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Analysis of *stunter1*, a Maize Mutant with Reduced Gametophyte Size and Maternal Effects on Seed Development

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ABSTRACT

Many higher eukaryotes have evolved strategies for the maternal control of growth and development of their offspring. In higher plants this is achieved in part by postmeiotic gene activity controlling the development of the haploid female gametophyte. *stunter1* (*stt1*) is a novel, recessive, maternal effect mutant in maize that displays viable, miniature kernels. Maternal inheritance of *stt1* results in seeds with reduced but otherwise normal endosperms and embryos. The *stt1* mutation displays reduced transmission through the male and female parents and causes significant changes in the sizes of both male and female gametophytes. *stt1* pollen grains are smaller than wild type, have reduced germination efficiency, and reduced pollen tube growth. *stt1* embryo sacs have smaller central cells and abnormal antipodal cells that are larger, more vacuolated, and fewer in number than wild type. Embryos and endosperms produced by fertilization of *stt1* embryo sacs develop and grow more slowly than wild type. The data suggest that the morphology of mutant embryo sacs influences endosperm development, leading to the production of miniature kernels in *stt1*. Analysis of seeds carrying a mutant maternal allele of *stt1* over a deletion of the paternal allele demonstrates that both parental alleles are active after fertilization in both the endosperm and embryo. This analysis also indicates that embryo development until the globular stage in maize can proceed without endosperm development and is likely supported directly by the diploid mother plant.

THE maize female gametophyte (embryo sac) follows the Polygonum-type pattern of development, which results in an eight-nucleate, seven-celled megagametophyte (Reiser and Fischer 1993; Christensen *et al.* 1997). Differentiation of the cells produces a mature embryo sac consisting of antipodal cells at the chalazal end, two synergid cells and the egg cell at the micropylar end, and the homodiploid central cell in the middle. In maize, the antipodal cells proliferate after cellularization, producing a cluster of 30–100 cells (Bedinger and Russell 1994). Seed production is accomplished through double fertilization, during which the male gametophyte (pollen) contributes two sperm cells that fuse with the female gametes, the egg cell and central cell to give rise to the sporophytic tissues of the seed—the diploid embryo and the triploid endosperm, respectively (for reviews see Sheridan and Clark 1994; Walbot and Evans 2003).

Following fertilization, the maize embryo develops through three distinct phases (for reviews see Sheridan and Clark 1994; Vernoud *et al.* 2005): formation of the proembryo; establishment of radial symmetry, the embryonic axis, and the shoot and root meristems; and maturation and dehydration of the embryonic structures. Maize endosperm development begins just after fertilization and occurs in four stages. First there is a period of free nuclear divisions to produce a syncytium consisting of >250 nuclei [1–2 days after pollination (DAP)] (Randolph 1936). Following syncytial development, the endosperm cellularizes, starting at the periphery and finishing in the center of the endosperm (3–4 DAP). The endosperm then begins differentiating into four distinct domains: the aleurone, the basal endosperm transfer layer (BETL), the central starchy endosperm (CSE), and the embryo-surrounding region (ESR) (6 DAP) (Olsen *et al.* 1999; Olsen 2004). The CSE undergoes rapid mitotic growth followed by endoreduplication before undergoing programmed cell death (PCD), beginning near the crown and finally encompassing all of the CSE (16–40 DAP) (Young *et al.* 1997).

Several large mutant studies have been conducted in maize to try to understand the number and types of genes involved in seed development in general (Neuffer and Sheridan 1980; Sheridan and Neuffer 1980; Clark and Sheridan 1991; Scanlon *et al.* 1994; McCarty *et al.* 2005; Settles *et al.* 2007) and endosperm development in particular (for reviews see Bommert and Werr 2001; Olsen 2001, 2004). Additionally, many

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studies have been done in an attempt to elucidate the genetic makeup of the female haploid plant in maize and Arabidopsis (Patterson 1978; Cordts *et al.* 2001; Le *et al.* 2005; Pagnussat *et al.* 2005; Sprunck *et al.* 2005; Yu *et al.* 2005; Yang *et al.* 2006; Jones-Rhoades *et al.* 2007; Steffen *et al.* 2007; Wuest *et al.* 2010). Many of these mutant and gene expression studies helped determine that early seed development is largely under the control of the maternal genome (Vielle-Calzada *et al.* 2000). Mutations in genes required in the embryo sac for proper seed development can be identified by mode of inheritance (Grossniklaus and Schneitz 1998; Evans and Kermicle 2001). Although required in the female but not male gametophyte for normal seed development, many gametophytic maternal effect mutations also influence development of the male gametophyte, reducing male transmission without paternal effects on seed development (Pagnussat *et al.* 2005; Boavida *et al.* 2009).

To date, several gametophytic maternal effect mutants have been identified in both Arabidopsis and maize (Olsen 2004; Kohler and Grossniklaus 2005; Pagnussat *et al.* 2005; Pien and Grossniklaus 2007). Studies of these mutants and others have revealed several causes for maternal effects. Possible mechanisms include: changes in functional gene dosage in the endosperm (*e.g.*, *floury3*; Ma and Nelson 1975), abnormal embryo sac morphology (*e.g.*, *baseless1*; Gutierrez-Marcos *et al.* 2006), loss of embryo sac proteins normally stored cytoplasmically to act after fertilization (perdurance) (*e.g.*, *PROLIFERA*; Springer *et al.* 2000), or imprinting (*e.g.*, *Polycomb* group and other genes in maize and Arabidopsis (recently reviewed in Huh *et al.* 2008; Johnson and Bender 2009; Jullien and Berger 2009). Known maternal gametophyte effect mutants are involved in basic cellular process, such as DNA demethylation, DNA repair, and cell cycle regulation, as well as seed specific processes such as the suppression of autonomous endosperm development (Golden *et al.* 2002; Holding and Springer 2002; Ngo *et al.* 2007; Andreuzza *et al.* 2009).

Maternal effect mutants have been described in maize that affect the endosperm and embryo [*e.g.*, *maternal effect lethal1* (*mel1*)] (Evans and Kermicle 2001), *bsl1*, (Gutierrez-Marcos *et al.* 2006), and *Dappled* (*Dap*) (Gavazzi *et al.* 1997). Despite lacking a paternal effect on seed development, these mutations often have a gametophytic effect on pollen development (*e.g.*, *mel1*, *bsl1*, and *Dap*). To better understand how maternal gene expression and embryo sac morphology can affect seed development, we have identified and characterized a novel maternal effect mutant in maize, named *stunter1* (*stt1*). The *stt1* mutation reduces the size of both male and female gametophytes, and the defective embryo sacs in turn produce seeds with smaller embryos and endosperms.

MATERIALS AND METHODS

Plant material and growth conditions: The *stt1* mutation arose spontaneously in a standard W23 inbred maize (*Zea mays*) plant. The *stt1* mutation was propagated as a heterozygote by transmission through the female and selection for miniature *stt1*/+ kernels. *stt1* was backcrossed by the M14 inbred maize stock through at least four generations prior to characterization of the effects of *stt1* on seed and gametophyte development. 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).

Male and female transmission of the *stt1* mutation and penetrance of the miniature kernel phenotype were partially assessed using plants carrying *stt1* linked in repulsion phase to the *waxy1* (*wx1*)-marked T2-9d reciprocal translocation in the M14 inbred background (Maize Genetics Cooperation Stock Center). Reciprocal crosses between F_1 plants and the *wx1* T2-9d tester were made to calculate transmission rates. The genetic distance between *stt1* and *wx1* on T2-9d was determined using the pollen phenotypes of *wx1* and *stt1*. Female transmission (Ft) of *stt1* was calculated on the basis of linkage to and transmission of the *wx1* marker. Transmission of *wx1* observed after crossing T2-9d/Normal chromosomes (N) +/*stt1 wx1*/+ as a female equals the number of nonrecombinant *wx1 Stt1*$^+$ kernels and recombinant *wx1 stt1* kernels divided by the total number of kernels. This relationship was used to calculate the Ft of *stt1* and therefore the percentage of embryo sacs carrying *stt1* that produced a detectable kernel. To test for male transmission of *stt1*, *Wx1*$^+$ kernels resulting from the cross of *wx1* T2-9d females by T2-9d/N +/*stt1 wx1*/+ were progeny tested to determine whether they carried *stt1* or *Stt1*$^+$.

Endosperms and embryos lacking maternal and paternal *stt1* function were produced by crossing plants carrying *stt1* and *anthocyaninless2* (*a2*) as females by the TB-5Sc-2L015-3 (TB-5S-2L) compound translocation carrying a segment of the short arm of chromosome 5 with *A2*$^+$ and the long arm of chromosome 2 with *Stt1*$^+$ translocated onto the supernumerary B chromosome (Bill Sheridan, University of North Dakota). Inheritance of the TB-5S-2L chromosome was assessed using the expression of anthocyanin in the endosperm or embryo as conferred by *A2*$^+$. Nondisjunction was inferred if one of the endosperm or embryo was pigmented while the other was colorless. Kernels expressing anthocyanin in the endosperm were further analyzed in testcrosses with *a2/a2* to verify the presence or absence of *A2*$^+$ (and hence B-5S-2L) in the embryo. The rate of nondisjunction for the TB-5S-2L stock was determined by crossing *white3*/+ heterozygotes as females by the TB-5S-2L stock, which carries the wild-type allele of *white3* (*w3*) on the long arm of chromosome 2. *w3* homozygous seeds have white endosperms and viviparous embryos. Seeds with *w3* hypoploid endosperms and hyperploid embryos have white endosperms and dormant embryos, and seeds with *w3* hypoploid embryos and hyperploid endosperms have viviparous embryos and yellow endosperms.

A +/+/*stt1*, +/*a2*/*a2*, triB-5S-2L/N/N tertiary trisomic stock was identified by making reciprocal crosses between *a2* homozygotes and plants from the purple kernels of the above cross carrying *stt1* (verified by production of miniature kernels when crossed as a female) and the TB-5S-2L translocation (verified by nondisjunction when crossed as a male). Trisomic individuals carrying *stt1* were identified from these crosses by growing plants from the purple kernels (those inheriting B-5S-2L) of this cross and making reciprocal crosses with *a2*. Trisomic plants carrying *stt1* segregate miniatures as females, do not undergo nondisjunction, and have reduced, non-

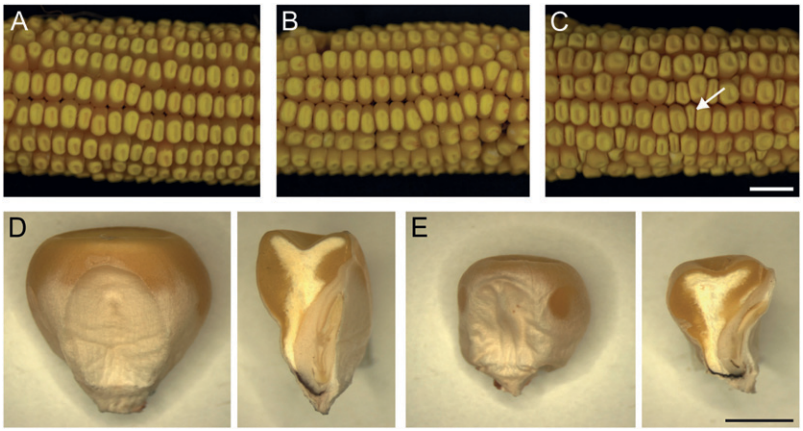


FIGURE 1.—Seed phenotypes of *stt1*. (A) Ear from a homozygous wild-type female pollinated by a homozygous wild-type male. (B) Ear from a homozygous wild-type female pollinated by a *stt1*/+ heterozygous male. (C) Ear from a *stt1*/+ heterozygous female pollinated by a homozygous wild-type male (arrow indicates a *stt1*/+ miniature kernel). Bar, (A–C) 1 cm. (D and E) Germinal (embryo) face (left) and a median longitudinal section (right) of wild-type (D) and *stt1*-affected (E) kernels at the same scale. Bar, (D and E) 0.25 cm.

identical male and female transmission of $A2^+$ (AUGER and BIRCHLER 2002).

Mapping with simple sequence repeat and insertion/deletion markers: *stt1* mapping populations were generated by crossing $stt1^{W23}/+^{M14}$ or $stt1^{W23}/+^{B73}$ hybrid females by wild-type M14 or B73 males, respectively. DNA was extracted from seedlings by minor modification of the method of SAGHAI-MAROOF *et al.* (1984), and PCR reactions were performed as described (EVANS and KERMICLE 2001). Twenty simple sequence repeat (SSR) markers from bin locations across the genome were tested against 48 miniature kernels to determine which markers cosegregated with the kernel phenotype, revealing linkage only on the long arm of chromosome 2. Markers that demonstrated linkage were then tested on a larger population of miniature and normal kernels, and progeny testing was conducted to verify whether recombinant individuals were *stt1*/+ or wild type. Map position was refined using additional SSR and insertion/deletion markers designed for genes within the chromosomal region that showed polymorphisms between M14 or B73 and the original *stt1* chromosome.

Pollen staining and germination and tube growth assays: Pollen was analyzed from *stt1*/+ heterozygotes grown in field conditions or in the greenhouse under long-day conditions. Old anthers were removed 2 hr before pollen was collected in tassel bags and pollen was stained or spread on pollen germination medium (WALDEN 1994). Pollen was allowed to germinate at 28° for 0 or 60 min before fixation with FAA (50% ethanol, 10% formalin, 5% glacial acetic acid) and staining with I_2/KI (0.1% I_2, 1% KI reagent). Digital images were acquired using a Leica DFC320 camera attached to a Leica MZ125 stereomicroscope and analyzed using Image Pro Express 6.0. For 4′-6-diamidino-2-phenylindole (DAPI) staining, pollen coats were digested according to JEWELL and ISLAM-FARIDI (1994). Pollen was collected by centrifugation, resuspended in 100% EtOH, placed on a microscope slide, and allowed to dry. Pollen was stained with 1 μg/ml of DAPI (Sigma) in H_2O for 5 min before visualization and imaging on a Nikon Eclipse E600 UV fluorescent microscope equipped with a Fujifilm FinePix S5 Pro camera.

Confocal microscopy and histology: Embryo sacs were analyzed from *stt1*/+ heterozygous and wild-type M14 ears collected at similar stages of development. Endosperms and embryos from developing kernels were analyzed from *stt1*/+ heterozygotes crossed as females by M14 or TB-5S-2L males, and ears were fixed at multiple DAP. Samples were processed according to GUTIERREZ-MARCOS *et al.* (2006) and visualized on a Leica SP5 (Wetzlar, Germany) laser scanning confocal microscope. Excitation was performed at 405 nm, 488 nm, and 561 nm and emission was collected at 410–480 nm, 495–555 nm, and 565–730 nm for the merged images. Images were analyzed and processed using ImageJ and Adobe Photoshop CS3.

For the reporter assays, *stt1*/+ heterozygous plants were crossed as females by males carrying either $_{Pro}$*Bet1::B-glucuronidase* (*GUS*) or $_{Pro}$*VP1::GUS* transgenic reporters (HUEROS *et al.* 1999; CAO *et al.* 2007). Normal and miniature kernels were cut along the longitudinal axis and either stained for GUS activity as previously described (COSTA *et al.* 2003) or stained with 0.1% Evans Blue for 2 min and washed two times with water to

TABLE 1

Male and female transmission of the *stt1* mutation

	Seed phenotype after female transmission of the mutation				Seed genotype after male transmission of the mutation	
	Mutant *stt1*/+ (♀) × wild type (♂)				Wild type (♀) × mutant *stt1*/+ (♂)	
Cross no. (*N*)	Miniature (*stt1*) (%)	Normal (*stt1*) (%)	Normal (WT) (%)	Cross no. (*N*)	Heterozygous *stt1*/+ (%)	Homozygous WT (%)
1 (177)	4	15	81	1 (77)	0	100
2 (203)	29	3	68	2–13 (466)	0	100
3–27 (1127)	8	25[a]	67[a]			

When *stt1* is crossed as a heterozygous mutant female by a wild-type male <50% of the progeny are abnormal, demonstrating incomplete penetrance of the maternal effect phenotype. Transmission of the mutation through the pollen was not detected, suggesting *stt1* is required for normal pollen development and/or function.

[a] Calculations made on the basis of transmission of *wx1* on T2-9d in repulsion to *stt1*.

TABLE 2

***stt1* is a recessive maternal effect mutation**

	Seed phenotypes			
	Normal, purple	Normal, yellow	mn, purple	mn, yellow
Expected if *stt1* is a dominant maternal effect mutation	B-5S-2L + + N a2 + N a2 + (and due to IP B-5S-2L + + N a2 *stt*1 N a2 +)	N a2 + N a2 + (and due to IP N a2 *stt*1 N a2 +)	B-5S-2L + + N a2 *stt*1 N a2 +	N a2 *stt*1 N a2 +
	~24%	~36%	~16%	~24%
Expected if *stt1* is a recessive maternal effect mutation	B-5S-2L + + N a2 + N a2 + (and due to IP B-5S-2L + + N a2 *stt*1 N a2 +)	N a2 *stt*1 N a2 + (and due to IP N a2 *stt*1 N a2 +)	—[a] B-5S-2L + - N a2 *stt*1 N a2 +	N a2 *stt*1 N a2 +
	~39%	~36%	Rare (~1%)	~24%
Observed number of progeny	162 normal, purple(43%)	124 normal, yellow(33%)	4 mn, purple(1%)	88 mn, yellow(23%)

Possible seed phenotypes and genotypes of the progeny from a *stt1* trisomic stock crossed as a female by *a2/a2* as a male. Expected outcomes if *stt1* is a dominant or recessive maternal effect mutation. Observed number of each type of progeny. The transmission of B-5S-2L as a trisome through the female is only ~40% instead of 50%, so in both scenarios <50% of the progeny are purple (*i.e.*, inherit the trisome with *A2*$^+$). IP, incomplete penetrance of the miniature phenotype.

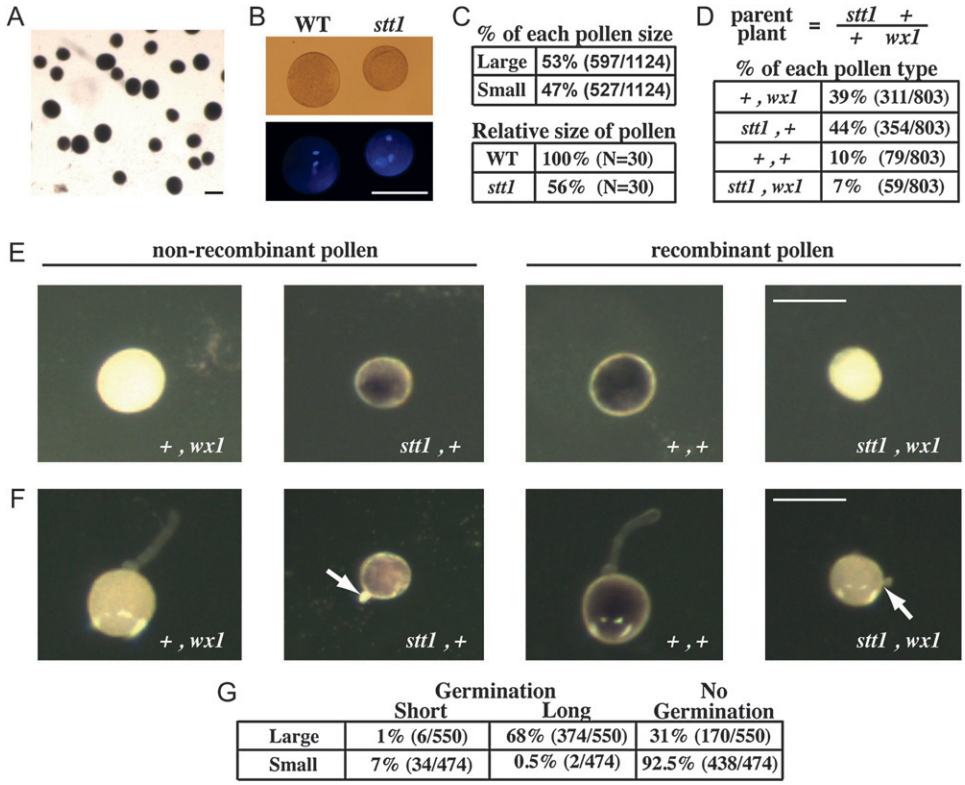
[a] Result of rare recombination events between the B-5S-2L chromosome and the normal 5 chromosome.

visualize programmed cell death. Images were collected as described above for the pollen germination assay.

RESULTS

***stunter1* is a novel recessive maternal effect mutation in maize:** The *stt1* mutation was originally isolated from a single cross (standard W23 inbred female crossed by a W22 inbred male) that was segregating miniature kernels. The miniature kernels were viable, and, in reciprocal crosses with wild type, miniature kernels were only seen when mutants were used as females, suggesting a maternal effect mutant. The *stt1* seeds display reduced but otherwise normal endosperms and embryos (Figure 1) that produce normal seedlings and mature plants. *stt1* was mapped to the long arm of chromosome 2 in Bin 2.08, 1.2% (14/1141) away from *bnlg1233* and 1.0% (3/304) away from *GRMZM2g077823* (annotation based on the Maize Genome Sequencing Project, Release 4a.53, http://maizesequence.org/). The mutation was crossed twice to several inbred lines and found to exhibit strong phenotypic expression in M14. Therefore the mutation was backcrossed for two more generations to M14 before further analysis.

The percentage of miniature seeds that result by pollinating *stt1*/+ plants with wild-type pollen ranges from 4 to 29% (Figure 1 and Table 1), indicating incomplete penetrance of the miniature kernel phenotype or failure of a subset of *stt1* embryo sacs to produce a seed. To aid in the assessment of male and female transmission rates of *stt1*, the *wx1* T2-9d reciprocal translocation stock was crossed to *stt1* to link *wx1* as a visible endosperm and pollen marker in repulsion to *stt1*. F_1 plants were subsequently crossed reciprocally with the *wx1* T2-9d tester. Progeny testing of the normal kernels and/or scoring for *wx1* in crosses using *stt1*/+ as the female revealed that 3–25% of the phenotypically normal kernels were carrying the *stt1* mutation. Total *stt1*/+ mutant progeny (miniature plus normal kernels carrying *stt1*) comprises <50% of all progeny from a *stt1*/+ female, indicating that a subset of *stt1* embryo sacs are never fertilized or produce seeds that abort at a stage too early to identify by visual examination. Using the *wx1* female transmission rate in the kernels and the *wx1 stt1* recombination rate determined from the pollen (below), we calculated that approximately half (47%) of *stt1* embryo sacs made an identifiable seed while the other half (53%) did not.



G

	Germination Short	Germination Long	No Germination
Large	1% (6/550)	68% (374/550)	31% (170/550)
Small	7% (34/474)	0.5% (2/474)	92.5% (438/474)

FIGURE 2.—Effect of *stt1* on pollen. (A) I_2/KI -stained pollen from a *stt1*/+ heterozygote. (B) DAPI staining to show nuclei of pollen from a *stt1*/+ heterozygote. (C) The percentage of pollen grains of each size and their relative sizes (to large pollen) are indicated. (D–F) Pollen from T2-9/N *wx1*/+ +/*stt1* heterozygotes with *wx1* and *stt1* in repulsion phase (parent plant). (D) The percentage of recombinant and nonrecombinant pollen classes ($n = 803$). (E and F) Pollen from T2-9/N *wx1*/+ +/*stt1* plants stained with I_2/KI without germination (E) or after 60-min incubation on pollen germination medium (F). Arrows point to arrested pollen tubes. (G) The percentage of nongerminated pollen and germinated pollen with long and short pollen tubes. Bars, (A, B, E, and F) 100 μM.

To determine whether the maternal effect of the *stt1* mutation is recessive and therefore more likely to be a loss of function, we generated a *stt1* trisomic stock (+/+/*stt1*, +/*a2*/*a2*, triB-5S-2L/N/N; see MATERIALS AND METHODS), which will produce some disomic *stt1*/+ embryo sacs. We crossed miniature, purple individuals (carrying *stt1* and the B-5S-2L chromosome) by *a2*/*a2*. From this cross, four seed phenotypes are expected in the progeny: normal, purple; normal, yellow; miniature (mn), purple; and mn, yellow. Because the unpaired, triB-5S-2L chromosome is not transmitted as efficiently as the normal chromosomes 2 and 5, only ~40% instead of 50% of the seeds are expected to inherit B-5S-2L and thus be purple. Table 2 lists the expected ratios of each seed phenotype and the causal genotypes for dominant or recessive action of *stt1*. From this cross, we observed 162 normal, purple seeds (43%); 124 normal, yellow seeds (33%); 4 mn, purple seeds (1%); and 88 mn, yellow seeds (23%). This ratio is not consistent with *stt1* being dominant ($\chi^2 = 108.2$, $P < 0.0001$), but is consistent with *stt1* being recessive ($\chi^2 = 2.69$, $P = 0.44$). The key frequency is that of miniature purple seeds (*i.e.*, those inheriting both *stt1* and $A2^+$). If *stt1* were dominant, these would be almost as common as the yellow miniatures that lack the trisome. Instead, this class was very rare, which is consistent with *stt1* being recessive, and likely resulted from rare recombination between B-5S-2L and one of the normal chromosomes.

***stunter1* affects the size and germination efficiency of pollen:** Segregation distortion of *wx1* and progeny testing in crosses using *stt1*/+ as the male revealed a strong defect in *stt1* pollen transmission (Table 1). Consistent with this observation, *stt1*/+ heterozygotes produce ~50% small pollen grains consistent with a heterozygous gametophytic mutation (Figure 2, A–C, $\chi^2 = 0.36$, $P = 0.55$, $n = 1124$). The smaller grains are approximately half the size of the larger grains. As visualized by DAPI staining, the small pollen had normal vegetative and sperm nuclei, suggesting mitosis I and II proceeded normally. I_2/KI staining of the pollen from a *stt1*/+ heterozygote revealed that the small pollen grains were also fully filled with starch like wild type.

To determine whether the smaller grains are a consequence of the *stt1* mutation, we utilized the T2-9/N *wx1*/+ +/*stt1* translocation stock described above. Pollen grains carrying the $Wx1^+$ allele dye purple when stained with I_2/KI reagent, while pollen carrying the *wx1* allele stain red (Figure 2, D–F, purple *vs.* yellow appearance with transmitted light) (BRINK and MACGILLIVRAY 1924; DEMEREC 1924). After staining, 80% of the large pollen grains were red and 86% of the small pollen grains were purple, which confirms that the small pollen phenotype is linked to *wx1* (~17 cM away) and so likely results from the *stt1* mutation.

Despite having normal nuclei and starch filling, the small pollen was not functional. *In vitro* germination of pollen from *stt1*/+ heterozygotes revealed that the small *stt1* pollen grains had reduced germination (8%) compared to the large wild-type grains (69%) (Figure 2G), and, of the small *stt1* pollen grains that did

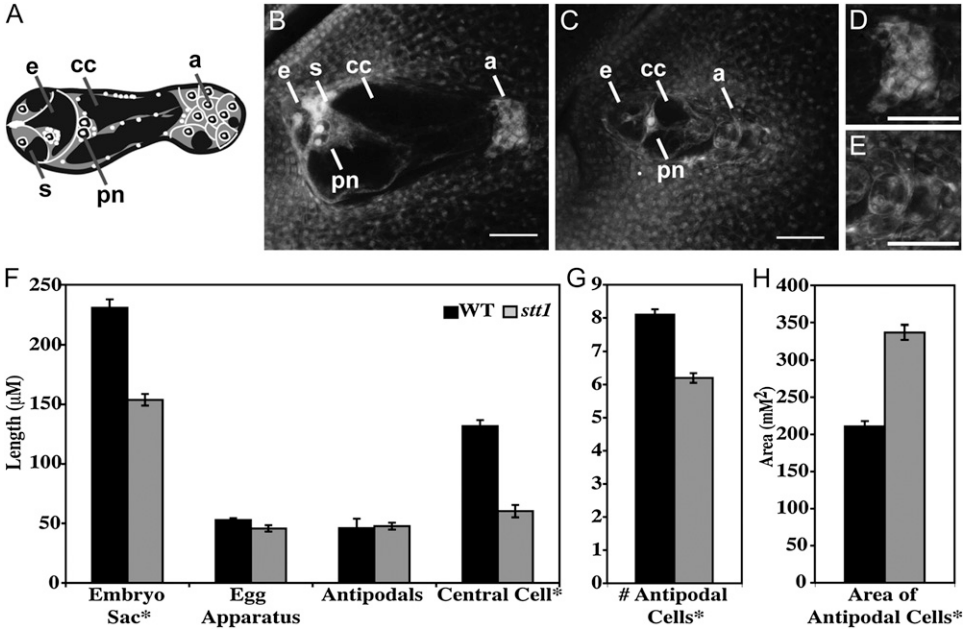


FIGURE 3.—Effect of *stt1* on embryo sac morphology. (A) Diagram of a mature normal embryo sac. (B–E) Confocal micrographs of embryo sacs of wild type (B and D) and *stt1* (C and E). (B and C) Whole embryo sacs with the micropyle to the left. a, antipodal cells; cc, central cell; pn, polar nuclei; e, egg cell; s, synergid. (D and E) Close up view of the antipodal regions. Bars, 50 μM. (F) The length (μM) of the whole embryo sac, egg apparatus, central cell, and antipodal cell region of normal and mutant embryo sacs ($N = 21$ for WT and $N = 25$ for *stt1*). (G) The number of antipodal cells in wild-type and mutant embryo sacs ($N = 21$ for WT and $N = 25$ for *stt1*). (H) The size of individual antipodal cells in wild-type and mutant embryo sacs (μM² in cross-section) ($N = 60$). For F, G, and H comparisons are made between sibling embryo sacs from *stt1*/+ heterozygotes. Error bars ± 1 SEM. *Significantly different from WT ($P < 0.0001$).

germinate, only 6% extended a long pollen tube while 94% had a short arrested pollen tube. This is in contrast to the germinated, large pollen grains that extended long pollen tubes 98% of the time and short pollen tubes only 2% of the time ($n = 1024$).

To see whether competition with wild-type pollen caused the reduced *stt1* pollen transmission, we crossed *wx1* T2-9d ears by T2-9/N *wx1*/+ +/*stt1* heterozygous males using varying amounts of pollen. In six paired crosses, ears were pollinated either sparsely or heavily creating less and more competition between wild-type and *stt1* sibling pollen grains, respectively. Male transmission rates of *wx1* in repulsion to *stt1* were not significantly different between the paired sparse (8.9%) and heavy (12.3%) pollinations ($\chi^2 = 1.07$, $P = 0.30$; supporting information, Table S1). Taken together, these data support the hypothesis that *stt1* pollen is never functional.

***stunter1* embryo sacs are smaller than wild type:** In *stt1*/+ heterozygotes, two types of embryo sacs (Figure 3) segregated ~1:1 (40/75 mutant:35/75 normal), consistent with a gametophytic mutation with high penetrance ($\chi^2 = 0.44$, $P = 0.51$). Mutant embryo sacs (Figure 3C) are significantly smaller than wild type (Figure 3B) with abnormal antipodal cells and synergids.

The difference in size between the two types of embryo sacs is largely due to a smaller central cell in *stt1*—approximately half that of wild type (Figure 3F). Both the egg apparatus (measured from the micropyle to the polar nuclei) and antipodal cell region are similar in size in the mutant embryo sacs and wild type, but mutant antipodal cells appear larger, less cytoplasmically dense, and fewer in number than wild type (Figure 3, D–F). Additionally, the mutant synergids are less distinguishable than wild-type synergids, which fluoresce brightly under these fixation conditions since they degenerate at maturity (Figure 3, B and C).

Maize ovules are initiated at the base of the ear first, so the tip of the ear has the least mature ovules, and ovules at the base of the ear are the most mature. Because of this

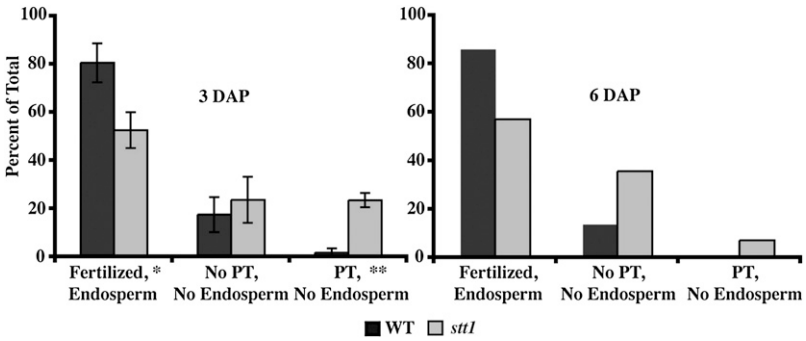


FIGURE 4.—Fertilization rate of *stt1* embryo sacs. Heterozygous *stt1*/+ ears were pollinated by wild type and fixed 3 or 6 DAP. Embryo sacs were classified as WT or *stt1* and as fertilized with endosperm proliferation, lacking both PT entry and endosperm proliferation, or having PT entry without endosperm proliferation. For 3 DAP, average ratios ± SEM are shown, $N = 4$ independent crosses for a total of 162 WT individuals and 148 *stt1* individuals. For 6 DAP, ratios are shown for 1 cross ($N = 36$ for WT, $N = 42$ for *stt1*). Significantly different from WT (*$P < 0.05$ or **$P < 0.001$).

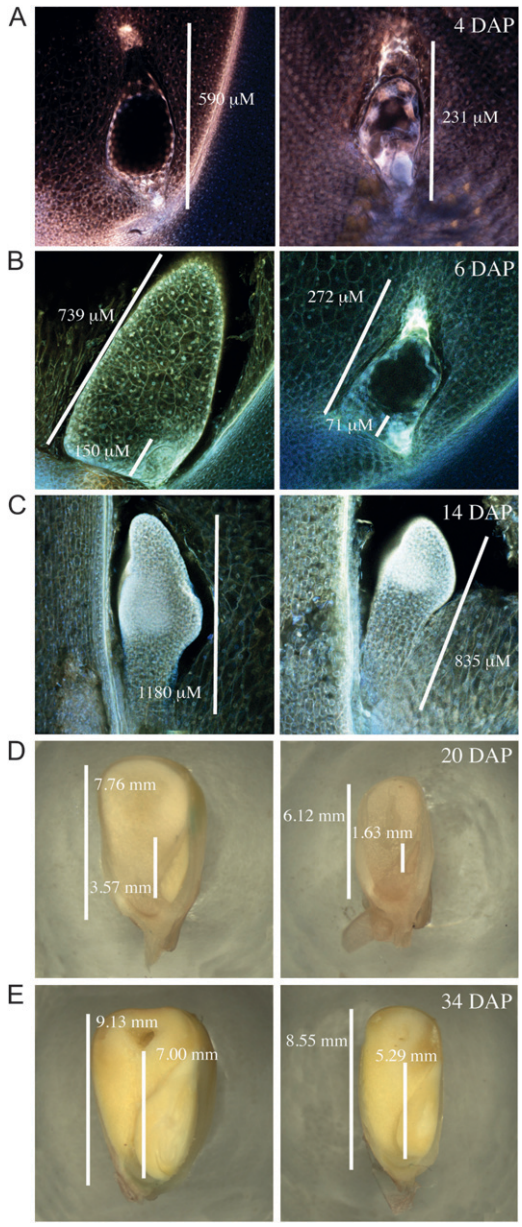


FIGURE 5.—*stt1* seeds are developmentally delayed compared to wild type. Embryos and/or endosperm 4 DAP (A), 6 DAP (B), 14 DAP (C), 20 DAP (D), and 34 DAP (E). Left panels depict wild type-like kernels and right panels depict mutant kernels. Bars, length of each embryo or endosperm.

progressive development, adjacent ovules should be at the same developmental stage and close in size. In *stt1*/+ heterozygotes, we were able to identify neighboring embryo sacs that were either two distinct sizes or at two different stages of development at least as early as the two-nucleate stage (Figure S1). These results indicate that the *stt1* mutation acts very early in development prior to embryo sac maturation to affect the size of the embryo sac.

Fertilization of *stt1* embryo sacs: Since fewer than half of the seeds of a *stt1*/+ heterozygote inherit *stt1*, some *stt1* embryo sacs are never fertilized or produce seeds that arrest so early they are not distinguishable from unfertilized ovules. To distinguish between these two scenarios, we examined embryo sacs from *stt1*/+ heterozygotes crossed by wild type at 3 DAP (Figure 4, left graph). At 3 DAP, wild type and *stt1* can be distinguished by size and antipodal cell morphology. Fertilization was inferred by syncytial endosperm development. At 3 DAP, a ring of cortical nuclei is evident in cross-section of both *stt1* and wild type with significantly fewer nuclei in *stt1* (Figure 5A). Pollen tube entry into the embryo sac was inferred either by intense fluorescence of the penetrated synergid (Figure S2) or by endosperm growth. We imaged and categorized 310 total ovules at 3 DAP from four independent crosses (Figure 4, left graph). Of the wild-type ovules, 81% had attracted a pollen tube and been fertilized, 18% were unfertilized without evidence of pollen tube (PT) entry, and 2% showed evidence of PT entry without endosperm growth. For *stt1* ovules, we observed that 53% were fertilized, 24% remained unfertilized without evidence of PT entry, and the remaining 23% showed PT entry into the synergid with no visible signs of endosperm growth, making it unclear whether these ovules remained unfertilized or were fertilized but developing slowly. These results confirm that while PT attraction to *stt1* embryo sacs is normal, a significant percentage of *stt1* embryo sacs are either never fertilized or initiate development very slowly.

To determine whether the ovules that had evidence of PT entry without endosperm development at 3 DAP were fertilized but delayed in initiation of seed development, we examined 78 embryo sacs from a *stt1*/+ heterozygote crossed by wild type at 6 DAP (Figure 4, right graph). At 6 DAP, it was rare to detect PT entry without endosperm development when compared to 3 DAP, possibly because the distinctive bright fluorescence of the penetrated synergid had faded. For wild-type embryo sacs at 6 DAP, there were no significant changes in the percentage of ovules for each class when compared to 3 DAP. The percentage of *stt1* embryo sacs that had initiated endosperm development at 6 DAP was also unchanged from 3 DAP, suggesting that some *stt1* embryo sacs never initiate seed development despite pollen tube entry. That this phenotype is due to a lack of fertilization of these *stt1* embryo sacs is consistent with these observations, but alternate models are also possible.

Immaturity of *stt1* embryo sacs at the time of pollination could explain a reduced fertilization rate. To test this hypothesis, pollination of the T2-9/N *wx1*/+ +/*stt1* females by *wx1* T2-9d males was performed on ears at different stages of maturity. Allowing embryo sacs more time to mature did not increase the transmission rate of *stt1*, making it less likely that the reduced female transmission of *stt1* is a simple consequence of delayed maturation (Table S2).

***stunter1* embryos and endosperms are developmentally delayed:** We examined developing kernels from

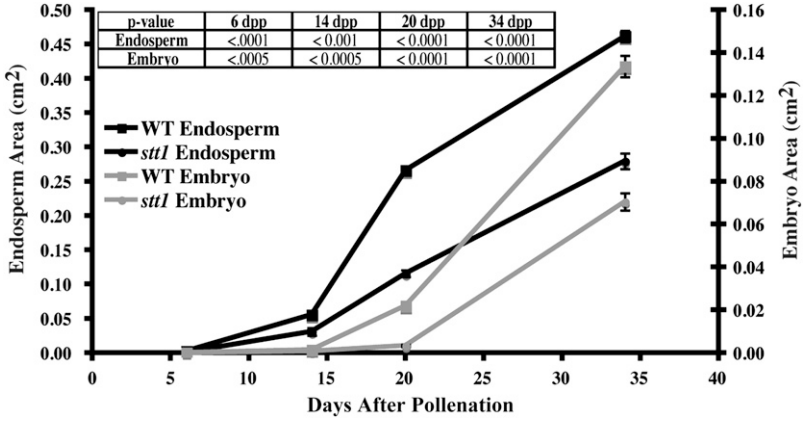


FIGURE 6.—Growth rate and size of *stt1* seeds. The area of the endosperm and embryo at the median longitudinal cut face for WT and miniature kernels from *stt1*/+ ears at 6, 14, 20, and 34 DAP. Error bars are ±1 SEM, $N \geq 10$ for each data point. The table inset lists the *P* values for comparisons of *stt1* and wild type at each pair of data points.

crosses of *stt1*/+ heterozygous females by homozygous wild-type males at 3, 4, 6, 14, 20, and 34 DAP (Figures 4 and 5). At all time points we could clearly identify two classes of kernels, where one class had significantly smaller embryos and endosperms (Figure 6). The endosperms in the *stt1*/+ kernels were 30–66% smaller than in the wild-type kernels, while the embryos were 45–85% smaller in *stt1*/+. Retarded development of *stt1* embryos likely caused their reduced size (*e.g.*, 14 DAP wild-type embryos were at the coleoptile stage of development while the *stt1*/+ embryos were still in transition stage; Figure 5C). Though developmentally delayed, no gross morphological differences in the embryo or endosperm of the *stt1*/+ seeds were apparent. All regions of the differentiated endosperm (aleurone, CSE, BETL, and ESR) developed typical cell shapes and sizes (SCANLON and TAKACS 2009).

To determine the effects of *stt1* in more detail we examined several markers of endosperm development. Since some maternal effect mutants cause kernels to develop patches lacking aleurone (GAVAZZI *et al.* 1997) or BETL (GUTIERREZ-MARCOS *et al.* 2006), and some zygotic mutants ectopically express aleurone tissue (COSTA *et al.* 2003), we examined the expression of markers for these two tissues. For aleurone, we crossed *stt1*/+ females by males carrying $_{Pro}$*Vp1::GUS* and stained for GUS activity in mutant and wild-type endosperms at 20 and 34 DAP (Figure 7) (CAO *et al.* 2007). At 20 DAP, while the larger wild-type kernels showed GUS activity in the entire aleurone layer and embryo, the miniature kernels only showed expression in the embryo and in the aleurone layer at the very crown of the seed where *VP1* expression begins. By 34 DAP, both large and miniature seeds showed *VP1* expression throughout the aleurone layer, indicating that *VP1* expression in the aleurone is delayed in the mutant kernels but eventually develops the same pattern as wild type.

For the BETL, we crossed *stt1*/+ females by males carrying $_{Pro}$*BET1::GUS*, which should be active throughout the basal domain of the endosperm (HUEROS *et al.* 1999). At 34 DAP, the area of GUS staining in the kernels was similar between normal and miniature kernels, but the area was smaller in the miniature kernels at 20 DAP, suggesting that the development of the BETL, like that of the aleurone, is delayed in *stt1* kernels (Figure 7).

Programmed cell death of the CSE, which typically begins at the crown of the kernel and progresses to the base, was examined using Evans Blue staining, which stains dead cells (YOUNG *et al.* 1997; YOUNG and GALLIE 2000). At 34 DAP, a smaller region of less intense staining was seen in the miniature kernels compared to the normal kernels (Figure 7), suggesting that PCD progression was delayed by *stt1*. Taken together, these data suggest that the *stt1* mutation causes significant

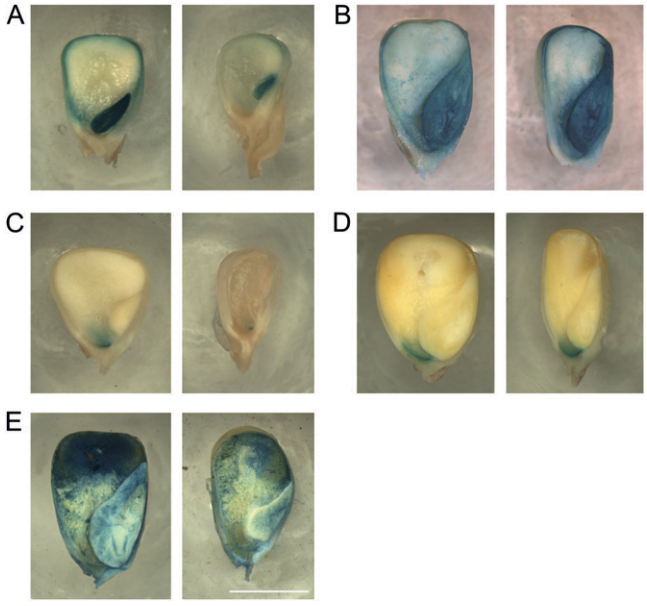
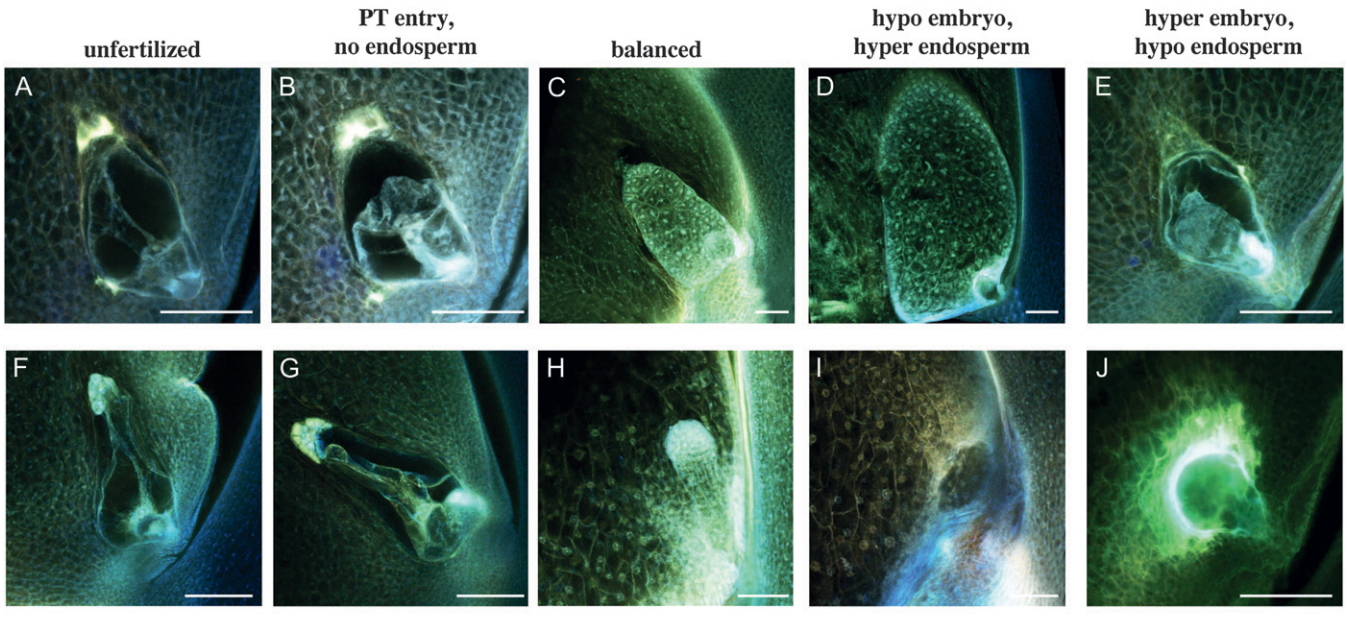


FIGURE 7.—*stt1* effects on cell type differentiation and maturation. (A and C) 20 DAP; (B, D, and E) 34 DAP. Kernels from a *stt1*/+ ear expressing (A and B) $_{Pro}$*VP1::GUS* in the aleurone layer and embryo or (C and D) $_{Pro}$*Bet1::GUS* in the BETL. (E) Kernels from a *stt1*/+ ear stained with Evans Blue to indicate the extent of PCD in the CSE. Left panels depict wild type-like kernels and right panels depict *stt1* kernels. Bar, 5 mm.



K

	WT	*stt1*
fertilized, endosperm	85.5% (71/83)	59.1% (39/66)
no PT, no endosperm	13.3% (11/83)	24.2% (16/66)
PT, no endosperm	1.2% (1/83)	16.7% (11/66)

	WT*	*stt1***
balanced	53.8%	51.3% (20/39)
hypo embryo, hyper endosperm	23.1% (14/121 x 2)	30.8% (12/39)
hyper embryo, hypo endosperm	23.1% (14/121 x 2)	17.9% (7/39)

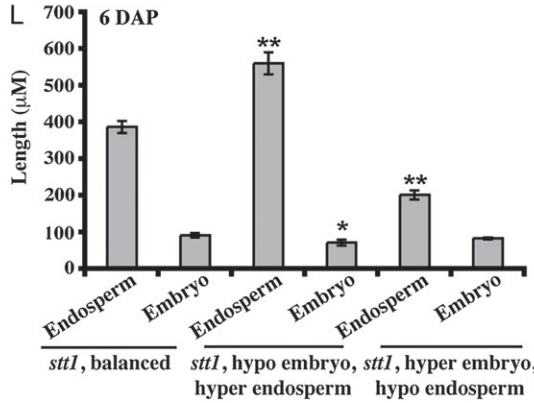


FIGURE 8.—Lethality of hypoploid *stt1*/− embryos and endosperms. Confocal micrographs of embryo sacs and developing seeds from *stt1*/+ crossed as a female by TB-5S-2L males at 6 DAP (A–H) or 10 DAP (I and J). (A–E and I and J) *stt1*. (F–H) WT. Bars, 100 μM (A–J). (K) Fertilization rates (top) and frequency of kernels with each genetic constitution (bottom) resulting from *stt1*/+ crossed as a female by TB-5S-2L as a male 6 DAP. Rates of hypoploidy for *WT calculated from a cross of *w3*/+ females by the same TB-5S-2L males and ***stt1* calculated from the frequency of novel *stt1* phenotypes. (L) Embryo and endosperm length (±SEM) of *stt1* individuals from *stt1*/+ by TB-5S-2L for each genetic constitution. *Significantly different from balanced *stt1* individuals (*$P < 0.05$ or **$P < 0.001$).

delays in all aspects of endosperm and embryo development beginning at fertilization.

Embryos and endosperms lacking both maternal and paternal wild-type *stt1* activity arrest early: Lack of *stt1* pollen transmission precluded an analysis of *stt1* homozygotes for possible mutant phenotypes. To test for sporophytic roles of *stt1*, we crossed *stt1* females with plants carrying a reciprocal translocation between the supernumerary B chromosome and the long arm of chromosome 2, carrying the wild-type allele of *stt1*. Nondisjunction of the B-A chromosome during the second pollen mitosis produces a pollen grain carrying one sperm cell with zero doses of the long arm of chromosome 2 (*i.e.*, no copies of wild-type paternal $Stt1^+$) and another sperm carrying two copies of the long arm of chromosome 2. Fertilization of *stt1* embryo sacs by such a pollen grain produces one progeny (either the embryo or endosperm) with no paternal copies of $Stt1^+$ (hypoploid) and the other progeny (either the endosperm or embryo) with two copies (hyperploid). The hypoploid embryos and endosperms produced by fertilization of *stt1* embryo sacs consequently have no functional *stt1* alleles.

To identify *stt1*/− hypoploid individuals, we crossed *stt1*/+; *a2*/+ heterozygotes as females by males carrying the compound B-A chromosome, TB-5S-2L, which is carrying $A2^+$ and $Stt1^+$. Fertilization of *a2* embryo sacs by pollen grains that undergo nondisjunction would produce seeds that either contain a hypoploid embryo (anthocyaninless with no paternal $A2^+$ or $Stt1^+$) with a hyperploid endosperm (purple with two paternal copies of $A2^+$ and $Stt1^+$) or a hyperploid embryo and a

hypoploid endosperm. Fertilization of embryo sacs by pollen grains in which the chromosomes disjoin normally produces seeds with one paternal dose of both $A2^+$ and $Stt1^+$ in the endosperm and the embryo causing both to be purple, a balanced individual similar to pollination by stocks without B-A translocations.

Within the class of seed with $A2^+$ in the endosperm, seeds potentially carrying *stt1* hypoploid embryos were identified by an aborted embryo/germless kernel phenotype that was more severe than the miniature *stt1*/+ seed phenotype. Since identification of seeds lacking anthocyanin in the embryo was difficult when the endosperm was purple, other candidate seeds for *stt1* hypoploid embryos having miniature, purple (*i.e.*, $A2^+$) endosperms and normal embryos were progeny tested for *stt1* and *a2* in the embryo. Fourteen plants from seeds with purple miniature endosperms segregated miniature seed when crossed by *a2*, but none were *stt1*/− hypoploids, on the basis of the lack of the spindly growth habit characteristic of B-5S-2L hypoploids and the segregation of $A2^+$ in the progeny (Beckett 1994; Birchler and Guo 1997). For comparison, 23 normal, purple seeds were tested and 7/23 exhibited the hypoploid syndrome. The difference in the percentages of hypoploid individuals in these two classes of seed is statistically significant (Fisher's exact test; $P = 0.01$), demonstrating that there is a deficit of *stt1* kernels with hypoploid embryos.

Due to the potential lethality of *stt1*/− hypoploid embryos, we tested for a similar phenotype for *stt1*/− hypoploid endosperms (rare miniature seeds with yellow endosperms and purple embryos). Of the eight miniature seeds tested, all had hyperploid embryos producing >90% $A2^+$ progeny on the resultant ears, but none segregated *stt1*. The lack of *stt1*/+ ears from the eight miniature seeds tested is statistically significant (likelihood that *stt1*/+ plants were missed by random chance $P = 0.04$), indicating that *stt1* hypoploids in the endosperm are not recoverable as mature seeds. The small seed size in the kernels tested was likely caused by the variable effects of hypoploidy for the B-5S-2L chromosome in the endosperm rather than by maternal inheritance of the *stt1* mutation.

To identify the phenotype of *stt1*/− embryos and endosperm, we examined ovules of *stt1*/+ females crossed by TB-5S-2L males at 6 DAP using confocal microscopy. The ratios of wild-type and *stt1* ovules fertilized (Figure 8, C and H), unfertilized without evidence of PT entry (Figure 8, A and F), or with evidence of PT entry but without endosperm development (Figure 8, B and G) were similar to the ratios observed at 6 DAP when *stt1*/+ was crossed by the standard diploid stocks (Figures 4 and 8K), indicating that sperm cells lacking *stt1* function can fertilize *stt1* mutant female gametes as efficiently as wild-type sperm. However, within the class of fertilized ovules we observed several types of developing seeds that differed from what we observed in the standard cross (Figure 5B). In addition to the typical wild-type and *stt1* seeds (presumably genetically balanced) (Figure 8, C and H), we also observed ovules that had well-developed *stt1* endosperms with very small, aborted, or absent embryos (Figure 8D) and ovules that had developing embryos with little or no endosperm proliferation (Figure 8E). These novel phenotypes likely represent individuals with hypoploid *stt1*/− embryos (31% hypoploid embryos) and hypoploid *stt1*/− endosperms (18% hypoploid endosperms), respectively (Figure 8K). We calculated the expected rates of nondisjunction from a cross of a *w3*/+ female by the same TB-5S-2L plants and found that the rates of nondisjunction as measured by the frequency of hypoploidy of *w3* in the endosperm and embryo, identified as described in materials and methods, were not statistically different from the observed rates of hypoploidy of *stt1* ($\chi^2 = 4.04$, $P = 0.13$).

At 10 DAP, the individuals with *stt1*/− hypoploid embryos were not very different from 6 DAP and would likely form seeds that were germless or had aborted embryos (as supported by the novel mature seed phenotype) (Figure 8I). However, those individuals with *stt1*/− hypoploid endosperms had already begun to abort at 10 DAP and would likely never form an identifiable seed (in agreement with the absence of these individuals at ear maturity) (Figure 8J).

Interestingly, despite abortion of *stt1*/− seeds at 10 DAP, the *stt1* hyperploid embryos could develop until 6 DAP without the support of a developing endosperm (Figure 8E). At 6 DAP, the globular-stage embryos of *stt1* seeds with nonproliferating *stt1*/− endosperms are not significantly different in size from balanced *stt1* individuals (Figure 8L). This phenotype suggests that early embryo development in maize is supported directly by the mother plant but later embryo development requires a functional endosperm.

DISCUSSION

***stunter1* is a new maternal effect mutant in maize:** Seed development in angiosperms is a highly regulated process requiring gene activity not only in the genomes of the embryo and endosperm but also that of the female gametophyte (for reviews see Olsen 2004; Kohler and Grossniklaus 2005; Pien and Grossniklaus 2007; Jullien and Berger 2009). Analyses of maternal effect mutants, such as *baseless1* in maize, have demonstrated that the morphology of the embryo sac can have a significant effect on seed development (Gutierrez-Marcos *et al.* 2006). Our studies have identified a new maternal effect gene, *stt1*, which is required in the female gametophyte for proper embryo sac development. Similar to *baseless1*, improper development of the female gametophyte is likely the primary cause for the maternal effects of *stt1*. *stt1* mutant seeds are develop-

mentally delayed and reduced in size but are patterned correctly with the typical constitution of cell types.

***stunter1* affects female gametophyte development:** The *stt1* mutation causes abnormal and delayed development of the female gametophyte prior to fertilization such that the mature embryo sac is smaller, most notably in the size of the central cell. Additionally, the antipodal cells in *stt1* embryo sacs are larger and more vacuolated than in wild type. To date, little is known about the functions of the antipodal cells in fertilization and seed development. It has been speculated that in maize these cells play a specialized role in nutrient transfer from the nucellus of the mother plant into the female gametophyte, because they possess papillate cell walls (Diboll and Larson 1966; Huang and Sheridan 1994). Consequently, changes in the morphology of the antipodal cells and thus the ability to transfer nutrients into the female gametophyte could result in the smaller size of the *stt1* embryo sacs. However, *stt1* embryo sacs are smaller than wild-type embryo sacs prior to cellularization making it likely that factors acting early in megagametogenesis lead to the reduced size of *stt1* embryo sacs. Whether or not these factors are involved in nutrient transport into embryo sacs at the syncytial stage and later into antipodal cells remains to be determined. Despite these abnormalities, *stt1* embryo sacs attract pollen tubes at a normal frequency but some may fail to be fertilized despite pollen tube entry.

***stunter1* affects male gametophyte development:** Like mutant embryo sacs, *stt1* pollen grains are smaller than wild type and also germinate poorly and extend small, stunted pollen tubes. These mutant pollen grains are likely nonfunctional, as we are unable to detect *stt1* male transmission even when competition with wild-type pollen grains is reduced. It is possible that, similar to megagametogenesis, microgametogenesis in *stt1* is slower and mutant pollen grains never fully mature, despite having normal starch filling and sperm morphology.

Of the known pollen mutants in maize and Arabidopsis, many are affected during pollen development and a number have been identified as playing a role in the progamic phase, including mutants in maize and Arabidopsis pollen specific Rop GTPases (reviewed in Bedinger and Fowler 2009). Mutations in maize *rop2* or Arabidopsis *ROP1* result in defective pollen tube growth, reduced ability to compete with wild-type pollen, and shorter, broader pollen tubes (Li *et al.* 1999; Arthur *et al.* 2003). These phenotypes are distinct from the pollen tubes of *stt1* both in their appearance and in their ability to function when competition from wild-type pollen is reduced.

Vesicle fusion is very dynamic in the growing pollen tube (Hicks *et al.* 2004), and vesicle and vacuole formation play significant roles in development of both the embryo sac and pollen and may contribute to the size of both gametophytes (for reviews see Bedinger and Fowler 2009; Evans and Grossniklaus 2009). The SABRE-like proteins encoded by *aberrant pollen transmission1* (*apt1*) in maize and *KINKY POLLEN* (*KIP*) in Arabidopsis are critical for membrane trafficking in the pollen tube tip (Procissi *et al.* 2003; Xu and Dooner 2006). Mutations in *apt1* and *KIP* result in pollen grains that germinate, but extend short, twisted, or kinky pollen tubes, which, like pollen of *rop* mutants, are distinct from the effects of *stt1* in appearance and their ability to achieve fertilization. However, a more severe impairment in membrane trafficking could explain the arrested pollen tubes, smaller pollen grains, and smaller central cells in *stt1* mutants. In addition to identification of the *stt1* gene, a more detailed characterization of the developing pollen and the individual cell types of the embryo sac will help to determine the causes of these phenotypes.

Role of *stunter1* in the developing seed: We have shown that *stt1* continues to have an effect during development of the seed after fertilization. *stt1* endosperms develop more slowly than wild type in growth, in onset of expression of *Vp1* and *Bet1*, and in initiation of PCD. Although *Vp1* and *Bet1* show delayed expression, the aleurone and BETL do eventually develop completely in *stt1* seeds. The reduced size of *stt1* seeds likely results from the slower growth and delayed development of the endosperm. The maternal effects of *stt1* on the embryo may be an indirect effect of an abnormal endosperm; alternatively, maternal *stt1* may be required in both the embryo and the endosperm independently.

Since the defects in pollen function precluded generation of *stt1* homozygotes, we took advantage of nondisjunction of maize B-A chromosomes to produce embryos and endosperms lacking the paternal allele of *stt1*. Our data from these crosses indicates that postfertilization expression of *stt1* is essential for seed development and that both maternal and paternal alleles are active in the embryo and endosperm. In these crosses we saw novel phenotypes in the endosperms or embryos of different seeds, consistent with the absence of maternal and paternal wild-type *stt1* function (from maternal inheritance of the *stt1* mutation and paternal inheritance of a chromosome arm deletion). Seeds with cellularized endosperms and arrested or degenerating embryos suggest that there is postfertilization expression of both *stt1* alleles in the embryo. Similarly, seeds with globular-stage embryos developing with no endosperm proliferation suggest that there is postfertilization expression of both *stt1* alleles in the endosperm. Since a subset of these endosperms undergo one or two rounds of free-nuclear divisions, it is likely that arrest of *stt1*/− endosperm development produces this class of seeds rather than failure of *stt1*-deficient sperm to fertilize *stt1* central cells. These phenotypes also demonstrate that requirements for postfertilization *stt1* function in the embryo and endosperm are partially independent of each other.

It is striking to note how much the embryo will develop without the support of a developing endosperm, even endosperms that undergo no free nuclear divisions. Our findings in maize are similar to those seen in several Arabidopsis gametophyte mutants, which produce embryos without producing endosperm (*e.g.*, *retinoblastoma related1*, *cdka-1*, and *msi1* mutants) (Iwakawa *et al.* 2006; Chen *et al.* 2008; Ingouff *et al.* 2009). In all cases of embryo development without endosperm formation in Arabidopsis, the embryos arrest at the globular stage, similar to *stt1*/+/+ embryos with a hypoploid *stt1*/− endosperm, suggesting that in monocots and dicots the early stages of embryo development are supported directly by the mother plant and that postglobular stage development is supported by the developing endosperm.

A parsimonious model for *stt1* action in seed development is that the abnormal morphology of the embryo sacs is responsible for the slower development of the seed. However, we cannot at this time rule out direct, postfertilization effects on seed size caused by inheritance of *stt1* rather than an indirect effect of *stt1* embryo sac abnormalities. *stt1* can be classified as a new maternal effect *miniature* seed mutant with novel gametophyte phenotypes. Of known maize mutants, only *Mn::Uq* bears any resemblance to *stt1* (Pan and Peterson 1989). The *Mn::Uq* mutation is only transmitted maternally, and heterozygotes segregate large and small, nonfunctional pollen, also like *stt1*, but *Mn::Uq* seeds are more severely abnormal than *stt1*. Further investigation is necessary to determine whether *stt1* and *Mn::Uq* are allelic or affect the same genetic pathway.

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Analysis of *stunter1*, a Maize Mutant with Reduced Gametophyte Size and Maternal Effects on Seed Development

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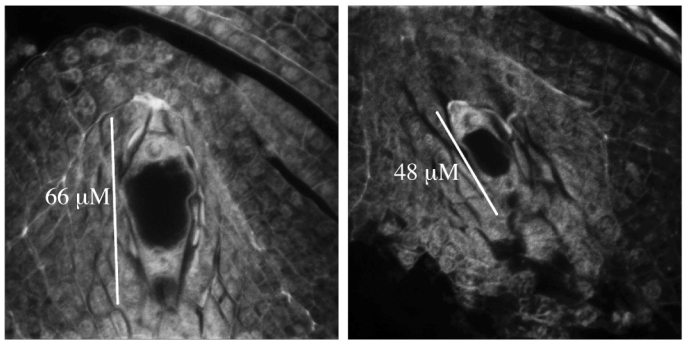

FIGURE S1.—*stt1* embryo sacs are smaller compared to wild type early in development. Neighboring embryo sacs from a *stt1/+* heterozygote at the FG 2,3 stage. Scale bars indicate the size of each embryo sac.

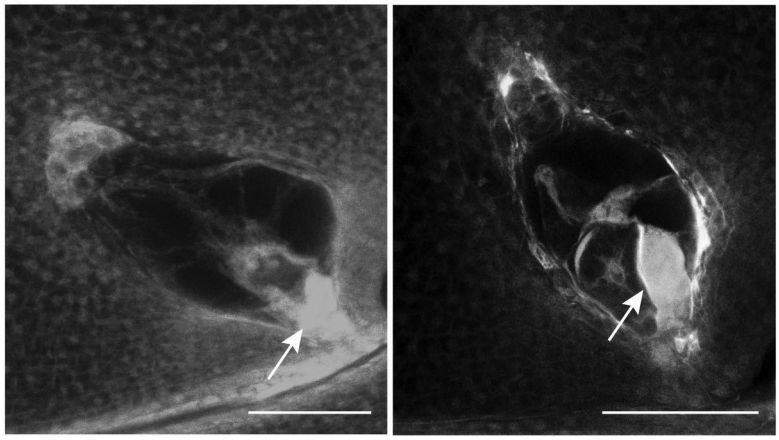

FIGURE S2.—WT and *stt1* embryo sacs with evidence of PT entry. WT embryo sac on left, *stt1* embryo sac on right. Arrows point to fluorescence associated with PT entry. Size bars represent 100 μM.

TABLE S1

Male transmission of the *stt1* mutation with sparse and heavy pollinations

	sparse pollinations		heavy pollinations	
Cross #	+, *wx1*	+, *Wx1*+	+, *wx1*	+, *Wx1*+
1	16	3	214	26
2	75	11	164	24
3	69	3	110	18
4	55	6	189	23
5	45	3	171	31
6	7	0	128	15
Total (%)	267/293=91.1%	26/293=8.9%	976/1113=87.7%	137/1113=12.3%
***stt1* Transmission (% *Wx1*+)**		8.9%		12.3%

No significant difference in *stt1* transmission rates between sparse and heavy pollinations ($\chi 2=1.07$, $p=0.30$).

wx1 T2-9d ears were crossed as females by the *wx1*/+; +/*stt1* translocation stock as males. The *stt1* transmission rate was calculated by scoring mutant waxy (*wx1*) versus normal (*Wx1*$^+$) kernels and totaling the kernels carrying *Wx1*$^+$, which is linked to *stt1*.

TABLE S2

Female transmission of the *stt1* mutation on immature and mature ears

		mn, *wx1*	mn, *Wx1+*	+, *wx1*	+, *Wx1+*	
Immature Ears	1	1	8	27	9	***stt1***
	2	5	12	27	14	**Transmission**
	3	4	13	58	23	**(% *Wx1+*)**
	4	2	21	66	32	
Total (%)		12/322=3.7%	54/322=**16.8%**	178/322=55.3%	78/322=**24.2%**	**41.0%**
Mature Ears	1	7	37	85	24	
	2	5	34	65	23	
Total (%)		12/280=4.3%	71/280=**25.3%**	150/280=53.6%	47/280=**16.8%**	**42.1%**

No significant difference in *stt1* transmission rates between immature and mature pollinations (χ^2=0.05, p=0.82).

Immature and mature ears from the *wx1*/+; +/*stt1* translocation stock were crossed as females by the *wx1* T2-9d tester as males. The *stt1* transmission rate was calculated by scoring miniature (mn) versus normal (+) and mutant waxy (*wx1*) versus normal (*Wx1*$^+$) kernels and totaling the kernels carrying *Wx1*$^+$, which is linked to *stt1*.