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Natural variation for gene expression responses to abiotic stress in maize

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SUMMARY

Plants respond to abiotic stress through a variety of physiological, biochemical, and transcriptional mechanisms. Many genes exhibit altered levels of expression in response to abiotic stress, which requires concerted action of both *cis*- and *trans*-regulatory features. In order to study the variability in transcriptome response to abiotic stress, RNA sequencing was performed using 14-day-old maize seedlings of inbreds B73, Mo17, Oh43, PH207 and B37 under control, cold and heat conditions. Large numbers of genes that responded differentially to stress between parental inbred lines were identified. RNA sequencing was also performed on similar tissues of the F_1 hybrids produced by crossing B73 and each of the three other inbred lines. By evaluating allele-specific transcript abundance in the F_1 hybrids, we were able to measure the abundance of *cis*- and *trans*-regulatory variation between genotypes for both steady-state and stress-responsive expression differences. Although examples of *trans*-regulatory variation were observed, *cis*-regulatory variation was more common for both steady-state and stress-responsive expression differences. The genes with *cis*-allelic variation for response to cold or heat stress provided an opportunity to study the basis for regulatory diversity.

Keywords: regulatory variation, gene expression, abiotic stress, allele-specific expression, *Zea mays*.

INTRODUCTION

Plants experience a myriad of abiotic stresses over the course of one life cycle including extreme temperatures, drought, and nutrient deficiencies. Because plants are sessile, they utilize multiple mechanisms to respond to, and survive, in these diverse environments. These stresses can have negative effects on plant health, survival, and in the case of crops, a significant loss of yield. For example, temperature stress has been shown to negatively affect germination, stunt growth, increase leaf chlorosis and necrosis, and reduce yield (Yadav, 2010; Hasanuzzaman *et al.*, 2013). Constitutive responses to adverse environments can also limit growth and fitness (Gilmour, 2000; Dubouzet *et al.*, 2003). Therefore, plants have evolved physiological, biochemical, and transcriptional processes to sense and respond to environmental stresses (Mickelbart *et al.*, 2015). While there are important biochemical and physiological responses to abiotic stress (Ashraf and Hafeez, 2004; Crafts-Brandner and Salvucci, 2002; reviewed in Hasanuzzaman *et al.*, 2013), underlying regulation of gene expression plays an important role for tolerance to many different stress responses (Wang *et al.*, 2004; Miura and Furumoto, 2013). Many QTL studies have highlighted the importance of altered gene expression levels or patterns in driving functional variation among alleles. We were interested in understanding the mechanisms that drive allelic variation for response to environmental conditions in order to understand the sources of functional allelic variation that might contribute to local adaptation.

Expression of genes coding for key components in stress response pathways is essential for tolerance to abiotic stress. Several transcription factors (TFs) directly affect responses to temperature stress by inducing transcription of important stress response genes (Abe *et al.*, 1997; Hasanuzzaman *et al.*, 2013; reviewed in Qu *et al.*, 2013). In *Arabidopsis thaliana* a set of TFs called dehydration-responsive element binding (DREB) factors or C-repeat

binding factors (CBF) plays an important role in tolerance to various abiotic stresses including tolerance to cold, drought, and heat (Jaglo-Ottosen, 1998; Schramm *et al.*, 2008; Sakuma *et al.*, 2006). CBF/DREBs play similar roles in many flowering plants including maize, rice, and soybean (Dubouzet *et al.*, 2003; Chen *et al.*, 2007; Liu *et al.*, 2013). There is also evidence from *Arabidopsis thaliana* studies that expression of cold-responsive (COR) genes and heat shock protein (Hsp) families are important for cold and heat tolerance, respectively (Yadav, 2010; Hasanuzzaman *et al.*, 2013).

Multiple studies have described *cis*-regulatory elements upstream of known stress-induced genes, including dehydration response element (DRE), abscisic acid (ABA) responsive promoter elements (ABREs), and heat shock elements (HSEs, reviewed in Raghavendra *et al.*, 2010; Yamaguchi-Shinozaki and Shinozaki, 1994; Guiltinan *et al.*, 1990). These elements provide the necessary binding site for TFs to confer stress-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 1993; Hovarth *et al.*, 1993; Schoffl *et al.*, 1998; Mittal *et al.*, 2011; Raghavendra *et al.*, 2010). The cold and drought-responsive genes DREB1A and DREB2A also bind to *cis*-DREs upstream of responsive genes, which resulted in expression of downstream genes. These two DREB genes increase in expression independently under different stresses and induce the up-regulation of different downstream genes. This situation suggests that the DREB genes function and are induced independently (Liu, 1998). Two recent studies have explored sources of regulatory variation for gene expression responses to drought conditions on a genome-wide level. One study, in *Arabidopsis thaliana,* monitored both steady-state and drought-responsive *cis*- and *trans*-regulatory variations (Cubillos *et al.*, 2014). A study in switchgrass also detected signatures of steady-state and stress-responsive regulatory variations in response to drought (Lovell *et al.*, 2016). Both studies found a bias toward *cis*-regulatory variation when contrasting accessions, as well as treatment and control plants (Cubillos *et al.*, 2014; Lovell *et al.*, 2016).

Maize (*Zea mays*) exhibits substantial genomic and transcriptomic diversity between inbred lines (Chia *et al.*, 2012; Hirsch *et al.*, 2014). Both *cis*- and *trans*-regulatory variations contribute to this diversity and have been shown to play important roles in processes including domestication and heterosis (Stupar and Springer, 2006, Holloway *et al.*, 2011; Li *et al.*, 2013; Swanson-Wagner *et al.*, 2009; Lemmon *et al.*, 2014; Hirsch *et al.*, 2014). The sources and prevalence of *cis*- and *trans*-regulatory variations and their role in stress response remain poorly characterized in most species including maize. One recent study suggested transposable element (TE) polymorphism may be one source of *cis*-regulatory variation for the allele-specific response to abiotic stress (Makarevitch *et al.*, 2015).

In this study we utilized the extensive natural variation of maize to study variation in the abiotic stress response among different genotypes. By monitoring allele-specific gene expression in heterozygous plants it was possible to identify examples of *cis*-regulatory variation for responsiveness to cold or heat stress. In particular, first we confirmed that there is genetic variation for transcriptional responses to stress between our parents. Next we showed that while *cis*-regulatory variation is more common than *trans*-regulatory there is ample evidence for both *cis*- and *trans*-regulatory variations both at the steady-state and in response to stress. Our results provide a comprehensive picture of the genetic variation that contributes to the evolution of stress responses to the abiotic environment.

RESULTS

RNA-seq was used to survey natural and allelic variation in global gene expression in response to cold and heat stress in several maize inbred and hybrid genotypes. The inbred parents (B73, Mo17, PH207 and Oh43) were selected because they have genetic/genomic resources, including whole genome assemblies (B73 and PH207) or deep re-sequencing data (Mo17 and Oh43). These inbred lines also represent four different population groups of maize (Nelson *et al.*, 2008) and provide transcriptome diversity (Stupar *et al.*, 2008). The F_1 hybrids B73xMo17, B73xPH207, and Oh43xB73 were used to examine the relative expression of alleles in hybrids. The transcriptome data were produced in two separate experiments. The first experiment included B73, Oh43 and Oh43xB73. The second experiment included B73, PH207, Mo17, B73xMo17 and B73xPH207. In total, this provided three triplet combinations of two parents and their F_1 off-spring for all conditions with data from the same experiment. For all seven genotypes, RNA-seq was performed for three biological replicates of 14-day-old seedlings subjected to control, cold or heat treatments (Table S1). Gene expression levels were estimated from the RNA-seq data by aligning the reads to single nucleotide polymorphism (SNP)-corrected reference genomes and counting uniquely aligning reads (Dataset S1). For the hybrid genotypes, the allele-specific expression (ASE) ratios were determined using SNPs within the transcripts to count reads derived from each allele (Dataset S1). This dataset provides data for 25 433–25 641 expressed genes in each genotype by treatment combination and approximately 74% of these genes had ASE data in the hybrid.

Transcriptome changes induced by stress highlight roles of known regulatory pathways

Stressed samples were collected immediately following the treatment to reduce gene expression differences due to morphological changes and developmental differences between control and stressed plants that could arise

during subsequent growth (Makarevitch *et al.*, 2015). There were no apparent morphological differences immediately following the stress treatment, but leaf necrosis and reduced growth of stressed plants is apparent several days after the stress treatment. In order to confirm that the cold and heat stress treatments were triggering cold or heat responses we investigated the gene ontology (GO) terms over-represented in up-regulated genes following heat or cold treatment (see Experimental procedures for details). As expected, we found that 72% and 52% of the 25 most significant over-represented GO categories were associated with response to stress in cold and heat conditions, respectively (Tables S2 and S3).

The importance of transcription factors (TFs) for stress response in *Arabidopsis thaliana,* maize, rice, and wheat is well documented (Abe *et al.*, 1997; Qin *et al.*, 2004; Wang *et al.*, 2004; Lata and Prasad, 2011; Mizoi *et al.*, 2012). We assessed whether maize TF families were enriched for up-regulated genes in response to heat or cold stress. In total, 20 of the 57 annotated maize TF families (Yilmaz *et al.*, 2009) were enriched for up-regulated genes in cold and/or heat stress relative to control conditions in at least three of the inbred genotypes (Figure S1a). Eight families of TFs enriched for genes that were up-regulated in response to heat stress included the heat shock (HS) and the myeloblastosis (MYB) TF families (Figure S1a). Many (15/29) of the HSF TFs showed a significant (FDR corrected *P*-value <0.01) increase of expression in B73 plants exposed to heat stress (Figure S1b). There were 18 families of TFs enriched for up-regulation in response to cold stress, including the APETALA2/Ethylene Responsive Factor (AP2/EREB), which contains the maize orthologs of *Arabidopsis* DREB/CBF genes. Many (8/13) of the maize CBF/DREB orthologs (Liu *et al.*, 2013) exhibit up-regulation in response to cold or heat stress (Figure S1c).

Prior research has provided evidence that genes responding to abiotic stress often contain proximal *cis*-elements, including DRE/CRT, ABRE, HSE, NACR, WRKY, MYBR, MYCR, or LTRE motifs, which provide binding sites for DREB/CBF, ABA, HSF, NAC, WRKY, MYB, MYC, or LTR proteins, respectively (Xiao and Lis, 1988; Guiltinan *et al.*, 1990; Yamaguchi-Shinozaki and Shinozaki, 1994, 2006). The genomic distribution of DRE/CRT, ABRE, HSE, NACR, WRKY, MYBR, MYCR, or LTRE motifs in the B73 reference genome was used to assess whether these motifs are enriched near up-regulated genes in response to heat (n = 1627) or cold (n = 1858) stress in B73. The HSEs were enriched near genes that are responsive to heat stress, but not cold-induced genes (Figure S1d). The DRE/CRT, ABRE, MYCR and WRKY binding sites were all enriched in the 1 kb regions upstream of both cold- and heat stress induced genes (Figure S1d). The MYBR and LTRE sites are enriched in distal promoter regions for cold-induced genes. NACR elements were not enriched in any of the regions tested (Figure S1d). These results provide evidence for expected *cis*-/*trans*-regulatory pathways leading to the observed changes in gene expression in response to cold/heat stress.

Variation for transcriptional responses to abiotic stress among maize inbreds

Prior studies have documented numerous examples of *cis*- and *trans*-regulatory variation for steady-state transcript abundance in maize (Stupar and Springer, 2006, Holloway *et al.*, 2011; Li *et al.*, 2013; Swanson-Wagner *et al.*, 2009; Lemmon *et al.*, 2014; Hirsch *et al.*, 2014). In this study we were particularly interested in documenting examples of variation for responsiveness to heat or cold stress for specific alleles. Clustering of the gene expression levels revealed significant treatment effects on the transcriptome, but also found evidence that genotypes have slightly different responses (Figure S2a, b), with hybrids often exhibiting intermediate responses in both batches of samples (Figure S2b). Clustering was also performed using genes that are differentially expressed (DE) in at least one inbred genotype in response to cold (n = 7838) or heat (n = 7155) (Figure 1). Visual examination of the clustering suggests

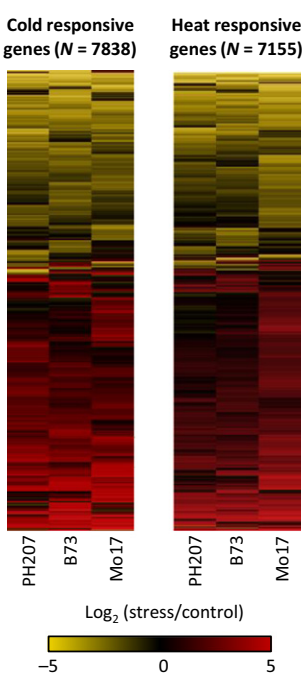


Figure 1. Many number of genes show significant genotype and/or treatment effects.
The non-redundant set of cold (left) or heat (right) responsive genes from genotypes in batch 2 were used to perform hierarchal clustering (UPGMA method) using the gene expression response values (log_2 stress RPM divided by control RPM). Genes that are up-regulated (red) or down-regulated (yellow) in response to stress in at least one inbred comparison are visualized. A subset of genes are DE in all three inbred comparisons, but some genes only have strong responses in one or two genotypes and have no change in gene expression (black) in other genotypes.

that many genes had consistent responses to heat or cold stress in all inbreds. However, there were also a substantial number of genes with variable responses to cold or heat treatments among genotypes. Both clustering methods revealed large transcriptional changes in response to cold or heat as well as suggesting some variation in those responses among genotypes. Thus, we next used two complementary statistical approaches to assess if there was evidence for genetic variation between parents in transcriptional responses to stress.

For our first approach, we utilized a two-way factorial analysis of variance (ANOVA) on RPM (reads per million mapped reads) levels of each gene. This parses expression variation due to genotype (G), treatment (T) and genotype by treatment (G $\times$ T) interactions. We ran this model twice for each pair of inbred parents; once comparing control treatment to heat treatment and once comparing control to cold. (Table 1a; see Experimental procedures for details). Many expressed genes (21–52%) exhibited significant genotype and/or treatment effects after controlling for multiple comparisons. A smaller subset of genes (1–28%) showed evidence of genotype-specific responses to treatment (Table 1(a). There were notable differences in the frequency of G $\times$ T effects in the batch 1 samples including B73 and Oh43 compared to the genotypes in batch 2. It is not clear whether this is due to biological differences in these genotypes or technical differences among batches of samples. A visualization of the amount of variation (η^2 values) explained by genotype, treatment and G $\times$ T reveal large clusters of genes predominantly affected by either genotype or environment with fewer genes with strong G $\times$ T effects (Figure S3). Thus while genotype and treatment explained much of the variation between parents, there is evidence for genetic variation in response to stress.

For our second approach we used a negative binomial generalized linear modeling (GLM) framework to model differential expression of normalized count data. This method also provided an opportunity to model ASE levels of the hybrids enabling an assessment of patterns of gene expression consistent with environmentally sensitive *cis*- and *trans*-variation (approach adapted from Lovell *et al.*, 2016). It is important to note that this approach only utilizes reads that can be mapped specifically to one allele in each parental/hybrid contrast and thus uses a different dataset than the one described above (see Experimental procedures for details). We included treatment (control or stress), allele (parental allele source), and generation (parental or F_1) as factors in the model as well as all possible interactions between them. As expected, allelic identity and treatment were the most common sources of variation in expression (Table 1b). However, several hundred genes exhibit significant variation due to allele by treatment effects suggesting differential regulation of some alleles in response to heat or cold stress. A similar number of genes exhibit significant allele by generation effects suggesting that these hybrids exhibit differences in expression of certain alleles compared to the parents. An even smaller number of genes exhibit significant generation by treatment interactions that would indicate steady-state trans-responses or three way interactions *trans* variationbetween treatment, allele, and generation, which indicate environmentally sensitive. This suggests relatively rare differences for expression responses to heat or cold stress in hybrids compared to inbred parents. The B73/Oh43 contrast showed a much larger number of significant interaction terms and this could be due to batch effects or increased power for this experiment due to lower variation among replicates. This analysis provides evidence for *cis*-

Table 1 Number of genes with significant effects for total expression levels or allelic expression levels

	Control and cold treatment			Control and heat treatment		
	B73/Mo17	B73/PH207	B73/Oh43	B73/Mo17	B73/PH207	B73/Oh43
(a) ANOVA (RPMs)						
Genotype	8691	7851	17 135	9030	7834	16 049
Treatment	8291	6439	16 119	9461	9543	20 461
Genotype by treatment	584	308	8075	918	472	10 950
Total genes with data	39 656	39 656	39 656	39 656	39 656	39 656
(b) GLM (ASE counts)						
Allele	5758	5630	5032	5482	5679	4185
Treatment	6482	6554	5821	5835	5992	4857
Generation	296	386	709	244	413	682
Allele by generation	275	297	2273	302	316	1097
Allele by treatment	273	507	1930	236	202	1594
Treatment by generation	1	3	1176	52	5	1181
Treatment by Generation by Allele	1	0	276	2	0	880
Total genes with data	15 997	15 360	15 609	15 837	15 283	15 458

Significance for both tests: FDR < 0.01.

regulatory variation both for steady-state expression and for responsiveness to stress.

High levels of *cis*-regulatory variation contribute to gene expression differences among genotypes

Previous research has documented high levels of *cis*-regulatory variation, particularly for genes with strong expression variation between maize inbred lines (Stupar and Springer, 2006; Holloway *et al.*, 2011; Li *et al.*, 2013). In this study we had the opportunity to evaluate whether the contributions of *cis*- and *trans*-regulatory variation are similar in different environmental conditions. The relative contribution of *cis*- and *trans*-regulatory variation was estimated for each trio of lines within each environmental treatment. Biased allelic expression in the F_1 provided evidence of *cis*-regulatory variation, while balanced expression of the two alleles suggested *trans*-regulatory variation, despite differences in expression in the parental genotypes (Figure 2). Genes were classified as having *cis*- or *trans*-regulatory variation by performing a chi-square analyses of the observed read counts relative to either the *cis*- or *trans*-expectation and identifying the genes with significant ($\chi^2 < 0.01$) from one expected value but not the other (Table S4). The observed allelic proportions in the F_1 hybrids were compared to the expected allelic proportions predicted from the relative expression level of the parents for each gene in each treatment (see methods for details) in order to visualize the relative contributions of *cis*- and *trans*-regulatory variation (Figures 3 and S4). *Cis*-regulatory variation is quite prevalent in all conditions with fewer examples of *trans*-regulatory variation although higher rates of *trans*-regulatory variation are observed for the B73-Oh43 comparison from batch 1 (Table S4). Genes that are classified as subject to *cis*-regulatory variation in the control experiments frequently exhibit *cis*-regulatory variation in both of the stress treatment conditions with relatively few examples of the same genes switching to *trans*-regulatory variation (Figure S5a). In contrast, it was more common for genes classified as having *trans*-regulatory variation in control conditions to exhibit *cis*-regulatory variation in heat or stress conditions (Figure S5b).

Allelic variation for responsiveness to abiotic stress

Given the high levels of transcriptional variation in response to abiotic stress among maize inbreds and the frequent *cis*-regulatory variation within a treatment, we expected that there would also be examples of *cis*-regulatory variation for gene expression responses to an abiotic stress. The analysis of allelic variation for response to heat or cold stress may help uncover the mechanisms that underlie gain or loss of stress-induced gene expression. In order to identify examples of genes with regulatory variation in response to heat or cold stress we found genes with ASE data with equivalent levels of expression in the two inbred parents in control conditions, but exhibiting significant differences in expression following heat or cold stress (Figure 4). These include genes with both up- and down-regulation in response to the stress, as well as genes that exhibited low or no expression in control conditions. These could include examples of *cis*- or *trans*-regulatory variation for responsiveness. If a gene has purely *trans*-responsive

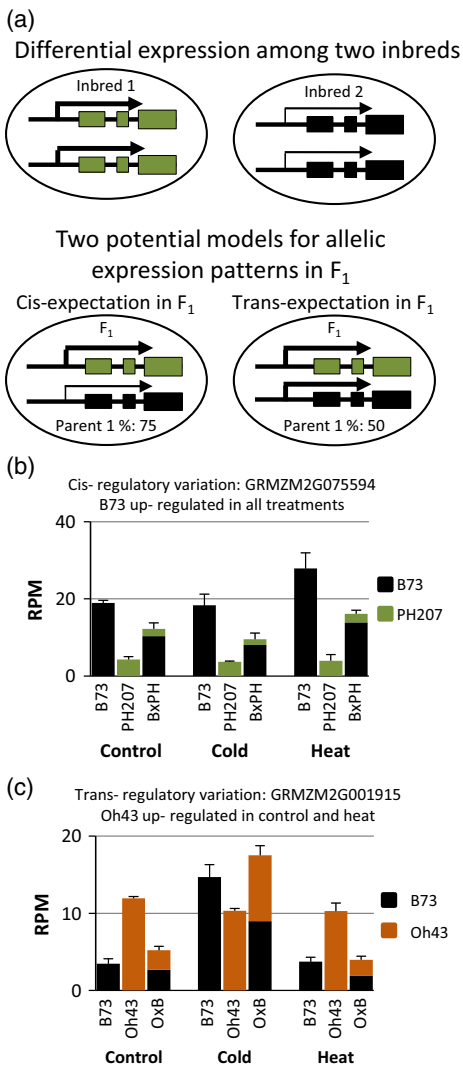


Figure 2. Genes with *cis*- or trans-regulatory variation between genotypes within a condition were observed.
(a) In this example the allele of inbred 1 is more highly expressed than the allele of inbred 2, which is indicated by the thickness of the arrow. If a gene exhibits *cis*-regulatory variation then the allelic ratio in the F_1 would be biased toward inbred 1. Whereas, if the gene exhibited *trans*-regulatory variation both alleles from the parents would be equivalent.
(b, c) The reads per million mapped reads (RPMs) for a trio of samples across all treatments are shown for an example of *cis*-regulatory (b) and *trans*-regulatory (c) variation. (b) Shows an example of *cis*-regulatory variation where the B73 (black) allele is preferentially expressed in all conditions as compared to the PH207 allele (green). (c) An example of trans-regulatory variation for a gene where the Oh43 allele (orange) is more highly expressed in control and heat treatments. However, in the F_1 plants the B73 (black) and Oh43 (orange) alleles exhibit equivalent expression levels.

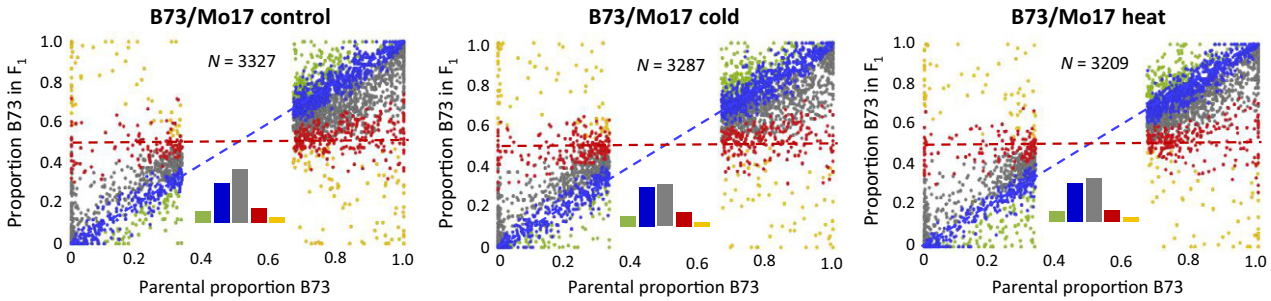
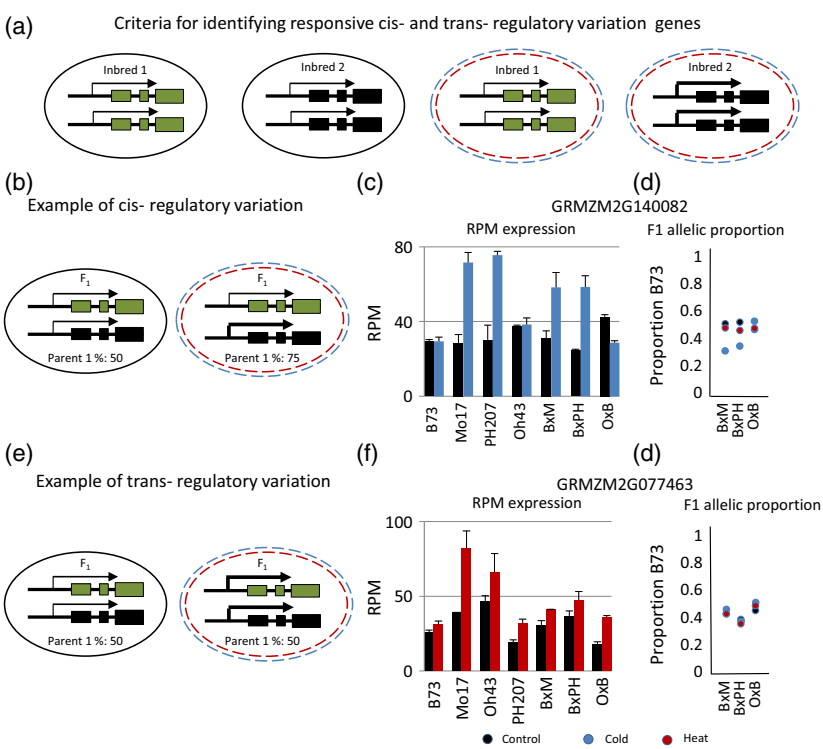


Figure 3. Classification of differentially expressed genes into regulatory variation categories.
Genes that are differentially expressed (DE) between inbreds were identified for each condition. The gap in the middle of each figure is the result of the requirement that genes have differential expression in the parents. Each scatterplot shows the entire set of DE genes between B73 and Mo17 for control (left), cold (middle), and heat (right). Genes with purely *cis*-regulatory variation are expected to plot along the diagonal blue dashed line while genes subject to *trans*-regulatory variation will plot along the horizontal red dashed line. The inset bar charts in each scatterplot show the distribution of genes in five different categories. These include genes that are not statistically distinguishable from *cis*-regulatory (blue) or *trans*-regulatory (red) variation expectations. In addition, some genes exhibit a greater allelic bias in the expected direction (green) or a bias toward the opposite allele (yellow) than predicted by parental ratios. The remaining genes did not fit the statistical cutoffs for *cis*- or trans-regulatory variation and have an observed allelic proportion that falls between expected *cis*- and *trans*-expected values (grey).

Figure 4. Examples of responsiveness *cis*- and *trans*-regulatory variation.
(a) Responsiveness regulatory variation candidates were identified by assessing genes that have similar expression in the control (black circles) and variable response to stress (blue and red dashed circles) between two inbreds (thickness of arrows).
(b) *Cis*-regulatory responsiveness variation would result in approximately equally expression of both alleles in control conditions, but biased allelic expression in stress conditions (thicker arrow).
(c) The expression (RPMs) for all genotypes in control (black) and cold (blue) conditions. Error bars are standard error for all replicates for each batch, except for B73 which is the standard error between batch 1 and batch 2.
(d) Is the proportion of B73 in the F_1 hybrids for a gene that is biased toward Mo17 and PH207 alleles under cold conditions is shown.
(e) Conversely, if a gene exhibits *trans*-regulatory responsiveness variation then both parental alleles would be equivalently expressed in both control and stress conditions in the hybrid.
(f, g) (f) The expression levels and (f) proportion of B73 allele in the hybrids for a gene that is bias toward Mo17 when contrasting B73 and Mo17 (PH207 was not DE between control and heat) under heat conditions.



regulatory variation, then the two alleles in the stress F_1 hybrid should not be statistically different than the proportion observed in the F_1 control. Whereas, a biased expression of alleles in the F_1 stress sample suggests responsive *cis*-regulatory variation (Figure 4b). For example, by contrasting ASE in the F_1 hybrids for gene GRMZM2G140082 we observed that both Mo17 and PH207 alleles showed biased expression compared with the B73 allele, whereas the Oh43 allele showed equivalent expression in the cold condition (Figure 4c). The observed bias expression of the Mo17 and PH207 alleles in the hybrids was in the direction we would predict given the parental expression values (Figure 4d). An example of *trans*-regulatory variation for stress response (Figure 4e) was observed for gene GRMZM2G077463, which showed equivalent parental expression for B73 and Oh43, but higher expression of Mo17 and PH207 when exposed to heat (Figure 4f). Allelic ratios of B73 to Mo17 and B73 to PH207 were equivalent in the respective hybrids under control and stress conditions (Figure 4g). Hundreds of genes exhibited variation in stress-induced expression between two parents (Figure 5). Overall, more genes had altered allelic proportions in the

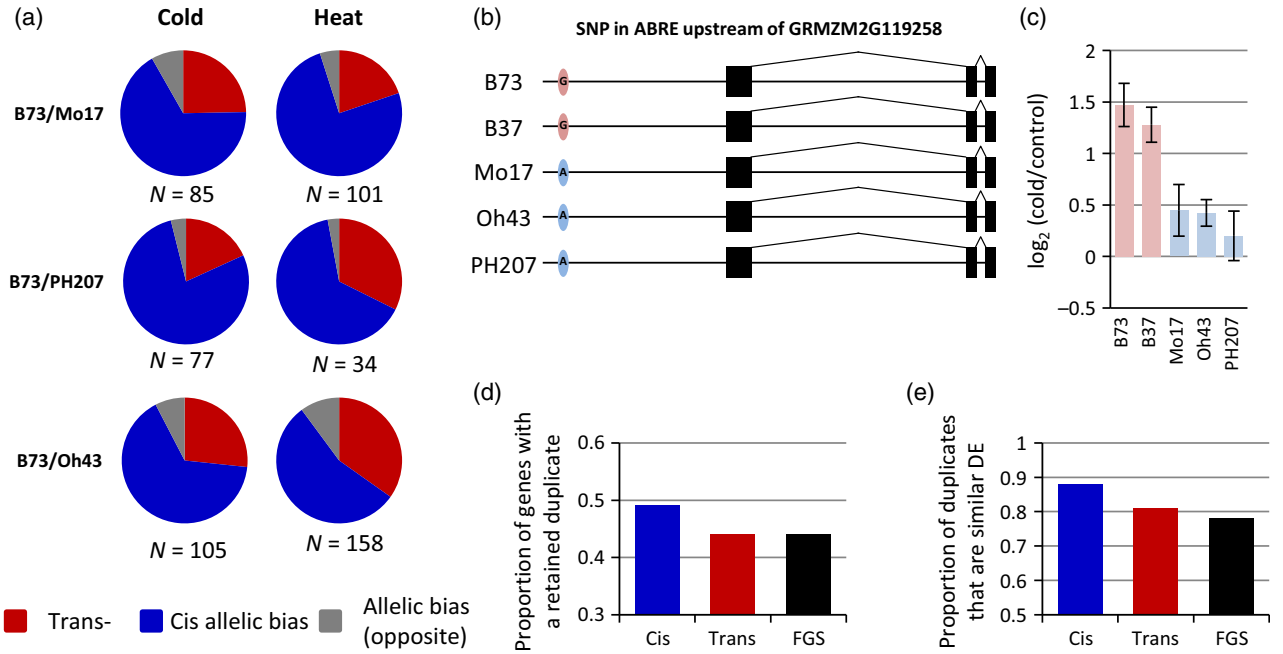


Figure 5. Genes with stress-induced differential expression more often exhibit *cis*-regulatory variation.
(a) Categorization of *cis*- and *trans*-regulatory variation for response to cold and heat stress (gene number listed under the pie charts indicates the number of genes that could be classified). Each gene was categorized into one of three categories. A majority of genes exhibit biased allelic proportions between the control and stress F_1 samples (blue and grey). Of the genes that show allelic bias a majority showed a bias in the direction we would predict given parental expression values (blue). This pattern is consistent with *cis*-regulatory variation. A small proportion of genes (4–16%) showed an allelic bias toward the opposite parental allele (grey). The rest of the genes exhibit *trans*-regulatory variation patterns (red) in which the proportion of alleles were not statistically different ($\chi^2 > 0.01$) between control and stress conditions in the F_1.
(b) An ABRE (coloured oval) located upstream of gene GRMZM2G119258 has a SNP in Mo17, Oh43 and PH207 relative to B73 and B37 (Bukowski *et al.*, 2015).
(c) The relative fold change in gene expression in cold relative to control is shown for GRMZM2G119258 in each of the five genotypes.
(d) The proportion of genes that have a retained duplicate are shown the filtered gene set (FGS, black), classified as *cis*-regulatory (blue), or *trans*-regulatory (red) variation. Maize paralogs (as reported in Schnable *et al.*, 2011) for genes that are expressed, have at least 25 allele-specific expression reads, and are DE between control and stress in at least one genotype were utilized for this analysis.
(e) The proportion of paralogs in (d) that are also DE in the same direction (up or down) under the same stress conditions are plotted for the three categories: FGS (black), *cis*-regulatory (blue), and *trans*-regulatory variation (red).

F_1 between control and stress treatments, which would suggest *cis*-regulatory variation (Figure 5a). Of the genes that exhibited allelic bias 84–96% were in the direction we would predict based on parental expression.

Allelic variation for stress-induced expression can be the result of a loss or gain of expression activation in response to stress. Variation in important *cis*-regulatory elements upstream of genes could provide a potential mechanism for loss of expression activation. SNPs within important *cis*-binding elements (ABRE, DRE/CRT, and HSE) were identified within the promoter region for two groups of genes. The first group of genes exhibited increased expression following a stress treatment in B73, but no difference in expression for the other genotype in the trio, whereas the second group showed an increase in both alleles. If SNPs in binding motifs are associated with loss of stress-induced expression, then we would predict a greater rate of SNPs in binding regions upstream of the genes with variable response than those with consistent responses for both alleles. Genes with allelic variation for response to stress showed a higher rate of SNPs in nearby *cis*-regulatory elements than genes where both alleles are up-regulated for most of the stress by motif combinations (Table S5). One example, GRMZM2G119258, was categorized as a gene exhibiting *cis*-regulatory variation for response to cold stress between B73 and Oh43. B73 had an intact ABRE element upstream of the gene, while Oh43 and Mo17 contained a SNP (Bukowski *et al.*, 2015) in that element. The expression level and genotype for PH207 and B37 (RNA-seq data from Makarevitch *et al.*, 2015) were also determined (Figure 5b). If the SNP in this motif is associated with expression differences, then genotypes with the B73-like haplotype should increase in expression under stress conditions, and non-B73 haplotypes should not increase. Analysis of expression showed that B73 and B37 are DE following cold stress while Mo17, Oh43, and PH207 do not respond to a cold treatment (Figure 5b).

The allelic variation for responsiveness to an abiotic stress could be the result of loss of regulatory information for one allele, or the result of acquisition of novel regulatory information for the other allele. The presence of retained duplicates from a recent whole genome

duplication event provides an opportunity to assess the relative frequency of gains and losses for responsive expression. Maize underwent a whole genome duplication event approximately 6–12 million years ago (Gaut and Doebley, 1997). Subsequent fractionation has resulted in frequent loss of paralogs or *cis*-regulatory elements (Freeling *et al.*, 2015). A subset of maize genes have retained both syntenic paralogs (Schnable *et al.*, 2011), which can be used to assess the likely ancestral state for expression responses. We can look at the responses of the retained syntenic duplicates if a gene exhibits allelic variation with some alleles responding to a stress, and other alleles that do not respond. If the retained duplicate responds, then we infer that some alleles have likely lost the expression response. Alternatively, if the retained duplicate does not respond to the abiotic stress, we would infer that some alleles for the gene with a variable response have gained novel responsiveness. Genes that exhibited *cis*- or *trans*-regulatory variation retain paralogs at a similar rate (44–49%) to that of other expressed maize genes (44%, Figure 5c). For the genes classified as exhibiting *cis*- or *trans*-regulatory variation for responses to abiotic stress, we determined whether the retained duplicates also responded to the same stress (Figure 5d). The majority of these genes exhibited stress responses for the retained duplicates, suggesting that most examples of allele variation reflect recent loss of stress-responsive expression.

Previous work has shown that genes exhibiting steady-state changes and *cis*-regulatory variation patterns tended to have a higher fold change between inbreds than trans-regulatory variation (Stupar and Springer, 2006). Comparing the fold change between stress and control treatments for genes that have responsive *cis*- or *trans*-regulatory variation patterns allowed us to test whether this pattern holds true for stress-induced expression. A non-redundant list of genes exhibiting *cis*- and *trans*-regulatory variation was created by combining all genes categorized as *cis* or *trans* from all three trio comparisons. GO enrichment analysis was performed using the top *Arabidopsis thaliana* BLAST hit for this set of genes, and the only enriched biological process was response to stimulus. In contrast to steady-state expression patterns, we did not observe a bias of genes with strong expression variation also exhibiting *cis*-regulatory variation for any of the comparisons made in this experiment (Figure S6). Interestingly, in the contrast of B73 and Oh43 from batch 1 data we observed a higher mean and larger variation for genes exhibiting *trans*-regulatory variation then for the genotypes monitored in batch 2 (Figure S6). This result could reflect a biological difference among these genotypes or technical variation among batches. Many transcription factor families were enriched in our stress-induced expression gene sets, therefore we were interested in which TFs that show stress-responsive *cis*- or *trans*-regulatory variation. Although some TF families overlap between *cis*- and *trans*-genes (EREB and Orphan), a subset of important abiotic stress response TF families (MYB and NAC) were only present in the *cis*-regulatory variation category (Table S6).

DISCUSSION

The expression of many genes is influenced by cold (Miura and Furumoto, 2013) or heat stress (Wang *et al.*, 2004) and we found that many of these expected changes in gene expression were observed in our cold or heat stressed samples. Expression variation is a major contributor to phenotypic variation within species and divergence among species. In particular, the developmental timing of gene expression or responsiveness of expression to environmental stimuli can vary among species. Differential regulation of alleles can result from changes at proximal regulatory regions (*cis*), changes in unlinked loci that encode regulatory molecules such as TFs or regulatory RNAs (*trans*), or a combination of both. While researchers appreciate the importance of variation at *cis*-regulatory regions for contributing to differences in both steady-state expression variation and differences in responsiveness to environmental stimuli, we lack a detailed understanding of the sequence changes underlying this variation. In this study we utilized natural variation in maize to assess the regulatory variation for steady-state expression levels, as well as expression responses to heat or cold stress in several maize genotypes. Given the previous evidence for abundant *cis*- and *trans*-regulatory variation contributing to differences in steady-state expression levels (Stupar and Springer, 2006; Holloway *et al.*, 2011; Li *et al.*, 2013), we were interested in documenting the contribution of *cis*- and *trans*-regulatory variation for gene expression responsiveness to specific treatments such as heat or cold stress. The relative expression for both parental lines, as well as the two alleles in hybrid plants grown in control or stressed conditions were used to identify examples of differential response to environment and then to partition these into *cis*- or *trans*-regulatory variation. We found hundreds of genes with variable expression responses to heat or cold stress, with much of differences due to *cis*-regulatory variation.

These examples of *cis*-regulatory variation for stress responsiveness can be utilized to learn about the mechanisms leading to expression variation within species. The allelic variation for stress-responsive expression could arise from the loss of responsiveness for an allele or through the acquisition of novel responsiveness. An analysis of the expression response for paralogs from a recent whole genome duplication event suggested that in many cases the variation is likely due to loss of responsiveness for one allele (Figure 4d, e). This variation can occur due to SNPs or deletions in binding sites for TFs that are stress-induced. We were interested in the rare cases of novel

acquisition of stress responsiveness. These examples may shed light on the mechanisms that could lead to novel variation for gene expression responses. Previous research has suggested that transposon polymorphisms may contribute to stress-responsive expression in maize (Makarevitch *et al.*, 2015) and further research into the haplotypes of genes identified in this study will document the role of transposon variation at these loci.

Several recent studies (Cubillos *et al.*, 2014; Lovell *et al.*, 2016) have also studied variation in expression responses in Arabidopsis and switchgrass. While Lovell *et al.* (2016) focused on parental varieties exhibiting strong phenotypic differences for tolerance to drought stress, both Cubillos *et al.* (2014) and this study utilized parental varieties without strong phenotypic differences for tolerance to the treatment conditions. There are some differences in the sensitivity of B73, Mo17, Oh43 and PH207 to cold and heat stress in terms of relative growth rates in the days following the stress, but these varieties tolerate our stress regimes and recover. This suggests the abundant molecular differences for stress-responsive expression are not contributing to a long-term, major phenotypic difference. This study was not designed to identify genes that would provide tolerance to cold or heat stress in maize. Instead, our goal was to begin to document regulatory features that contribute to the ability of a gene to respond to cold or heat stress in maize, and to be able to monitor how variation in promoter regions may contribute to changes in responsiveness to stress. Studying regulatory mechanisms on a large scale is quite complicated, but improvements in sequencing technology, annotation of genomes, and novel analytic tools are providing a platform to begin understanding complex regulatory mechanisms. This information may be useful in developing predictive models for gene expression responses and for engineering novel responses to abiotic stress in crops.

EXPERIMENTAL PROCEDURES

Stress conditions, tissue collection, and RNA extraction

Inbred and hybrid maize seedlings were grown under 16 h of light at 24°C in growth chambers for 14 days. Plants were cold stressed, heat stressed, or remained in control conditions. Plants that were cold stressed were placed in a cold room (7°C) for 16 h. Heat stressed plants were placed in an incubator (50°C) for 4 h. Above-ground seedling tissue was collected immediately following each stress treatment with six plants pooled per sample and the tissue kept at −80°C. Biological replicates were grown 5 days apart for all treatments. After tissue collection each pooled sample was ground in liquid nitrogen. Total RNA was extracted in Trizol (Life Technologies, NY, USA) and then purified with LiCl.

Sequencing and data processing

RNA-seq libraries were prepared by the University of Minnesota Genomics Center in accordance with TruSeq library creation protocol (Illumina, San Diego, CA, USA). Samples were sequenced using the Illumina HiSeq-2000 platform outputting 15–30 million reads per sample. All replicates and conditions of B73 were aligned to the maize reference genome (B73 AGPv2). To improve mapping quality of the non-reference inbreds, SNP-corrected references were made for Mo17, PH207, and Oh43. The SNP-corrected references were made by taking the B73 reference fasta file and replacing the nucleotides where a SNP was present between the two inbreds. Each sample of Mo17, PH207, and Oh43 was aligned to their respective corrected reference. All alignments were made using Tophat 2 (version 2.1.0, Kim *et al.*, 2013) and bowtie2 (version 2.0.10, Langmead and Salzberg, 2012). To keep alignments consistent across all samples, only reads that mapped without any mismatches were retained and used for counts and reads per million mapped reads (RPM) calculations (Tophat2 option -N 0). Bamtools (version 2.3.0) was used to filter out reads that mapped with indels or to multiple places in the genome (Barnett *et al.*, 2011). Inbred and hybrid samples were aligned to both parental references using the same criteria (e.g. B73xOh43 was aligned to both B73 and Oh43 corrected references). Any SNPs that were not fully validated for the inbred samples were removed from subsequent analyses. To obtain ASE counts for hybrids, reads from the two alignments for each hybrid were compared and reads that mapped uniquely to one parent were retained. All uniquely mapping reads from inbred parents and the ASE counts from the hybrids were run through HTSeq (version 0.6.1p1, Anders *et al.*, 2014) to get per gene counts and per gene ASE counts, respectively. RPM values, were calculated for all inbred parents by using the count files from HTSeq. Hybrid RPM values were calculated by summing the parent specific reads and the reads that mapped equally to both parents.

ANOVA and eta-squared

A pairwise two-way factorial ANOVA was run for each gene on all biological replicates for a pair of inbreds and conditions (e.g. control and cold) using the aov function in R (Chambers, 1992) to look for significant genotype, treatment, and genotype by treatment effects. RPMs for each sample were used. Statistical significance was determined for each factor by calculating FDR corrected *P*-values. For a factor to be considered significant the adjusted *P*-value had to be <0.05. Eta-squared values were used to estimate the effect size of each factor from the ANOVA (G, T, and G × T) for each gene. Eta-squared values were also calculated for each gene with at least one significant factor from the ANOVA. Eta-squared values were calculated in R using the eta-Squared function within the lsr package in R (Navarro, 2015).

Generalized linear model (GLM)

Allele-specific counts (see the Sequencing and data processing section for details) for both the inbreds and F_1 hybrids in a trio for control and one stress condition were used to assess which factors contribute to the variation in allelic expression. (similar to Lovell *et al.*, 2016). The allele-specific counts for genes that have a mean ASE count >5 for both alleles were fit to a negative binomial model using DESeq2 (Love *et al.*, 2014) with:

$$\log y_{ij} = \beta_0 + \beta_T T_i + \beta_A A_i + \beta_G G_i + \beta_{TA} T_i * A_i + \beta_{TG} T_i * G_i + \beta_{AG} A_i * G_i + \beta_{TAG} T_i * A_i * G_i$$

where for individual i at gene j, T_i describes treatment where control is the baseline and the treatment (cold or heat) is the alternate set, A_i is the allele where the baseline is B73 and the alternate is non-B73, G_i represents the generation where the baseline is the inbreds (F_0) and the alternate is the hybrid (F_1). In order to test whether any factor or interaction between factors were significant

we applied nested likelihood ratio tests (LRT) and used FDR corrected *P*-values (<0.01). We tested the significance of all possible individual factors and interactions beginning with the highest order interaction. If the highest order interaction was not significant, we dropped that term and tested each subsequent two-way interaction while controlling for the other two interactions. If none of the two-way interactions was significant, we tested for each individual factor while controlling for the other two individual factors.

GO analysis

Genes that were up-regulated in response to cold or heat for each genotype were independently run through the BinGO plug-in from Cytoscape to assess enrichment of biological processes (Maere *et al.*, 2005). The top Arabidopsis BLAST hit for each gene was used to run the enrichment analysis using the default statistical options and *Arabidopsis thaliana* database. The entire list of the enriched biological processes for each genotype was combined and a non-redundant (NR) list was created. The corrected *P*-value for each inbred was pulled for the top 25 most significant NR GO categories.

Transcription factor and motif enrichment

The entire set of available characterized maize transcription factors (TFs, Yilmaz *et al.*, 2009) was used to test whether certain TF families were associated with stress-induced DE genes. Enrichment of TFs were calculated by contrasting the number of up-regulated genes in at least three genotypes under cold or heat treatments to the expected number (ratio of genes that are up-regulated in at least three genotypes in the entire filtered gene set). A chi-squared test was used to determine which families were statistically enriched ($\chi^2 < 0.01$) in our up-regulated gene sets.

The Find Individual Motif Occurrences (FIMO, Grant *et al.*, 2011) tool, which is part of the MEME suite (version 4.4, Bailey and Elkan, 1994) was used to count the number of DRE/CRT, ABRE, HSE, NACR, WRKY, MYBR, MYCR, and LTRE motifs throughout the B73 reference genome. The number of motifs per bin upstream, downstream, and within each gene was calculated by summing the number of motifs overlapping the specified bin using the BEDTools (version 2.25.0, Quinlan and Hall, 2010) window tool. Each motif was counted per bin (5–2.5, 2.5–1, and 1 kb for both 5′ and 3′ ends) and within the gene for up-regulated genes in response to cold or heat and across the entire filtered gene set. Enrichment of motifs was calculated for each bin by contrasting the proportion of up-regulated genes that have at least one motif in a particular bin to the proportion of genes that have at least one motif in that bin in the genome. A chi-squared test was run to see if the observed number of motifs per bin was statistically different from expected.

Differential expression

Raw read counts were run through the DESeq package in R (Anders and Huber, 2010) to run statistical tests for differential expression between control and stress (stress-induced DE) for each parent comparing control to cold or heat as well as between inbred genotypes within each condition (genotype DE). Differentially expressed genes were identified by having an adjusted *P*-value (FDR) <0.01, an RPM >0.5 for both parents (genotype DE) or in control and stress (stress-induced DE), and at least a two-fold difference in expression between genotypes (genotype DE) or between control and stress (stress-induced DE). Genes that turned 'on' or 'off' in response to stress have an FDR < 0.01 and exhibited a 10-fold difference in expression between genotypes (genotype DE) or between control and stress (stress-induced DE).

Identifying *cis*- and *trans*-regulatory variation

Genotypic regulatory variation. Each gene that is DE between two inbred parents was run through independent chi-squared tests to test for *cis*- and *trans*-regulatory variation. Genes that exhibit *cis*-regulatory variation will show a biased allelic proportion in the F_1, where the expected value is the total number of allele-specific reads for a given condition multiplied by the proportion of the parental alleles, and the observed values are the number of allele-specific read counts for both alleles in the F_1. The test for *trans*-regulatory variation used the total number of allele-specific reads in the hybrid divided by two for the expected value and the same observed values as the test for *cis*-regulatory variation. Genes that are not statistically different than expected for *cis*- or *trans*-regulatory variation patterns ($\chi^2 > 0.01$) were categorized as examples of *cis*- or *trans*-regulatory variation.

Stress-responsive regulatory variation. Genes that exhibited variation in response to stress between two inbreds were used to assess the contribution of *cis*- and *trans*-regulatory variation. A chi-squared test was applied to these gene sets to test for *trans*-regulatory variation patterns. If the proportion of alleles in the control F_1 sample was not statistically different from the proportion in the stress F_1, then those genes would be classified as *trans*-regulatory variation. Genes whose allelic proportion differed between the two F_1 samples would be classified as *cis*-regulatory variation examples. Genes with allelic bias were further binned into genes whose allelic bias is in the direction we would predict given parental expression values or toward the opposite allele.

ACCESSION NUMBERS

The RNA-seq reads used in this study are deposited at the National Center for Biotechnology Information Sequence Read Archive under project numbers PRJNA244661.

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AUTHOR CONTRIBUTIONS

A.J.W., I.M. and N.M.S. designed the research, A.J.W., J.N., and I.M. preformed the research, C.N.H, C.D.H. and L.B. contributed analytic/computational tools, A.J.W. and J.N. analyzed the data, and A.J.W. and N.M.S. wrote the article.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. A subset of transcription factors and regulatory motifs are enriched in cold and heat DE gene sets.

Figure S2. Transcriptional responses in maize seedlings exposed to heat or cold treatment.
Figure S3. Genotype or treatment explain most of the variation observed in ANOVA analysis.
Figure S4. Classification of differentially expressed genes into regulatory variation categories for additional trios.
Figure S5. Conservation of *cis*- and *trans*-regulatory variation calls.
Figure S6. Cis- and *trans*-regulatory variation genes exhibit similar fold change between stress and control.
Table S1. Sequencing depth for samples used in this study.
Table S2. GO enrichments for up-regulated genes in response to cold treatment.
Table S3. Enrichment of GO categories for genes that are up-regulated in response to heat stress.
Table S4. Distribution of *cis*- and *trans*-regulatory variation calls for genes DE between genotypes within a condition.
Table S5. Proportion of genes that have *cis*-elements within 5 kb upstream and have SNPs within elements.
Table S6. Stress response genes that are characterized transcription factors.
Dataset S1. Gene and allele-specific expression counts for all samples used in the study. For each gene the reads per million (RPM) and allele-specific counts are provided for each of the RNA-seq samples.

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