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Report

Equivalent Parental Contribution to Early Plant Zygotic Development

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Summary

Hybrid vigor or heterosis results from the combination of genetically distant genomes at fertilization, and as well as being of major commercial importance, it is held to contribute significantly to fitness [1]. Activation of the paternal genome marks the transition from maternal to zygotic control of development, but a reported delay of paternal-genome activation in flowering plants [2–4] and animals [5, 6] excludes heterosis from impacting on very early development. We have analyzed the allele-specific expression of 25 genes after fertilization of the egg in maize and show immediate equivalent parental genomic contribution to the zygote. Every gene expressed before the first cell division of the zygotes showed paternal transcripts. Sequence comparisons indicate that these genes are involved in a range of processes and are distributed throughout the genome. Our findings confirm that some plant species have evolved a strategy to activate the paternal genome immediately after fertilization, in contrast to the situation in other plants and in animals. Such an extensive activation of the paternal genome very early in development is consonant with observations of high levels of heterosis in early hybrid maize embryos [7, 8], indicating a significant impact of this sexual strategy on fitness.

Results and Discussion

After fertilization, the activation of both maternal and paternal genomes signifies the assumption of true zygotic control over development. However, results to date from *Arabidopsis* and maize suggest that seed development is largely maternally regulated for several days after fertilization [2–4]. This cannot hold for all genes because embryos of *knolle keule* double mutants of *Arabidopsis* can be rescued by wild-type pollen at the two-cell stage [9], and the expression of the highly sensitive transgenic marker gene *BARNASE* can be detected at this stage [3]. Nevertheless, these examples of paternal expression were assumed to be exceptions to a general rule that is otherwise supported by the identification of a number of genes preferentially maternally expressed during *Arabidopsis* embryo and endosperm development [10]. The situation might differ in maize, where the expression of paternally inherited transgenes in zygotes and early endosperm indicates contributions from both parental genomes on early kernel development [11]. However, recent allele-specific reverse transcription-polymerase chain reaction (RT-PCR) experiments in maize have failed to detect paternal transcripts for up to 3 days after fertilization [4]. Taken together, current gene expression data thus point to the predominantly maternal control of early plant embryo development, as it is the case in animals [5, 6].

Such uniparental dominance in early embryogenesis fits uncomfortably with studies of hybrid vigor. For example, hybrid maize embryos at 8 [7], or even 6 [8] days after pollination (dap) display striking increases in vigor, confirming a major contribution of both parental genomes to this early phase of kernel development. To resolve this apparent paradox, we compared inbred lines (UH005, UH301) with their corresponding reciprocal hybrids (005×301, 301×005; the maternal parent is denoted first), generating morphological data by confocal laser-scanning microscopy (CLSM) of 6 dap embryos stained to reveal cell walls (Figure 1A). The analysis of this material indicated that the increased size of the hybrid embryos resuletd from an increased cell number, rather than from cell expansion—as occurs during maize root growth after germination [12]. Because all embryos originated from a single-celled zygote and because embryo length and cell number were tightly correlated for all four combinations of genotypes (Figure 1B), the size differences between the hybrid and inbred embryos can only be explained by a higher rate of cell division. Further confirmation comes from the striking lack of significant differences in mean cell sizes between hybrids and their respective maternal lines (see Tables S1 and S2 in the Supplemental Data available online). Although the molecular basis of heterosis is not understood, heterotic traits clearly stem from the coexpression of two genetically diverse genomes [1]. Because tremendous differences in size between inbred and hybrid embryos were evident at 6 dap, the developmental changes that determined the higher rate of cell proliferation must have occurred at an earlier stage—pointing to a major contribution by the paternal genome very early in development.

To explore this possibility of a significant paternal involvement in early embryogenesis, we measured the relative abundance of allele-specific transcripts for 25 genes in zygotes at 1 dap (before the first cell division), in embryos at 3 dap (after three to four rounds of cell division), and in embryos at 6 dap (embryos consisting of some 100 cells) with primer-extension assays and then matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry [13]. Maternal transcripts from surrounding tissue were eliminated by the microdissection of all samples. To distinguish between parental and allele-specific effects of the two inbred lines, we analyzed reciprocal hybrids for all stages. Initial gene selection was based on copy DNA (cDNA) microarray-derived gene expression patterns associated with

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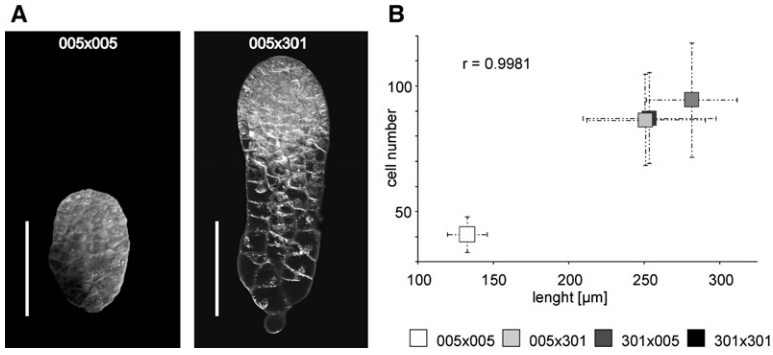

Figure 1. Enhanced Growth Rate of maize F1 Hybrids

(A) Midplane confocal images of cell wall stained UH005 inbred and 005×301 hybrid embryos, 6 days after pollination (dap). Scale bars represent 100 μm.

(B) Correlation between cell number and length of hybrid and inbred embryos 6 dap. The mean embryo length of inbred line UH005 and UH301 and reciprocal hybrids plotted against mean cell numbers of the same genotypes is shown. The standard deviation is given by error bars. A strong correlation of both features was found by PEARSON analysis.

heterosis at 6 dap (S.M. and S.S., unpublished data). Genes with expression levels ranging from differential to near equal between the parental genotypes were randomly chosen so that a bias resulting from allele specific regulatory effects could be avoided. For 20 genes selected on the above criteria, we identified suitable polymorphic sites to facilitate allelic discrimination. Quantitative, real time RT-PCR (QPCR) confirmed the diversity of expression differences in extent and direction between the parental lines (Figure S1). Furthermore, we identified suitable polymorphic sides to assay the allelic contribution for another five genes. In a previous study, paternal expression of four of these genes (DR798944, AW066927, DT649185, and AB073081) was not detectable in maize ovules at 3 days after pollination [4]. The fifth gene (AJ313531) encodes ZmPRL, a maize homolog of the *Arabidopsis* PRL with 77% amino acid identity [14].

Paternal transcripts of all 24 active genes were detected in the zygotes, demonstrating distinct transcriptional activation of the paternal genome before the first zygotic division (Figure 2). Transcripts of one gene (AB073081) were first detectable at 6 dap. Strong differences in expression between the parental lines UH005 and UH301 detected by QPCR correspond for four genes (DW475393, EH038219, EH038217, and EH038211) with predominant expression of one allele in zygotes and early hybrid embryos, irrespective of the parental origin. Interestingly, for another two genes (EH038207, EH038206) with strong expression differences between the parental lines (Figure S1), the exclusive expression of the UH005 allele occurred in both reciprocal hybrids (Figure 2). These data thus confirm an equivalent transcriptional potential for each of the parental genomes, because the alleles themselves determine transcript levels in the hybrid state, irrespective of their parental origin. Our observations that paternal transcripts of all active genes were present in the early embryo clearly challenge the current model of predominant maternal allelic expression during early seed development in maize [4]. Contrary results for the repeated analyses of four genes (DR798944, AW066927, DT649185, and AB073081) most probably reflect the employment of different experimental strategies. Comparing the early parental expression of the *Arabidopsis* and maize homologs of the DNA replication licensing factor MCM7, a highly conserved protein found in all eukaryotes, provide an indication for species specific differences. The *Arabidopsis* homolog, *PRL,* was shown to be exclusively maternally expressed until 3 dap [2]. In contrast, we detected equal amounts of paternal and maternal transcripts of the maize homolog *ZmPRL* (AJ313531), in zygotes at 1 dap and further embryo development at 3 and 6 dap.

Parental effects on transcription were determined by the comparison of allele-specific transcript abundance of reciprocal hybrids, which are genetically identical except for the reversed parental origin of the alleles. Expressed in terms of the relative expression of one parental allele, the parental effect can range from 100% for exclusive expression to 0%, if the relative expression level of the alleles is unaffected. Whatever the level of paternal activity or inactivity, a preponderance of maternal transcripts will always be expected in the very young zygotes because the male genome must require time to decondense to acquire transcriptional competence [11, 15]. Certainly, both gametes store transcripts [16, 17], but egg cells massively exceed sperm cells in size [18]. The discovery that the expression of as many as 13 of the 25 genes analyzed showed no significant parental effect is thus surprising. Even the maternal effect is smaller than anticipated, with ten genes showing predominantly maternal allelic expression in zygotes, but with levels ranging between 8% and 37.4% (mean 19.8%). The unexpectedly low level of maternal transcript predominance signifies a high level of transcriptional activity by paternal alleles prior to the first division of the zygote. Indeed, one gene was expressed at a significantly greater level (3.2%) from the paternal allele. At 3 dap, the maternal predominance decreased, with just eight genes showing a higher level of maternal transcription at a mean level of 15.9%. At this time point, the paternal allele from one gene generated relatively more transcripts, which in turn caused an effect of 26.4%. By 6 dap, any parental effect was negligible, with five genes showing a maternal and two a paternal effect, with means of 7.8% and 6.2%, respectively. These data, summarized in Figure 3, confirm that parent-specific transcription is exhibited by only a small subset of genes during early embryo development in maize, and that this effect decreases within the first 6 days of development.

To investigate whether a particular subgroup of genes is involved in this pattern of early paternal activation, we studied sequences with the GenBank nucleotide and protein databases (Table 1). Sequences from six genes showed no similarities to sequences in the database, whereas the remaining 19 genes were predicted to have a range of functions. Some were putatively involved in basic cellular functions, such as intracellular transport, amino acid metabolism, or translation; others

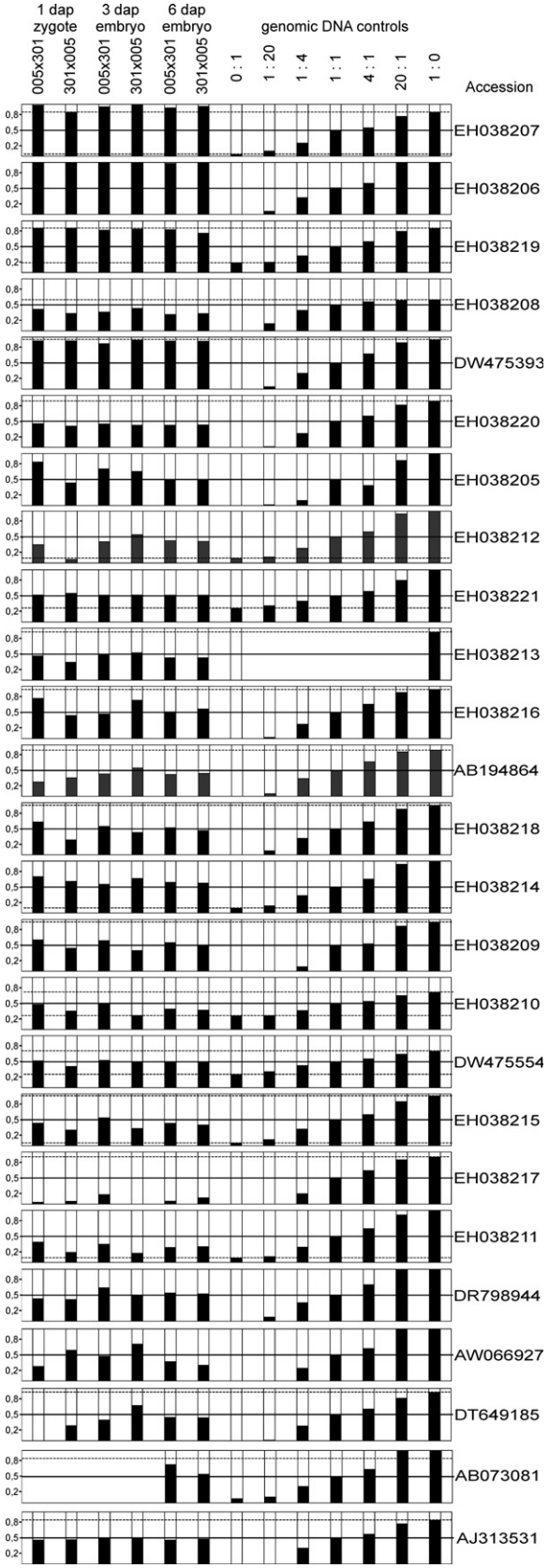

Figure 2. Parental-Genome Activities in Early Embryo Development

Relative allelic expression levels of 25 genes in the reciprocal F1-hybrid embryos 005×301 and 301×005 (the maternal line is denoted first) at 1, 3, and 6 dap. Transcript or genomic DNA amounts of both alleles were measured and displayed as a percentage of the total expression level. Black and white bar fractions indicate transcript abundance of the 005 and the 301 allele, respectively. The y axis scale refers to the portion of the 005 allele. Mixes of gDNA [1:20, 1:4, 4:1, 20:1] of the inbred lines [005:301] were used so that the assay performance concerning allelic ratio reproduction could be controlled. Inbred line gDNA 005 [1:0] and 301 [0:1] was used for the definition of the thresholds of each assay for both alleles. The detection limits are indicated by dashed lines. Hybrid gDNA [1:1 allelic ratio] was used for the normalization of the data for each assay. Continuous lines indicate 50% allelic proportion. For EH038213, cDNA of inbred lines served to define the allelic thresholds, and expression data were not normalized. For gDNA mixes [1:20, 1:4, 4:1, 20:1] of inbred lines one, for cDNA samples, hybrid gDNA [1:1] and both inbred gDNAs [1:0, 0:1] three independent replicates were analyzed by four mass spectrometry runs for analyte detection, and the mean values are displayed.

were predicted to encode signal-transduction proteins, transcription factors, or chromatin-remodeling factors. For ten sequences, chromosomal locations were identified by near identity to mapped B73 BAC sequences, and these genes were found to be sited on six out of the ten chromosomes of *Zea mays* (Table 1). The genes analyzed thus do not represent members of a specific family of proteins and are distributed across the *Zea mays* genome. Further, their expression pattern is not restricted to early embryo development; all sequences analyzed are expressed throughout development in diverse tissues such as leaves, roots, seedlings, and unpollinated ears (Figure S2).

Double fertilization in higher plants gives rise to an embryo and an endosperm. The functional relationship between these two structures parallels that of the placenta to the fetus in mammals, in that the endosperm nourishes the developing embryo [19]. The genetics of the endosperm are complicated because not only is the tissue triploid, but its development is also regulated by genetic mechanisms held to improve the evolutionary fitness of angiosperm sexual reproduction [20]. For example, one of the several evolutionary drivers behind imprinting, the uniparental allelic expression of particular genes [21], is regarded as the extension of maternal control over early seed development. The delayed, or at least attenuated, activation of the paternal genome in *Arabidopsis* has been proposed to prolong the maternal haploid phase [2, 19] to act as a global form of imprinting in both embryo and endosperm. The behavior of paternally encoded transgenes in *Arabidopsis* with generally late and attenuated expression [3] is supportive of such a mechanism. Whatever the system operating in *Arabidopsis,* our data presented here and the near immediate and strong expression of paternally encoded transgenes [11] confirm that no such extension of the maternal haploid phase occurs in maize, suggesting that fundamental differences in these mechanisms exist between plant species.

The rationale for these differences is unclear. It might be associated with the transitory nature of the dicotyledonous endosperm, which requires a higher degree of maternal control during its rapid development and demise in the young seed [19, 22]. Alternatively, it might relate to differing sexual strategies. For example, *Arabidopsis* is an inbreeder and self pollinates at a high frequency [23], resulting in high levels of

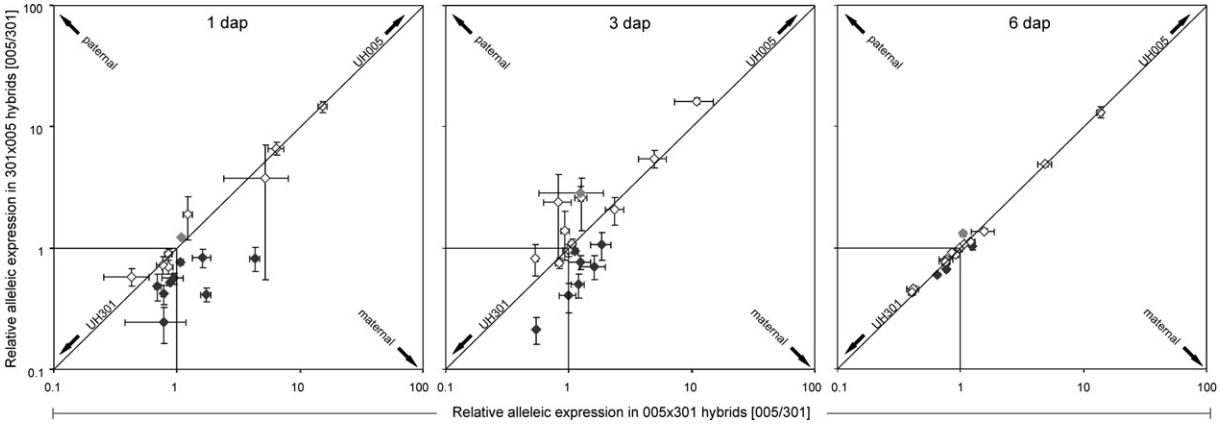

Figure 3. Equivalent Parental-Genome Activities in Early Embryo Development

Relative allelic expression ratio from hybrids of crosses of maize inbred line UH301 females and inbred line UH005 males (005/301, y axis) plotted against ratio of hybrids from the reciprocal crosses (005/301, x axis). Expression ratios are plotted on a logarithmic scale, with error bars indicating 95% confidence intervals. 005/301 = 1 demonstrate equal expression of both alleles; the y = x line highlights the relationships without parental effects. Maternal or paternal effects lead to opposed deviation from the line (marked by arrows). The deflection of y = x = 1 along the y = x line specifies UH005 or UH301 allelic preference, also indicated by arrows. Points are grayscale coded according to significance of a two-sided Student's t test, where H_0: allele 005 (301×005) = allele 005 (005×301). No parental influence (white boxes), paternal influence ($p < 0.01$) (gray boxes), and maternal influence ($p < 0.01$) (black boxes) are indicated. EH038212, EH038217, EH038207, AW066927, DT649185, AB073081; and EH038206 are not displayed because of monoallelic or absent expression, at least at one time point.

homozygosity—under which circumstance early activation of the paternal genome would confer relatively little advantage. In contrast, maize is an outcrossing species and, in the wild, possesses high levels of heterozygosity. The early exploitation of this heterozygosity in the form of increased hybrid vigor might significantly improve evolutionary fitness [1].

In animals, maternal gene products drive early development while the newly formed embryo is transcriptionally inactive, and the activation of the embryonic genome is accompanied by the degradation of maternal transcripts [24]. These mechanisms might also function in plants, and maternally provided transcripts might dominate early stages. However, our data indicate that some plants have evolved mechanisms to permit paternal genomes to contribute to development at an earlier stage than in animals. The earliest reported paternal-genome activation in animals occurs in the mouse at the two-cell stage [6, 25], with the uncoupling of transcription and translation preventing the translation of a small number of early paternal transcripts expressed at the one-cell stage [26]. By contrast, nascent paternal transcripts are effectively translated in maize zygotes within the first 6 hr after fertilization [11], with the main bulk of the paternal genome being transcriptionally active in zygotes.

The data reported here reveal that even prior to the first zygotic division, both parental alleles of many genes in maize are active. Together with earlier observations that nascent transcripts are effectively translated in maize zygotes, these findings provide evidence that maize evolved a different genetic mechanism than animals and other plant species to control embryo development in the initial phase after fertilization. The early paternal contribution is consonant with observations of high-level heterosis in early hybrid maize embryos, indicating a significant impact of this sexual strategy on fitness.

Experimental Procedures

Plant Material and Growth Conditions

Plant growth conditions and crossbreeding for hybrid production have been described [9]. Inbred lines UH005 (national listing of plant varieties [NLPV], accession no [AC]: M9379, European flint) and UH301 (NLPV AC: M8652, Iodent) were obtained from A. Melchinger, University of Hohenheim.

Laser-Scanning Confocal Microscopy

Embryos were isolated as described [9], fixed in 50% ethanol:formalin:acetic acid solution (90:2:5, per volume), and washed twice with 70% ethanol. Cell walls were stained with calcofluor white (3 mg/ml) and washed with 650 mosmol mannitol solution. The stained embryos were visualized with a confocal laser-scanning microscope CLSM-510META (C. Zeiss [Göttingen, Germany]). An argon laser line of 488 nm was used for excitation. CLSM-510META software was used for image acquisition and all size measurements.

Quantitative and Standard RT-PCR

Quantitative RT-PCR of inbred lines UH005 and UH301 6 dap embryos were performed with three independent biological replicates and normalized against *actin* expression as described [8]. Nonquantitative, standard RT-PCR experiments were performed the same way, except that an 18T oligonucleotide was used for the reverse transcription of mRNA of leaves, ears, roots, and tassels. The produced cDNAs were used as templates for PCR with 35 cycles. Primer sequences, designed with Lasergene software (GATC Biotech AG [Germany]) are available on request.

Detection of Sequence Polymorphism

Gene-specific oligonucleotides were designed with Lasergene software (GATC Biotech AG) and used for the amplification of cDNA of 6 dap embryos or genomic DNA of inbred lines UH301 and UH005. Primer sequences are available on request. The PCR products were sequenced. Sequences were aligned manually so that insertion, deletions, or SNPs could be identified.

Allele-Specific Expression Analysis

Isolation of mRNA and cDNA synthesis from three independent samples each of 20 to 25 zygotes, 3 dap embryos, and 6 dap embryos, isolated by microdissection, have been described [8, 17]. The genomic DNA of UH005, UH301, and the hybrid 301×005 was isolated from leaves of young seedlings as described [11]. All cDNA

Table 1. BlastX Sequence Similarity and Genomic Localization of the Genes Investigated

Accession	BlastX Sequence Similarity[a] (Accession Number)	E Value	Localization[b]
EH038207	None	-	Chromosome 4
EH038206	(→ DV492610.1) Putative helicase [Oryza sativa] (AAM76346.1)	4e-36	-
EH038219	None	-	-
EH038208	(→ AY107974.1) RNA polymerase Rpb1, domain 2 family protein [Oryza sativa] (ABA91105.2)	5e-09	Chromosome 5
DW475393	Plastid ATP/ADP transporter [Oryza sativa] (AAX58120.1)	2e-32	-
EH038220	Putative proliferating cell nuclear protein P120 [Oryza sativa] (BAD12915.1)	3e-36	Chromosome 4
EH038205	Mitochondrial elongation factor G [Oryza sativa] (AAK53868.1)	8e-160	-
EH038221	(→ CF636186.1) Nucleotid binding [*Arabidopsis thaliana*] (NP_189852.1)	9e-04	-
EH038213	Putative translation initiation factor eIF-2B epsilon subunit [Oryza sativa] (BAD22043.1)	1e-122	-
EH038212	ACT domain-containing protein [Oryza sativa] (ABA95833.1)	3e-76	-
EH038216	None	-	Chromosome 4
AB194864	CCAAT-binding transcription factor subunit B family protein [Oryza sativa] (ABA99882.1)	5e-27	-
EH038218	Chromatin-remodeling factor CHD3 [Oryza sativa] (AAL47211.1)	2e-60	-
EH038214	NAM-like protein [Oryza sativa] (BAD37987.1)	4e-22	-
EH038209	Putative brain and reproductive organ-expressed protein [Oryza sativa] (BAD53578.1)	1e-89	-
EH038210	(→ CO447404.1) Putative RING-H2 zinc finger protein [Oryza sativa] (AAP85546.1)	4e-09	Chromosome 2
DW475554	Adenosine kinase [*Zea mays*] (CAB40376.1)	2e-52	Chromosome 2
EH038215	Putative CBL-interacting protein kinase 1 [Oryza sativa] (BAA96628.1)	5e-25	Chromosome 3
EH038217	None	-	Chromosome 8
EH038211	None	-	-
DR798944	CDPK-related protein kinase [*Zea mays*] (BAA12691)	2e-54	-
AW066927	None	-	-
DT649185	Putative beta 1-3-glucanase [Oryza sativa] (AAO64485)	2e-76	Chromosome 1
AB073081	Legumin 1 [*Zea mays*] (AAL16994)	0.0	-
AJ313531	Replication licensing factor MCM7 homolog [*Zea mays*] (CAC44902)	0.0	Chromosome 1

[a] Accession numbers of sequences used for BlastX searches instead of the original sequence are indicated by "→".
[b] Chromosomal localization of BACs with near BlastN sequence identity (e value < $1e^{-42}$).

samples were shown to be free of genomic DNA (gDNA) contaminations by PCR with primers spanning introns (see Figure S3). All cDNA and gDNA solutions were adjusted to 100 ng/μl final concentrations previous to the use in relative allele-specific expression analyses on the MassARRAY system (Sequenom) by N. Storm at Bioglobe (Hamburg, Germany) applying the MassEXTEND (hME) biochemistry and MALDI-TOF mass spectrometry for analyte detection.

Parental effects were deduced by significance of a two-sided Student's t test, where H_0: allele 005 (301×005) = allele 005 (005×301). If $p > 0.01$, no parental influence is indicated. A paternal influence is given if allele 005 (301×005) > allele 005 (005×301), $p < 0.01$; a maternal influence is given if allele 005 (301×005) < allele 005 (005x301), $p < 0.01$. The number of measurements for each gene is given in Table S3.

Supplemental Data

Experimental Procedures, five figures, and four tables are available at http://www.current-biology.com/cgi/content/full/17/19/1686/DC1/.

Acknowledgments

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Accession Numbers

The 17 cDNA sequences reported in this paper have been deposited in GenBank with the accession numbers EH038205 to EH038221.