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Paternal Dominance of Trans-eQTL Influences Gene Expression Patterns in Maize Hybrids

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Heterosis refers to the superior performance of hybrid progeny relative to their inbred parents, but the mechanisms responsible are unknown. Hybrids between the maize inbred lines B73 and Mo17 exhibit heterosis regardless of cross direction. These reciprocal hybrids differ from each other phenotypically, and 30 to 50% of their genes are differentially expressed. We identified ~4000 expression quantitative trait loci (eQTL) that allowed us to identify markers linked to variation in expression. We found that over three-quarters of these eQTL act in trans (78%) and that 86% of these differentially regulate transcript accumulation in a manner consistent with gene expression in the hybrid being regulated exclusively by the paternally transmitted allele. This result suggests that widespread imprinting contributes to the regulation of gene expression in maize hybrids.

Heterosis, the enhanced agronomic performance of a hybrid relative to its inbred parents (*1*), is widely exploited, but the underlying molecular mechanisms have not been deciphered (*2*). There are widespread differences in gene expression in the maize Mo17 × B73 hybrid relative to its inbred parents, B73 and Mo17 (*3*–*5*). Because maize is monoecious, with a physical separation between the male and female flowers on a single plant, any given plant can be used as both male and female parents of hybrids. Reciprocal B73 × Mo17 (female × male) and Mo17 × B73 hybrids are both highly heterotic but, despite having identical nuclear genomes, exhibit statistically significant differences in development (table S1). Reciprocal effects on phenotypes have been documented in plants (*6*) but have not been widely investigated at the molecular level (*7*, *8*).

Genome-wide transcript accumulation in the B73 × Mo17 and Mo17 × B73 hybrids was measured with a cDNA microarray (*9*), and the analysis identified 1515 (~11%) significantly differentially expressed genes with a 5% false discovery rate (FDR) cutoff (table S2). Similar results were obtained from an independent RNA-sequencing experiment (tables S2 and S3). Although the magnitude of these effects (expression fold changes) were modest (fig. S1), we estimated (*9*) that >50% (N = 7325/14,118) of the genes on the array and ~33% (N = 871/2640) of the highly expressed genes in the RNA sequencing experiment were differentially expressed. Smaller proportions of genes showing reciprocal expression effects have been previously reported in several species (*10*–*13*).

Expression QTL (eQTL) experiments (*14*) are typically conducted with recombinant inbred lines (RILs) or other inbred genotypes and thus are unable to determine the effects of heterozygosity on gene expression patterns (*10*). We examined eQTL in a set of 29 IBM (intermated B73 × Mo17) RILs derived from a cross between the maize B73 and Mo17 lines and hybrids generated by crossing each RIL onto B73 (B73 × RIL) and Mo17 (Mo17 × RIL). These hybrids provided a contrast between gene expression regulation at heterozygous and homozygous genotypes across all loci that are polymorphic between B73 and Mo17 (Fig. 1A).

Separate eQTL analyses were conducted within each cross type (B73 × RIL, Mo17 × RIL, and RIL) by scanning the genome with 1064 highly informative markers (*9*) and comparing the genetic map positions of differentially regulated genes relative to the genetic positions of the regulating eQTL. We defined an eQTL as acting in cis if a regulated gene and its corresponding eQTL were within 5 cM of each other. Trans-eQTL were defined as those located on different chromosomes than the genes they regulate.

P values and estimated FDRs (*9*) resulted in 1334 to 1904 significant eQTL associations within each cross type (table S4). About 25% of the significant genes were regulated in multiple cross types. In the majority of such cases, the same genomic region regulated the gene among multiple cross types (table S5 and figs. S2 and S3).

Only 10% of the detected eQTL acted in cis, which on average showed larger effects than the trans-eQTL (table S4 and fig. S4), consistent with previous reports for maize (*4*, *14*) and other species (*15*). Trans-eQTL with large effects may be rare because they can regulate many genes or entire pathways that, if substantially up- or down-regulated, could be detrimental (*16*, *17*). Many cis-regulated genes detected within the RIL cross type were previously (*5*) identified as differentially expressed between the B73 and Mo17 inbred lines (79/114) and exhibited consistent directions of effect in the current study. Because the RILs used for this study were mosaics of the B73 and Mo17 genomes, this validates the stability of these cis-eQTL across genetic backgrounds.

Nearly 80% of the eQTL acted in trans (table S4 and fig. S3), consistent with reports from other species (*15*, *18*–*23*) and of trans-regulation of diverse biological processes in maize (*24*–*28*). Previous studies may have overestimated the number or proportion of cis-regulated genes (*3*, *29*) due to ascertainment bias because they only analyzed genes containing single nucleotide polymorphisms between B73 and Mo17 and/or had limited statistical power to detect the more subtle effects of trans-eQTL (*15*).

Clustering of expression patterns of all genes regulated by eQTL distinguished cis- and trans-regulated genes (Fig. 1B). Most (93%) genes with additive gene action are cis-regulated and have similar amounts of expression in the two heterozygotes (Mo17 × RIL_{BB} and B73 × RIL_{MM}; clusters 1 and 2, Fig. 1, A and B). Instead of showing additive gene action, most (86%) trans-eQTL exhibit a mode of gene action we term "paternal dominance" that is consistent with imprinting, wherein expression values in those heterozygotes with a RIL_{BB} as the paternal parent matched expression values in lines that were homozygous for the B allele of the trans-eQTL; similarly, heterozygotes with a RIL_{MM} as the paternal parent had expression values that matched the expression values in lines that were homozygous for the M allele of the trans-eQTL (i.e., MB was not equal to BM; clusters 3 and 4, Fig. 1B). Because our experimental design held the maternally contributed allele in hybrids constant, we could detect variation in paternally contributed alleles (Fig. 1A) while controlling for maternal or cytoplasmic effects. Hence, these results suggest that gene expression in the hybrids is regulated exclusively by the paternally transmitted allele of these trans-eQTL.

An eQTL marker (interval 377) associated with the differential regulation of more than 20 genes was back-crossed into the Mo17 inbred genetic background for multiple generations (fig. S5). Seedlings of the BM and MM genotypes generated at this interval in genetic backgrounds that were otherwise 87.5% homozygous for the Mo17 genotype showed significant differences in expression that were consistent with patterns observed in the eQTL analysis. Thus, this trans-eQTL is stable across generations and technologies (table S6).

Because no direct comparisons were performed between B73 × RIL_{MM} and Mo17 × RIL_{BB}, we validated the unusual paternal dominance patterns of gene expression for five genes regulated in trans by interval 377 by using a combination of quantitative reverse transcription polymerase chain reaction and Sequenom (Sequenom, San Diego, CA) quantitative gene expression (QGE) assays (table S6).

It is not possible to conclude how widely distributed paternal dominance is across species because previous studies have most commonly been performed with only inbred genotypes; such

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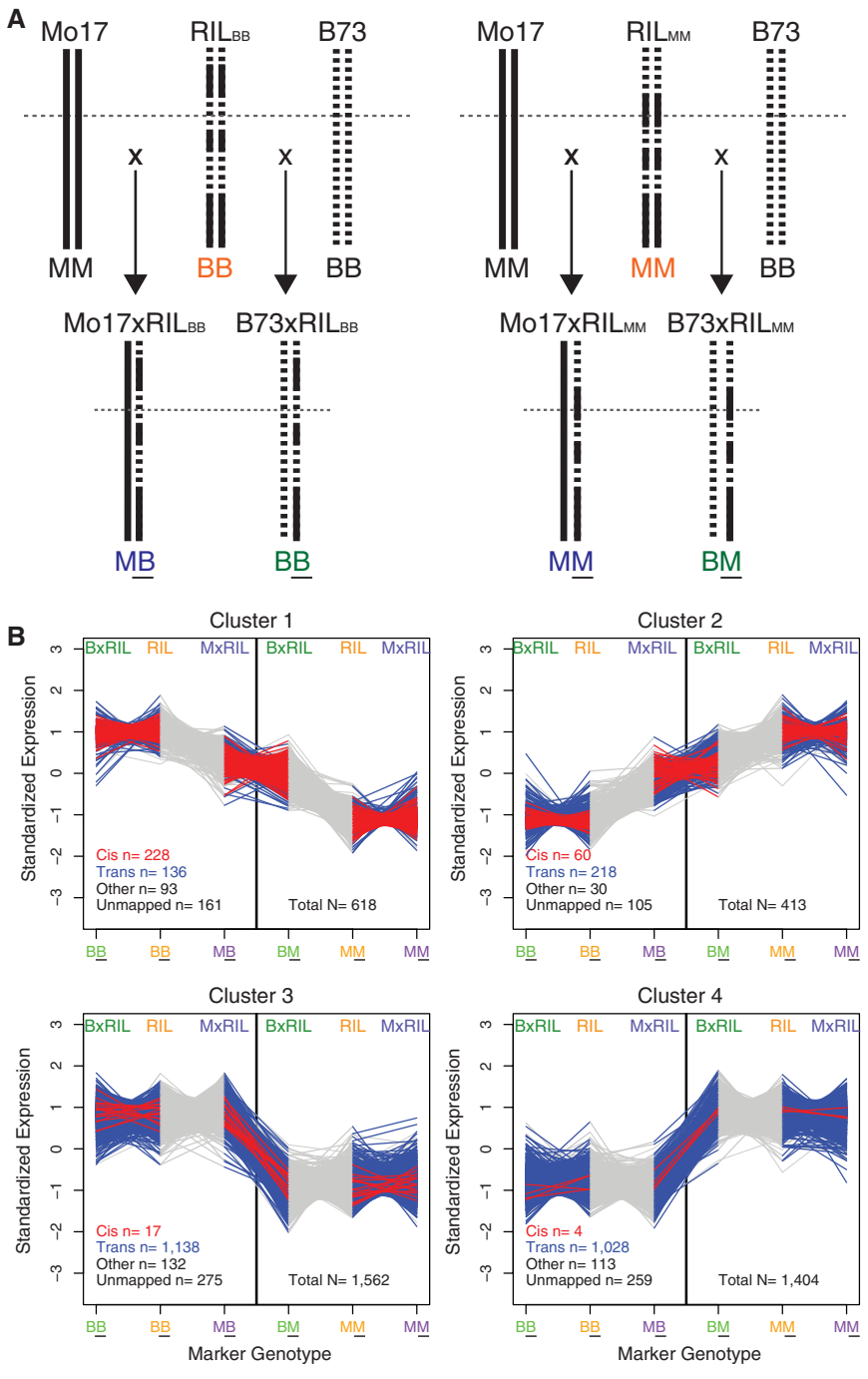

Fig. 1. Cis- and trans-regulated genes exhibit different patterns of expression. (**A**) Because the inbred IBM RILs are derived from a cross between B73 and Mo17, a given IBM RIL is homozygous for either the B (B73) or M (Mo17) allele of a given marker (denoted by a gray dashed line). We designate such RILs as RIL_{BB} and RIL_{MM}, respectively. When crossed onto B73 (BB), RIL_{BB} and RIL_{MM} RILs will generate hybrids that are homozygous and heterozygous for the marker in question, respectively. Similarly, when crossed onto Mo17 (MM), RIL_{BB} and RIL_{MM} RILs will generate hybrids that are heterozygous and homozygous for the marker in question, respectively. Thus, each cross type provided mean expression values for each of two genotypic groups. The allele inherited from the paternal parent is underlined. (**B**) Standardized expression values (*y* axis) were clustered to group genes with similar expression patterns. For clustering purposes, each nonredundant gene-marker association ($N = 3997$) was represented by a vector of six expression means. The four resulting clusters were plotted according to the genotype of the associated regulating eQTL marker (*x* axis). Red lines denote cis-eQTL, and blue lines denote trans-eQTL. The cross type in which the expression was measured is indicated at the top of each plot (B73 × RIL, green; RIL, orange; and Mo17 × RIL, purple), and the allele inherited from the paternal genome is underlined in the *x*-axis labels. Clusters 1 and 2 both show additive gene action but differ as to whether lines homozygous for the B allele of an eQTL locus show higher expression than lines homozygous for the M allele or vice versa. Clusters 3 and 4 both show paternal dominance but have opposite modes of gene action as described for clusters 1 and 2.

experiments cannot define the mode of gene action and cannot detect genomic imprinting because the effects of heterozygosity and reciprocally generated genotypes are not typically investigated. If we had only examined one of the two hybrid cross types, the gene action of the paternally dominant trans-eQTL would have incorrectly been classified as Mendelian dominant. Lastly, sufficient statistical power is needed to detect the modest effects of trans-eQTL.

We hypothesize that at least some paternally dominant trans-eQTL are small RNAs, because small RNAs regulate gene expression in trans (*30*, *31*) and can be subject to parent-specific genomic imprinting (*32*, *33*). Because there are many paternally dominant trans-eQTL in maize, and many of these regulate multiple genes, their effects are broadly propagated throughout the transcriptome. Paternal dominance may, therefore, contribute to the observed phenotypic differences between reciprocal hybrids.

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Supporting Online Material
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Materials and Methods
Figs. S1 to S6
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Symbiotic Nitrogen Fixation in the Fungus Gardens of Leaf-Cutter Ants

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Bacteria-mediated acquisition of atmospheric N_2 serves as a critical source of nitrogen in terrestrial ecosystems. Here we reveal that symbiotic nitrogen fixation facilitates the cultivation of specialized fungal crops by leaf-cutter ants. By using acetylene reduction and stable isotope experiments, we demonstrated that N_2 fixation occurred in the fungus gardens of eight leaf-cutter ant species and, further, that this fixed nitrogen was incorporated into ant biomass. Symbiotic N_2-fixing bacteria were consistently isolated from the fungus gardens of 80 leaf-cutter ant colonies collected in Argentina, Costa Rica, and Panama. The discovery of N_2 fixation within the leaf-cutter ant–microbe symbiosis reveals a previously unrecognized nitrogen source in neotropical ecosystems.

Ants play a critical role in shaping terrestrial ecosystems. They make up at least one-third of the global insect fauna biomass and 86% of the arthropod biomass in tropical forest canopies, and, in the Amazon forest, they represent four times more biomass than do all land vertebrates combined (*1*–*3*). Among ants, the leaf cutters (tribe Attini: genera *Atta* and *Acromyrmex*) play an important role as one of the most dominant herbivores in New World tropical ecosystems, stimulating new plant growth and facilitating nutrient cycling (*4*). Mature *Atta* colonies are among the largest of any social insect, consisting of up to 8 million workers and occupying an underground volume of more than 20 m^3 (Fig. 1, A and B) (*5*). These "superorganisms" harvest more than 240 kg dry weight of leaf material per year (*4*), which they use to cultivate a fungus for food (*6*). This ability to grow a specialized fungal crop using freshly cut plant material is a key factor in the ecological success of leaf-cutter ants (*7*). In addition to their relationship with fungal mutualists (family Lepiotaceae), the ants engage in a second mutualism with Actinobacteria (genus *Pseudonocardia*), which produce antibiotics to help defend the fungus garden from parasites (*8*, *9*). We explored the possibility that leaf-cutter ants engage in mutualistic associations with N_2-fixing symbionts to supplement the nitrogen budget of their fungus gardens.

Nitrogen is expected to be a growth-limiting resource in leaf-cutter ant agriculture: The primary nutrient input into their colonies is fresh leaves, which have a much lower nitrogen-to-carbon (N:C) ratio than is required by insects (*10*, *11*). In contrast to this expectation, several field studies have shown that the exhausted leaf substrate removed from the bottom of fungus gardens by ant workers contains higher proportions of N than either freshly harvested leaf material or surrounding leaf litter does, indicating that N enrichment occurs as the plant substrate is processed by the colony (*4*, *12*, *13*). Although these findings suggest the presence of N_2-fixing symbionts, potential additional sources are mineralized N from the soil and compensatory feeding by the ants (*13*, *14*). We analyzed the N content of laboratory-maintained colonies of *Atta cephalotes* in which we prevented N input from these alternate sources (*15*). We found an increase in N content as leaf substrate passes through the system: N content was lowest in fresh leaf cuttings, significantly higher in the fungus

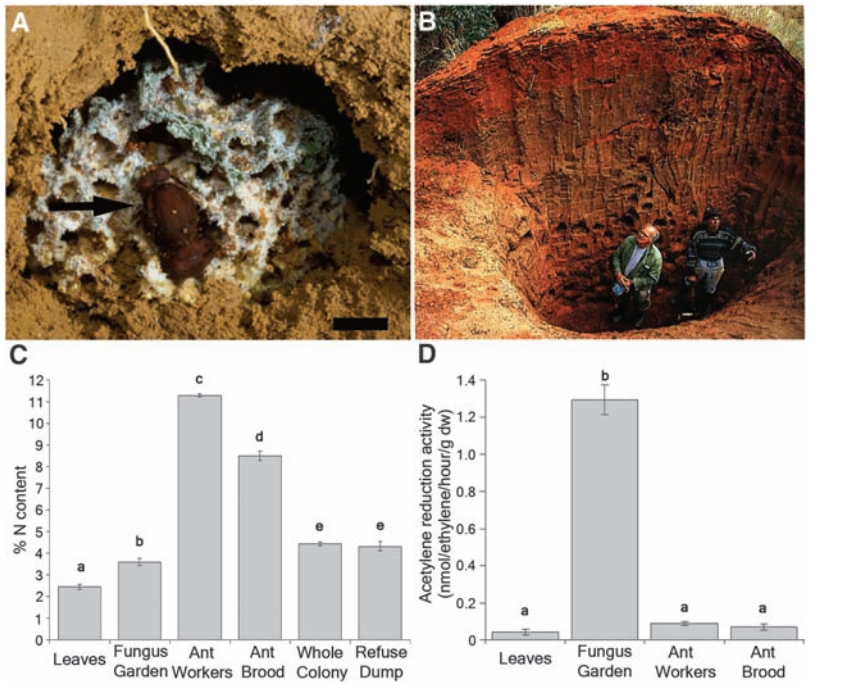

Fig. 1. Evidence for N_2 fixation in the fungus gardens of leaf-cutter ants. (**A** and **B**) The agricultural system of leaf-cutter ants is extremely efficient, allowing colonies to grow from a single fungus garden chamber [(A) incipient *At. cephalotes* colony with queen (black arrow) on top of fungus garden; scale bar = 1 cm] to a massive underground operation with hundreds of chambers, intricate tunnel systems, and millions of workers [(B) partially excavated nest of a mature *Atta* colony]. (**C**) Nitrogen content of the different components of five *At. cephalotes* colonies. (**D**) N_2-fixation activity measured by acetylene reduction for different components of 10 *Atta* spp. colonies. All results are shown as means ± SEM. Means labeled with different letters (a to e) are statistically different ($P < 0.05$). [Photo credits: (A) Graham D. Anderson, (B) M. Moffett/Minden Pictures]

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