Deletion Mutagenesis Identifies a Haploinsufficient Role for γ-Zein in opaque2 Endosperm Modification

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Quality Protein Maize (QPM) is a hard kernel variant of the high-lysine mutant opaque2. Using γ-irradiation, we created opaque QPM variants to identify opaque2 modifier genes and to investigate deletion mutagenesis combined with Illumina sequencing as a maize (Zea mays) functional genomics tool. A K0326Y QPM deletion mutant was null for the 27- and 50-kD γ-zeins and abolished vitreous endosperm formation. Illumina exon and RNA sequencing revealed a 1.2-megabase pair deletion encompassing the 27- and 50-kD γ-zein genes on chromosome 7 and a deletion of at least 232 kb on chromosome 9. Protein body number was reduced by over 90%, while protein body size is similar to the wild type. Kernels hemizygous for the γ-zein deletion had intermediate 27- and 50-kD γ-zein levels and were semivitreous, indicating haploinsufficiency of these gene products in opaque2 endosperm modification. The γ-zein deletion further increased lysine in QPM in its homozygous and hemizygous states. This work identifies 27-kD γ-zein as an opaque2 modifier gene within the largest QPM quantitative trait locus and may suggest the 50-kD γ-zein also contributes to this quantitative trait locus. It further demonstrates that genome-wide deletions in nonreference maize lines can be identified through a combination of assembly of Illumina reads against the B73 genome and integration of RNA sequencing data.

Maize (Zea mays) opaque endosperm mutants have been studied because of the nutritional improvement that they often show, as well as for the insight they provide into the kernel hardening, an essential quality trait for most of the grain’s uses. The most well known opaque mutant, opaque2 (o2), has been widely studied because of its increased lysine and Trp level (Mertz et al., 1964) associated with the reduced synthesis of α-zeins. The O2 gene encodes a transcription factor that regulates α-zein gene expression (Schmidt et al., 1990), although all zein proteins are indirectly reduced. O2 also regulates pyruvate orthophosphate dikinase (Maddaloni et al., 1996) and many other genes (Jia et al., 2013). Although the soft kernels and yield penalty of o2 inhibited its commercial success, subsequent breeding projects, including those in Mexico (Vasal et al., 1980) and in South Africa (Geetha et al., 1991; Lopes and Larkins, 1991), led to the development of hard kernel o2 varieties called Quality Protein Maize (QPM). QPM kernels generally have low levels of α-zeins and high levels of Lys and Trp, but the genetic basis of o2 endosperm modification is poorly understood.

One prominent feature of QPM endosperm is accumulation of the 27-kD γ-zein at two- to three-fold higher levels than in the wild type and o2 (Wallace et al., 1990; Geetha et al., 1991; Lopes and Larkins, 1991). Although the genetic and biochemical mechanisms responsible for this increase are unknown, the degree of endosperm vitreousness in QPM correlates with the level of 27-kD γ-zein protein (Lopes and Larkins, 1991). Furthermore, the 27-kD γ-zein gene, along with the closely linked 50-kD γ-zein, maps to the most significant quantitative trait loci (QTL) for endosperm modification located on chromosome 7 (Lopes and Larkins, 1995; Lopes et al., 1995; Holding et al., 2008, 2011). QPM endosperm accumulates larger numbers of small, γ-zein-rich protein bodies. This is proposed to allow the formation of a rigid, glassy matrix similar in texture to mature wild-type endosperm. RNA interference (RNAi) reduction of both 27- and 16-kD γ-zeins in QPM caused opaque reversion and thus supported the suggestion that 27-kD γ-zein contributes to endosperm modification (Wu et al., 2010). However, the extent to which 27-kD γ-zein is alone sufficient as a modifier is unknown. Furthermore, the possible role of the 50-kD γ-zein in modification has not been previously addressed. The molecular characterization of opaque mutants has shown that vitreous endosperm formation depends on more than just correct abundance and spatial organization of zein proteins.
potential of deletion mutagenesis for analysis of gene function and creating desirable changes for breeding programs, a radiation mutagenesis approach is limited by lack of knowledge of the extent and size range of deletions induced. We used γ-irradiation of K0326Y QPM as a means to identify genes corresponding to α2 modifiers and genes generally required for vitreous endosperm formation. For identification of DNA sequence deletions in the induced mutants, we combined exon-capture DNA sequencing and RNA sequencing (RNA-seq). Among the opaque variants induced in QPM, one had a deletion that eliminates both the 27- and 50-kD γ-zein loci, which are within a significant QPM QTL on chromosome 7 (Holding et al., 2008, 2011). By demonstrating the dosage-dependent action of 27-kD γ-zein in α2 endosperm modification, this mutant highlights the potential of this approach for identifying other α2 modifier genes as well as genes involved in a variety of aspects of endosperm development.

RESULTS
Identification of a 27- and 50-kD γ-Zein Protein Null among Novel Opaque Deletion Mutants

We recovered 305 M2 ears of variable seed fill, of which 293 were successfully propagated to M3 families (approximately 20 ears each). Although single M2 ears were potentially affected by nongenetic environmental factors, screens of adequately filled M2 ears were conducted for dominant seed mutant phenotypes and identified more than 30 lines having a substantial proportion of opaque, small, defective, empty pericarp, or rough kernels. All 293 lines were propagated to the M3 generation, and 27 lines exhibited clear segregation for opaque mutants. Of these 27 lines, opaque kernels in 17 lines were not viable and presumed to be pleiotropic mutations. The other 10 lines had fully viable opaque kernels and were thus candidates for factors specifically involved in α2 endosperm modification and/or vitreous endosperm formation in general. Three of these 10 confirmed mutants, including line 107 (Fig. 1), line 198 (Supplemental Fig. S1), and line 12 (not shown) exhibited mutant kernels in the M2 ears, while the others were first observed in the M3 and resulted from M2 ears that either appeared normal or had too few kernels to screen. Segregating M3 ears for examples of these lines is shown in Supplemental Figure S1. Analysis of zein proteins revealed several different phenotypic classes affecting zein composition (Supplemental Fig. S1). These included no apparent change in zeins (line 8), generalized reduction of all zeins (lines 66 and 112), loss of residual 22-kD α-zein present in K0326Y QPM (lines 44 and 133), and loss of 19-kD α-zein (line 198). Line 107 exhibited a striking absence of the 27- and 50-kD γ-zeins (Fig. 2A), which was confirmed with immunoblot analysis (Fig. 2B). Given the suggested role of 27-kD γ-zein in endosperm modification in QPM, we focused on the characterization of this mutant. As expected, 27-kD γ-zein was significantly increased in K0326Y QPM compared with the W64A wild type (Fig. 2A). Interestingly, although 50-kD γ-zein is not
as abundant as the 27-kD γ-zein protein and does not appear to be involved in protein body initiation (Guo et al., 2013), similar to 27-kD γ-zein, we consistently observed the protein to be increased in QPM compared with the wild type (Fig. 2, A and B).

Complete Lack of 27-kD γ-Zein in Line 107 Dramatically Reduces Protein Body Initiation But Further Improves Protein Quality

To investigate the effects of the γ-zein deletion on protein body initiation, morphology, and zein distribution, we conducted immunogold transmission electron microscopy (TEM) of 18-d after pollination (DAP) developing endosperm. Low magnification (1,000×) of the W64A wild-type control showed that protein bodies are densely clustered by the fourth subaleurone cell layer (Fig. 3A). Thus, for quantitative counts and qualitative analysis protein body morphology and composition, we focused on the fourth subaleurone cell layer. Starch grains in this layer remained smaller and less abundant than in more central endosperm regions, which would have impaired the ability to quantify protein body number. Cell layers internal to the fourth layer could often not be reliably deciphered because of the oblique and irregular cell wall planes as well as the relatively lower contrast of the immunogold TEM compared with standard TEM. Nonmutagenized QPM accumulated smaller protein bodies 30% to 40% of the W64A wild-type size but in higher number than the wild type (Figs. 3B and 4, D–F; Table I), which is consistent with the suggested role of increased 27-kD γ-zein in QPM (Geetha et al., 1991). Protein bodies in QPM are darker staining due to deposition of γ-zein throughout the protein body (Fig. 4E), where it colocalizes with α-zeins (Fig. 4F). Elimination of 27- and 50-kD γ-zein proteins in line 107 resulted in almost undetectable numbers of protein bodies at low magnification (Fig. 3C) due in part to their lack of contrast compared with wild-type or QPM protein bodies (Fig. 4, G and H). At high magnification, and with the aid of α-zein labeling (Fig. 4I), line 107 protein bodies were identified as being similar in size to the wild type and with irregular, undulating surfaces. Protein body number in line 107 is dramatically reduced compared with the wild type and QPM (Fig. 3C; Table I). This is similar to results obtained by RNAi suppression of 27-kD γ-zein synthesis (Guo et al., 2013), except that suppression of protein body initiation is even more severe in the complete absence of 27-kD γ-zein (Fig. 4, G–I). Consistent with RNAi results (Guo et al., 2013), this shows that 16-kD γ-zein is not sufficient for supporting the normal level of protein body initiation. The increased protein body size in line 107, relative to its QPM progenitor (Fig. 4, G–I), is likely due to the need to package substantial 19-kD α-zein in a smaller number of protein bodies. The irregular undulating protein body shape (Fig. 4, G–I) may be due to the improper packaging of the hydrophobic 19-kD α-zein. The γ-zein antibody, which was raised against the 27-kD γ-zein, does not detect γ-zein in line 107 protein bodies, which explains the lack of protein body contrast.

We measured Lys and Trp content in mature kernels of the 107 mutant and compared it with the K0326Y QPM progenitor, the W64A wild type, and W64Ao2. Comparison of W64A wild-type and o2 kernels showed the expected levels of improvement for these amino acids (Table II). Improvement of the Lys and Trp percentage in QPM was not as pronounced as W64A o2, probably as a result of higher level accumulation of Lys-devoid 27-kD γ-zein. However, when 27-kD γ-zein was absent, the content of these amino acids was similar to W64A o2. Consistently, the partially modified semiopaque 107 kernels had a higher level of Lys than QPM, suggesting that partial accumulation γ-zein can present a breeding strategy to balance protein quality and endosperm texture.

Figure 1. M3 ear phenotypes of K0326Y QPM control ear and segregating and homozygous K0326Y deletion line 107. Inserts in A and C show light box phenotypes.

Figure 2. A, SDS-PAGE analysis of zein proteins from mature kernel of deletion line 107 compared with K0326Y QPM and the W64A wild type and W64Ao2. B, Western analysis of zein proteins loaded at 1:1,000 dilution of gel shown in A, probed with total α-zein antiserum (1:10,000) and anti-27-kD γ-zein antiserum (1:2,000). WT, Wild type.

[See online article for color version of this figure.]
Line 107 Shows Absence of 27- and 50-kD γ-Zein Genes and Haploinsufficiency for α2 Endosperm Modification

We sought to determine if the absence of 27- and 50-kD γ-zein proteins resulted from an indirect effect on zein synthesis in general, a regulatory defect, or the physical loss of the genes. Reverse transcription (RT)-PCR showed that transcripts of both genes were undetectable in endosperm RNA of line 107 (Fig. 5A). Levels of the unlinked 16-kD γ-zein were normal, ruling out the possibility that zein gene expression was generally affected. Genomic PCR did not detect the 27- or 50-kD zein genes, while the 16-kD γ-zein gene was present (Fig. 5B), showing that the protein absence was not a regulatory effect but due rather to the loss of these genes. Given that the 27- and 50-kD γ-zein genes are closely linked (being separated by approximately 27 kb; Holding et al., 2008), the most likely scenario was that a deletion covered both genes, which was confirmed by sequencing exon-captured genomic DNA (below).

While most of the heritable opaque mutants we recovered appeared to segregate according to Mendelian ratios in the M3 (Supplemental Fig. S1), line 107 had more than 25% opaque kernels and obvious variability.
Table I. Protein body number in fourth subaleurone layer of the wild type, K0326Y QPM, and K0326Y line 107

| Protein body no. | W64A Wild Type | K0326Y QPM | K0326Y Line 107 |
|------------------|----------------|------------|-----------------|
| so               | 50.0           | 87.6       | 6.2             |
| P value          | ±3.0           | ±8.4       | ±0.8            |
|                  | —              | 1.6 × 10⁻⁵ | 4.4 × 10⁻⁵      |

Table II. Lys and Trp content in mature kernels

Mature kernel amino acid contents for Lys and Trp are shown in grams per 100 g protein ± so (n = 3).

|                  | W64A Wild Type | W64A o2 | K0326Y QPM | K0326Y Line 107 Opaque | K0326Y Line 107 Semiopaque |
|------------------|----------------|---------|------------|------------------------|---------------------------|
| Lys              | 2.39 ± 0.08    | 4.96 ± 0.04 | 4.09 ± 0.00 | 4.93 ± 0.17            | 4.42 ± 0.01               |
| Trp              | 0.61 ± 0.05    | 1.00 ± 0.00 | 0.92 ± 0.00 | 0.97 ± 0.13            | 0.92 ± 0.05               |

Role of γ-Zein in opaque2 Endosperm Modification

in the level of opacity (Fig. 1B). With careful examination on a light box, the number of fully opaque kernels (Fig. 6) was found to be approximately 25% (46/190, 9/38, and 14/56 in three M3 segregating ears). Kernels in this class were homozygous for the deletion mutation because they gave rise to 100% opaque kernels when propagated (Fig. 1C). Another class of kernels, making up less than 50%, was opaque with a vitreous cap (Fig. 6). These were determined to be hemizygous for the γ-zein deletion, because they gave rise to segregating ears when propagated (Fig. 1B). The remainder of the expected 50% hemizygous kernels from these ears comprised less severe semioopaque, which could not be reliably scored. SDS-PAGE analysis of zein proteins from vitreous, semiopaque, and fully opaque kernels from a segregating ear showed that partial opacity results from reduced accumulation of the 27- and 50-kD γ-zein proteins (Fig. 6). This suggests that expression from a single allele of these two genes is haploinsufficient for full γ2 endosperm modification. Importantly, unlike the majority of mutants that first segregated in the M3 ears, the M2 ear for line 107 segregated 1:1 for normal and opaque with vitreous cap (hemizygous) kernels, which supports the haploinsufficiency explanation and is consistent with the ontology of the mature embryonic shoot apical meristem (Johri and Coe, 1983; Poethig et al., 1986); see “Discussion”).

Exon- and RNA-Seq Identifies a Deletion Spanning the 27- and 50-kD γ-Zein Genes

To confirm that the 27- and 50-kD γ-zeins were within a single deletion and define the size of that deletion, we used Illumina sequencing of exon-enriched genomic DNA. Deletions were called according to an algorithm that considered both the number of reads for a given exon in the nonmutagenized QPM and the ratio of reads between nonmutagenized QPM and a mutagenized line (see “Materials and Methods”). The relatively nonstringent criteria returned deletion candidates on all chromosomes apart from chromosome 7 (Supplemental Table S1). We tested all of the candidates using genomic PCR and RT-PCR (data not shown) and established that all except those genes within the chromosome 7 and 9 deletions described below were false positives. As further confirmation, none of the genes identified as false positives were identified as differentially expressed using RNA-seq (Supplemental Tables S1 and S2).

By combining exon read data with genomic and RT-PCR, we identified two deletions in line 107. One of these is in chromosome 7, Bin 7.02, and is approximately 1,236,000 bp (Fig. 7). Using genomic PCR on exon deletion candidates, we showed that there are at least 11 predicted genes in this region that are absent in line 107. RT-PCR revealed that at least seven of these genes are expressed in normal developing endosperm but not detectable in RNA of line 107 (Fig. 7). Two genes close to the 5’ end of the deletion encode the 27- and 50-kD γ-zeins. Four of the 11 genes are either endosperm nonexpressed or expressed at a low level or are pseudogenes. Four other genes were confirmed as deleted in this region by comparing exon sequencing (exon-seq) and RNA-seq reads (Supplemental Table S2). We used primers within flanking genes (Fig. 7) as well as intergenic primers to estimate the extremities of this deletion. A smaller deletion of at least 231,968 bp was identified on chromosome 9, Bin 9.03 (Fig. 8). This region contains five genes, four of which have expression abolished based on RT-PCR. Expression of the fifth gene was not detectable in the nonmutagenized control. Genomic and RT-PCR tests of other exon deletion candidates (Supplemental Table S1) identified elsewhere in the genome by low or absent read number did not confirm additional single or multiple exon deletions.

The RNA-seq comparison of K0326Y QPM and line 107 had three purposes: confirmation of deletions identified with exon-seq identification of additional deletions that exon-seq may have missed; and potential identification of indirect transcriptional affects of putative gene deletions, as a consequence of defective transcription factors. For this, we mixed RNA in equal proportions extracted from whole developing seeds (endosperm and embryo), juvenile leaf, and juvenile root. This potentially allowed us to identify transcript alterations resulting in sporophytic tissues as well as seed tissues. Because our goal was to identify complete losses or substantial changes in expression, rather than minor fold changes, a single RNA-seq library was prepared from K0326Y QPM and line 107, and this allowed bar coding and pooling with other deletion mutants. The genes with transcripts that were decreased or increased
in line 107 based on RNA-seq, along with their exon-seq reads, are shown in Supplemental Table S2. Twenty genes were increased in abundance more than 4-fold in line 107 compared with K0326Y QPM, whereas 70 genes had transcripts more than 4-fold reduced. Within these 70 genes within the chromosome 7 and 9 deletions (Supplemental Tables S3 and S4). Of the five genes within the chromosome 9 deletion, only one gene, GRMZM2G099648, a ubiquitin ligase, had RNA-seq reads (Supplemental Table S4). Of the other differentially expressed genes with chromosome coordinates not within the chromosome 7 and 9 deletions, none qualified as candidate deletions. However, several differentially expressed genes identified with RNA-seq were not detected with exon-seq in either K0326Y or line 107 (Supplemental Table S2, pink highlights). We tested all of these genes with at least two pairs of genomic PCR primers each and showed that they were not gene deletions missed during exon-seq (not shown). Zein genes are expressed at high levels, and this was reflected in the reads measured for the 27- and 50-kD y-zein genes in K0326Y QPM (386,441 and 180,567 reads, respectively). As expected, reads for these genes in line 107 were drastically reduced to 0.2% and 0.1% of control, respectively, but surprisingly, they were not completely absent as might be expected for deletion of single copy genes (869 and 284 reads, respectively). These genes share extensive identity with the 16-kD y-zein, but at a lower level than our allowable threshold for RNA-seq reads (98-bp perfect match of the 100-bp read length). However, some nucleotides within the y-zein reads had suboptimal scores, and if some had been incorrectly called for 16-kD y-zein reads, this could give rise to reads incorrectly annotated as 50- or 27-kD reads. Alignment of K0326Y sequences to the B73 reference genome is also a possible source of errors. In addition, because K0326Y and line 107 were sequenced in the same pool in the same flow cell lane, these reads could, in theory, be the result of very low-level incorrect parsing of bar code sequences.

Three α-zein genes were around six- to 16-fold down-regulated in line 107 (Supplemental Table S2). One of these is annotated as a Z1A (19-kD) family member and two are Z1C (22-kD) family members. Z1A genes are not regulated by O2, and the Z1C genes identified here are also likely O2 independent. The basis for this conclusion is that their expression in K0326Y (modified o2/o2) is still substantial (Supplemental Table S2), and the O2 transcript in both K0326Y and line 107 was undetectable by RT-PCR and quantitative RT-PCR (data not shown). Consequently, these α-zeins may be repressed through an unknown feedback mechanism that results from reduced levels of y-zein synthesis. Such a mechanism was also proposed as a result of the recent observations of indirect suppression of α-zein gene expression caused by various γ-zein RNAi constructs (Guo et al., 2013).

**DISCUSSION**

The 27-kD y-Zein Acts in a Dosage-Dependent Manner to Modify o2 Endosperm

The feasibility of radiation mutagenesis to identify o2 modifier genes was demonstrated because one line of a population of 293 M2 families had a deletion of a suspected modifier gene, the 27-kD y-zein as well the nearby 50-kD y-zein, and caused complete opaque reversion in QPM. Null mutant alleles have not been previously reported for either of these genes, although knockdown lines have been generated with RNAi by ourselves and others (Wu and Messing, 2010; Guo et al., 2013). RNAi studies have allowed dissection of the overlapping and distinct roles of the different y-zeins, but their interpretation is not simple because the transgenic lines are not null. However, their phenotypes have suggested that the 27-kD y-zein has a specialized role in protein body initiation (Guo et al., 2013). Crossing an RNAi event that reduces both the 27- and 16-kD y-zeins to K0326Y QPM caused complete opaque kernel reversion and thus is consistent with abundant previous data suggesting that 27-kD y-zein plays a key role in endosperm modification (Geetha et al., 1991; Lopes and Larkins, 1991, 1995; Lopes et al., 1995; Wu et al., 2010).

**Figure 5.** A, RT-PCR showing y-zein transcript accumulation. B, Genomic PCR analysis of y-zein genes.

**Figure 6.** SDS-PAGE and kernel opacity analysis showing haploinsufficiency of y-zein for endosperm modification. Selected kernels from a segregating ear are shown. [See online article for color version of this figure.]
The 27- and 50-kD γ-zein null line reported here provides more definitive proof of essentiality of the 27-kD γ-zein for QPM development. It also invokes the possibility that the 50-kD γ-zein is involved in endosperm modification. Despite the relatively lower abundance of the 50-kD γ-zein, it is readily detectable in wild-type endosperm and becomes almost undetectable in O2 endosperm (Fig. 2). Furthermore, during endosperm modification in QPM, this protein is not only restored but is increased relative to the wild type. Like that of increased 27-kD γ-zein transcript and protein (Holding et al., 2011), the mechanism causing this increase is unknown. It is possible that pre- or posttranscriptional regulation could affect these genes independently or that they could be coregulated. In either case, these genes are so closely linked that it is not currently possible to separate their contributions to the QTL on chromosome 7.02.

The 27-kD γ-zein deletion also extends our knowledge of its role in protein body initiation. While protein body number is reduced to about 40% of the wild-type number by 27-kD γ-zein RNAi (Guo et al., 2013), the knockout mutant reduced the protein body number to about 12% of the wild-type and about 7% of the QPM protein body number. Vitreous endosperm formation in modified O2 is thought to be driven, at least in part, by the initiation of a larger number of protein bodies as a result of increased 27-kD γ-zein accumulation (Dannenhoffer et al., 1995;...
| Gene ID         | Predicted function          | Exon coordinates          | Illumina reads | Genomic PCR | RT-PCR |
|----------------|----------------------------|----------------------------|----------------|-------------|--------|
| GRMZM2G398628  | unknown                    | 49068066-49069246          | 6.6            |            |        |
|                |                            | 49069377-49069481          | 1.2            |            |        |
|                |                            | 49089516-49089538          | 0.0            |            |        |
|                |                            | 49069732-49069782          | 13.2           |            |        |
|                |                            | 49069916-49070000          | 1.4            |            |        |
|                |                            | 49070243-49070310          | 22.17          |            |        |
|                |                            | 49070705-49070954          | 4.1            |            |        |
|                |                            | 49071037-49071242          | 0.0            |            |        |
| GRMZM2G435380  | Polygalacturonase activity | G9132685-49133047         | 16.2           |            |        |
|                |                            | G9133156-49133346          | 9.3            |            |        |
|                |                            | G9133437-49133640          | 6.3            |            |        |
|                |                            | G9133837-49134016          | 0.0            |            |        |
|                |                            | G9134122-49134256          | 2.0            |            |        |
|                |                            | G9134481-49134663          | 0.0            |            |        |
| GRMZM2G099648  | ubiquitin ligase complex   | 49177017-49178036         | 135.1          |            |        |
|                |                            | 49178335-49179500         | 157.0          |            |        |
|                |                            | 49185658-49185658         | 10.0           |            |        |
|                |                            | 49185904-49186089         | 13.0           |            |        |
|                |                            | 49187841-49187932         | 1.0            |            |        |
|                |                            | 49188881-49189071         | 0.0            |            |        |
| GRMZM2G040605  | Clone 323008 mRNA sequence | 49308671-49309734         | 26.0           |            |        |
| GRMZM2G583994 | unknown                    | 49376092-49376278         | 0.0            |            |        |
| GRMZM2G160098 | Unknown                    | 49378158-49378477         | 0.0            |            |        |
|                |                            | 49385304-49385415         | 0.0            |            |        |
|                |                            | 49385522-49385558         | 0.0            |            |        |
| GRMZM2G319760 | ATP binding                | 49408086-49408985         | 160.0          |            |        |
|                |                            | 49385555-49396708         | 5.0            |            |        |
|                |                            | 49397325-49397325         | 5.0            |            |        |
|                |                            | 49397540-49397681         | 1.0            |            |        |
|                |                            | 49397931-49398029         | 1.0            |            |        |
|                |                            | 49398632-49398652         | 9.0            |            |        |
|                |                            | 49398846-49398974         | 1.0            |            |        |
|                |                            | 49403154-49403229         | 22.0           |            |        |
|                |                            | 49403313-49403402         | 10.0           |            |        |
|                |                            | 49408846-49409624         | 7.0            |            |        |
|                |                            | 49407025-49407104         | 13.0           |            |        |
|                |                            | 49407185-49407259         | 1.0            |            |        |
| Intergenic region | NA                        | 49377028-49377856       |               |            |        |
| Intergenic region | NA                        | 49386750-49386351       |               |            |        |
| Intergenic region | NA                        | 49395768-49396313       |               |            |        |
| GRMZM2G499214 | Unknown                    | 49681106-49681854         | 0.0            |            |        |
| GRMZM2G108302 | unknown                    | 49746399-49746452         | 0.0            |            |        |
|                |                            | 49746703-49746943         | 1.0            |            |        |
|                |                            | 49747014-49747126         | 0.1            |            |        |
|                |                            | 49747208-49747321         | 0.0            |            |        |
|                |                            | 49747644-49747839         | 0.0            |            |        |
|                |                            | 49747930-49747958         | 0.1            |            |        |
|                |                            | 49748370-49748465         | 0.5            |            |        |
|                |                            | 49752124-49752366         | 0.0            |            |        |

**Figure 8.** Exon-seq and genomic and RT-PCR verification of deletion on chromosome 9.03. Illumina reads column and PCR columns show nonmutagenized K0326Y versus line 107. Most gene identifications show absence of Illumina reads in more than one exon (denoted by multiple sets of coordinates). GRMZM2G398628 and 435380 are the two nondeleted genes flanking the 5’ end of the deletion, and GRMZM2G499214 and 108302 are the two nearest nondeleted genes flanking the 3’ end of the deletion. Genes with an asterisk are either low-abundance transcripts, endosperm nonexpressed genes, or pseudogenes.
Gibbon and Larkins, 2005). These protein bodies remain small, with γ-zein located throughout (Fig. 4), and have an even round shape due to their appropriate packaging of residual α-zein (Fig. 4). However, in the absence of the 27- and 50-kD γ-zeins, the protein body size is similar to the wild type, with highly irregular shape. The size increase likely results from the packaging of residual α-zein in less than 10% of the number of protein bodies (Fig. 4), and the distortions likely result from hydrophobic α-zeins, especially the abundant 19-kD α-zeins, not being correctly packaged by the remaining γ-zeins. Protein body shape distortions also result from dominant negative α- and γ-zein mutants, which interfere with correct zein trafficking into the lumen of the endoplasmic reticulum (Coleman et al., 1997; Kim et al., 2004, 2006). Similarly, low-level accumulation of an unprocessed α-kafrin protein causes protein body reticulation in the high digestibility high lysine mutant of Sorghum bicolor (Wu et al., 2013).

Unlike the highly duplicated α-zein genes, whose expression collectively results in massive levels of α-zein protein, the γ-zeins are encoded by single copy genes and, in the case of 27-kD γ-zein, protein levels approach those of α-zeins. What drives this high-level expression as well as its greater expression in QPM is not known. The degree to which γ-zein protein levels are limited by pre- and posttranscriptional factors is also not known. Our results show that one copy of the 27-kD γ-zein gene is not sufficient for full modification because hemizygous seeds accumulate intermediate amounts of the protein and are only partially modified. This haploinsufficiency suggests that maximal transcription from both 27-kD γ-zein gene copies is required to create a fully vitreous endosperm. However, the synthesis of 27-kD γ-zein is also likely limited by amino acid availability and translational capacity because 27-kD γ-zein overexpression constructs do not further increase the protein (D. Holding, unpublished data). The γ-zein deletion is currently being introgressed into the B73 wild type to separate it from the effects of the o2 mutation and the modifier genes. This will provide insight into whether this same critical threshold for γ-zein abundance also exists for normal vitreous endosperm formation as it does for endosperm modification in QPM.

The increased Lys and Trp content of the QPM γ-zein deletion emphasizes one of the drawbacks of QPM in relation to unmodified o2. Because 27-kD γ-zein is devoid of Lys, its increased level in QPM reduces the nutritional quality compared with starchy unmodified o2. Our results show that this particular QPM inbred has Lys and Trp levels intermediate between W64A o2 and the wild type. However, hemizygous γ-zein deletion kernels had similar amounts of these amino acids to unmodified o2 while exhibiting greater levels of vitreousness. This may suggest that selection of lines with partially accumulating γ-zein may be a novel strategy for breeding maize lines with both increased Lys and an acceptably vitreous kernel texture.

The γ-Zein Deletion Line Demonstrates the Potential for Identifying Other o2Modifiers as Well as for General Maize Seed Functional Genomics

We used γ-irradiation mutagenesis to study genes affecting vitreous endosperm formation (modification) in the hard kernel o2 variant K0326Y QPM. In addition to o2 modifier genes, this approach potentially allows functional genomic dissection of genes more generally involved in endosperm formation as well as seed and plant development. In a population of 293 M3 families, we identified 27 mutants segregating for opaque kernel phenotypes. Although M2 screens did identify at least three dominant opaque kernel mutants, which were true breeding from the M2 to the M3 and M4, the majority of opaque mutants were first observed segregating in the M3 ears. The likely reason for this is that different populations of cells within the mature maize embryo shoot apical meristem give rise to the ear and tassel (Johri and Coe, 1983; Poethig et al., 1986). A hemizygous DNA deletion in an ear progenitor cell within the shoot apical meristem of a mature embryo will ultimately result in a mixture of hemizygous and wild-type kernels in an M2 ear because the tassel progenitor cells giving rise to the pollen would not have the mutation. Thus, only dominant mutations would be visible in the M2 ears. The number of tassel and ear progenitor cells in the meristem was given as four (±1) for the tassel and one to three cells for the ear (Johri and Coe, 1983). These numbers would be expected to produce variable phenotypic ratios for dominant mutants in M2 ears. Line 107 and line 12 (not shown) had apparent 1:1 ratios of normal to mutant seeds (19:19 and 164:174, respectively) and may suggest that the mutation arose in one meristem initial cell that gave rise to the whole ear. The other dominant mutant (line 198) had a lower ratio of M2 mutant kernels (230:52), perhaps suggesting that the mutation arose in one of several ear or tassel initial cells.

Using mutants that had viable kernels taken from segregating M3 ears, and gave rise to 100% mutant M4 ears, we were able to assume homozygosity and directly assay for gene loss using Illumina sequencing of genomic DNA. To simplify the sequencing template, we enriched for the exon fraction, using exon capture hybridization to a Nimblegen B73 maize exome array. This simplification of sequencing template enabled pooling of five mutants and sequencing in a single Genome Analyzer II flow cell lane. Despite this suboptimal Illumina platform (compared with the Hi-Seq 2000 platform) and assembling K0326Y QPM sequences against the nonsogenic B73 genome, we were able to call and test deletions ranging from single to multiple genes. RNA-seq allowed verification of the deleted genes and identified genes with expression that was indirectly affected by DNA deletions.

Prospects and Limitations for Maize Seed Functional Genomics Using Radiation Mutagenesis

The data presented here strengthen genetic, biochemical, and transgenic evidence suggesting that the 27-kD
γ-zein, possibly acting with the 50-kD γ-zein, is an o2 modifier gene. As with transposon or EMS mutagenesis, in which multiple genes are mutated, deletion mutagenesis requires reverse genetics to formally link mutations to phenotype. Typically, this involves isolation of independent mutant alleles in the gene suspected of causing the phenotype or complementation or knock-out using transgenic constructs. The knockout mutants of the 27- and 50-kD γ-zeins described herein are the only known mutants in these genes. However, we showed the essentiality of 27-kD γ-zein in modification of K0326Y QPM endosperm using RNAi (Wu et al., 2010). Furthermore, RNAi lines that dramatically reduce the level of 27-kD γ-zein support the role of this protein in vitreous endosperm formation in a normal vitreous endosperm formation (Wu and Messing, 2010; Guo et al., 2013).

In addition to identifying genes involved in vitreous endosperm formation, characterizing more opaque mutants in this QPM population will allow appraisal of the heterogeneity and frequency of radiation-induced deletions and their utility for maize functional genomics when combined with current reverse genetics resources and ever-improving sequencing capabilities. In addition to large deletions, radiation can also cause subgene scale mutations (Naito et al., 2005), but we could not detect these without a K0326Y QPM reference genome. A second, much larger deletion population has been created that has 1,784 mutagenized families in the B73 reference genetic background. Many apparent opaque and small kernel seed mutants are currently being tested for heritability. Because of the isogenicity of these mutants with the B73 reference genome, we will be able to detect subgene mutations in this population, and exon-seq and RNA-seq reads can be assembled and compared for identification and appraisal of the power of deletion mutagenesis for creating and utilizing mutations ranging from a few base pairs to mega base pairs.

MATERIALS AND METHODS

Radiation Dosage Testing and γ-Radiation Mutagenesis

K0326Y QPM seed were equilibrated to 13% moisture as previously described (Hossain et al., 2004) and treated in batches of approximately 50 seeds with various dosages of γ-radiation (15–35 Gray [Gy]). After irradiation, the seeds were immediately planted in the greenhouse in potting soil. Plant survival was determined as the proportion of seedlings compared with nonirradiated control seeds 1 month after sowing. Radiation dosage was set at 25 Gy based on a 75% survival rate and was used for full-scale mutagenesis of 1,909 seeds. These seeds were planted in the field at the University of Nebraska East Campus farm in summer 2010.

A population of 1,909 K0326Y QPM seeds, treated with 25 Gy of γ-radiation, were propagated in the field in 2010 and 1,108 germinated (58%). The radiation dosage was previously optimized to produce 75% survival rate under controlled greenhouse condition using a small-scale (approximately 50 seeds per dosage) experiment.

Screening of M2 and Heritability Testing in M3

Adequately filled M2 ears (more than approximately 30 kernels) that did not have fungal contamination were screened for dominant kernel phenotypes before shelling and after shelling on light box. Zein profiles were obtained from putative opaque mutant kernels to prioritize lines for more urgent greenhouse heritability testing. Twenty kernels from all M2 ears were propagated in the field in summer 2011. This enabled heritability testing of dominant M2 phenotypes, rescue of M2 ears with few kernels, and identification of recessive mutants appearing in the M3 generation.

Genotyping, RT-PCR, Zein Protein Analysis, and Fixation for Microscopy

Plants or seeds were selected for the γ-zein deletion using leaf or embryo genomic PCR using DNA extracted according to the urea method (Holding et al., 2008). For RT-PCR, RNA was extracted and DNase-treated complementary DNA synthesized as previously described (Holding et al., 2011). All primers used for genotyping and RT-PCR are shown in Supplemental Table S5. Zeins were extracted from mature and developing endosperm as previously described (Wallace et al., 1990). Endosperms of kernels genotyped for the deletion were processed as previously described (Guo et al., 2013). For the 18 DAP stage, kernels were removed, without damaging the base of the kernels, using a razor blade and treated as follows. Kernels were placed embryo-side down on a clean glass plate and sliced in half longitudinally through the embryo. The embryo halves were placed immediately into DNA extraction buffer on ice, one-half of the endosperm was frozen in liquid nitrogen for protein analysis, and the other half kept was dissected for fixation. Keeping the pericarp intact, the half kernel was placed cut-side down, and a 1- to 2-mm longitudinal central section was taken and placed into 5 mL fixative (2% [w/v] Suc, 0.1 M sodium cacodylate, pH 7.4, and 5% [v/v] glutaraldehyde). Samples were fixed at 4°C for at least 1 week while genotyping, and SDS-PAGE of zeins was conducted to identify transgenic kernels for further processing. Embryo DNA was extracted using microcentrifuge scale urea DNA extraction.

Immunogold TEM and Protein Body Counts

Selected kernel segments were further dissected following fixation to remove the apical and basal kernel portions leaving two 1-mm midkernel pieces with pericarp still attached for orientation. Samples were infiltrated in LR White resin (Electron Microscopy Sciences) before sectioning. Ninety-nanometer sections were cut using a Diatome diamond knife and a LKB Ultrotome III microtome and attached to form and Formvar-coated grids (EMS). For immunogold labeling, grids were hybridized in 50-μL drops on Parafilm strips as follows. The grids were incubated for 30 min in blocking solution, pH 8.2, containing 0.2% (w/v) bovine serum albumin and 0.06% (v/v) Tween 20 in 20 μL Tris-HCl and 500 μL NaCl. Rabbit primary antiserum were added (γ-zein at 1:2,000 and γ-zein at 1:100) and incubated overnight at 4°C before three 10-min washes in 100 μL blocking solution. Secondary antibody (goat anti-rabbit, 15 nm, gold conjugated from EMS) was added to 1:20 dilution for 1 h at room temperature. The grids were washed three times, rinsed three times in deionized water and air (Holding et al., 2008), and viewed on a Hitachi 7500 TEM.

Protein body images were standardized by viewing the fourth subaleurone starchy endosperm cell layer. At the 18-DAP kernel stage, the first starchy endosperm cell layer had few protein bodies and increased in size and number in the layers internal to this. Estimations of protein body number were standardized by making counts within 100-μm square regions within fourth subaleurone cell layer cells away from the cell wall and nucleus.

Exon-Seq

Genomic DNA was extracted from leaf tissue using a modified urea extraction protocol (Holding et al., 2008). DNA concentration and purity was assayed with NanoDrop and 100 μg crude DNA purified using Qiagen DNA clean kit. Ten-microgram samples of purified DNA was used for exon capture DNA library construction, exome capture, and quality control validation were carried out as a paid service by the Genomic Technologies Facility at Iowa State University. For DNA library construction, 3-μg DNA samples were sheared with nebulizers and prepared incorporating bar codes according to Illumina TruSeq DNA Sample Prep kit’s protocol (catalog no. FC–121–2001). For quality control of the precapture libraries, a Bioanalyzer 2100 was used to establish library size and quantity. For hybridization, each library was diluted to 30 ng μL−1. Fragmented DNA from four opaque deletion mutants and the progenitor K0326Y QPM parent were used to make Illumina TruSeq DNA libraries, which allowed incorporation of bar codes within the adaptors and pooling of the five samples. The five libraries (300 ng per library) were 128 Plant Physiol. Vol. 164, 2014
combined together for one array hybridization using the Nimblegen Zm B73 HX3 exome capture array (1.5μg total per array). The procedure of hybridization followed NimbleGen Array User’s Guide (Plant Sequence Capture Illumina Optimized protocol (version 1, 2010). The eluted libraries were subjected to 10 cycles of amplification using the TrueSeq enrichment PCR kit. For validation of hybridization efficacy, the ratios of five random genes were tested by real-time quantitative RT-PCR in the pre- and postcaptured libraries as part of the service at the Iowa State University genomics facility (not shown). Libraries were then sequenced (100-bp single-end reads) using the Illumina Genome Analyzer 2 service at the University of Nebraska genomics facility. Small reads were discarded if their average quality scores were smaller than 30. The adapter sequence on 5′ end and six nucleotides on the 5′ end of 7-base bar codes were used to determine the trimming position and deconvolute the reads. After trimming the adapter sequence and bar codes, the grouped short reads were mapped against the B73 genome (ZmB73_Ref-Gen_v2 from http://www.maizesequence.org) using Bowtie (Langmead et al., 2009), allowing up to two base mismatches per read. Reads mapped to multiple locations were discarded. Numbers of reads in exons were counted using the HTSeq-count tool using B73 gene annotations (ZmB73_Sb_FGS from http://www.maizesequence.org), and the ‘union’ resolution mode was used. For pairwise comparisons, the edgeR package (Robinson et al., 2010) with trimmed mean of M-values normalization method (Robinson and Oshlack, 2010) was used to analyze the numbers of reads aligned to exons.

The use of a NimbleGen exome array to purge repetitive and intron sequences and specifically select for exon sequences resulted in the template size of each mutant being reduced from 3.2 gigabase pairs to approximately 50 megabase pairs (64-fold simplification) and allowed us to sequence this pool in a single Genome Analyzer II flow cell lane, which has a coverage of 3.6 gigabase pairs. After deconvolution of reads from the five lines, they were assembled against the B73 genome. The non-isogenic nature of the QBK background compared with B73 precluded the possibility to assemble reads and infer subexon mutations in this population, but we were able to effectively score for presence/absence of exons. Deletions were called according to an algorithm that considered both the number of reads for a given exon in the nonmutagenized QPM and the ratio of reads between nonmutagenized QPM and a mutated exon. An exon was automatically called a deletion candidate if its P value was smaller than $10^{-15}$ (50% of candidates fit this criterion). The other 70% of called candidates had P values ranging between $10^{-15}$ and $10^{-5}$. These candidates were only called when the number of reads for the mutated line were not larger than 0, 1, 2, 3, and 5 when the numbers of reads for the nonmutagenized reference line were in ranges of (30–50), (50–70), (70–100), (100–150), and (150 and above), respectively. For example, a candidate with 125 reads in nonmutagenized line must have three or less reads in the mutated line.

RNA-Seq

Even though we are mostly interested in the loss of endosperm-specific transcripts, total RNA was extracted as previously described (Holding et al., 2011) from leaf, root, and whole seed. RNA was purified using Qiagen RNeasy cleanup kits, and a pool was made for RNA-seq to identify transcripts abolished from multiple tissues. Ten micrograms of RNA was used for preparation of Illumina complementary DNA libraries, and 100-bp reads were obtained from Illumina Genome Analyzer 2 platform using RNA from nonmutagenized K0326Y, line 107, and one other deletion mutant per flow cell lane. For the RNA-seq reads, the same method as exon reads was used to deconvolute the bar codes, trim the adapter sequences, map to the reference genome, and analyze the abundance of reads in each gene. A threshold value for fold change differential expression was set at log2 greater than 1 (2-fold actual value) with adjusted p values < 0.001 for the null hypothesis, although we show gene changes at least log2 greater than 2 (4-fold actual values). Differentially expressed genes were annotated using the Gene Ontology database.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF371263, DQ400403, AF371261, and NM001174912 for primer design in the 50′-16′, and 27′-kD gamma-zein genes and the tubulin control gene, respectively. All other genes are listed by their maize genome descriptors (e.g. GRMZM2C398628).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. M3 phenotypes of selected segregating K0326Y opaque deletion mutants.

Role of γ-Zein in opaque2 Endosperm Modification

Supplemental Table S1. Candidate gene deletions in deletion line 107 from exon-seq.

Supplemental Table S2. RNA-seq showing genes identified as at least 4-fold differentially expressed between K0326Y QPM and deletion line 107.

Supplemental Table S3. Exon reads and RNA-seq reads for genes within the chromosome 7 deletion.

Supplemental Table S4. Exon reads and RNA-seq reads for genes within the chromosome 9 deletion.

Supplemental Table S5. Primers for genomic and RT-PCR.

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