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Title

The maize Zmsmu2 gene encodes a putative RNA splicing factor that affects protein synthesis and RNA processing during endosperm development

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Abstract

We characterized two maize (Zea mays) mutants, *zmsmu2-1* and *zmsmu2-3*, that result from insertion of a *Mutator* transposable element in the first exon of a gene homologous to the nematode gene, *smu-2*, which is involved in RNA splicing. In addition to having a starchy endosperm with reduced levels of zein storage proteins, homozygous *zmsmu2-1* mutants manifest a number of phenotypes, including defective meristem development. The *zmsmu2* mutants have poor seedling viability, and surviving plants are sterile. The gene encoding ZmSMU2 is expressed in the endosperm, embryo and shoot apex, which explains the pleiotropic nature of the mutation. We found that proper expression of *Zmsmu2* is required for efficient ribosomal RNA (rRNA) processing, ribosome biogenesis and protein synthesis in developing endosperm. Based on the pleiotropic nature of the mutations and the known function of animal *Zmsmu2* homologues, we propose a possible role for ZmSMU2 in the development of maize endosperm, as well as a mechanism by which mis-regulation of *zmsmu2* causes the mutant phenotypes.
Introduction

The texture of maize (Zea mays) endosperm is an important quality trait, as it influences the shipping characteristics of the grain, its susceptibility to insects, the yield of grits from dry milling, energy costs during wet milling, and the baking and digestibility properties of the flour. However, factors contributing to texture, i.e. hardness and vitreousness, are poorly understood (Mestres and Matencio, 1996; Chandrashekar and Mazhar, 1999). Kernel texture is partially related to the formation of a vitreous, or glassy, endosperm, which is influenced by the protein content of the seed and the conditions of the kernel during desiccation and storage. There appears to be a causal relationship between kernel hardness and the formation of zein protein bodies in the endosperm, because mutations that affect protein body size and the organization of zein proteins within them result in soft, starchy endosperms that cause an opaque kernel phenotype (Holding and Larkins, 2006). For example, Opaque2 (O2) encodes a transcription factor that regulates genes expressed in the endosperm, in particular those encoding 22-kDa α-zeins (Schmidt et al., 1990). Protein bodies in o2 endosperm are smaller than wild type, and this has generally been considered to be the basis for the opaque kernel phenotype (Geetha et al., 1991; Segal et al., 2003). Several opaque mutants, e.g. floury2 (fl2) and Defective endosperm B30 (De-B30) (Coleman et al., 1997; Kim et al., 2004a) are caused by defective signal peptides in α-zein proteins that disrupt protein body assembly (Zhang and Boston, 1992), leading to increased expression of genes involved in the unfolded protein response (Hunter et al., 2002). These observations are consistent with the hypothesis that the size, number and structure of zein protein bodies influence the texture and vitreous phenotype of the mature endosperm (Coleman and Larkins, 1999).

Although studies of the o2, fl2, and De-B30 mutants make it clear that zein proteins influence endosperm texture, other opaque mutants suggest there must be additional factors that
contribute to this phenotype. For example, the *opaque1* mutation (Nelson et al., 1965) has no detectable effect on zein synthesis, and the zein level in several others, e.g. *opaque5*, *opaque9* and *opaque11*, is only marginally reduced (Hunter et al., 2002). Unfortunately, most of the known opaque mutants were identified as spontaneous mutations or were induced by EMS mutagenesis, which makes it difficult to discover their molecular basis.

To facilitate the identification and cloning of additional mutant genes that cause an opaque kernel phenotype, we screened a large population of *Mutator* (*Mu*)-tagged maize mutants created as part of Pioneer Hi-Bred’s TUSC collection (Bensen et al., 1995). Ears were inspected for evidence of segregating mutant phenotypes, and subsequently kernel samples were viewed with a light box to confirm possible opaque mutations. From this screen we identified a number of potential *Mu*-tagged opaque (*mto*) mutants that were later confirmed by genetic testing. One of these, *mto38*, was found to contain a *Mu*-tagged genomic fragment that co-segregated with the mutant phenotype. Characterization of the gene associated with this fragment showed that it encodes a homologue of mammalian RED proteins, which are known components of spliceosomes (Neubauer et al., 1998; Makarov et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002). The comparable mutation in *C. elegans*, *smu-2* (Lundquist and Herman, 1994), was shown to play a role in pre-mRNA splicing (Spartz et al., 2004). Hence, we designated the mutant maize gene *zmsmu2* (*Zea mays* homologue of nematode *smu*-2). Phenotypic comparison of independent mutant alleles indicated that the *zmsmu2* mutation is responsible for a number of *mto38* phenotypes, including abnormal meristem development, poor germination and reduced accumulation of seed storage proteins. Although the *Mu* insertions in *zmsmu2* do not create null mutations, they affect the activity the ZmSMU2 protein. We found that *zmsmu2-1* endosperm manifests defective rRNA processing and ribosome biogenesis, as well as inefficient protein synthesis on a global level.
Results

Identification of mto38 and a candidate gene responsible for the opaque kernel phenotype

The mto38 mutant was discovered during a screen of Pioneer Hi-Bred’s TUSC mutant collection (Bensen et al., 1995). A subset of these mutants, mto38 through mto50, showed a similar pattern of reduced zein synthesis, and they were later found to have originated from the same F1 cross of a Mu-active and a Mu-inactive parental line. Thus, mto38 to mto50 are most likely independent isolates of the same Mu-induced mutation. Several of the mutant isolates, including mto38, were out-crossed to W64A+ and then back-crossed six generations. In all subsequent experiments we used the W64A-introgressed mto38 allele.

During the backcrossing process, the mutant phenotype was monitored using a light box to screen for opaque kernels in segregating ears. Approximately 25% of the progeny from an F2 ear segregating for the mto38 mutation manifested an opaque kernel phenotype (Figure 1A), suggesting the mutation is due to a recessive allele. SDS-PAGE analysis of the zein and non-zein protein fractions from endosperms of these kernels revealed a marked reduction of the major zein storage proteins in the mutant (Figure 1B, c.f. lanes 1 and 2) and quantitative differences in some non-zein proteins (Figure 1B, c.f. lanes 3 and 4).

The mto38 kernels germinated poorly and rarely survived in the field. When the seeds were placed on MS medium (Murashige and Skoog, 1962), their rate of germination improved, but the seedlings still had poor viability. Surviving plants were shorter than wild type, and developed a sterile ear and a short, poorly branched sterile tassel. Because of the sterility, we were unable to propagate homozygous mto38 mutants; consequently, we maintained the mutant allele in heterozygotes.

We frequently observed an unusual proliferation of leaves in homozygous mto38
seedlings (Figure 2A and 2B), suggesting that meristem development was also affected. To investigate this, developing F2 kernels from a self-pollinated +/mto38 ear were harvested 16 days after pollination (DAP) and the endosperm and embryo dissected. (Note, embryos were genotyped by PCR after the nature of the Mu insertion was known.) By 16 DAP, wild-type embryos formed five to seven leaves and had a small, distinct shoot apex (Figure 2C). In contrast, mto38 embryos were smaller and the apical meristem produced fewer leaves, as though development was delayed (Figure 2D-F). Some mto38 embryos had two shoot (Figure 2D) and two root apices (Figure 2E), while others possessed a single shoot and root apex (Figure 2F). Thus, it appeared the gene responsible for mto38 is required for proper regulation of meristematic activity, and this may explain the low rate of germination and poor viability of the mutant seedlings.

To identify the Mu insertion responsible for the mto38 mutant, we used a TAIL-PCR approach (Liu et al., 1995). DNA was extracted from 70 homozygous wild-type and 70 homozygous mto38 seedlings, and two sequential PCRs were carried out to amplify target DNA sequences with an arbitrary degenerate primer and nested Mu-specific primers that correspond to the Mu terminal inverted repeat (Settles et al., 2004; see Methods). Upon gel electrophoretic separation of the products, we identified a DNA band of approximately 150 bp that was present in all the homozygous mto38 mutants (Figure 3A, lanes 1-6), but not homozygous wild type seedlings (Figure 3A, lanes 13-16). We also analyzed a few seedlings from non-genotyped vitreous F2 kernels (Figure 3A, lanes 7-12). As predicted from the recessive nature of the mto38 allele, the 150-bp band was detected in some of these plants (Figure 3A, lanes 8, 9 and 10), indicating they were probably heterozygous for the Mu insertion.

DNA sequence analysis of the 150 bp fragment revealed that approximately 90 bp corresponded to the Mu terminal inverted repeat, while 60 bp were non-Mu DNA. To further
characterize the \textit{Mu}-associated DNA sequence, we designed two gene-specific primers, F1 and F2 (Figure 3C, primers represented by arrowheads) to amplify the corresponding 5’- and 3’- ends (5’-RACE and 3’-RACE). While no product was obtained with the 5’-RACE, the 3’-RACE produced a cDNA containing a 1698-bp open reading frame; the 5’ end of this sequence was identical with the 60-bp non-\textit{Mu} DNA sequence. Using this cDNA sequence, we designed a downstream gene-specific primer, R6, so we could easily genotype F2 individuals for the wild-type and mutant alleles. For genotyping the locus, PCR primers R6 and MuTir6 (Figure 3C, gray arrowheads) were used to detect the mutant allele, while primers F3 and R6 were used to amplify the wild-type allele. With the F3-R6 primer pair, only the W64A+ but not the \textit{mto38} mutant allele could be amplified by PCR (Figure 3B, lanes with a plus sign). Genotyping plants from vitreous and opaque kernels showed that all the F2 seedlings with an opaque phenotype (n=45) were homozygous for the \textit{Mu}-tagged fragment, while those from vitreous kernels (n=136) were either homozygous wild type or heterozygous. This result confirmed that the \textit{Mu}-tagged sequence was tightly linked with the opaque kernel phenotype.

Nucleotide sequence analysis showed the cDNA encoded a hydrophilic protein consisting of 565 amino acids (Figure 4). BLASTX comparison of the amino acid sequence against the NCBI protein database revealed a high degree of identity with RED-related proteins, which are conserved in higher eukaryotes. These proteins were named based on the RED domain, which contains several repeats of either arginine (R) and glutamate (E) or arginine and aspartate (D). However, many of the homologous proteins, including all the plant accessions, do not contain distinct RE or RD repeats. Two highly conserved regions in the N- and C-termini were identified in the sequence alignment of the homologous proteins (Figure 4), although no function has been assigned to these regions. We did not identify any of the other conserved domains, including those for binding to nucleic acids.
Among RED proteins, the human and nematode homologues are the best characterized. The human RED protein was identified in several independent proteomic studies where components of human spliceosomes were isolated (Neubauer et al., 1998; Makarov et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002). The nematode homologue, *smu-2*, was isolated from a screen for genetic suppressors of the *mec-8; unc-52* double mutant (Lundquist and Herman, 1994). SMU-2 plays a role in pre-mRNA splicing, since the *smu-2* mutant accumulates *unc-52* transcripts that lack exon 17, which contains a premature stop codon, thereby preventing the *mec-8; unc-52* double mutant from being lethal (Spartz et al., 2004). The gene product of another suppressor, *smu-1*, physically interacts with SMU-2, and a human protein homologous to SMU-1 is also found in spliceosomes (Jurica and Moore, 2003). Because of the high level of sequence identity among *smu-2* homologues and the biochemical properties of animal *smu-2* homologues, we designated the *mto38* candidate gene as *Zmsmu2*.

In the *mto38/zmsmu2-1* mutant, the *Mu* element appeared to be inserted in the first exon, which encodes the putative 5’ UTR of the mRNA. To verify this, it was necessary to define the structure of the gene. Using primers derived from the *Zmsmu2* cDNA sequence, we amplified a 6.5-kb W64A+ genomic DNA fragment. Comparison of its sequence with that of the cDNA revealed the gene contains 11 exons (Figure 3C). Because the *Mu* insertion made it difficult to obtain additional 5’ genomic DNA sequence from W64A+, we screened a B73+ BAC library with a *Zmsmu2* cDNA probe. A comparison of the 5’ ends of the W64A+ and B73+ *Zmsmu2* genes revealed a poor conservation of nucleotide sequence near the *Mu* insertion site in *zmsmu2-1* (boxed sequence in Figure 3D), which sharply contrasted with their nearly identical downstream exonic and intronic sequences. Several potential *cis*-acting elements for binding of sequence-specific transcription factors were identified in the 5’ sequence preceding the coding region of *Zmsmu2* (underlined sequences in Figure 3D). Thus, there did not appear to be
additional exon or intron sequences upstream of the Mu insertion site in the W64A+ Zmsmu2 gene.

**Expression of the zmsmu2-1 gene**

Based on the tight linkage of the zmsmu2-1 allele with the opaque kernel phenotype, we hypothesized this gene is responsible for the pleiotropic effects of the mutation. To test this, we investigated whether the zmsmu2-1 mutant exhibits defects in the expression of the Zmsmu2 gene, and whether additional mutant alleles of Zmsmu2 result in similar phenotypes. Since the Mu insertion occurs in the first exon of Zmsmu2, it is possible this alters expression of the gene (Lisch, 2002). We performed a semi-quantitative reverse transcriptase PCR (RT-PCR) analysis of zmsmu2-1 RNA in developing endosperm. F2 kernels from self-pollinated heterozygous (+/zmsmu2-1) ears were genotyped for the zmsmu2 locus, and RNA was isolated from endosperms genotyped as homozygous wild type or mutant. For RT-PCR, we used three pairs of primers (see Figure 3C): F5 and S1 amplified the partial 3’ coding sequence of Zmsmu2 RNA; F1 and R5 amplified the 5’ UTR and part of the coding sequence; and MuTir6 (gray arrowhead in Figure 3C) and R5 amplified the Mu terminal inverted repeat (MuTIR) as well as 5’ UTR and part of the coding sequence. The result with the F5-S1 primer pair indicated the zmsmu2-1 transcript level in mutant endosperm is slightly higher than its wild-type sibling (Figure 5A). In contrast, we were unable to detect any RT-PCR product from zmsmu2-1 endosperm with primers F1 and R5. Likewise, no RT-PCR product was detected from wild-type endosperm with the mutant-specific primer pair, MuTir6 and R5 (Figure 5A). These results indicated that the mutant endosperm accumulates a somewhat higher level of zmsmu2 RNA than wild type, although the mutant transcripts appear to have a slightly different 5’ UTR sequence. To verify this, we determined the nucleotide sequences of the RT-PCR products. The coding sequence of the
zmsmu2-1 RNA was identical to its wild-type counterpart, indicating that the primary amino acid sequences of the proteins are most likely the same. However, comparison of the 5′ UTR sequence of the mutant and wild-type RT-PCR products confirmed that the 5′ end of the mutant transcript contains a portion of the Mu element (see also Figure 3D, sequence in gray). A higher level of Zmsmu2 RNA in the mutant was also confirmed by Northern blot analysis (data not shown). We did not detect potential splice variants of Zmsmu2 transcripts, but the resolution and intensity of the RNA band was insufficiently distinct to exclude the possibility of minor splice variants.

To investigate the level of ZmSMU2 protein in wild type and mutant endosperm, ZmSMU2 antibodies were prepared by injecting a rabbit with antigen prepared after enzymatic removal of GST (glutathione-S-transferase) from a recombinant GST::ZmSMU2 fusion protein. The resulting antiserum recognized the recombinant fusion protein, but did not bind GST (Figure 5B, lanes 1 and 2). Immunoblotting of W64A+ endosperm proteins with these antibodies detected three polypeptides (Figure 5B, lane 3) with apparent sizes of approximately 85-, 75- and 55-kDa. To determine which of these bands correspond to the ZmSMU2 protein, we took advantage of the cross-reactivity of these antibodies with the homologous Arabidopsis (Arabidopsis thaliana) protein, AtSMU2, and its fusion protein GST::AtSMU2 (Figure 5B, lane 4). We identified two Arabidopsis mutants, atsmu2-1 and atsmu2-2 (Chung et al., manuscript in preparation), in which a T-DNA is inserted in the eighth exon and the sixth intron, respectively, of the single-copy AtSMU2 gene. These appear to be knockout mutants, since we were unable to detect a full-length cDNA from their reverse transcriptase reaction products (data not shown). Immunoblotting of extract from Arabidopsis seedlings at 7 days after germination (DAG) also identified three protein bands (Figure 5B, lane 5). Based on the absence of the 100-kDa band in extracts of homozygous atsmu2-1 and atsmu2-2 mutants (Figure 5B, lanes 6 and 7), we
concluded that the band of approximately 100-kDa most likely corresponds to the AtSMU2 protein. Since ZmSMU2 is predicted to be 20 amino acids shorter than AtSMU2 (see Figure 4 and also cf. lanes 2 and 4 in Figure 5B), the 85-kDa band in lane 3 must correspond to the ZmSMU2 polypeptide, even though its apparent size is significantly larger (85- vs 64-kDa) than the predicted mol wt. The slower migration of this protein during SDS-PAGE presumably results from an unusual secondary structure or some type of post-translational modification. We did not determine the nature of the smaller (75- and 55-kDa) proteins, but they could share an epitope in common with the 85-kDa ZmSMU2 band or represent degradation products.

The level of ZmSMU2 protein in homozygous wild-type endosperm (Figure 5C, lane 1) appeared to be slightly lower than in the zmsmu2-1 mutant (Figure 5C, cf. lanes 1 and 3). Furthermore, SDS-PAGE of 16 DAP endosperm extract of heterozygous and homozygous zmsmu2 genotypes revealed ZmSMU2 proteins with slightly different mobility. The wild-type protein appeared to be slightly larger than that in the mutant (Figure 5C, c.f. lanes 1 and 3), and heterozygous (+/zmsmu2-1) endosperm contained proteins of both sizes (Figure 5C, lane 2). This co-dominant pattern of ZmSMU2 inheritance was observed in nearly all the heterozygous F2 endosperms examined, with rare exceptions showing a banding pattern like the wild type (data not shown).

Immunoblotting of proteins from different maize tissues was done to determine the spatial expression of the Zmsmu2 gene. This analysis showed comparable levels of the protein in developing endosperm (Figure 5D, lane 1), embryo (Figure 5D, lane 2), and the shoot apex (Figure 5D, lane 3). Unfertilized ovules (Figure 5D, lane 6) contained lower but detectable amounts of ZmSMU2 protein, while none was found in pollen (Figure 5D, lane 7). Very weak immunodetection was obtained with proteins from developing tassels, roots and leaves, even when larger amounts of proteins were analyzed (data not shown). These results indicate that the
maize Zmsmu2 gene is most highly expressed in mitotic/developing tissues, although it could be expressed throughout the plant.

In summary, the Mu insertion in the first exon of the Zmsmu2 gene alters the 5’ UTR of the mRNA transcript and increases its level. The difference in Zmsmu2 RNA levels between the wild-type and mutant endosperm is positively correlated with the protein levels. Significantly, the ZmSMU2 protein in mutant endosperm has a slightly smaller apparent mol wt.

**Shared phenotypes of zmsmu2-1 and zmsmu2-3 mutants**

If the zmsmu2-1 mutation creates the diverse phenotypes of the mto38 mutant, additional mutant alleles of this gene should do the same. To test this, a reverse genetic screen of Pioneer Hi-Bred’s TUSC population was conducted to identify additional Mu insertions in the Zmsmu2 gene. We were able to identify several Mu insertions at the 5’ end of the Zmsmu2 gene, but nowhere else, even though we tested PCR primers corresponding to multiple intron and exon sequences. One of the Mu-tagged mutants, zmsmu2-3, had a Mu insertion in the first exon, 266 bp before the start codon, which is 59 bp downstream of the zmsmu2-1 insertion site (see Figure 3C and 3D). The other Mu insertions, zmsmu2-2 and zmsmu2-4, occurred in the first and second introns, respectively. No mutant phenotypes were observed for zmsmu2-2 and zmsmu2-4, suggesting introns containing these Mu insertions are removed by splicing during transcription.

To eliminate Mu elements and obtain a more uniform genetic background, we introgressed the zmsmu2-3 allele into W64A+ by two or three generations of backcrossing. Subsequently, F2 progeny from the ear of a self-pollinated heterozygous zmsmu2-3 plant was compared with those from a self-pollinated heterozygous zmsmu2-1 plant (Table I). The genotype for each of the F2 progeny was determined by PCR, and the number of leaves was counted at 10 DAG as an index for growth and development. Notably, homozygous zmsmu2-3
mutant kernels often failed to germinate or exhibited delayed seedling growth when compared to homozygous wild type and heterozygous zmsmu2-3 siblings. Poor germination and delayed seedling growth were also observed in homozygous zmsmu2-1 mutants (Table I). After germination, homozygous zmsmu2-3 plants grew slowly, similar to the zmsmu2-1 mutant. Tassels and ears of the homozygous zmsmu2-3 plants were either absent or poorly developed, as with zmsmu2-1. Thus, in terms of germination and seedling growth, the zmsmu2-3 mutation appeared to phenocopy zmsmu2-1.

To determine if the Mu insertion in the zmsmu2-3 allele altered its expression, as well as that of other genes, we analyzed of RNAs and proteins in wild type and homozygous zmsmu2-1 and zmsmu2-3 mutant endosperms at 16 DAP. Compared to homozygous and heterozygous wild-type endosperm, there was a reduction in the level of zmsmu2-3 RNA (Figure 6A) and protein (Figure 6B) in the homozygous mutant. This contrasted with homozygous zmsmu2-1 endosperm, where the gene was more highly expressed than in wild type (cf. Figure 6A and B; see also Figure 5). SDS-PAGE analysis showed that the mobility of the ZmSMU2-3 protein, like that of zmsmu2-1, was altered relative to wild type (Figure 6B). In both cases, the change in size appeared to be small, but the difference was reproducible. The nucleotide sequence of the zmsmu2-3 cDNA was only slightly different from the wild type and zmsmu2-1 alleles as a result of substitutions of Glu107 and Ala233 with Asp107 and Ser233 (see asterisks in Figure 4). However, these amino acid substitutions do not explain the change in mobility detected by SDS-PAGE, since the mol wt predicted for the protein is increased only 2-Da.

An opaque kernel phenotype was observed in kernels developed from some of the zmsmu2-3 backcross populations, but a starchy endosperm was not as penetrant in this mutant as in zmsmu2-1. Nevertheless, zmsmu2-3 resulted in a reduction in zein synthesis, particularly α-zeins, as was true of developing zmsmu2-1 endosperm (Figure 6C).
Ribosome biogenesis and protein synthesis are major targets of the zmsmu2-1 mutation

We observed about a 50% decrease in total RNA per gram fresh weight of developing zmsmu2-1 endosperm compared with wild-type, in spite of only a slight difference in their mRNA levels (Table II). Furthermore, transcript profiling of zmsmu2-1 endosperm indicated that genes encoding ribosomal proteins and many ribosome biogenesis factors, especially rRNA processing proteins, appeared to be up-regulated in the mutant (Chung et al., manuscript in preparation). Consequently, we decided to assess rRNA processing in zmsmu2-1 endosperm.

Figure 7A illustrates the primary transcript of a maize rDNA gene. Although rRNA processing sites have not been defined in the primary transcript, nucleotide sequences for rDNA gene repeats and their rRNA components - 18S, 5.8S and 25S rRNA - were available. Based on this information, we designed six probes for Northern blot analysis (Figure 7A), which allowed a comparison of mature and unprocessed rRNAs between wild-type and zmsmu2-1 endosperm. An rRNA intermediate containing the 5.8S rRNA, internal transcribed spacer 2 (ITS2) and 25S rRNA accumulated at higher levels in zmsmu2-1 endosperm (Figure 7B, band b in blots 4, 5 and 6), suggesting that the mutation leads to defective rRNA processing at cleavage sites in ITS2. An effect on rRNA processing in the mutant was also shown by the accumulation of unprocessed primary rRNA transcript (Figure 7B, band a in all blots). Based on these observations, we concluded that the reduced yield of rRNA and the induction of ribosome biogenesis genes in zmsmu2-1 endosperm resulted from defective rRNA processing.

To test if the zmsmu2-3 mutant also manifests defective rRNA processing, we performed a similar experiment with 16-DAP zmsmu2-3 endosperm. RNA blot analysis revealed that rRNA processing in zmsmu2-3 endosperm resulted in a slightly higher level of unprocessed rRNA than in wild-type or heterozygous sibling endosperms (Figure 7C).
To investigate whether the *zmsmu2-1* mutation affected the translational efficiency of mRNAs, polysomes from equal amounts of 16 DAP wild type and mutant endosperms were analyzed by sucrose density gradient centrifugation. Using an ISCO 640 density gradient fractionator, the size distribution of polysomes was monitored by continuous UV absorbance as the sucrose gradient was divided (top to bottom) into 0.8 ml fractions. Comparison of the wild type and mutant polysome profiles provided additional evidence of a lower concentration of ribosomes in *zmsmu2-1* endosperm (Figure 8A). There was approximately a 15% reduction in total ribosomal material (monosomes plus polysomes) in *zmsmu2-1* endosperm, although this value was most likely underestimated, because some of the large polysomes in the wild type sample pelleted to the bottom of the gradient. The highest absorbing polysome size-class in wild type endosperm contained 9-10 ribosomes, while that in *zmsmu2-1* endosperm contained only 4-5 ribosomes per mRNA. This reduction in polysome size was observed in four independent pools of mutant endosperms, suggesting that the *zmsum2-1* mutation not only results in fewer ribosomes per cell, but also that their translational activity is reduced.

An RT-PCR analysis was done to determine the distribution of selected RNAs among the polysome size classes. Based on the concentration of ribosomal RNA (Figure 8B, panels 1 and 2) and mRNAs (Figure 8B, panels 3-8), gradient fractions 6-8 had the highest amount of monosomes, while mRNAs in polysomes were recovered in fractions 9-13 (Figure 8B). The highest concentration of ubiquitin conjugase (*Ubc*) mRNA, which was used as an internal control for semi-quantitative RT-PCR, was found in fractions 10 and 11 of wild-type polysomes, compared with fractions 9 and 10 for polysomes from the *zmsmu2-1* mutant. Evidence for less efficient mRNA translation was also found by the analysis of ribosomal protein S29 (*Rps29*) transcripts (Figure 8B, panels 5 and 6). The total amount of *Rps29* transcripts in monosome and polysome fractions was larger in the mutant. However, the peak concentration of mRNA in wild
type polysomes was in fractions 9 and 10, while in zmsmu2-1 the peak was in fractions 7-9, and a large portion of the RNA was in fractions 5 and 6, which corresponds to monosomes and, potentially, ribonucleoproteins (RNPs). Similarly, the peak concentration for mRNA encoding one of the 22-kD α-zeins was in fractions 12 and 13, while the corresponding mRNA in zmsmu2-1 peaked in fractions 10 and 11. In contrast, the Zmsmu2 RNA appeared to be similarly distributed in large polysomes in both wild type and mutant endosperm (Figure 8B, panels 7 and 8), although the level of transcripts was higher in the mutant. This result is consistent with previous analyses, which showed a higher level of zmsmu2-1 RNA and protein in the mutant endosperm (see Figure 5C).

To determine if altered rRNA processing in the mutant might have a functional significance, we hybridized the polysome-associated rRNA with the rRNA ITS2 probe. This analysis showed that more rRNA intermediates retaining the ITS2 sequence were found in the monosome fraction of the mutant than wild type endosperm, while polysomes of the mutant contained very little of this intermediate (Figure 8C). Thus, it is possible the presence of unprocessed rRNA contributes to the reduced translational efficiency of the ribosomes.

Discussion

The opaque kernel mutant we identified as mto38 was found to result from a Mu insertion in the 5’ UTR of a gene having a high degree of sequence identity with the “RED” proteins in mammals and C. elegans. The human RED protein is a component of spliceosomes (Neubauer et al., 1998; Makarov et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002). In C. elegans, SMU-2 and a related protein, SMU-1, were shown to play an important role in splice site selection during alternative RNA splicing (Spartz et al., 2004). Consequently, we designated the maize mutant gene Zmsmu2. In maize, the zmsmu2 mutation has pleiotropic effects on gene
expression that affect both endosperm and embryo development. The \textit{zmsmu2} mutation affects embryo morphogenesis with consequent effects on shoot and root meristem development, and the mutation negatively affects seedling viability, making it impossible to propagate homozygous mutant plants. Since we were most interested in the effects of the mutation on endosperm development, we did not pursue analysis of the mutation on gene expression during embryogenesis.

Several lines of evidence support the conclusion that \textit{zmsmu2} is responsible for most if not all of the mutant phenotypes of \textit{mto38}. First, we observed tight linkage between the \textit{Mu}-tagged \textit{zmsmu2}-1 allele and the opaque kernel phenotype (Figure 3B). Second, through a reverse genetics screen we identified additional \textit{Mu} insertions in this gene, one of which, \textit{zmsmu2}-3, manifested many of the same phenotypes as \textit{zmsmu2}-1, including reduced germination and seedling viability, abnormal development of the shoot apical meristem, leading to proliferation of small leaves, and reduced levels of zein synthesis during endosperm development. Like \textit{zmsmu2}-1, the \textit{zmsmu2}-3 mutation also resulted in inefficient rRNA processing in endosperm (Figure 7C) as well as the up-regulation of ribosomal protein genes (Chung et al., manuscript in preparation).

Comparison of the \textit{Zmsmu2} genes in \textit{W64A+} and \textit{B73+}, relative to \textit{Zmsmu2} cDNAs, indicated the \textit{zmsmu2}-1 and \textit{zmsmu2}-3 mutations are created by \textit{Mu} insertions in the first exon of the gene, which encodes the 5' UTR of the mRNA. Initially, it was difficult to determine the correct start codon for \textit{Zmsmu2} transcripts, since the ~250 bp sequence corresponding to the proposed 5' UTR (see Figure 3D) does not contain either a start or a stop codon. Based solely on the sequence analysis, we could not exclude the possibility of a start codon further upstream, i.e. additional exon/intron sequences. However, several lines of evidence suggest this is unlikely. First, when we searched the protein database using the 80 amino acid residues deduced from the 5' UTR, we were unable to find sequence similarity to any proteins; notably, they did not align...
with the N-terminal amino acid sequences of animal SMU-2 homologues. Second, all the plant
SMU-2 homologues aligned very nicely at their N-terminal ends when the proposed initiation
codon was used to deduce the ZmSMU2 amino acid sequence (see Figure 4). It is difficult to
conceive that only the maize protein would have a longer N-terminal sequence. Third, we were
unable to further extend the sequence of the 5’ end of the wild type TAIL-PCR product by 5’
RACE. Finally, comparison of the W64A+ genomic sequence with that of B73+ showed that
their nucleotide sequence identity quickly disappeared prior to the beginning of the 5’ UTR.
Since the nucleotide sequences of the W64A+ and B73+ alleles are identical in the first intron
and subsequent sequences, this effectively argues against the likelihood there are exon or intron
sequences further upstream of the proposed 5’ UTR. Furthermore, we identified potential cis-
elements for sequence-specific transcription factors in this region, implying it is part of the
Zmsmu2 promoter sequence.

Previous studies have shown that Mu insertions in a genomic sequence corresponding to
the 5’ UTR of a transcript can cause changes in gene expression (Barkan and Martienssen, 1991;
Arthur et al., 2003). Consistent with this, our data indicate that the Mu insertions in zmsmu2-1
and zmsmu2-3 result in mis-regulation of gene transcription. Neither of the Mu insertions block
gene expression in the endosperm; rather, in the case of zmsmu2-1 there is an increase in the
level of RNA, while in zmsmu2-3 there is a slight decrease in RNA transcripts relative to wild
type. In addition to differential RNA accumulation in the endosperm, zmsmu2-1 and zmsmu2-3
transcripts have different 5’ UTR sequences: a portion of the zmsmu2-1 5’ UTR is derived from
the Mu terminal inverted repeat (Figure 3D). In contrast, the zmsmu2-3 5’ UTR appears to be
shorter and contains little, if any, Mu-derived sequence, since we could not amplify zmsmu2-3
transcripts by RT-PCR with the MuTir4 and R5 primers (data not shown; the binding site of the
MuTir4 primer is only 34 nucleotides away from the end of the Mu sequence). Based on these
observations, the difference in the level of \textit{zmsmu2} RNAs in the two mutants can be explained in several ways. In \textit{zmsmu2-1}, transcription could be enhanced by the \textit{Mu} insertion abolishing a \textit{cis}-acting repressor element, or the \textit{Mu} sequence could act as an enhancer of transcription. It is also possible the \textit{Mu}-derived 5’ UTR sequence influences mRNA stability. In \textit{zmsmu2-3}, the transcription initiation site(s) may be less efficient than in wild type. Alternatively, a shorter 5’ UTR may make the \textit{zmsmu2-3} mRNA unstable.

Our analysis of maize BAC clones showed that the \textit{Zmsmu2} gene maps in bin 10.01 of chromosome 10, although putative paralogues, represented by weaker hybridization signals, were found on chromosomes 3 and 8 (data not shown). Most of the maize expressed sequence tags (ESTs) encoding the ZmSMU2 protein are identical to the \textit{Zmsmu2} sequence, indicating that this gene encodes the predominant ZmSMU2 protein. The potential \textit{Zmsmu2} paralogous genes on chromosomes 3 and 8 could be non-functional copies, as a search of genomic databases showed that the rice genome has only one functional copy of the \textit{OsSMU2} gene (Os11g04950) and two pseudogenes, Os12g04780 and Os12g04940, the latter of which could encode a truncated paralogue of \textit{OsSMU2} (supported by an EST CK041467.1).

The \textit{zmsmu2-1} and \textit{zmsmu2-3} mutations create a change in the ZmSMU2 protein that leads to altered mobility during SDS-PAGE (Figure 5C and Figure 6B), although the mechanism is unclear. Since we were unable to identify any differences between the deduced primary amino acid sequences of the wild type and ZmSMU2-1 mutant proteins, there are at least two plausible explanations for the altered mobility. First, variation in the 5’ UTR of the \textit{zmsmu2-1} and \textit{zmsmu2-3} RNAs could affect usage of a downstream start codon, such as Met13. The size differences of the proteins and their co-dominant expression pattern in heterozygous endosperm are consistent with this explanation. An alternative explanation is that the variation in sizes of the wild-type and mutant proteins results from some type of post-translational modification. While we do not know
the nature of this modification, phosphorylation is one possibility. Nevertheless, it is unclear what would bring about a change in the mutant protein’s conformation that would lead to differential post-translational modification.

We cannot pinpoint which aspect of zmsmu2 gene expression is responsible for the opaque kernel phenotype. The difference in ZmSMU2 protein level between the wild type and zmsmu2 mutants could be responsible for mutant phenotypes. Many splicing regulators can affect processing of their target pre-mRNAs in a concentration-dependent manner (Smith and Valcarcel, 2000; Matlin et al., 2005), and their over-expression often results in severe developmental defects (Kraus and Lis, 1994; Longman et al., 2000; Allemand et al., 2001). However, this model alone cannot explain why the zmsmu2-1 and zmsmu2-3 mutations show similar phenotypes, since their effects on the zmsmu2 RNA level in the endosperm are opposite.

A second, but not mutually exclusive, explanation is that the mutant phenotypes are due to some type of modification to the ZmSMU2 protein, which is suggested by the slight change in ZmSMU2 protein mobility during SDS-PAGE. It is noteworthy that the ZmSMU2 protein level in the zmsmu2-1 embryo at 16 DAP appeared to be similar to or lower than the wild-type embryo (data not shown), in contrast to what was observed in the endosperm. Nevertheless, ZmSMU2 protein from the mutant embryo showed the same increase in mobility during SDS-PAGE compared to that of the wild-type embryo. Thus, the Mu insertion in zmsmu2-1 does not appear to have the same effect on zmsmu2 transcript levels in different tissues, suggesting a stochastic effect on control of downstream gene expression. For example, either too low or too high a level of ZmSMU2 protein during early endosperm development could disrupt a regulatory network for pre-mRNA splicing and lead to auto-regulation of its activity by post-translational modification. This model predicts a change in the activity of splicing regulators such as SR proteins, which is implied based on the transcript profile of zmsmu2-1 endosperm (Chung et al., manuscript in
Our data are consistent with the hypothesis that ZmSMU2 is a splicing factor. Among the evidence supporting this conclusion are the following observations: (i) mutations in zmsmu2 cause pleiotropic mutant phenotypes (Figures 1 and 2); (ii) the deduced amino acid sequence of ZmSMU2 is highly similar to a human splicosomal protein and the nematode SMU-2, which is involved in alternative splicing (Figure 4); and (iii) ZmSMU2 showed a high level of tissue-specificity, as is true of many SR proteins (Figure 5D). Furthermore, we identified differential pre-mRNA splicing events in zmsmu2 endosperm, as well as protein interactions that imply a role for ZmSMU2 in pre-mRNA splicing (Chung et al., manuscript in preparation).

zmsmu2-1 is the first plant mutant showing defective processing of non-organellar rRNA (Figure 7). Inefficient rRNA processing in this mutant is associated with a reduction in rRNA and, presumably, ribosomes, which could explain the global change in polysome sizes (Figure 8A and Figure 8C). The mutant endosperm appears to accumulate increased levels of ribosomal protein transcripts that could be translated into ribosomal proteins (Figure 8B). However, these proteins might not be assembled into functional ribosomes, because we observed a smaller pool of ribosomes in mutant than wild type endosperm (Figure 8A). The unassembled ribosomal proteins might be degraded, as occurs in yeast (Warner et al., 1985).

We do not know how the zmsmu2-1 mutation causes defective rRNA processing in the endosperm. One explanation is that ZmSMU2 may participate in rRNA processing, independently of its function in pre-mRNA splicing. This is a possibility, since yeast Prp43p was recently found to have dual functions in pre-mRNA splicing and ribosome biogenesis (Combs et al., 2006; Leeds et al., 2006). Alternatively, the zmsmu2-1 mutation could affect the splicing pattern for a pre-mRNA encoding either an rRNA processing factor or a small nucleolar RNA.
(snoRNA). Notably, *zmsmu2-1* leaf tissues, compared to wild-type leaves, showed only a marginal reduction of total RNA yield and accumulation of abnormal rRNA intermediates (data not shown). This suggests that the degree of defective rRNA metabolism reflects the abundance of ZmSMU2 protein in a tissue, which could determine the tissue-specific pre-mRNA splicing pattern.

**Materials and Methods**

**Cloning of maize (Zea mays) zmsmu2-1 by Mu-Tail PCR**

To identify the *Mu* insertion responsible for the opaque kernel phenotype of *mto38*, genomic DNA was prepared from leaf tissue of 40 homozygous wild-type and 40 homozygous *mto38* seedlings, and DNA sequences flanking the *Mu* elements were amplified by TAIL-PCR (Liu et al., 1995). Primary *Mu*-Tail PCR reactions (20 µL) contained 1X PCR buffer, 2 mM MgCl₂, 200 µM each of dNTPs, about 100 ng of genomic DNA, one unit of Taq polymerase (Invitrogen, Carlsbad, CA), 0.2 µM of the *Mu*-specific degenerate primer MuTIR6 (5’-agagaagccacaagcctcctc-3’) and 1 µM of arbitrary degenerate primer AMS2 (5’-gwsidramsctgctc-3’) (Settles et al., 2004). The primary *Mu*-TAIL PCR was performed as described in Supplementary Table I with a Peltier Thermal Cycler-200 (MJ Research, Waltham, MA). Aliquots (2 µL) from a 50-fold dilution of the primary PCR products were used directly for secondary *Mu*-TAIL PCR reactions (20 µL) containing 1X PCR buffer, 2.0 mM MgCl₂, 200 µM each of dNTPs, 1.0 unit of Taq polymerase, 0.2 µM of the *Mu*-specific degenerate primer, MuTIR4 (5’-gccawcgctctccttcaat-3’), and the arbitrary degenerate primer used in the primary reaction. The PCR-amplified products from the second reaction were analyzed by electrophoresis in 1.5% agarose or 5% native acrylamide gel. The latter was stained with 0.2%
AgNO$_3$ in 28% formaldehyde to visualize DNA bands. A 150-bp PCR product was excised from the agarose gel, purified with a GeneClean kit (QBiogene, Carlsbad, CA) and inserted into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Nucleotide sequence analysis was performed by the University of Arizona DNA Sequencing Service; the nucleotide sequence of all PCR-constructed clones was verified by sequencing isolates of multiple clones.

3’ RACE analysis of the Zmsmu2 cDNA

Total RNA was isolated from homozygous W64A+ and zmsmu2-1 endosperms at 8, 10, 12, 14, 16, 18 and 20 days after pollination (DAP). A 3’ RACE system kit (GIBCO BRL, Gaithersberg, MD) was used to amplify the Zmsmu2 cDNA (GenBank Accession EF460507), and the product was cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) for DNA sequence analysis. Primary 3’ RACE-PCR was carried out with the primer F1 (5’-caacagcggaacagggccaaatcg-3’) based on the 5’ sequence of the 150 bp fragment associated with the Mu insertion and the UAP primer that came with the RACE kit. Primary PCR reactions were performed according to the manufacture’s instructions. Aliquots (2 µL) from 100-fold dilutions of the primary PCR products were used directly for secondary 3’ RACE-PCR reactions (20 µL). These were initiated by denaturing the cDNA at 94°C for 5 min, followed by 30 cycles of PCR as follows: 94°C, 30 sec; 58°C, 30 sec; 72°C, 1.5 min, using upstream primer F2 (5’-atcacttcgctccgctc-3’) and the UAUP downstream primer in the RACE system kit. The final cycle was extended at 72 °C for 5 min. The 1.6-kb cDNA obtained from this reaction was cloned into pCR4-TOPO and its nucleotide sequence subsequently determined.

Genotyping of wild-type and mutant zmsmu2 alleles

Genotyping of the Zmsmu2 wild-type alleles was carried out by genomic PCR, using the
upstream primer, F3 (5’-acacagggccaaatcgaaaaaatcactt-3’), and the downstream primer R6 (5’-gcatacaacctctcttatagtagttcctc-3’), while genotyping for \textit{zmsmu2-1} and \textit{zmsmu2-3} alleles utilized the upstream primer, MuTir6, and the downstream primer, R6. Genomic DNA was denatured at 94°C for 5 min, followed by 35 cycles of PCR as follows: 94°C, 30 sec; 54°C, 45 sec; 72°C, 45 sec. The final cycle was extended at 72°C for 5 min. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

**Identification of the \textit{Zmsmu2} gene from W64A+ and B73+**

To PCR amplify the \textit{Zmsmu2} gene from W64A+, we designed the primers S1 (5’-tcccccgggggatcagccacgctttcttcgagct-3’) and E1 (5’-ggaattcatgtcatcgaagaactataag-3’). PCR conditions were as follows: 5 min at 94°C (first cycle); 30 sec at 94°C, 30 sec at 56°C and 5 min at 72°C (35cycles); 5 min, 72°C (last cycle). The DNA products were inserted into pCR4-TOPO (Invitrogen, Carlsbad, CA) for sequencing (GenBank Accession EF460506). To obtain B73+ BAC clones containing \textit{Zmsmu2}, we screened a maize genomic library ZMMBBb (http://www.genome.arizona.edu/orders/) with a radiolabeled probe from a 353-bp KpnI fragment corresponding to the 3’ portion of the \textit{Zmsmu2} cDNA. Hybridization and identification of positive BAC clones were performed as described in http://www.genome.arizona.edu/information/protocols/addressnew.html. Of the 10 BAC clones with strongest hybridization signals, 6 clones, b0013K09, b0078C03, b0152G15, b0018M23, b0232J06 and b0098O15, were located in contig 392 of the maize FPC map (http://www.genome.arizona.edu/fpc/maize/). After the BAC clone, b0013K09, was digested with EcoRI, HindIII or BamHI, the fragments were introduced into pBluescript. A radiolabeled genomic PCR product amplified with primers F3 and R6 was used for the secondary screen of the 5’ UTR and promoter region of \textit{Zmsmu2} gene (GenBank Accession EF460508).
Identification and characterization of additional *zmsmu2* alleles by reverse genetic analysis

To identify additional *zmsmu2* alleles, approximately 42,000 F1 maize plants obtained from a cross with a *Mu*-active line were analyzed. DNA pools from this population were screened using the TUSC procedure at Pioneer Hi-Bred (Bensen et al., 1995; Meeley and Briggs, 1995). Pool screening was performed with *Zmsmu2*-specific primers 61699 (5’-tatcaagccacgtgctgctcatctt-3’) and 61700 (5’-gtgcccactgcattgaacaaggaatgc-3’) and a MuTIR primer (5’-agagaagccaaagccawcgcctcyatttcgtc-3’). The primary screen identified seven progeny lines with putative *Mu* insertions at the 5’ end of the *zmsmu2* gene. No insertions could be confirmed toward the 3’ end of the coding sequence. To identify the positions of the *Mu* insertions, PCR reactions with primers MuTIR6 and MTO38F1 were performed for 30 cycles as follows: 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min. The PCR products were inserted into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) for DNA sequencing.

Anatomical characterization of developing *zmsmu2-1* embryos

Embryos were dissected from 16-DAP kernels and fixed in 4% formaldehyde in PHEM-DMSO buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, 5% DMSO, pH 6.9) overnight at 4°C. They were then dehydrated in an ethanol series and infiltrated with Steedman’s wax (Brown and Lemmon, 1995). Ten-µm sections were obtained, adhered to ProbeOn Plus® slides (Fisher scientific), dewaxed with ethanol and stained with 1% toluidine blue O in deionized water.

Analysis of RNA

Total RNA was isolated with TRIZOL reagent (Invitrogen, Carlsbad, CA) according to
manufacturer’s instructions. When RNA was extracted from developing endosperm, 50 mg samples were homogenized in SDS-LiCl buffer (50 mM Tris-Cl, pH 8.0, 150 mM LiCl, 5 mM EDTA, 1% SDS) and phenol-chloroform extracted, followed by conventional TRIZOL extraction. After precipitation, RNA pellets were resuspended in 50 µl of RNase-free water (1 µl/mg fresh wt). For RT, 2.0 µg of total RNA and 0.5 µg of oligo-dT12-18 were used in a 20 µl Superscript II reaction (Invitrogen, Carlsbad, CA). When RT was performed using RNA extracted from \textit{zmsmu2-1} endosperm, the same volume of RNA solution was used as wild-type. This resulted in a more uniform amplification of \textit{Ubc} control transcripts among the samples, because the yield of total RNA per gram fresh weight was lower from mutant than wild type endosperm, but the mRNA yield was only slightly reduced in the mutant. Reproducibility of the RT-PCR results was verified by three repetitions of independent experiments involving at least two biological replicates. Nucleotide sequences for the RT-PCR primers were as follows: \textit{ubc}, ZmUBC\_F1 (5’-aagatgcaggcatctaggcaagg-3’) and ZmUBC\_R1 (5’-aggtcttggctgtgacatgttc-3’); \textit{rps29}, ZmrpS29\_F1 (5’-atgggacactccagttggaac-3’) and ZmrpS29\_R2 (5’-gttgcatgtgctcagctcata-3’); 22-kD \textit{α}-zein, 22az\_FW (5’-acagctgcaacagttatgccagtgc-3’) and 22az\_RV (5’-aaacacatgtgcttgagggcacc-3’); and \textit{Zmsmu2}, F1 (5’-caacagcggaacaggggcaaatcg-3’) and R5 (5’-tccttagcagatgcggtcgtttgtaatc-3’) or F5 (5’-tctgctcaagcttgcaacaaccg-3’) and S1 (5’-tcccccggggatcagcagctgctbctcagct-3’).

For Northern blot analysis of rRNA, probes were designed based on the nucleotide sequences of the maize rDNA gene (GenBank accession numbers K02202, U46605 and AJ309824). \(^{32}\)P-labeled probes were prepared by genomic PCR using W64A+ DNA as template and various pairs of primers as follows: 5’ ETS, ZmrDNA\_5ETS\_F1 (5’-tcggatgtggctacgcttgaaggc-3’) and ZmrDNA\_5ETS\_R2 (5’-tagcaagtctcgcgacaggggcca-3’); 18S rRNA, ZmrDNA\_18S\_F3 (5’-cgtaacgaacagacgcagct-3’) and ZmrDNA\_18S\_R4 (5’-tcggatgcttggctagc-3’).
ctgatgactcgegttactaggca-3’); ITS1, ZmrDNA_ITS1_F1 (5’-cagaccgcaacgagtagc-3’) and ZmrDNA_ITS1_R2 (5’-tgcctaggtgtaaccgcggc-3’); 5.8S rRNA, ZmrDNA_5.8S_F1 (5’-acgctctggacagacgtctc-3’) and ZmrDNA_5.8S_R1 (5’-tgacggcaggcagtagtctc-3’); ITS2, ZmrDNA_ITS2_F1 (5’-aagacactccaacccccccgcg-3’) and ZmrDNA_ITS2_R2 (5’-agggcaagctcggtgtc-3’); and 25S rRNA, ZmrDNA_25S_F3 (5’-gaccgcgccggttactaggca-3’) and ZmrDNA_25S_R4 (5’-tgcctgcaaggattcagacgc-3’). RNA was separated by agarose gel electrophoresis and transferred to nylon membrane as described in Sambrook and Russell (2001).

**Isolation and analysis of polysomes**

Polysome isolation was based on the methods described by Larkins (1985) and Kim et al. (2004b). Briefly, 16 DAP kernels were dissected and genotyped to obtain homozygous wild type and *zmsmu2-1* endosperms. Five hundred mg of pooled endosperms of each genotype were ground in 1 ml of polysome extraction buffer (0.2 M Tris·HCl, pH 8.5, 35 mM MgCl2, 60 mM KCl, 0.2 M sucrose, 25 mM EGTA, 5 mM DTT, 50 µg/ml cycloheximide). The homogenate was maintained at 4°C and centrifuged at 2,000g for 5 min. The supernatant was decanted into a new tube, adjusted to 1% (v/v) Triton X-100, and incubated on ice for 5 min. After centrifugation at 16,000g for 10 min, 0.8 ml of the supernatant was loaded onto 10-ml linear 10-60% sucrose gradients in buffer B (40 mM Tris·HCl, pH 8.5, 20 mM MgCl2, 20 mM KCl). Polysomes were separated by centrifugation at 330,000g in a Beckman SW41Ti rotor for 3.5 hr at 4°C. Fractions of 0.8 ml were recovered with an ISCO Model 640 gradient fractionator, while the absorbance at 254 nm was monitored continuously. RNA was extracted from 0.6 ml of each fraction with phenol-chloroform-isoamyl alcohol (25:24:1). After isopropanol precipitation, RNA was dissolved in 30 µL of nuclease-free water. For each sample, absorbance at 260 nm was measured with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies,
Wilmington, DE) and 2 µL was analyzed by gel electrophoresis. For semi-quantitative RT-PCR, an aliquot of 3 µL was used for a 20 µL reverse transcription reaction, and subsequent steps were performed as described in the previous section.

**Analysis of proteins**

Zein and non-zein proteins from maize endosperm were prepared as described previously (Wallace et al., 1990). Five µL aliquots of the zein and non-zein protein extracts were separated by 12.5% and 7.5% SDS-PAGE, respectively. Various other tissues, including 16 DAP embryo, 20 day after germination (DAG) shoot apex, roots (20-DAG), leaf blade (20-DAG), ovules, pollen and tassel were ground in Laemmli buffer, and 4 µg of protein was separated by 7.5% SDS-PAGE.

**Preparation of ZmSMU2 antiserum**

A Zmsmu2 cDNA containing the complete coding region was amplified by PCR with primers 38GEXE1 (5’-cggaattccatgtcatcgaagaactac-3’) and 38GEXN1 (5’-agaatgcggccgctaatcagccacgctgtttcttcgag-3’). The DNA product was cloned into the EcoRI and NotI sites of pGEX4T-3 (GE Healthcare, Piscataway, NJ) to produce the fusion protein GST::ZmSMU2, and the plasmid was used to transform *E. coli* strain BL21(DE3) codon+ (Stratagene, La Jolla, CA). Bacterial cell extract in high-salt lysis buffer (10 mM phosphate buffer, pH 7.0, 0.5 M NaCl, 1 mM DTT, 1 mM EDTA and 0.2 mM PMSF) containing 1% Triton X-100 was incubated with glutathione-agarose beads (Sigma, St. Louis, MO) at 4 °C. After two washes with the high-salt lysis buffer and two washes with phosphate buffered saline (pH 7.3), the fusion protein on the beads was digested with thrombin (Sigma, St. Louis, MO) to remove the GST tag. Following SDS-PAGE, 1 mg of ZmSMU2 protein was purified by gel excision and
used as antigen for preparation of custom rabbit polyclonal antibodies (Strategic BioSolutions, Windham, ME). Immunoblots were incubated with a 1:2,000 dilution of rabbit ZmSMU2 antiserum, and then with 1:50,000 dilution of goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Pierce, Rockford, IL).

Production of recombinant GST::AtSMU2 protein and acquisition of Arabidopsis

(\textit{Arabidopsis thaliana}) atsmu2-1 and atsmu2-2 mutants

The coding sequence of At2g26460 was amplified by RT-PCR using primers F+60BspHI and R+3924BamHI (5’-cgggatccgctaagttgccgagc-3’) and the DNA product was introduced into pCR4blunt-TOPO (Invitrogen, Carlsbad, CA). The resulting plasmid, pCR4b-AtSMU2, was digested with EcoRI, reinserted into pGEX4T-3 (GE Healthcare, Piscataway, NJ) and used to transform the E. coli strain BL21(DE3) codon+ (Stratagene, La Jolla, CA). The recombinant protein, GST::AtSMU2, was purified by the same procedure as GST::ZmSMU2. The Arabidopsis mutants, atsmu2-1 (stock # SALK_039202) and atsmu2-2 (stock # WiscDsLox320H09), were obtained from Arabidopsis Biological Resource Center (Columbus, OH).

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number(s) EF460507, EF460506, EF460508.

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Figure Legends

Figure 1. Kernel and protein phenotypes of mto38/zmsmu2-1. (A) Mature F2 kernels from a heterozygous zmsmu2-1/+ ear that segregated for vitreous and opaque (arrowheads) phenotypes at a 3:1 ratio. (B) SDS-PAGE of zein (lanes 1 and 2) and non-zein proteins (lanes 3 and 4) from vitreous (lanes 1 and 3) and opaque (lanes 2 and 4) F2 endosperms.

Figure 2. Phenotypes of zmsmu2-1 mutants. (A) Wild-type (left) and zmsmu2-1 (right) seedlings at 20 days after germination (DAG); note the mutant seedling produced more leaves than wild type. (B) A mature zmsmu2-1 mutant plant showing numerous leaves and a poorly branched tassel. (C-F) Sagittal longitudinal sections through 16-DAP embryos of wild-type (C) and zmsmu2-1 (D-F). The mutant embryos (D and F) are typically smaller than wild-type (C). Though many mutant embryos have a single shoot and root meristem (F), like the wild type, some have twin plumules (D) and twin radicles (E). A magnified image of the section in panel D. S, scutellum; Cp, coleoptile; R, radicle; Cr, coleorhiza; bars = 0.2 mm.

Figure 3. Identification of a Mu insertion in the maize Zmsmu2 gene that is tightly linked with the opaque kernel phenotype. (A) Mu TAIL-PCR products from genomic DNA of seedlings of opaque F2 kernels (lanes 1-6), seedlings from vitreous F2 kernels (lanes 7-12), and homozygous wild type W64A+ seedlings (lanes 13-18). A 150-bp fragment (arrow) was detected in DNA of all the opaque mutants (lanes 1-6) but none of the homozygous wild types (lanes 13-18). This DNA was detected in some of the vitreous F2 progeny (lanes 9-11), which may be heterozygous for zmsmu2-1. (B) Linkage analysis of zmsmu2-1 with the opaque kernel phenotype. Genomic PCR using gene-specific primers F3 and R6 (lanes with a plus sign; see panels C and D for the
position of the primers in the Zmsmu2 gene) yielded a DNA band specific for the wild-type allele at the Zmsmu2 locus, whereas another PCR using primers MuTir6 and R6 (lanes with a minus sign) produced a zmsmu2-1-specific band. The lane numbers correspond to F2 individuals that provided genomic DNA templates for the PCR. (C) Structure of the maize Zmsmu2 gene. White and black boxes correspond to exon sequences in the 5’ and 3’ UTRs and the coding region, respectively, and gray bars between the boxes represent introns. The Mu element (not to scale) in alleles zmsmu2-1 to zmsmu2-4 is illustrated by reverse triangles; the allele numbers are specified in the black triangles. Black and gray arrowheads mark the positions of gene-specific primers and of a Mu-specific primer, MuTir6, respectively, used for PCR amplification. (D) Alignment of the Zmsmu2 genomic DNA sequence from two maize inbreds, W64A+ and B73+. Only the 5’ ends of the available sequences are shown. The nucleotide sequence in gray corresponds to the Mu-containing sequence obtained from zmsmu2-1 cDNA. Transcription may start around the F1 primer site, since we were able to detect Zmsmu2 cDNA from wild-type RT products using the F1 and R5 primer pair (see Figure 5A). Intron sequences are italicized. Capital letters over the W64A+ sequence are the first 16 amino acid residues of the deduced protein. The three sequences in boxes show the target duplication sites for Mu insertion in zmsmu2-1, zmsmu2-3 and zmsmu2-4. Primer sequences are shown in arrows. Underlined nucleotide sequences are conserved cis-elements identified by TESS: Transcription Element Search System (http://www.cbil.upenn.edu/tess).

**Figure 4.** Deduced amino acid sequences of ZmSMU2 and its homologues in eukaryotes.

Multiple sequence alignment obtained from CLUSTALX (Thompson et al., 1994) revealed identical (in black) and conservative (in gray) amino acid residues among SMU-2 homologues. ZmSMU2 (translated from mRNA EF460507), OsSMU2 (ABA91466.2), LeSMU2 (translated
from mRNA BT013452), AtSMU2 (NP_180214.1), HsSMU2 (NP_006074.1), DmSMU2 (NP_649865.1) and SMU-2 (NP_494559.1) correspond to maize, rice, tomato, Arabidopsis, human, fruit fly and nematode proteins, respectively. Solid lines indicate amino acid residues comprising the RED domain in HsSMU2 and SMU-2. Two asterisks mark sequence variations in the zmsmu2-3 allele, Glu107 to Asp107 and Ala233 to Ser233.

**Figure 5.** The zmsmu2-1 mutant shows altered gene expression. (A) Semi-quantitative RT-PCR comparing transcript levels of Zmsmu2 and ubiquitin conjugase (Ubc) control genes in homozygous wild-type and zmsmu2-1 mutant endosperm. For Zmsmu2 transcripts, 3 different primer pairs were used (refer to Figure 3C depicting location of primers and the Mu insertion in zmsmu2-1). The F5 and S1 primers detected RNAs corresponding to the 3’ end of the coding sequence; the F1 and R5 primers detected RNAs corresponding to the wild-type-specific 5’ UTR and coding region; the MuTir6 and R5 primers detected RNAs corresponding to the zmsmu2-1-specific 5’ UTR, plus 5’ coding region. +/+, endosperm dissected with a homozygous wild-type embryo; -/-, endosperm dissected with a homozygous zmsmu2-1 mutant embryo. (B) An immunoblot with maize ZmSMU2 antibodies detects the corresponding protein (indicated by an arrow in lane 3) and the homologous Arabidopsis protein (AtSMU2) (arrow in lane 5). Lane 1, 1 ng of GST; lane 2, 1 ng of GST::ZmSMU2; lane 3, non-zein proteins from W64A+ endosperm 14 DAP; lane 4, 1 ng of GST::AtSMU2; lane 5, protein extract from three seedlings of Arabidopsis Col-0 (7 DAG); lane 6, protein extract from three seedlings of Arabidopsis mutant atsmu2-1; lane 7, protein extract from three seedlings of Arabidopsis mutant atsmu2-2. (C) ZmSMU2 immunoblot of non-zein endosperm proteins from 16 DAP kernels of an F2 ear segregating for zmsmu2-1. Lane 1, homozygous wild-type; lane 2, heterozygote; lane 3, homozygous zmsmu2-1. (D) Tissue-specific expression of ZmSMU2 protein based on
immunoblot assay. Each lane contained about 4 μg of proteins from various tissues of W64A+.
lane 1, 14 DAP endosperm; lane 2, 14 DAP embryo; lane 3, shoot apex from a 20 DAG seedling;
lane 4, roots from this seedling; lane 5, leaf blade from this seedling; lane 6, unfertilized ovule;
lane 7, mature pollen; lane 8, developing tassel.

Figure 6. zmsmu2-1 and zmsmu2-3 mutants share similar molecular phenotypes. Heterozygous
(zmsmu2-1/+ or zmsmu2-3/+) plants were self-pollinated and 16 DAP kernels dissected into embryo and endosperm tissues. Embryo DNA was used to genotype the Zmsmu2 locus, and protein and RNA were extracted from homozygous wild-type, heterozygous, and homozygous mutant endosperms. +/-, endosperm with a homozygous wild-type embryo; +/-, endosperm with a heterozygous embryo; -/-, endosperm with a homozygous zmsmu2-1 mutant embryo. RNA was used for semi-quantitative RT-PCR (panel A). Protein was separated into zein and non-zein fractions, separated by SDS-PAGE, and immunoblotted with ZmSMU2 antibodies (panel B, non-zeins) or stained with Coomassie Blue (panel C, zeins). A minimum of three replicated experiments for each homozygous zmsmu2-1 and zmsmu2-3 genotype was analyzed. (A) Comparison of Zmsmu2 transcripts in F2 kernels segregating for zmsmu2-1 and zmsmu2-3. Relative to wild type, Zmsmu2 transcripts were more reduced in zmsmu2-3. Primers F5 and S1 were used for the amplification of Zmsmu2 transcript. (B) Immunodetection of ZmSMU2 protein in zmsmu2-1 and zmsmu2-3. Although this protein was synthesized in both zmsmu2-3 and zmsmu2-1, its migration was altered during SDS-PAGE. (C) Comparison of zein proteins in zmsmu2-1 and zmsmu2-3 endosperm. Both zmsmu2-1 and zmsmu2-3 showed reduced synthesis of zein proteins, particularly α-zeins. Zein protein designations are shown on the right; each lane contained 1/120 of the total zeins extracted from 50 mg of 16-DAP endosperm.
**Figure 7.** Evidence of defective rRNA processing in *zmsmu2-1* endosperm. (A) Diagram illustrating the maize rRNA transcription unit. Solid bars indicate sequences present in mature 18S, 5.8S and 25S rRNAs after primary transcript processing. Lines between the bars correspond to the 5’ external transcribed spacer (5’ ETS), internal transcribed spacers 1 and 2 (ITS 1 and 2) and the 3’ ETS. Gray lines below the transcription unit show the location of probes used for Northern blots. (B) Northern blot analysis comparing levels of mature and unprocessed rRNAs in wild-type and *zmsmu2-1* endosperm. Each lane contained one µg of total RNA extracted from genotyped endosperm. +/+, endosperm from a kernel with a homozygous wild-type embryo; -/-, endosperm from a kernel with a homozygous *zmsmu2-1* embryo. Number above each blot indicates the probes used for the Northern blot and correspond to the number in the diagram in A. The blots were exposed to X-ray film for 1 hour (blots 2, 4 and 6) or overnight (blots 1, 3 and 5). Four major bands (arrowheads; labeled a, b, c and d) were detected, and the diagrams on the right show the intermediate and mature rRNA transcripts predicted from the band pattern. Note that bands corresponding to 5.8S rRNA are not shown here due to their small size. (C) Northern blot analysis comparing the ITS2 levels in wild type, *zmsmu2-1* and *zmsmu2-3* mutants. The arrowheads indicate unprocessed rRNA species containing ITS2. Each lane contained one µg of total RNA. Ethidium bromide-stained 25S and 18S rRNA are shown below as the loading control.

**Figure 8.** Reduced efficiency of mRNA translation in *zmsmu2-1* mutant endosperm. Polysomes were isolated from 16 DAP endosperm as described in Methods, separated by centrifugation in 10-60% sucrose gradients, and the absorbance at 254 nm monitored continuously during fractionation with an ISCO 640 fractionator. +/+, endosperm from a kernel with a homozygous wild-type embryo; -/-, endosperm from a kernel with a homozygous *zmsmu2-1* embryo. (A) Polysome profiles from 16-DAP wild type (upper panel) and *zmsmu2-1* mutant endosperm...
(lower panel). The numbers above the peaks indicate the monosome (1) and number of ribosomes in polysomes (2-6 or more). The arrow indicates the direction of centrifugation. (B) RT-PCR analysis of RNA obtained from polysomes in panel A. Aliquots of 0.8 ml were collected during gradient fractionation, and RNA was purified by phenol-chloroform extraction. Equal volumes of RNA solution were used for RT-PCR to detect ubiquitin conjugase (Ubc), ribosomal protein S29 (Rps29), a 22-kD α-zein gene (22kD α-zein) and Zmsmu2 transcripts. For Zmsmu2, primers F5 and S1 were used. (C) Northern blot analysis of monosomal (M) and polysomal (P) fractions with a probe for the ITS2 of the rRNA transcription unit. Approximately 0.5 and 1.0 µg of RNA was used for the monosomal and polysomal fractions, respectively. Ethidium bromide-stained 25S and 18S rRNA are shown below as the loading control. Total RNA (T) was analyzed for comparison. The rRNA precursors corresponding the two bands detected are labeled as in Figure 7.
Table I. Seed germination and seedling growth in *zmsmu2-1* and *zmsmu2-3*

| F2 population | Genotype  | N    | 0  | 1  | 2 or more |
|---------------|-----------|------|----|----|-----------|
| *zmsmu2-1*    | +/+       | 25   | 1  | 2  | 22 (88%)  |
| (n=81)        | +/zmsmu2-1| 36   | 2  | 1  | 33 (92%)  |
|               | zmsmu2-1/zmsmu2-1 | 20   | 6  | 1  | 13 (65%)  |
| *zmsmu2-3*    | +/+       | 26   | 2  | 1  | 23 (88%)  |
| (n=84)        | +/zmsmu2-3| 42   | 3  | 2  | 37 (88%)  |
|               | zmsmu2-3/zmsmu2-3 | 16   | 8  | 0  | 8 (50%)   |

*a* Genotypes were determined by genomic PCR, as described in Materials and Methods. Based on an expected genotypic ratio of 1:2:1, calculated chi square values for *zmsmu2-1* and *zmsmu2-3* were 1.62 (P>0.05) and 2.38 (P>0.05), respectively.

*b* Number of leaves was determined at ten days after germination.
Table II. Total RNA and mRNA extractable from wild-type and zmsmu2-1 endosperm

|            | a Endosperm F.W. (g) | b Total RNA yield (mg RNA/g FW) | c mRNA yield (ng mRNA/g FW) |
|------------|----------------------|---------------------------------|------------------------------|
| Wild type  | 0.31 ± 0.06          | 0.97 ± 0.17                     | 6.8 ± 1.3                    |
| zmsmu2-1   | 0.25 ± 0.04          | 0.52 ± 0.14                     | 5.4 ± 0.2                    |

a. Four wild-type and four mutant half-endosperms were weighed, and total RNA was extracted from the individual endosperms as described in Materials and Methods. Values are shown as mean ± standard deviation of four biological replicates.

b. Obtained by dividing amount of extractable total RNA by values in column a. Total RNA yield from wild-type is significantly higher than that from zmsmu2-1 (P<0.01).

c. Obtained by dividing amount of purified mRNA by values in column a. mRNA yield from wild-type endosperm is not significantly higher than that from zmsmu2-1 (P>0.05).
A

|          | zmsmu2-1 |          |          | zmsmu2-3 |
|----------|----------|----------|----------|----------|
| +/+      | ![Ubc](image1) | +/+      | ![Ubc](image2) | +/+      | ![Ubc](image3) |
| +/-      |          | +/-      |          |          |          |
| -/-      |          | -/-      |          |          |          |
| -/-      |          | -/-      |          |          |          |

B

|          | zmsmu2-1 |          |          | zmsmu2-3 |
|----------|----------|----------|----------|----------|
| +/+      | ![ZmSMU2](image4) | +/+      | ![ZmSMU2](image5) | +/+      | ![ZmSMU2](image6) |
| +/-      |          | +/-      |          |          |          |
| -/-      |          | -/-      |          |          |          |
| -/-      |          | -/-      |          |          |          |

C

|          | zmsmu2-1 |          |          | zmsmu2-3 |
|----------|----------|----------|----------|----------|
| +/+      | ![50kD γ-zein](image7) | +/+      | ![50kD γ-zein](image8) | +/+      | ![50kD γ-zein](image9) |
| +/-      | ![27kD γ-zein](image10) | +/-      | ![27kD γ-zein](image11) | +/-      | ![27kD γ-zein](image12) |
| -/-      | ![22kD α-zein](image13) | -/-      | ![22kD α-zein](image14) | -/-      | ![22kD α-zein](image15) |
| -/-      | ![19kD α-zein](image16) | -/-      | ![19kD α-zein](image17) | -/-      | ![19kD α-zein](image18) |
### B

| Fraction # | Monosome | Polysome |
|------------|----------|----------|
| 5          | ![rRNA](rRNA.png) | +/+      |
| 6          | ![rRNA](rRNA.png) | -/-      |
| 7          | ![Ubc](Ubc.png)   | +/+      |
| 8          | ![Ubc](Ubc.png)   | -/-      |
| 9          | ![Rps29](Rps29.png) | +/+      |
| 10         | ![Rps29](Rps29.png) | -/-      |
| 11         | ![22kD α-zein](22kD_α-ztein.png) | +/+      |
| 12         | ![22kD α-zein](22kD_α-ztein.png) | -/-      |
| 13         | ![Zmsmu2](Zmsmu2.png) | +/+      |
|            | ![Zmsmu2](Zmsmu2.png) | -/-      |

### C

| +/+  | -/-  | +/+  | -/-  |
|------|------|------|------|
| ![ITS 2](ITS_2.png) | ![rRNA](rRNA.png) | ![ITS 2](ITS_2.png) | ![rRNA](rRNA.png) |