Maternal Gametophyte Effects on Seed Development in Maize

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ABSTRACT Flowering plants, like placental mammals, have an extensive maternal contribution toward progeny development. Plants are distinguished from animals by a genetically active haploid phase of growth and development between meiosis and fertilization, called the gametophyte. Flowering plants are further distinguished by the process of double fertilization that produces sister progeny, the endosperm and the embryo, of the seed. Because of this, there is substantial gene expression in the female gametophyte that contributes to the regulation of growth and development of the seed. A primary function of the endosperm is to provide growth support to its sister embryo. Several mutations in Zea mays subsp. mays have been identified that affect the contribution of the mother gametophyte to the seed. The majority affect both the endosperm and the embryo, although some embryo-specific effects have been observed. Many alter the pattern of expression of a marker for the basal endosperm transfer layer, a tissue that transports nutrients from the mother plant to the developing seed. Many of them cause abnormal development of the female gametophyte prior to fertilization, revealing potential cellular mechanisms of maternal control of seed development. These effects include reduced central cell size, abnormal architecture of the central cell, abnormal numbers and morphology of the antipodal cells, and abnormal egg cell morphology. These mutants provide insight into the logic of seed development, including necessary features of the gametes and supporting cells prior to fertilization, and set up future studies on the mechanisms regulating maternal contributions to the seed.

KEYWORDS maize; embryo sac; maternal effect; endosperm transfer layer; gametophyte

THE process of double fertilization is unique to flowering plants and results in the formation of the seed. The two sperm cells of the pollen grain fertilize the egg and central cell of the female gametophyte, or embryo sac, to form the diploid (1 maternal:1 paternal) embryo and typically triploid (2 maternal:1 paternal) endosperm, respectively (Sheridan and Clark 1994; Walbot and Evans 2003). The endosperm is thus a genetic sister of the embryo and is functionally equivalent to the mammalian placenta, acting as a nutritive tissue that supports the growth of the developing embryo and seedling. The maize endosperm consists of several morphologically and transcriptionally distinct domains: the aleurone, the basal endosperm transfer layer (BETL), the starchy endosperm, the conducting zone (CZ), the basal intermediate zone (BIZ), and the embryo-surrounding region (Olsen et al. 1999; Olsen 2004; Leroux et al. 2014; Li et al. 2014).

Haploid gene expression and patterning of the female gametophyte prior to fertilization can significantly affect the development of both the endosperm and embryo (Drews et al. 1998; Walbot and Evans 2003; Marton et al. 2005; Vernoud et al. 2005). The maize embryo sac is produced from a single megaspore by three rounds of free nuclear divisions generating an eight-nucleate syncytium which then cellularizes to produce seven cells of four types (Evans and Grossniklaus 2009): the egg cell, two synergids, the central cell, and three antipodal cells. Division of the antipodal cells, associated with auxin signaling, produces a cluster of 20–100 antipodal cells in maize (Chettoor and Evans 2015). The function of the
antipodal cells is undetermined but they are hypothesized to act as a transfer tissue based on the presence of cell wall invaginations on the surfaces facing the maternal nuellus (Diboll 1968). Alternatively, they could act as a signaling center by providing positional information for the embryo sac, or even for the endosperm since they persist in the maize seed after fertilization (Weatherwax 1926; Randolph 1936).

Two types of mutants with maternal effects on seed development can be distinguished based on their mode of inheritance: those in genes required in the maternal sporophyte (Li and Berger 2012; Li and Li 2015) and those in genes required in the maternal female gametophyte (Luo et al. 2014). They can be distinguished from each other by the mode of transmission (Grossniklaus and Schnetz 1998; Evans and Kermicle 2001). Recessive maternal sporophyte effect mutants will only have consequences if parent plants are homozygous. Both maternal gametophyte effect mutants and dominant maternal sporophyte effect mutants produce abnormal seeds when heterozygotes are crossed as females; but in the case of gametophyte mutants the abnormal seeds inherit the mutant allele because the embryo sac must carry the mutation to cause an effect, while the allele present in the embryo sac (and hence the seed) is irrelevant in the case of maternal sporophyte effects. Consequently, in the case of dominant maternal sporophyte effects, wild-type and mutant alleles are equally represented in both abnormal and normal seeds.

Gametophytic maternal-effect mutants have been identified in both Arabidopsis and maize (Gavazzi et al. 1997; Evans and Kermicle 2001; Grini et al. 2002; Olsen 2004; Köhler and Grossniklaus 2005; Pagnussat et al. 2005; Gutierrez-Marcos et al. 2006b; Pien and Grossniklaus 2007; Phillips and Evans 2011). Although not affecting postfertilization seed development when transmitted through the pollen, many gametophytic maternal-effect mutations in Arabidopsis (Pagnussat et al. 2005; Boavida et al. 2009) and maize (Evans and Kermicle 2001; Gutierrez-Marcos et al. 2006b; Phillips and Evans 2011) have reduced male transmission, indicating a separate role for the gene in pollen development/function.

Studies of these mutants have revealed several causes for maternal effects, as identified through genetic and cellular analysis. Maternal gametophyte effects can be caused by defects in functional gene dosage in the endosperm (Singletary et al. 1997), embryo-sac morphology (Lin 1978), cytoplasmic storage of gene products (Sprunger et al. 2000), and imprinting (Kinoshta et al. 1999; Vielle-Calzada et al. 1999). Sporophytic maternal effects can occur through disruption of maternal transfer tissues or integuments (Felker et al. 1985; Garcia et al. 2005), nonreduction of gametes (which leads to endosperm parental ploidy imbalance) (Barrell and Grossniklaus 2005; Singh et al. 2011), or microRNA production (Golden et al. 2002). Although the two types of maternal effects have distinct modes of inheritance and time of action, there is evidence of interaction between the imprinting pathway (typically a gametophyte effect) and maternal sporophyte effects (Dilkes et al. 2008; FitzGerald et al. 2008).

Nonequivalence of the maternal and paternal genomes in endosperm development was identified through the analysis of interlopid cross (e.g., tetraploid by diploid) in multiple species of plants, and these data contributed to the formation of the parental conflict theory (Haig and Westoby 1989). According to this theory, maternal and paternal alleles in the endosperm have different activities, leading to restriction or promotion of the growth of the endosperm, respectively. The endosperm phenotypes of seeds with maternal or paternal genome excess are in agreement with this theory (Haig and Westoby 1991; Charlton et al. 1995; Scott et al. 1998). In maize, the BETL is particularly sensitive to maternal or paternal genome excess (Charlton et al. 1995). Nonequivalent expression of the parental alleles of many genes is present in the embryo as well, primarily before the midgloabular stage (Vielle-Calzada et al. 2000; Baroux et al. 2001; Grimanelli et al. 2005; Autran et al. 2011; Baroux and Grossniklaus 2015). Analysis of the early phenotypes of embryo-lethal mutants corroborated these studies and demonstrated that early embryogenesis is largely under maternal control (Del Toro-De León et al. 2014).

RNA sequencing has enabled the identification of hundreds of genes with parent-specific and parent-biased expression in the seed of several plant species (Gehring et al. 2011; Hsieh et al. 2011; Waters et al. 2011; Wolff et al. 2011; Zhang et al. 2011; Xin et al. 2013; Pignatta et al. 2014). Many genes have only a subset of their naturally occurring alleles imprinted. Frequently, imprinting is stage-specific, with expression being uniparental early in endosperm development and biallelic later. Gametophytic maternal-effect mutants in Arabidopsis frequently show defects during this early period of development (Pagnussat et al. 2005; Ngo et al. 2012).

The imprinted status of these genes is regulated, at least in part, by parent-specific DNA methylation, polycomb group-mediated repression, and small RNA pathways (Köhler et al. 2003; Gutierrez-Marcos et al. 2004; Köhler et al. 2005; Gutierrez-Marcos et al. 2006a; Haun and Springer 2008; Makarevich et al. 2008; FitzGerald et al. 2009; Jahnke and Scholten 2009; Hsieh et al. 2011; Wolff et al. 2011; Vu et al. 2013; Pignatta et al. 2014; Zhang et al. 2014); and is often associated with repetitive DNA elements (Gehring et al. 2009; Villar et al. 2009; Pignatta et al. 2014). Molecular mechanisms that mark and maintain silenced alleles include a complex interplay between DNA methylation and histone modifications (Kawashima and Berger 2014).

While no functional data are available for most imprinted genes in plants, the maize maternally expressed meg1 gene has been shown to promote nutrient allocation to the seed by promoting differentiation of the BETL (Costa et al. 2012). However, the promotion of endosperm growth by a maternally active gene is the opposite of that predicted by parental conflict theory and demonstrates that there is maternal control of essential seed developmental processes unrelated to parental conflict theory. A different explanation for the function of imprinting in the seed is to generate functional diversity of genes in seed development (Bai and Settles 2014;
Pignatta et al. 2014). As these models are not mutually exclusive, selective pressure from both mechanisms (and others) could be operating during evolution to generate parent-of-origin-specific expression of genes for different purposes in the seed. For example, some paternally expressed genes are important for establishing interplodiy crossing barriers (Kradolfer et al. 2013; Wolff et al. 2015), while others are important for patterning of the embryo (Bayer et al. 2009; Costa et al. 2014).

Most maternal-effect mutants described in Arabidopsis do not have any prefertilization morphological defects (Grini et al. 2002; Pagnussat et al. 2005), except for those with fertilization-independent seed development (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998; Kiyosue et al. 1999). Some of the maternal-effect mutants in maize have abnormal gametophyte morphology that may contribute to their effects on seed development and pollen transmission (Gutierrez-Marcos et al. 2006b; Phillips and Evans 2011). Here we describe a set of maternal-effect mutants in maize with varying effects on seed development. A majority have visible morphological defects in the embryo sac before fertilization, and an overlapping majority affect patterning of BETL gene expression in the endosperm after fertilization. In most cases, the prefertilization defects are sufficient to explain the defects in seed development. Consequently, only a subset of these mutations may affect imprinted genes or imprinting processes. Whether or not any of these mutations have imprinting-specific effects or affect both imprinting and prefertilization embryo-sac development will be resolved after cloning and molecular analysis of the affected genes.

Materials and Methods

Plant material and growth conditions

This collection of maize maternal-effect mutants (mem) was developed from a variety of mutagenesis populations as follows: The Mn-Uq mutant was isolated previously (Pan and Peterson 1989). The sans scion1 (ssc1), heirless1 (hr1), no legacy1 (nol1), baseless2 (bsl2), and topknot1 (tpn1) mutants were identified as rare ears with 25–50% defective kernels after pollination of females with wild-type males during routine propagation of maize genetic stocks. The ssc1 mutation arose in a W22 inbred maize (Zea mays) plant carrying a mutable allele of enhancement of r1 (enr1) and a pale-aleurone-conferring allele, R1-r: (Venezuela), of the r1 gene (Stinard et al. 2009). hr1 arose in a W64A inbred line with active Mutator (Mu) transposons. nol1 arose in a line with active Ac/Ds transposons from a seed carrying a revertant to wild type of a vp1-m1::Ds mutant allele. bsl2 arose in an active Mu W64A/A158 hybrid line. topknot1 (tpn1) arose in an active Mu B73/W23 hybrid line. Two mutants, superbase1 (sba1) and maternally reduced endosperm1 (mrn1), were identified from an EMS mutagenesis as rare ears with a high frequency of defective kernels in an open pollinated population. One mutant, hr1, arose as a single defective kernel event in a W22 inbred line with active Ac/Ds transposons. The other mutants arose in UniformMu maize lines, inbred W22 (McCarty et al. 2005), as single defective kernel events on otherwise wild-type ears. Mutants were typically propagated as heterozygotes by transmission through the female and selection for miniature or defective kernels. Mutants and wild-type controls were grown side by side for each experiment, either in summer field conditions or in greenhouses under long-day conditions (16 hr light:8 hr dark cycles).

Most mapping populations were generated by crossing mem/+ Mo17 or mem/+ B73 hybrid females to wild-type Mo17 or B73 males, respectively. For hr1 and bsl2, the mutant phenotype was suppressed in F1 hybrids with B73 and Mo17, so the mapping populations were generated by crossing mem/+ B104 females to wild-type B104 males. For hr12, the mapping population was generated by crossing hr12W22/+ W64A females to wild-type W64A males.

Molecular mapping

DNA was extracted from seedlings by minor modification of the method of Sbaghi-Marof et al. (1984) or from mature seeds (Martin et al. 2010), and PCR reactions were performed as described (Evans and Kermicle 2001). Initial map position was determined from bulk segregant analysis by comparing DNA from a pool of 48 normal seeds (mostly wild-type homozygotes) to DNA from a pool of 48 defective seeds (mutant heterozygotes), using either SNP-based Sequenom mapping (Liu et al. 2010) or PCR with a set of polymorphic SSR markers (Martin et al. 2010). When bulk segregant analysis showed heterozygosity in the mutant pool but near homozygosity in the wild-type pool, PCR was performed using the same SSR markers or nearby SSR markers on 48 defective and 48 normal kernel individuals to verify cosegregation with the mutant phenotype. Map position was refined using additional SSR and indel PCR-based markers within the appropriate chromosomal interval.

Transmission and viability assays

For ssc1, map position was first identified based on linkage to the visible kernel mutant yellow endosperm1 (y1). Male and female transmission of the ssc1 mutation and penetrance of the defective kernel phenotype were partially assessed using plants carrying ssc1 linked in repulsion phase to y1. The genetic distance between ssc1 and y1 was determined using the kernel phenotypes of y1 and ssc1. Transmission of y1 was observed after making reciprocal crosses between + y1/ssc1 + plants and homozygous y1 plants. Similarly, for stt3 and mrn2, map position was identified based on linkage to the r1 gene. Male and female transmission of stt3 and mrn2 and penetrance of the defective kernel phenotype were partially assessed using reciprocal crosses between heterozygous mem R1-r:standard/+ r1-r plants and homozygous r1-r plants. For all other mutants, normal kernels from reciprocal crosses between mutant heterozygotes and wild-type plants were grown to maturity and progeny tested to determine what fraction had inherited the wild-type allele (i.e., were homozygous wild type), and what fraction had inherited the mutant allele (i.e., were heterozygous). For male crosses, this frequency produces the male transmission rate. For the female crosses, this frequency is
combined with the frequency of the defective kernels to calculate the female transmission rate using the percentages of all kernels that are homozygous wild type, all that are defective, and all that are heterozygous mutant but appear normal. To calculate the percentage of embryo sacs carrying the mutation that produced a detectable kernel (whether defective or normal), it was assumed that half of the embryo sacs inherited the mutation. If fewer than half of all kernels were mutant heterozygotes, then the number of embryo sacs that would need to be added to make the number of homozygous wild-type and heterozygous mutant kernels equal was assumed to consist of mutant embryo sacs that did not produce a detectable kernel. For viability assays, defective kernels, regardless of severity, were germinated on filter paper. If necessary, growing shoot tips were liberated by making an incision in the pericarp just beyond the tip of the shoot. Seeds with any root or shoot growth were transplanted to soil in small pots, and survivors were transplanted at the two- to three-leaf stage to the field and grown to maturity.

Confocal microscopy and histology

Embryo sacs were analyzed from mutant heterozygotes at mature stage (with a silk length ≥20 cm). Samples were processed and visualized on a Leica SP5 or Leica SP8 (Wetzlar, Germany) laser scanning confocal microscope as described previously (Gutierrez-Marcos et al. 2006b; Phillips and Evans 2011). Excitation was performed at 405, 488, and 561 nm and emission was collected at 410–2011). Excitation was performed at 405, 488, and 561 nm and emission was collected at 410

Results

Isolation of maternal-effect mutants

A total of 16 mutants with maternal gametophyte effects on seed development were collected and verified using reciprocal crosses and progeny testing to verify their strict maternal effects on seed development, and that these maternal effects depended on the genotype of the embryo sac (Figure 1). Five mutants arose spontaneously in standard maize stocks rather than as part of a screen for maternal-effect mutants. The ssc1, hrl1, bsl2, nol1, and tpn1 were first discovered as heterozygous plants segregating defective kernels, despite females being crossed with a wild-type male. Consequently, they were not expected to be recessive, zygotic mutants, but had to be maternal-effect mutants or dominant-zygotic mutants. ssc1 arose in a standard W22 inbred line carrying a mutable allele of enr1 (Stinard et al. 2009). In combination with certain alleles of r1, the enr1 mutation leads to more intense pigmentation in the aleurone of the germless mutant kernels than their normal siblings (Figure 1B). This facilitated identification of the ssc1 mutant plant, which would not otherwise have been as obvious because the embryos are covered and the endosperms are normal in appearance. The hrl1, bsl2, and tpn1 mutants all arose in lines with active Mutator transposons, and nol1 arose in a line with active Ac/Ds transposons.

The sba1 and mrn1 mutants were identified as heterozygous plants producing defective kernels when pollinated by wild-type males from a population of EMS-mutagenized families. ssc1 mutant kernels were not viable but the mutant was recovered from the normal siblings, a fraction of which carried the ssc1 mutation. The other mutants were recovered by growing the few viable defective kernels to maturity and making reciprocal crosses with them.

To screen for new, viable maternal-effect mutants, we took advantage of the fact that Mu and Ds insertions occur late in flower development and often affect only a single ovule. Each seed from a Mu active or Ac/Ds female can then be treated as a potentially independent event. Rare defective seeds were collected from Mu active UniformMu W22 females and Ac/Ds W22 females that otherwise produced normal seed (Supplemental Material, Figure S1). These could arise because of environmental reasons, because of aneuploidy in the seed, because of a new dominant zygotic mutation in the seed, or because of a new maternal-effect mutation in the embryo sac giving rise to the seed. Two dominant zygotic kernel mutants were isolated as part of these screens.

A total of 566 defective kernels of various types were collected from ~750 UniformMu ears. These ears were generated by crossing Mu active UniformMu females (MuDR; bs1-mum9::Mu; R1; W22) with males of lines without Mu activity (Mu off) (bs1-mum9::Mu; R1; W22 without MuDR). Defective seeds were germinated on filter paper and planted to soil after
root and shoot emergence. A total of 124 of the defective seeds produced viable seedlings and \( \approx 20\% \) of the survivors produced morphologically abnormal, male sterile plants consistent with aneuploid or haploid syndromes. Reciprocal crosses were made between the remaining 97 plants and a Mu off line to determine if the defective kernel phenotype was heritable and whether it behaved as a dominant zygotic mutant \( \text{[i.e., defective kernels were produced when crossed both as males or females (Figure 52)]} \) or as a maternal-effect mutant \( \text{(i.e., defective kernels were produced when crossed as a female but only normal kernels were produced when crossed as a male). Eight plants that produced} \geq 5\% \text{of defective kernels as females were retested in the next generation. Seven heritable maternal-effect mutants were identified from these. These mutants include no bet1 expression1 (nbe1), stunter2 (stt2), stt3, mnr2, mnr3, empty creche1 (ecr1), and ecr2.\\n
A similar mutagenesis was performed using the Ac/Ds transposable element system. A total of 378 defective kernels of various types were collected from \( \approx 1500 \) ears of Ac/Ds females. These ears were generated by crossing Ac/Ds females \( \text{(Ac; r-m3::Ds; W22) to males without Ac activity (r-m3::Ds; W22). Viable seedlings were produced by 244 defective seeds. Obvious haploids and/or aneuploids were not observed. These plants were pollinated as females by males without Ac activity (r-m3::Ds; W22). Eight plants that produced} \geq 5\% \text{of defective kernels as females were retested in the next generation. Reciprocal crosses were made between these eight lines and r-m3::Ds; W22 plants without Ac activity to determine if the defective kernel phenotype was heritable and whether it behaved as a dominant zygotic mutant or as a maternal-effect mutant. One heritable maternal-effect mutant, hrl2, was identified from this screen.\\n
All mutants were tested to distinguish between maternal sporophyte and maternal gametophyte effects. Plants grown from mutant kernels were crossed as females by wild type, and the progeny of these crosses (both normal and defective) were

Figure 1 Ears of maternal-effect mutants. (A) Wild-type W22 female pollinated by +ssc1; enr1-m with all normal kernels, showing the strict maternal effect. (B–Q) Maternal-effect mutant heterozygous females crossed by wild-type pollen. (B) +/ssc1; enr1-m W22 female by wild-type male. Germless mutant kernels with intense pigmentation of R1-r: (Venezuela). (C) +/Mn-Uq W22. (D) +/tpn1 Mo17. (E) +/bsl2 B104. (F) +/nol1 B104. (G) +/hrl1 B104. (H) +/hrl2 W22. (I) +/sba1 W22. (J) +/mm1 B73. (K) +/mm2 W22. (L) +/mm3 W22. (M) +/nbe1 W22. (N) +/stt2 B73. (O) +/stt3 Mo17, note the linkage between the small kernel phenotype and R1-r:standard (purple/brown kernels). (P) +/ecr1 W22. (Q) +/ecr2 W22. Arrowheads indicate representative defective kernels on each ear. Ear tips and hence the germinial sides of the kernels are all to the left. Bar, 1.0 cm. WT, wild type.
subsequently crossed as females by wild-type pollen. This backcross design ensures any phenotypes could not be the result of recessive maternal sporophyte effect mutants. Instead, defective kernels had to be maternal gametophyte effect or dominant maternal sporophyte effect mutants. The genotype of the embryo sac is irrelevant in dominant maternal sporophyte mutants, so both normal and defective kernels would be expected to segregate heterozygous mutant and homozygous wild-type plants in a 1:1 ratio. One dominant maternal sporophyte effect mutant was identified from this test from the EMS mutagenized population (data not shown). For all of the mutants described here, the defective kernels have almost exclusively inherited the mutant allele maternally and the normal kernels inherited the wild-type allele, indicating a maternal gametophyte effect on seed development (Table 1).

When crossed as females, mutants typically segregate <50% of the defective kernels. This could result either from incomplete penetrance, reduced female transmission due to failure of some of the mutant embryo sacs to be fertilized and produce a discernible seed, or a combination of both. Progeny testing of the normal kernels distinguishes these mechanisms. All of the mutants are incompletely penetrant. On a given ear, 4–32% of the seeds are mutant heterozygotes that are normal in appearance. For some mutants, this frequency is as high as that of the defective kernels (bsl2, mnr3, nbe1, hrl2, and sba1). In many cases, the penetrance of the mutant phenotype is variable from one cross to another (Table 1). Whether or not there is an environmental component contributing to the penetrance of the phenotype during ear development is unknown.

Female transmission was calculated by comparing the combined frequency of all heterozygous progeny to the frequency of homozygous wild-type progeny. Six of the mutants (mnr2, mnr3, ecr1, ecr2, hrl2, and stt3) produce significantly <50% mutant heterozygotes (P < 0.01 by χ²). These mutations likely cause sufficient defects in a subset of the embryo sacs to prevent their fertilization or arrest development very early before a seed is visible. For mnr3, ecr1, and ecr2, this is the majority of the mutant embryo sacs; while for stt3, mnr2, and hrl2 approximately half of the mutant embryo sacs do not produce a discernible seed.

The maternal-effect mutations were tested for reduced transmission through pollen as a measure for male gametophyte defects (Table 2). Mutant heterozygotes were crossed as males to wild-type females. A 50% transmission of the mutant allele in these crosses is indicative of no effect on male gametophyte function. For the ssc1 mutation, which is linked to the easily-scored y1 seed marker, transmission was assayed by effects on the transmission of y1 in repulsion to ssc1. Likewise, mnr2 and stt3 were assayed by their effects on the linked r1 gene. All three show reduced transmission of the mutant allele compared to wild type. For the rest of the mutants, seeds were grown to maturity and progeny tested. All but four of the mutants, hrl1, nol1, Mn-Uq, and sba1, showed significantly reduced transmission through the pollen (P < 0.01 by χ²). The next mildest effect was seen with bsl2 and hrl2. The strongest effects were seen with stt2, stt3, nbe1, and mnr3, which all had <10% transmission.

A total of 14 of the 16 mutations were mapped to chromosomal position using backcross populations and a combination of SNP and SSR markers (Figure 2; Table S1). The maternal-effect mutants are distributed over 8 of the 10 maize chromosomes. Based on the stt2 map position and phenotype, it is possible that stt2 is an allele of stt1 (Phillips and Evans 2011). Other potential allelic relations include hrl1 and nol1, which map very close together on chromosome 3; as well as hrl2 and nbe1, which map within the same bin on chromosome 6.
Table 2 Male transmission of maternal-effect mutants

| Mutant | mem/+ Heterozygotes |
|--------|---------------------|
| ssc1   | 15%* (362/2435)     |
| Mn-Uq  | 39% (31/80)         |
| tpn1   | 14%* (11/78)        |
| bsl2   | 33%* (60/180)       |
| nol1   | 45% (119/265)       |
| hrl1   | 47% (83/177)        |
| hrl2   | 28%* (46/162)       |
| sba1   | 42% (73/172)        |
| mn1    | 22%* (34/154)       |
| mn2    | 21%* (39/185)       |
| mn3    | 7%* (6/89)          |
| nbe1   | 4%* (5/144)         |
| stt2   | 8%* (10/125)        |
| stt3   | 1%* (8/963)         |
| ecr1   | 18%* (7/39)         |
| ecr2   | 14%* (6/44)         |

+/+ female × mem/+ male. * P < 0.01, significantly <50% by χ².

Phenotype of mutant kernels

The maternal-effect phenotypes can be divided into five classes: a normal endosperm with an abnormal, or aborted, embryo; a reduced endosperm with a loose pericarp (typically with some embryo abnormalities); an etched or pitted endosperm with an abnormal embryo; an empty pericarp; and a miniature, but otherwise normal, seed (Figure 3). The ssc1 mutant is the only one that exclusively produces kernels of the aborted embryo class (Figure 3B). While most mutants have variable expressivity, ssc1 does not. ssc1 has an aborted embryo every time it shows any phenotype. The most common phenotype is the loose pericarp class, which has a wide range of severity. The Mn-Uq, tpn1, bsl2, nol1, hrl1, sba1, mn1, mn2, mn3, and nbe1 mutants all fall into this category (Figure 1, C–M; Figure 3, C–M). The tpn1 mutant is unique in that the reduced expansion of the endosperm is limited to a collar near the crown of the endosperm, creating a protrusion on the top of the endosperm. It is possible that this is a consequence of the genetic background—tpn1 was the only mutant in a W23 inbred background (Figure 3D). The mn1 mutant more commonly produces kernels of the miniature class but these are unique in having a darker endosperm than wild type (Figure 1J; Figure 3J). The next most common phenotypic class of mutants has etched, pitted kernels; this includes nol1, hrl1, hrl2, sba1, and nbe1. Interestingly, nol1 and hrl1 endosperm defects are often limited to a groove on the abgerminale side of the kernel (Figure 1G). Many mutants also show a change in the ratio of vitreous endosperm to floury endosperm, with a reduction in vitreous endosperm being most common (Figure 3, E–G, M, and Q).

The bsl2, nol1, and Mn-Uq mutants all have extreme variability in the amount of endosperm in the defective seeds from different crosses (Figure 1, E and F). All three mutants can produce ears in which all of the abnormal kernels are only mildly affected (Figure 1G) or more severely affected (Figure 1, E and F). For these mutants, the most severe phenotype is the empty pericarp class, which may be the strongest version of the loose pericarp phenotype. The mn2 and mn3 kernel phenotypes are less variable from one ear to another, producing kernels of the loose pericarp, reduced endosperm class almost exclusively (Figure 1, K and L; Figure 3, K and L). The most common phenotype for the stt2 and stt3 mutants is a miniature endosperm with a normal embryo (Figure 1, N and O; Figure 3, N and O), similar to stt1 (Phillips and Evans 2011). These kernels are approximately half the size of their normal siblings. In some cases, stt3 mutants will also make kernels of the pitted class. The ecr1 and ecr2 mutants, like ssc1, have an aborted embryo, but, unlike ssc1, they typically have a smaller endosperm than wild type. While the miniature endosperms of stt2 and stt3 are typically normal in shape, the ren and pit phenotypes of most mutants are often associated with irregular growth of the endosperm, not just reduced growth. This can be seen in the uneven profile of the endosperm in cross section and invaginations present in many abnormal endosperms (e.g., Figure 3G).

All of the mutants have reduced viability, with ssc1 being the most severe (Table 3). In the least severe mutants, stt2, mn3, and ecr2, approximately half of the abnormal kernels are viable. The intermediate mutants, stt3, Mn-Uq, mn2, hrl2, nbe1, ecr1, sba1, mn1, and nol1, have approximately two-thirds inviable defectives; while in the more severe mutants, hrl1, bsl2, and tpn1, three-quarters or more of the defective seeds are inviable. In some cases, the viability varies widely between ears, particularly for nol1, bsl2, hrl1, and Mn-Uq. This is very similar to bsl1 which produces a lethal empty pericarp phenotype in some crosses but a viable, reduced endosperm phenotype in other crosses (Gutierrez-Marcos et al. 2006b). Only ssc1 causes complete loss of viability in affected seeds. Consequently, it would not have been recoverable through the screen for single defective kernels described above. All of the rest have partial viability, which presumably allowed for their recovery in the screen. It also suggests that many true maternal-effect mutants were lost to inviability of primary defective kernels.

Effects of mutations on embryo-sac morphology before fertilization

To determine if there were any effects of these mutants on embryo-sac morphology, mature embryo sacs from heterozygotes were examined using laser scanning confocal microscopy (Table 4; Figure 4). Seven different classes of phenotypes were seen in these mutants: mutants with only normal embryo sacs, miniature embryo sacs, embryo sacs with misplaced polar nuclei, embryo sacs with abnormal antipodal cells, embryo sacs with extra cells, embryo sacs with abnormal egg cells, and embryo sacs which had arrested or aborted early in development.

Five mutants, Mn-Uq, tpn1, hrl2, nbe1, and ecr2, have normal embryo-sac morphology with aborted embryo sacs at a wild-type background frequency (from 0 to 10% depending on plant vigor, growth conditions, and amount of transposon activity). Abnormal egg cells were seen in ssc1, but in only one of the three ears tested (Table 4; Figure 4, B and R). These egg
cells have all cytoplasmic contents against the micropylar pole of the egg cell (like a synergid) rather than having the nucleus in the center of the cell surrounded by plastids and cytoplasmic strands (Figure 4R). Five mutants have misplaced polar nuclei within the central cell: bsl2, nol1, hrl1, sba1, and mrn1 (Figure 4, C–I). In wild type, the partially fused polar nuclei are adjacent to the egg cell near the midline of the long axis of the central cell. As is the case for bsl1 (Gutierrez-Marcos et al. 2006b), the polar nuclei in these mutants are almost always adjacent to each other (except for one embryo sac in nol1), and in bsl2, nol1, hrl1, and sba1 they are adjacent to the egg cell. However, the polar nuclei are located off-center or against one of the lateral walls of the central cell. mrn1 is exceptional in having the polar nuclei displaced toward the chalazal end of the central cell in some embryo sacs; sometimes closer to the antipodal cells than the egg cell (Figure 4H). Like bsl1, sba1 typically has the polar nuclei against the future abgerminal side of the central cell (both mutants are in a W22 inbred background). nol1, bsl2, and hrl1 (in B73, W64A, and B104 inbreds, respectively) typically have the polar nuclei against the future adgerminal side of the central cell. In addition to chalazal displacement, mrn1 can also have the polar nuclei against the adgerminal or abgerminal side of the central cell (Figure 4I). Aborted embryo-sac development was observed in nol1, hrl1, and bsl2. However, based on the low frequency of this phenotype here and 50% transmission of the mutant allele through the female, this phenotype is likely unrelated to these mutations. Because nol1 and hrl1 map close to each other and have varying kernel phenotypes depending upon genetic background, they were examined in three different inbred lines each: B73, W23, and B104 for nol1 and W64A, Mo17, and B104 for hrl1. For nol1, B73 had the highest frequency of misplaced polar nuclei [nearly all mutant embryo sacs (half in a heterozygote) show the phenotype] followed by W23 [about one-third of the mutant embryo sacs (one-sixth in a heterozygote) show the phenotype] and B104. In hrl1, polar nuclei mislocalization is seen in B104 but not in W64A or Mo17. In B104, both kernel and embryo-sac phenotypes are similar between nol1 and hrl1.

Antipodal cell cluster defects were also observed in four of the mutants with polar nuclei misplacement: hrl1, nol1, sba1, and mrn1 (Figure 4, E–H). hrl1 affects the antipodal cells in the three different inbred backgrounds examined. Abnormal antipodal cell cluster morphology is shown in ~15% of the embryo sacs from heterozygotes, although the morphology varies depending upon inbred background. In W64A and B104 the antipodal cells are fewer in number but the same size as or smaller than wild type (Figure 4F), while in Mo17 the antipodal cells are also reduced in number but are larger than wild type (Figure 4F). In B104, nol1 has an antipodal cell phenotype very similar to that of hrl1 in W64A and B104. The antipodal cluster defects of sba1 and mrn1 in W22 and B73, respectively, are similar to those of nol1 and hrl1 in B104 (Figure 4, G–I). For sba1 this is the more common phenotype, with polar-nuclei displacement only occurring in a subset of the mutant embryo sacs with reduced antipodal cell cluster size (one-third of the embryo sacs with antipodal cell defects also have abnormal polar nuclei position). sba1 has no effect on polar nuclei position without an effect on antipodal cell cluster size. In mrn1 the opposite is true; a subset of the mutants with misplaced polar nuclei also have reduced antipodal cell cluster size (one-third of the embryo sacs with abnormal polar nuclei position also have abnormal antipodal cell clusters). mrn1 has no effect on antipodal cell cluster size when polar nuclei position is normal. These differences may result from genetic background differences or differences in the function of the mutated genes.

mrn2 and mrn3 show a significant frequency of embryo sacs that have aborted or arrested before cellularization (Figure 4, K and L). Approximately one-third of all embryo sacs in heterozygotes (or two-thirds of the mutant embryo sacs) fail to complete development. The frequency of this phenotype is consistent with the rate of reduced female transmission in these two mutants.

stt2 and stt3 have phenotypes similar to stt1 (Phillips and Evans 2011). Mutant embryo sacs are smaller, with a smaller central cell and fewer antipodal cells than wild type (Figure 4, M and N). Approximately half of the stt2/+ embryo sacs...
are small, suggesting that all mutant embryo sacs are affected. In contrast, only one-third of \textit{stt3}+/embryo sacs are small, suggesting that some \textit{stt3} embryo sacs are normal in size. Additionally, 10% of the embryo sacs from heterozygotes \textit{mrn2} and \textit{stt3} mutants have extra cells or a duplication of the entire embryo sac (Figure 4, J and O), but these phenotypes were less common than the other phenotypes described above. \textit{ecr1} mutant embryo sacs also have smaller central cells like \textit{stt1}, 2, and 3; but \textit{ecr1} is distinct from the \textit{stt} mutants in that half of the small embryo sacs also have small, unexpanded egg cells (Figure 4, P and S).

\textbf{Effects of mutations on expression pattern of a BETL marker}

Because the BETL is particularly sensitive to maternal control (Charlton \textit{et al.} 1995; Gutierrez-Marcos \textit{et al.} 2003; Gutierrez-Marcos \textit{et al.} 2006b), the expression of the maize BETL-specific gene reporter, \textit{p}_{\text{BET1}}::\textit{GUS} (Huerros \textit{et al.} 1999), was examined in all of the mutants to determine effects on patterning of BETL gene expression (Table 5; Figure 5; Table S2). Most crosses were made with \textit{p}_{\text{BET1}}::\textit{GUS}/- hemizygotes, but some crosses were made with homozygous \textit{p}_{\text{BET1}}::\textit{GUS} plants. Kernels were examined after establishment of \textit{p}_{\text{BET1}}::\textit{GUS} expression near maturity [between 33 and 37 days after pollination (DAP)] for all mutants, and at 20 DAP for a few mutants [still after establishment of the normal \textit{p}_{\text{BET1}}::\textit{GUS} expression pattern (compare Figure 5, A and B)]. Between 20 DAP and maturity in wild type, the \textit{p}_{\text{BET1}}::\textit{GUS} expression intensifies but does not change in pattern.

The penetrance of the effects of the mutations on \textit{p}_{\text{BET1}}::\textit{GUS} expression can be evaluated based on the frequency of the normal pattern of expression in the mutants (Table 5). \textit{ssc1}, \textit{mrn1}, \textit{mrn2}, and \textit{stt2} have no effects on \textit{p}_{\text{BET1}}::\textit{GUS} expression; showing neither an abnormal pattern nor an

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3.png}
\caption{Mature kernels of maternal-effect mutants. Germinal (embryo) face (left) and a median longitudinal section (right) through the center of the embryo (or where the embryo would be). Mutant kernels are from heterozygous mutant females crossed by wild-type pollen. The embryo in the longitudinal section is oriented to the left in all kernels. (A) Wild type, (B) \textit{ssc1}, (C) \textit{Mn-Uq}, (D) \textit{tpn1}, (E) \textit{bsl2}, (F) \textit{nol1}, (G) \textit{hrl1}, (H) \textit{hrl2}, (I) \textit{sba1}, (J) \textit{mrn1}, (K) \textit{mrn2}, (L) \textit{mrn3}, (M) \textit{nbe1}, (N) \textit{stt2}, (O) \textit{stt3}, (P) \textit{ecr1}, and (Q) \textit{ecr2}. e, embryo; f, floury endosperm; v, vitreous endosperm. All kernels are shown at the same scale. Bar, 0.5 cm.}
\end{figure}
increased likelihood of no expression of the reporter (Table 5; Figure 5, C, K, L, and O). An abnormal pattern was seen only once out of 18 kernels expressing the transgene in *mn3*. The next weakest effect was seen in *Mn-Uq, tnp1, stt3, nol1, bsl2,* and *hrl2*, with an effect 10–30% of the time; followed by *hrl1* and *sba1*, which affect *ProBet1::GUS* expression more than half the time. The strongest effect was seen with *nbe1* which produced no mutant kernels with normal *ProBet1::GUS* expression. Abnormal expression patterns can be grouped into four categories: (1) spots of expression unconnected to the base (e.g., at the crown, along the back of the endosperm, or in the center of the endosperm) (Figure 5, D–F, H, I, O, and R), (2) absence of expression in a portion of the normal domain (Figure 5G), (3) expression in an expanded domain at the base of the endosperm (Figure 5, E, J, and Q), or (4) absence of expression (Figure 5N). Some mutants have expression both in extra cell layers at the base of the endosperm and in ectopic spots unconnected to this domain.

A total of 8 of the 16 mutants have expanded *ProBet1::GUS* expression at the base of the endosperm (Table 5). This is most common in *sba1, hrl2,* and *ecr1*, which have this pattern in 20–28% of mutants showing this phenotype. This phenotype is also seen in *Mn-Uq, tnp1, bsl2,* and *hrl1* at a lower frequency (Table 5; and examples in Figure 5, E, F, J, and Q). *sba1* has the most extensive expansion of *ProBet1::GUS* expression into extra layers of cells at the base of the endosperm, with expression sometimes encompassing half of the endosperm (Figure 5J). More mutants (11 out of 16) show ectopic spots of *ProBet1::GUS* expression unconnected to any expression in the basal domain (Table 5; and examples in Figure 5, D–F, H, I, P, and R). On rare occasions, these ectopic spots are seen in the absence of any expression in the normal basal domain (once each in *stt3, ecr2,* and *nbe1*) (example in Figure 5R), but otherwise these spots are seen in combination with some form of basal expression. The ectopic spots have been detected most frequently in *hrl1, sba1,* and *stt3,* and less frequently in *nbe1, ecr2, bsl2, Mn-Uq, tnp1, hrl2, mn3,* and *nol1*. These ectopic spots are more frequently located at the periphery of the endosperm than in the center.

Like *bsl1*, four of the mutants with misplaced polar nuclei, *nol1, bsl2, hrl1,* and *sba1* (Figure 5, F–H, and J) have abnormal

### Table 3 Defective kernels that failed to produce a viable seedling

| Mutant          | Inviable seeds |
|-----------------|----------------|
| scc1/+          | 100% (108/108) |
| *Mn-Uq/+*       | 65% (117/180)  |
| tnp1/+          | 74% (127/171)  |
| bsl2/+          | 77% (132/172)  |
| nol1/+          | 68% (110/162)  |
| hrl1/+          | 78% (176/225)  |
| hrl2/+          | 66% (83/125)   |
| sba1/+          | 67% (80/120)   |
| mn1/+           | 69% (103/150)  |
| mn2/+           | 65% (66/102)   |
| mn3/+           | 43% (20/47)    |
| nbe1/+          | 71% (66/93)    |
| stt2/+          | 55% (33/60)    |
| stt3/+          | 68% (25/37)    |
| ecr1/+          | 65% (11/17)    |
| ecr2/+          | 50% (7/14)     |

*menI= female × +/- male.*

### Table 4 Morphology of embryo sacs in plants heterozygous for maize maternal-effect mutations

| Mutant (plants tested) | Normal | Misplaced polar nuclei | Early arrest/abortion | Small central cell | Abnormal antipodal cell cluster | Extra cells | Abnormal egg cell |
|------------------------|--------|------------------------|-----------------------|--------------------|-------------------------------|-------------|------------------|
| scc1/+; W22 (3)        | 74     | 0                      | 0                     | 0                  | 0                             | 0           | 5a               |
| *Mn-Uq/+; W22 (3)      | 61     | 0                      | 0                     | 0                  | 0                             | 0           | 0                |
| tnp1/+; W23 (4)        | 51     | 0                      | 4                     | 0                  | 0                             | 0           | 0                |
| bsl2/+; W64A (2)       | 36     | 18                     | 1                     | 0                  | 0                             | 0           | 0                |
| nol1/+; B73 (4)        | 32     | 27                     | 6                     | 0                  | 1                             | 0           | 0                |
| nbe1/+; W23 (1)        | 22     | 4                      | 1                     | 0                  | 0                             | 0           | 0                |
| nol1/+; B104 (2)       | 40     | 0                      | 1                     | 0                  | 6                             | 0           | 0                |
| hrl1/+; W64A (1)       | 27     | 0                      | 0                     | 0                  | 6                             | 0           | 0                |
| hrl1/+; Mo17 (1)       | 24     | 0                      | 0                     | 0                  | 3                             | 3           | 0                |
| hrl1/+; B104 (2)       | 71     | 4                      | 5                     | 0                  | 8                             | 0           | 0                |
| hrl2/+; W22 (1)        | 33     | 0                      | 0                     | 0                  | 0                             | 0           | 0                |
| sba1/+; W22 (1)        | 37     | 8                      | 0                     | 0                  | 26                            | 0           | 0                |
| mn1/+; B73 (2)         | 37     | 24                    | 0                     | 0                  | 9d                            | 0           | 0                |
| mn2/+; W22 (1)         | 19     | 0                      | 10                    | 0                  | 0                             | 3           | 0                |
| mn3/+; W22 (2)         | 22     | 0                      | 8                     | 0                  | 0                             | 0           | 0                |
| nbe1/+; W22 (3)        | 36     | 0                      | 0                     | 0                  | 0                             | 0           | 0                |
| stt2/+; W22 (1)        | 20     | 0                      | 1                     | 20                 | 0                             | 0           | 0                |
| stt3/+; W22 (2)        | 28     | 0                      | 3                     | 11                 | 0                             | 3b          | 0                |
| ecr1/+; W22 (2)        | 45     | 0                      | 2                     | 13                 | 0                             | 0           | 5f               |
| ecr2/+; W22 (2)        | 44     | 0                      | 0                     | 0                  | 0                             | 0           | 0                |

*a* Abnormal egg cells were only seen in one of three ears tested.

*b* These individuals are a subset of the embryo sacs with abnormal antipodal cells.

*c* The chalazal displacement of polar nuclei was seen in one of two ears tested, but the lateral displacement of polar nuclei was seen in both ears.

*d* These individuals are a subset of the embryo sacs with misplaced polar nuclei.

*e* Extra cells were only seen in one of two ears tested.

*f* These individuals are a subset of the embryo sacs with small central cells.
patterns of expression of the \( p_{\text{pro}}\text{Bet1}}::\text{GUS} \) reporter. \( \text{nol1} \) is more likely to lack expression within the basal region of the endosperm, while \( \text{bsl2}, \text{hrl1}, \) and \( \text{sba1} \) are more likely to have ectopic expression outside of the basal domain. Kernels that lack expression in the central domain but have it in the abgermineral and adgermineral domains—a pattern that is common in \( \text{bsl1} \)—are rare in these mutants. These differences may be a consequence of the polar nuclei typically being placed on the future abgerminal side of the central cell in \( \text{bsl1} \), but on the future adgerminal side of the central cell in \( \text{nol1}, \text{hrl1}, \) and \( \text{bsl2} \). \( \text{hrl1} \) and \( \text{sba1} \) did not produce kernels with partial \( p_{\text{pro}}\text{Bet1}}::\text{GUS} \) expression patterns, unlike \( \text{bsl1}, \text{bsl2}, \) and \( \text{nol1} \). All four of these mutants show expression of \( p_{\text{pro}}\text{Bet1}}::\text{GUS} \) outside of the normal BETL domain, with \( \text{sba1} \) having the strongest effect. \( \text{mrn1} \) is unique in having an effect on polar nuclei position but no effect on \( p_{\text{pro}}\text{Bet1}}::\text{GUS} \) expression (Table 5; Figure 5K). Whether all of the differences between these mutants are a consequence of differences in genetic background or differences in the functions of the mutated genes is unclear.

In \( \text{nbe1} \) mutants, expression of \( p_{\text{pro}}\text{Bet1}}::\text{GUS} \) was never detected in the normal basal domain in the 32 mutant kernels examined, and only once in ectopic puncta outside of this domain (Table 5; Figure 5N). The \( \text{hrl1} \) and \( \text{sba1} \) mutants also prevent \( p_{\text{pro}}\text{Bet1}}::\text{GUS} \) expression in some kernels (i.e., defective kernels lack expression more frequently than their wild-type siblings) (Table 5).

**Discussion**

Both mutant analyses (Gavazzi et al. 1997; Evans and Kermicle 2001; Grini et al. 2002; Olsen 2004; Köhler and Grossniklaus 2005; Pagnussat et al. 2005; Gutierrez-Marcos et al. 2006b; Pien and Grossniklaus 2007; Phillips and Evans 2011; Del Toro-De León et al. 2014) and gene expression studies (Vielle-Calzada et al. 2000; Baroux et al. 2001; Grimanelli et al. 2005; Autran et al. 2011; Baroux and Grossniklaus 2015) indicate a significant amount of both endosperm and embryo development is under maternal control. Differential timing of paternal allele activation would allow for some processes of embryogenesis to be controlled by the maternal genome longer than others.

In this study, we describe 16 maternal-effect mutations. Most of these cause morphological defects in the embryo sac prior to fertilization. Consequently, these mutations are likely affecting production of gametes with the correct structure to support normal development of the seed rather than affecting individual imprinted genes, or the imprinting process itself. Subcellular organization of the central cell in particular appears important for endosperm development. A connection between antipodal cell development and seed development is implied by some mutant phenotypes as well. These mutations identify at least 11 loci not previously indicated to play a role in the maternal regulation of seed development. Based on map
position and phenotype, stt2 could be an allele of stt1, and nol1 and hrl1 could be allelic with each other. hrl2 and nbe1 map within the same bin on chromosome 6 but have distinct seed and transmission phenotypes within the same inbred background, and so are less likely than the other two pairs to be allelic with each other. Because the action of these mutations is during the haploid phase of the life cycle, complementation tests are very difficult. Fine mapping and cloning of these genes will determine if they are indeed allelic. Although all of the mutants behave as maternal effects rather than zygotic dominants, mrn2 and mrn3 have dominant effects on sporophyte development. Tassels of +/mrn2 mutants have expanded glumes, giving them a brush-like appearance, and the tassels and ears of +/mrn3 mutants are smaller than wild type (Figure 1L; Figure S3). The mutant phenotypes in the sporophyte could either be caused by haploinsufficiency or gain-of-function mutations, raising the possibility that mrn2 and mrn3 may be causing gametophyte defects by gain of function rather than loss of function of the affected genes.

These maternal-effect mutants include several different types of effects on seed development and several classes of prefertilization embryo-sac defects. Although mutants were selected solely for their maternal effects, the majority also affect the male gametophyte as measured by reduced transmission of the mutant allele. The nature of the defects in the pollen grain are unknown, but for some mutants the effect is very severe with <10% male transmission efficiency. These results are in agreement with other screens for gametophyte mutants that indicate most mutants affect both sexes (summarized in Evans and Grossniklaus 2009).

Maternal-effect mutants usually affect both the endosperm and the embryo, but there are examples of specific effects on one or the other. Mild effects on the endosperm, such as a miniature kernel, often have no effect on the embryo. Severe endosperm defects, however, are almost always associated with abnormal embryo development or reduced viability. Whether the effects on the embryo for most of the mutants indicate a direct role for the mutated gene in embryo development or a downstream consequence of the embryo growing in the context of an abnormal endosperm is unclear. Severe defects in embryo development, in contrast, can occur in the absence of visible defects in the endosperm; although subtle defects in the ability of the endosperm to support growth of the embryo in these cases cannot be ruled out.

Many maternal-effect mutants are also associated with embryo-sac abnormalities prior to fertilization. Only a third of them have no morphological defects prior to fertilization. These defects fall into six distinct classes: misplacement of the polar nuclei within the central cell, abnormal antipodal cell cluster morphology or size, abnormal egg cell size/morphology, reduced embryo-sac size (particularly of the central cell), embryo sacs with extra cells, and embryo-sac arrest/abortion. Mutants can express multiple phenotypes, and phenotypes can vary depending upon genetic background. Some phenotypes have only been seen in one individual per mutant, and, for these, there is less confidence that they are caused by the mutation being analyzed. Examples of this are the abnormal egg cells in ssc1, the extra cells in stt3, or the chalazal displacement of polar nuclei in mrn1. These effects may be caused by a

Table 5 Endosperm ProBet1::GUS expression in maternal-effect mutants

| Normal ProBet1::GUS expression in defective kernels | Frequency of kernels expected to have ProBet1::GUS but lacking it in the BETL | Frequency of kernels with ProBet1::GUS only in part of the BETL | Frequency of kernels with ProBet1::GUS in extra cell layers | Frequency of kernels with ProBet1::GUS in ectopic spots |
|---------------------------------------------------|-------------------------------------------------------------------------|---------------------------------------------------------------|----------------------------------------------------------|---------------------------------------------------------|
| 100% normal                                        | nbe1 (100%), hrl1 (38%), sba1 (36%)                                       | nol1 (22%), bsl2 (6%)                                         | 20–28%                                                 | 10–25%                                                  |
| 94–96% normal                                      |                                                                         |                                                              |                                                         |                                                         |
| mm3 (42)                                           |                                                                         |                                                              |                                                         |                                                         |
| 60–85% normal                                      |                                                                         |                                                              |                                                         |                                                         |
| ecr2 (26)                                         |                                                                         |                                                              |                                                         |                                                         |
| tpn1 (41)                                          |                                                                         |                                                              |                                                         |                                                         |
| Mn-Uq (30)                                        |                                                                         |                                                              |                                                         |                                                         |
| stt3 (30)                                         |                                                                         |                                                              |                                                         |                                                         |
| nol1 (67)                                         |                                                                         |                                                              |                                                         |                                                         |
| ecr1 (33)                                         |                                                                         |                                                              |                                                         |                                                         |
| bsl2 (59)                                         |                                                                         |                                                              |                                                         |                                                         |
| hrl2 (39)                                         |                                                                         |                                                              |                                                         |                                                         |
| 25–45% normal                                      |                                                                         |                                                              |                                                         |                                                         |
| hrl1 (60)                                         |                                                                         |                                                              |                                                         |                                                         |
| sba1 (63)                                         |                                                                         |                                                              |                                                         |                                                         |
| 0% normal                                          |                                                                         |                                                              |                                                         |                                                         |
| nbe1 (32)                                         |                                                                         |                                                              |                                                         |                                                         |

See Table S2 for supporting data.

* Total defective kernels tested for expression in parentheses.
second mutation segregating in each line, or they may depend on a particular growth condition.

Misplacement of polar nuclei and reduced central cell size have previously been shown to occur in maize maternal-effect mutants (Gutierrez-Marcos et al. 2006b; Phillips and Evans 2011). Three additional mutants with small central cells and small seeds were identified here: \textit{stt2}, \textit{stt3}, and \textit{ecr1}. This aspect of the phenotype is very similar to that of \textit{stt1}. Indeed, \textit{stt2} may be an allele of \textit{stt1}, because these mutants map within the same chromosomal region. \textit{stt3} and \textit{ecr1} have additional kernel phenotypes not seen in \textit{stt1} or \textit{stt2}. \textit{stt3} shows occasional ectopic expression of \textit{pBet1::GUS} expression, and \textit{ecr1} kernels are typically germless. Both of these mutants also have embryo-sac phenotypes not seen in \textit{stt1} or \textit{stt2}. In one of the two individuals tested, \textit{stt3} had embryo sacs with extra cells or oocytes with a complete embryo-sac duplication. Half of the small \textit{ecr1} embryo sacs also have a small egg cell. The abnormal eggs of \textit{ecr1} might be unable to produce normal embryos in the seed, and may suggest that even the eggs without visible abnormalities are affected, since the frequency of germless kernels is higher than the frequency of egg defects. Since egg abnormalities segregated in one \textit{ssc1} ear, defects in the egg cell may contribute to embryo arrest in this mutant as well.

Five mutants have misplaced polar nuclei like \textit{bsl1} (Gutierrez-Marcos et al. 2006b). Four of these \textit{bsl2}, \textit{no1}, \textit{hr11}, and \textit{sba1} cause abnormal patterning of \textit{p\textsubscript{mr}Bet1::GUS} expression, also like \textit{bsl1}. These results support the model that there is a prepattern of an unknown factor(s) within the central cell that controls BETL development and that it is disrupted in these mutants. Alternatively, these genes may have multiple, independent roles during embryo-sac and seed development. For all four of these mutants, the effects on \textit{p\textsubscript{mr}Bet1::GUS} can be more severe than in \textit{bsl1}. The exception to this effect is \textit{mrn1}. \textit{mrn1} has no effect on \textit{p\textsubscript{mr}Bet1::GUS}, despite affecting polar nuclei placement. Therefore, the abnormal \textit{p\textsubscript{mr}Bet1::GUS} pattern is not a direct consequence of the abnormal position of the polar nuclei themselves. Several mutations have normal embryo-sac morphology but still affect \textit{p\textsubscript{mr}Bet1::GUS} expression pattern: \textit{Mn-Uq}, \textit{tpn1}, \textit{nbe1}, \textit{ecr2}, and \textit{hr12}. These mutations could be in genes required either before or after fertilization for establishing or maintaining such a BETL-determining pattern. Consequently, polar nuclei movement and the placement of this hypothetical determinant are genetically separable, with some regulatory factors shared between the two processes but others unique to one or the other.

Two types of misexpression of \textit{p\textsubscript{mr}Bet1::GUS} were observed: expression in extra cell layers of the endosperm, and in ectopic spots in the endosperm. Expression in extra cell layers at the base of the endosperm could be interpreted as a conversion of the domains adjacent to the BETL, the CZ,
and/or the BIZ, into BETL cells. More experiments are required to determine the extent to which markers for all three domains (Li et al. 2014) are affected in these mutants. The ectopic spots of \(p_{\text{Bet1}}::\text{GUS}\) are more commonly found at the periphery of the endosperm than the interior, possibly indicating a bias toward BETL formation at a boundary. Ectopic spots of expression are also much more common on the abgerminal side of the endosperm than the germinal side, suggesting there is inhibition of BETL gene expression near the embryo. Bias for BETL formation away from the center and toward the abgerminal side of the endosperm is consistent with the effects of overexpressing \(\text{meg1}\) (Costa et al. 2012).

All of the mutants with misplaced polar nuclei, except for \(bsl2\), also have abnormal antipodal cell clusters. The most common phenotype is a reduced number of antipodal cells of normal morphology. In \(hrl1\) and \(nol1\), effects on the antipodal cells are background dependent. In \(hrl1\), the antipodal cells are fewer in number but normal in appearance in W64A and B014, but larger than wild type in Mo17. In \(nol1\), the antipodal cells are usually normal in W23 and B73 but more frequently have reduced numbers in B104. \(nol1\) and \(hrl1\) have similar kernel and embryo-sac phenotypes in B104, which, coupled with their map positions, is consistent with them having mutations in the same gene. They also have similar transmission frequencies and are both likely homozygous viable (data not shown).

The antipodal cells have been proposed to transfer nutrients to the embryo sac (or early seed) or to act as a signaling center marking one pole of the endosperm (Diboll 1968; Chettoor and Evans 2015). The maize \(rgh^{*-1210}\) mutant indicates a connection between endosperm/embryo development and postfertilization morphology of the antipodal cells, although the cause-and-effect relationship of these phenotypes is undetermined (Clark and Sheridan 1988). In the maternal-effect mutants described here, the frequency of the antipodal cell phenotypes is often lower than that of the seed phenotypes. Consequently, there is not a 1:1 correspondence between seed phenotype and antipodal cell cluster phenotype. Reduced antipodal cell cluster size may contribute to reduced seed growth or abnormal endosperm patterning after fertilization, but the correlation is not clear. It is equally possible that the mutated genes promote proliferation of all cells, including the antipodals and the endosperm after fertilization. In this model, the maternal effect could be caused by delayed activation of the paternal allele. Under this model the mutations affect antipodal cell and seed development independently through the same mechanism, rather than affecting seed development via effects on antipodal cell development.

While a subset of these mutations may affect imprinting or be in imprinted genes, the majority seem to be involved in producing gametes with the correct structure to support normal development of the endosperm and/or embryo. While it cannot be ruled out that the mutants with abnormal gamete morphology also affect imprinting, the simplest model is one in which abnormal seed development is a direct consequence of gamete abnormalities. More data will be necessary to distinguish between these possibilities. Mutants with normal embryo-sac morphology (\(Mn-Uq, \text{tpn1}, hrl2, nbe1,\) and \(ecr2\)) are better candidates for affecting imprinted genes. These effects could be caused by mutations in genes with delayed or no paternal allele activation in the endosperm (or embryo) or with global effects on imprinting. These alternatives can be resolved once the mutant genes have been identified.

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**Note added in proof:** See Bai et al. 2016 (pp. 221–231) in this issue, for a related work.

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Figure S1. Example of a single defective kernel from a *Mu* active *UniformMu W22* female (arrowhead).
Figure S2. Example of a dominant mutant isolated from the maternal effect screen. (A) Mutant +/dek*-N6 female by wild type pollen. (B) Wild type female by +/dek*-N6 pollen. Note defective kernels segregating in both crosses, although the phenotype is more severe as a female than as a male indicating a potential dosage effect. Arrowheads indicate representative defective kernels on each ear.
Figure S3. Tassel phenotypes of *mrn2* and *mrn3*. (A) Tassel of wild-type W22. (B) Tassel of */mrn2* W22 heterozygote. (C) Tassel of */mrn3* W22 heterozygote.
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Table S1 Mapping data for maternal effect data. (.xlsx, 48 KB)

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Table S2 proBET1::GUS expression data. (xlsx, 36 KB)

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