0.05$; Supplementary Figure 4). It was expected that *cis*+*trans* regulated genes were as conserved as non-RD genes, considering the stabilizing, antagonistic effects of co-existing *cis* and *trans* variants to preserve expression levels. However, further study is required to understand how the observed higher substitution rates of *cis*-only and *trans*-only genes relate to selection. Overall, however, the data show that RD genes with *cis*-only variants generally evolve faster with a higher substitution rate in both promoter and coding regions.

When comparing sequence divergence between corresponding At and Dt homoeologs, modest Pearson correlations of 0.15-0.38 resulted, with the highest correlation in dN and the lowest in promoter indels. Comparable amounts of promoter and coding sequence change under domestication were also observed, except that Dt promoters accumulated more SNPs than did At promoters (6.4 *vs*. 5.7 SNPs on average within 2-kb upstream of the annotated transcription start sites; Student's t-test $P < 0.05$). This slightly higher number of promoter SNPs is not associated with any particular type of *cis* and *trans* regulatory divergence.

Supplementary Note 2. Functional implications of key TF RD genes in Maxxa versus TX2094 GRNs.

Of the 53 RD TFs in Figure 5, 33 have more predicted TGs in Maxxa, as represented by bigger node size, than their counterparts in TX2094. For example, 2,786 and 324 TGs were predicted for node 1 (Gohir.A01G033500, response regulator RR1) in Maxxa and TX2094, respectively, which is a *trans*-only gene with increased expression following domestication. This regulator gene functions in the cytokinin signaling pathway and auxin biosynthesis, and is critical for root and shoot development in *Arabidopsis* [1,2]. In vicinity of this hub gene in Maxxa, node 6 (Gohir.D10G215300, a POWERDRESS-like MYB), 7 (Gohir.A05G132500.1, GLABRA2), 12

(Gohir.D10G033800, MIKC_MADS family protein) and 27 (Gohir.D06G209500, homeobox-1) were also predicted to have more TGs in domesticated fibers; in contrast, fewer TGs were predicted for node 45 (Gohir.D05G08900 0, TANDEM ZINC FINGER PROTEIN 9). The regulatory link in the vicinity of node 7 are of particular interest, as GLABRA2 (GL2) is a key regulator downstream of the GL1/GL3/EGL3/TTG1 core complex in the *Arabidopsis* trichome development pathway [3]. Previously, a cotton GL2-like homolog *GhHOX3* has been found to play a central role in controlling cotton fiber elongation, which represents a basal and highly conserved component of the fiber developmental program [4]; it remains an open question whether and how human-mediated selection has acted on the core regulators of trichome development such as GL2.

Trans-only node 41 (Gohir.D03G133500) and *cis*-only node 43 (Gohir.A03G034800) are a pair of homoeologous genes that encode ethylene-insensitive-3 family proteins, which are known to initiate downstream transcriptional cascades for ethylene response (Figure 5 and Supplementary Data 2). It has been shown that exogenously applied ethylene promotes fiber cell expansion by increasing the expression of sucrose synthase, tubulin, and expansin genes, and fiber growth is suppressed by applying the ethylene biosynthetic inhibitor L-(2-aminoethoxyvinyl)-glycine [5]. A higher expression of Dt than At was consistently observed ($\log_2$(At/Dt) = -3.3 and -3.9 in Maxxa, -1.0 and -1.3 in TX2094 at 10 and 20 dpa, respectively; all with adjusted $P < 0.05$). In the Maxxa network, a much smaller number of TGs were inferred for both nodes 41 and 43 than in TX2094 (41 – 599 Maxxa TGs *vs*. 769 TX2094 TGs, 43 – 338 *vs*. 1526; Supplementary Data 2). In the vicinity of this pair of homoeologs, nodes 6 (Gohir.D10G215300, Duplicated homeodomain-like PWR superfamily protein), 13 (Gohir.A13G064000, FAR1-related sequence 5), 27 (Gohir.D06G209500, homoeobox-1), 28 (Gohir.A13G001500, bZIP transcription factor), 45 (Gohir.D05G089000, zinc finger CCCH-type family protein), and 47 (Gohir.D07G051500, auxin response factor 2) were found in the TX2094 GRN (Figure 5b), whereas nodes 6, 27, and 45 became detached in the Maxxa GRN while still remaining interconnected among themselves (Figure 5c). Although such gain and loss of predicted links awaits experimental validation to elucidate the underlying molecular mechanisms, these results demonstrate the power of GRNs and show that the integrated analysis with evolutionary analysis of *cis* and *trans* regulatory changes can help to identify candidate genes that may have been primary targets of selection during cotton fiber domestication. Other such candidates include three genes also detected in the QTL study [6] (also see Supplementary Data 2 column of fiber QTL genes), i.e., node 3 (Gohir.A08G027700, zinc finger CCCH-type/C3HC4-type family protein), 32 (Gohir.D04G041000, homeobox protein 31), and 50 (Gohir.A08G156400, a bHLH DNA-binding family protein MYC2), and one cell-wall synthesis related gene, node 48 (Gohir.D12G242500, NAC domain transcriptional regulator superfamily protein).

Supplementary References

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