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ORIGINAL PAPER

Non-Mendelian regulation and allelic variation of methionine-rich delta-zein genes in maize

Yongrui Wu · Wolfgang Goettel · Joachim Messing

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Abstract Sufficient methionine levels in the seed are critical for the supply of a balanced diet for feed and food. Currently, animal feed is supplemented with chemically synthesized methionine, which could be completely replaced with naturally synthesized methionine. However, insufficient levels of methionine are due to alleles of two genes in the maize genome that are expressed during seed development, which have a high percentage of methionine codons, ranging from 23 to 28%, while free methionine is very low. The two genes, *dzs10 and dzs18*, belong to the prolamin gene family that arose during the evolution of the grasses and were duplicated during a whole genome duplication event. We have found several *dzs10 and dzs18* null alleles caused either by transposon insertion or frame shift mutations. Maize seeds with null mutations of both genes have a normal phenotype in contrast to other prolamin genes, explaining the accumulation of methionine deficiency in normal breeding efforts. Moreover, the trans-regulation of these genes deviates from Mendelian inheritance. One allele of the regulatory locus *dzr1* is inherited in a parent-of-origin fashion, while another allele appears to prevent Mendelian segregation of the high-methionine phenotype in backcrosses.

Introduction

Zea mays, commonly known as corn, is largely used to produce animal protein. Livestock receives its major nutrients from corn and soybean, including essential amino acids. These in turn are derived from proteins in corn and soybean meal that act as storage for amino acids. Corn is like other cereals a member of the *Poaceae* and the most prolific storage proteins in the *Poaceae* are prolamins, rich in proline and glutamine (Xu and Messing 2008). In corn, prolamins are called zeins, which are encoded by a multigene family (Gibbon and Larkins 2005). Zeins are divided into four protein subfamilies, alpha, beta, gamma, and delta, based on their evolutionary relationship. The alpha zein protein subfamily is rich in glutamine and leucine and composes more than 50% of total zeins, proportionally reducing three important amino acids, i.e., methionine, lysine, and tryptophan (Osborne and Mendel 1914a, b). Although in animal feed, lysine, and tryptophan is increased by addition of a legume, the mixture is still methionine-deficient. Lack of methionine will result in severe malnutrition, e.g., reduced size of the fed animals and low yield of eggs (Messing and Fisher 1991). So the feed has to be further supplemented with synthetic methionine, which amounts to billions of dollars every year in additional costs. Therefore, for several decades, maize geneticists and breeders have taken great strides to increase the nutritional quality of corn for human and livestock.

One interesting approach to take advantage of natural variation in methionine content was based on a seedling screen. During germination seeds grown on media with 2 mM lysine plus threonine for 3 days would experience significantly retarded root growth due to feedback inhibition of the biosynthesis of methionine. A variant that would have enhanced protein resources for methionine, could

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Y. Wu · W. Goettel · J. Messing (✉)
Waksman Institute of Microbiology, Rutgers University,
190 Frelinghuysen Road, Piscataway, NJ 08854, USA
e-mail: messing@waksman.rutgers.edu

overcome inhibition through protein hydrolysis. Over 200 maize lines were screened for resistance to seedling growth inhibition on lysine-plus-threonine supplemented media. By comparing three resistant and four inhibited lines, BSSS53 was found to have the highest methionine level in its seed (Phillips et al. 1981), and it was further demonstrated that the 10-kDa delta zein contributed to the increased methionine content in the endosperm (Phillips and McClure 1985). In contrast to the alpha zein gene family, the 10-kDa delta zein gene (*dzs10*) is encoded by a single copy in the maize genome (Kirihara et al. 1988a, b) and was mapped to chromosome 9 (Benner et al. 1989). Differential mRNA accumulation of 10-kDa delta zein among inbred lines was ascribed to a post-transcriptional regulatory factor, designated DZR1 (delta zein regulator 1) (Cruz-Alvarez et al. 1991; Schickler et al. 1993), which was mapped to chromosome 4S (Benner et al. 1989; Chaudhuri and Messing 1995). Furthermore, it could be shown in transgenic maize plants that the target sequences of DZR1 are the untranslated regions (UTRs) of the *dzs10* mRNA (Lai and Messing 2002). To further characterize this regulatory factor, a series of crosses were performed between BSSS53, the high-methionine line, and other low-methionine lines. Surprisingly, two extremely different regulation patterns were identified, dosage and parent-of-origin dependent modes (Chaudhuri and Messing 1994). In hybrids of BSSS53 and W64A, the amount of 10-kDa delta zein was determined by dosage of the *Dzr1-BSSS53* allele. When crosses were performed between BSSS53 and Mo17, the accumulation of 10-kDa delta zein depended on the parental origin of the allele. When the specific allele, *Dzr1-Mo17*, was transmitted through the female parent, 10-kDa delta zein level was as low as the inbred parent, but when the *Dzr1-Mo17* allele was inherited from the male parent, 10-kDa zein level was as high as in inbred line BSSS53. To rule out that expression levels were due to the uneven contribution of parental alleles to the triploid endosperm, non-disjunction of a B-chromosome translocation during spermatogenesis, bearing chromosome arm 4S with the introgressed *Dzr1-Mo17* allele, was used in crosses to balance parental contributions of alleles, making the endosperm partially tetraploid or diploid for this chromosome arm (Chaudhuri and Messing 1994).

Genomic imprinting, of which the first case in flowering plants was observed for the *r* locus (Kermicle 1970), describes an epigenetic modification, where a gene inherited from its parents is differentially expressed in a parent-of-origin dependent pattern. Based on these results it was hypothesized that *Dzr1-Mo17* was negative dominant, but its default state was imprinted, hence its allele was not expressed, unless transmitted through female gametogenesis. If this is the case, one can ask whether *dzr1* regulates other storage protein genes as well and if so, would the *Dzr1-Mo17* allele also be negative dominant? For instance, the maize genome contains a second delta zein gene (*dzs18*) on chromosome 6L that has an internal duplication rendering its protein 18-kDa in size (Swarup et al. 1995). It also has been shown that the two delta zein genes originated from a common progenitor by allotetraploidization (Xu and Messing 2008). Curiously, the predicted mature 18-kDa delta zein contains one lysine and two tryptophan residues, both of which are absent in mature 10-kDa delta zein and normally underrepresented in other zeins. In this respect, DZS18 is agronomically more valuable than DZS10. Given the common origin of the delta zein genes, it would be not unexpected if they are regulated in a coordinated fashion. However, comparison of inbred lines has shown that protein levels of both delta zeins accumulate at variable levels (Kim and Krishnan 2003; Swarup et al. 1995). Here, our objectives were to investigate the differential accumulation of DZS10 and DZS18 in various inbred lines as the result of allelic variations of both structural and regulatory genes. By combining various *dzs10/18* with *dzr1* alleles in multiple crosses, we aimed to study the complex mechanisms of *dzs10/18* gene regulation.

Materials and methods

Nomenclature

We follow the nomenclature guideline for maize as outlined in http://www.maizegdb.org/maize_nomenclature.php. The abbreviation of a gene name is represented in lowercase letters and is italicized, for example *dzs10*. An allelic variant is referred to by its gene symbol, followed by a hyphen and a specific allele designation. Like genes, allele symbols are italicized. A functional or dominant allele is designated with a capitalized first letter, while a recessive allele begins with a lowercase letter. Throughout this text, we use the inbred line name as allele designation such as *Dzs10-B73* (functional allele) and *dzs10-A654* (nonfunctional allele). Gene products are not italicized. Proteins are written with capitalized letters, for instance DZS10.

Plant materials

Maize inbred lines BSSS53, B73, B37, Mo17, W64A, W22, A69Y, ILLIZE, A188 and A654, and genetic varieties SD-purple and p1-ww-1112 were from our own collection. The variety here referred to as p1-ww-1112 carries the *p1-ww-1112* null allele (Athma and Peterson 1991). Endosperm used for total RNA and protein extractions was dissected at 18 days after pollination (DAP) from BSSS53, B73, A654, Mo17, W64A and their reciprocal crosses.

Probe specificity test

The *dzs18*-specific probe was amplified from the non-conserved region between *dzs18* and *dzs10* with the primer pair dzs18F1, GGTATGATGCCACCGATG; dzs18R1, TGG CATCATACTAGCTATCG. The probe specificity test was performed by hybridization of 2 ng of *dzs10* and *dzs18* coding sequences with this probe.

Northern analysis

Total RNA was extracted by using TRIzol reagent (Invitrogen). For Northern analyses, 2 μg of total RNA was loaded for each sample. For the d*zs10* transcript, the autoradiography film exposure time was 6 h; because the *dzs18* gene was expressed at much lower levels than the *dzs10* gene, the autoradiography film for the *dzs18* transcript was exposed for 24 h with intensifying screens. The *actin* (Raizada and Walbot 2000) and *dzs27* probes were amplified by the following primer pairs, respectively: actinF, GCCGGTTTCGCTGGTGATGATGCGCC; actinR, GTG ATCTCCTTGCTCATACGATCGGC; dzs27F, TCTGCC ACCTCCGGTTCACC; dzs27R, TGTTGGCACGGGCAT GGGTG.

Misfit transposon insertion confirmation

A common primer pair for *dzs10* and *dzs18*, dzs18-10F1, CGCCATGGCAGCCAAGATG and dzs18-10R1, TATCTAGAATGCAGCACCAAC, was used to amplify *dzs18* and *dzs10* simultaneously. Because of the 10.9 kb transposable element *Misfit* insertion in between both primers, *dzs10* failed to be amplified in A654; the *Misfit* insertion was confirmed with another primer pair, one binds to *dzs10* and the other specifically to the *Misfit* sequence (dzs10F1, ATACTCTAGGAAGCAAGGAC; MisfitR, TGGCTGCGAGCATACAGTTC).

Cloning and sequencing of *dzs18* coding sequences

The primer pair (dzs18-10F1, described above; dzs18R3, ATGCCGACTTCATTATTGGG, based on *Dzs18-B73* (AF371265)) was used to amplify *dzs18* coding sequences from all the plant materials described above. The PCR products were cloned using the pGEM-TEasy Vector (Promega) and sequenced (ABI). The accession numbers for *dzs18* coding sequences are: BSSS53, FJ557101; B73, FJ557095; B37, FJ557103; Mo17, FJ557102; W64A, FJ557097; W22, FJ557100; P1-ww-1112, FJ557106; A69Y, FJ557104; ILLIZE, FJ557098; A188, FJ557105; SD-Purple, FJ557099; A654, FJ557096. The accession number for the *dzs10-A654* sequence is FJ627006.

RT-PCR analysis of transcript accumulation of mutated delta zein genes

The RT-PCR and 3′ race RT-PCR kits were purchased from Invitrogen. For the *dzs10-A654* transcript analysis, 3′ race RT-PCR was performed. The forward primer is dzs10F1, described as above, and the reverse one is the Generacer 3′ Primer. The primer pair for the analysis of *dzs18* mRNA accumulation in BSSS53 and B73 is: dzs18F2, AGGAAGCAAGGGAATCACATC and dzs18R2, TTCCTAAACAATGAGTCGCATG.

Total zein extractions

A total of 100 mg flour of finely ground mature kernels or endosperm at 18 DAPs was mixed and vortexed with 400 μl of 70% ethanol/2% 2-mercaptoethanol (vol/vol), then kept at room temperature for more than 2 h; the mixture was centrifuged at 1,300 rpm for 10 min, then 100 μl of the supernatant liquid was transferred to a new tube; 10 μl of 10% SDS was added to 100 μl of the extract, the mixture was dried by vacuum and resuspended in 100 μl of distilled water. The zein concentration was measured by the method of Bradford (Protein Assay Dye Concentrate, Bio-Rad).

Western analysis

A total of 0.2 μg of total zeins were separated on a 15% SDS-PAGE gel, and then transferred to an Immun-Blot PVDF membrane (Bio-Rad). The membrane was hybridized with a 1:3,000 diluted antibody against 10-kDa delta zein, which could also cross-react to 18-kDa delta zein (Swarup et al. 1995). The remaining procedures and reagents can be found at GE (GE ECL Western blotting detection reagents and analysis system).

Phenotype and genotype segregation test

For each individual immature kernel of the backcross of (Mo17 × B73) × Mo17 at 18 DAP, endosperm and embryo were dissected for protein extraction and tissue culture. The embryo was cultured on resting medium (Frame et al. 2002) for 7–14 days to generate callus. Then the callus was used for genomic DNA extraction.

For the phenotype segregation test, 0.2 μg of total zeins were analyzed by Western blot described as above. For the genotype segregation experiment, two SSR markers, umc2082 and umc1088, linked to *dzr1* from chromosome 4S were used to test *Dzr1-Mo17* and *Dzr1-B73* segregation. The sequences of the two primer pairs are available on http://www.maizegdb.org/cgi-bin/displaylocusrecord.cgi?

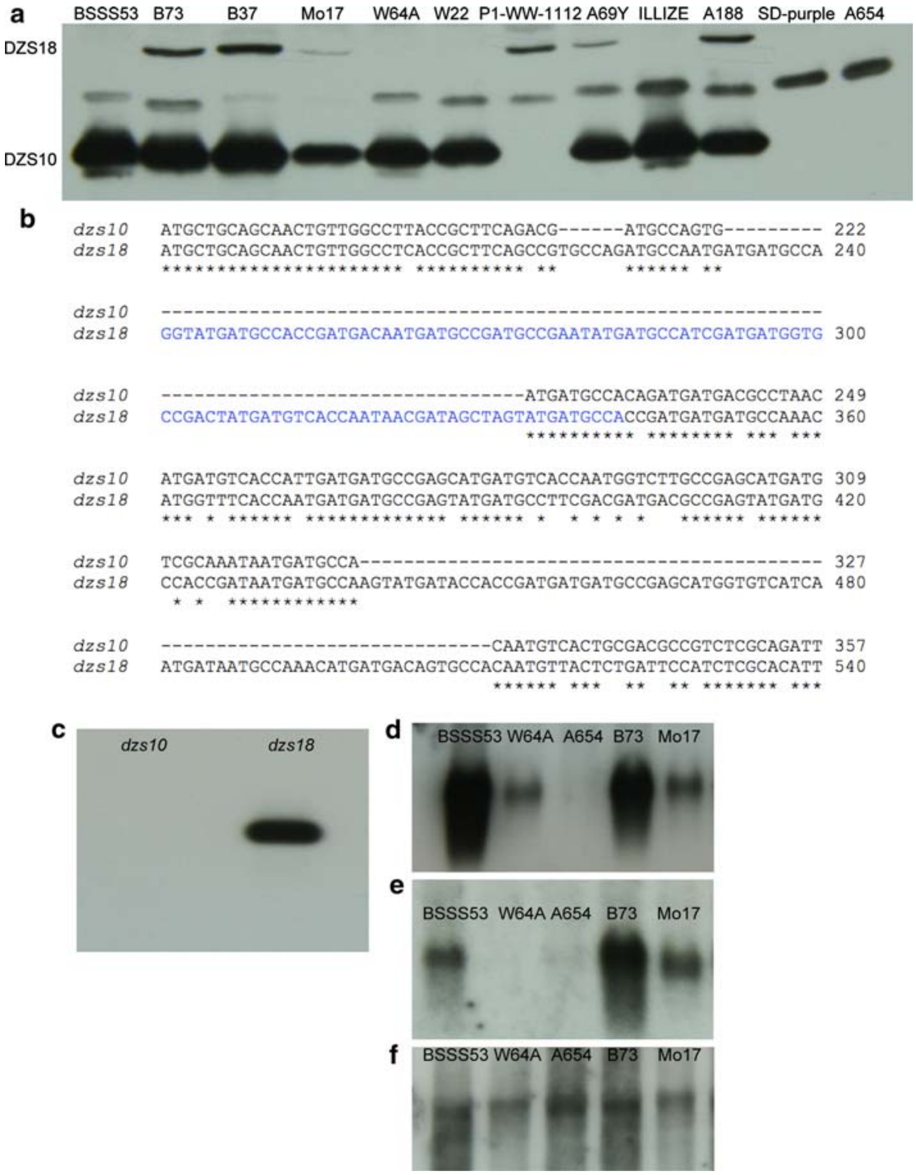
id=484887 and http://www.maizegdb.org/cgi-bin/display-locusrecord.cgi?id=12273, respectively.

Results

Unequal expression of delta zein genes in different inbred lines

Previously it was shown that different inbred lines of maize accumulate different levels of 10- and 18-kDa delta zeins, which could be due to allelic variations of the two genes. Therefore, we sought to correlate 10- and 18-kDa delta zein protein expression pattern with their genotype. Total zeins extracted from mature seeds of ten inbred lines and two genetic varieties were analyzed by Western blot to compare the expression pattern of 10- and 18-kDa delta zeins. As previously shown (Kim and Krishnan 2003; Swarup et al. 1995), protein levels indicated that the two orthologous gene copies are unequally expressed (Fig. 1a). While in BSSS53 the level of 10-kDa delta zein is very high, accumulation of 18-kDa delta zein protein is not detected. The opposite appears to be the case in p1-ww-1112, where 10-kDa delta zein remained undetectable. Out of twelve only five inbred lines exhibit protein accumulation from both genes, while the other seven lines missed one or both. However, the expression of the 10-kDa delta zein gene appears to be always higher than the 18-kDa delta zein gene consistent with EST data (Woo et al. 2001).

To correlate protein and mRNA accumulation, total endosperm RNA was isolated from a subset of the inbred lines including BSSS53, W64A, A654, B73, and Mo17 at



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dzs10    ATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGACG------ATGCCAGTG--------- 222
dzs18    ATGCTGCAGCAACTGTTGGCCTCACCGCTTCAGCCGTGCCAGATGCCAATGATGATGCCA 240
         ********************** ********** **      ****** **

dzs10    ------------------------------------------------------------
dzs18    GGTATGATGCCACCGATGACAATGATGCCGATGCCGAATATGATGCCATCGATGATGGTG 300

dzs10    ---------------------------------ATGATGCCACAGATGATGACGCCTAAC 249
dzs18    CCGACTATGATGTCACCAATAACGATAGCTAGTATGATGCCACCGATGATGATGCCAAAC 360
                                          ********** ******** *** ***

dzs10    ATGATGTCACCATTGATGATGCCGAGCATGATGTCACCAATGGTCTTGCCGAGCATGATG 309
dzs18    ATGGTTTCACCAATGATGATGCCGAGTATGATGCCTTCGACGATGACGCCGAGTATGATG 420
         *** * ****** ************* ****** *  * * * *   ****** ******

dzs10    TCGCAAATAATGATGCCA------------------------------------------ 327
dzs18    CCACCGATAATGATGCCAAGTATGATACCACCGATGATGATGCCGAGCATGGTGTCATCA 480
          * *  ************

dzs10    ------------------------------CAATGTCACTGCGACGCCGTCTCGCAGATT 357
dzs18    ATGATAATGCCAAACATGATGACAGTGCCACAATGTTACTCTGATTCCATCTCGCACATT 540
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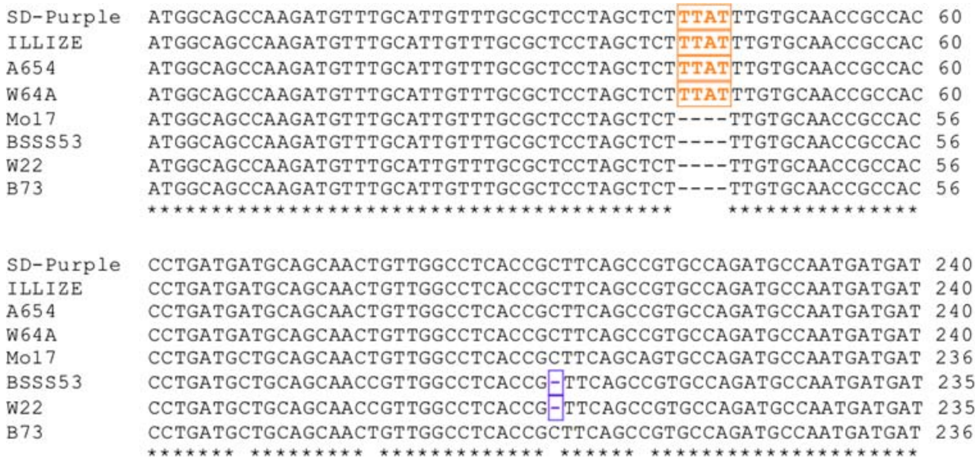
Fig. 1 Natural variation of 10- and 18-kDa zein mRNA and protein accumulation in twelve inbreds and genetic varieties: **a** protein levels. 0.2 µg of total zeins from mature seeds of ten inbreds and two genetic varieties were analyzed by Western blot using the 10-kDa zein antibody, which cross-reacts with 18-kDa zein. The band between 18- and 10-kDa delta zeins most likely represents the 15-kDa beta zein; **b** design of a specific *dzs18* probe for Northern blot analysis. Partial CDS of 10- and 18-kDa zein genes are aligned. The specific *dzs18* probe of 102 bp (colored in *blue*) is derived from one of the two insertion sequences relative to *dzs10*. The numbers beside the sequences indicate sequence position relative to their start codons; **c** Specificity test of the *dzs18* probe. Each lane was loaded with 2 ng of PCR-amplified *dzs10* or *dzs18* CDS, respectively; **d**–**f** Northern blot analyses of *dzs10* and *dzs18* mRNAs. 2 µg total RNA was isolated from endosperms of BSSS53, W64A, A654, B73, and Mo17 at 18 DAPs and probed with *dzs10* (**d**) and *dzs18* (**e**)**,** respectively; the actin gene was used as a control (**f**)

18 DAP and hybridized with *dzs10* and *dzs18* DNA probes, respectively. To distinguish between these highly homologous genes, the probe for *dzs18* was based on insertion sequences that also create the size differences in the two proteins (Fig. 1b). Indeed, no cross-hybridization could be detected by Southern blot analysis (Fig. 1c). Although the reciprocal test could not be applied with a *dzs10* probe, the level of *dzs10* mRNA was always significantly higher than that of the *dzs18* mRNA so that a distinction was possible, when a Northern blot was exposed for shorter time like 6 h; under those circumstances a *dzs18* probe did not exhibit a positive band (data not shown). Transcript levels of these inbred lines correlated well with protein levels except for BSSS53, indicating that differences in mRNA accumulation constitute a key regulatory step (Fig. 1d, e). Based on these results B73 appears to be an inbred with maximum expression of both delta zein genes, while in Mo17 both genes are also expressed, but at rather lower levels. W64A also had a reduced 10-kDa delta zein level, but in contrast to Mo17 no 18-kDa protein accumulation. A654 was a potential null mutant. Interestingly, BSSS53 that did not exhibit accumulation of any 18-kDa protein still had *dzs18* mRNA accumulation.

Maize variants with null mutations in delta zein genes

To investigate the absence or very low levels of gene expression of both genes in A654, genomic DNA was analyzed by restriction patterns in Southern blot analysis. While A654 appears to have a normal *dzs18* gene, a major shift in size occurred in the *dzs10* gene, indicating that it could have acquired an insertion (not shown). After cloning and sequencing the *dzs10-A654* allele, the sequence showed that the coding region of the *dzs10* gene in A654 is interrupted by the transposable element *Misfit* (Fu et al. 2002) with a size of 10.9 kb (Supplementary Fig. 1). While this gene disruption explains the lack of 10-kDa delta zein accumulation in A654 seeds, Southern blot analysis was not sufficient to explain the absence of 18-kDa delta zein mRNA. We therefore designed primers for PCR amplification of the *dzs18-A654* allele. Indeed, when this allele was sequenced, a four-base pair insertion TTAT caused an early frame shift in the *dzs18-A654* allele (Fig. 2), explaining the lack of 18-kDa protein in A654 seeds. Therefore, A654 represents a null mutant for delta zein genes, one based on a transposon insertion, the other due to a frame shift.

To test whether other natural variants exist as transposon disruption mutants, we took advantage of the different sizes of the two delta zein genes and designed primers to amplify both genes simultaneously (Fig. 3a). Previously, we have already shown that BSSS53 and B73 do not have discernable insertions and indeed their genomic DNA produced the two expected PCR products as well as did B37 (Fig. 3a). However, when p1-ww-1112, SD-Purple, and A654 genomic DNAs were amplified, only the *dzs18* band was detected. As shown in Supplementary Fig. 1, A654 had an insertion of about 11 kb in *dzs10*, explaining the lack of the lower band, suggesting that the other two lines might harbor the same allele. By selecting a primer specific for the *Misfit* transposon in the *dzs10-A654* allele, the expected PCR product showed up in both genetic varieties, p1-ww-1112 and SD-Purple, with A654 as a positive and B73 as a negative control (Fig. 3b). Sequence analysis of the p1-ww-1112 and SD-Purple PCR products indeed confirmed that these alleles are identical with the allele of A654 (data not shown).



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SD-Purple   ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCTTTATTTGTGCAACCGCCAC 60
ILLIZE      ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCTTTATTTGTGCAACCGCCAC 60
A654        ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCTTTATTTGTGCAACCGCCAC 60
W64A        ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCTTTATTTGTGCAACCGCCAC 60
Mo17        ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCT----TTGTGCAACCGCCAC 56
BSSS53      ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCT----TTGTGCAACCGCCAC 56
W22         ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCT----TTGTGCAACCGCCAC 56
B73         ATGGCAGCCAAGATGTTTGCATTGTTTGCGCTCCTAGCTCT----TTGTGCAACCGCCAC 56
            *****************************************    ***************

SD-Purple   CCTGATGATGCAGCAACTGTTGGCCTCACCGCTTCAGCCGTGCCAGATGCCAATGATGAT 240
ILLIZE      CCTGATGATGCAGCAACTGTTGGCCTCACCGCTTCAGCCGTGCCAGATGCCAATGATGAT 240
A654        CCTGATGATGCAGCAACTGTTGGCCTCACCGCTTCAGCCGTGCCAGATGCCAATGATGAT 240
W64A        CCTGATGATGCAGCAACTGTTGGCCTCACCGCTTCAGCCGTGCCAGATGCCAATGATGAT 240
Mo17        CCTGATGCTGCAGCAACTGTTGGCCTCACCGCTTCAGCAGTGCCAGATGCCAATGATGAT 236
BSSS53      CCTGATGCTGCAGCAACCGTTGGCCTCACCG-TTCAGCCGTGCCAGATGCCAATGATGAT 235
W22         CCTGATGCTGCAGCAACCGTTGGCCTCACCG-TTCAGCCGTGCCAGATGCCAATGATGAT 235
B73         CCTGATGCTGCAGCAACTGTTGGCCTCACCGCTTCAGCCGTGCCAGATGCCAATGATGAT 236
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Fig. 2 Partial alignments of *dzs18* coding regions isolated from different plant materials: *dzs18* coding regions were PCR-amplified and sequenced. Two insertions/deletions resulting in a frame shift are highlighted. The "TTAT" insertion is colored in *yellow* in A654, SD-Purple and W64A and the "C" deletion is colored in *blue* in BSSS53 and W22. Both frame shifts produce stop codons close to the INDELs. B73 and Mo17 that have intact 18-kDa zein genes are included as a reference. The numbers indicate positions in the CDS measured from the first nucleotide of their start codons

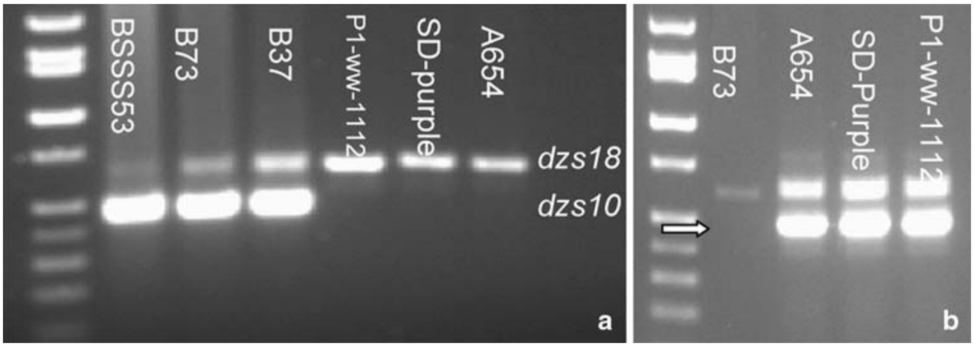

Fig. 3 Genomic PCR of delta zein sequences from different materials: **a** PCR amplification of *dzs18* and *dzs10* coding sequences. A common primer pair for *dzs10* and *dzs18* was used to amplify *dzs18* and *dzs10* sequences simultaneously from BSSS53, B73, B37, p1-ww-1112, SD-Purple and A654; **b** PCR confirmation of *Misfit* insertion. PCR reactions were repeated by using a 3′ primer that is specific to *Misfit*. Chimeric sequences for A654, SD-purple, and p1-ww-1112 are indicated by an *arrow*; the *upper* bands are non-specific fragments

To determine whether the lack of *dzs18* expression in other inbreds is due to the same allele as in A654, we cloned and sequenced *dzs18* coding sequences amplified from nine inbred lines and two genetic varieties. Indeed, lack of expression correlated with frame shifts in the coding region (Fig. 2). Like A654, ILLIZE, SD-Purple, and W64A had a four-base pair TTAT insertion causing a frame shift. BSSS53 and W22 had a single cytosine deletion, also causing a frame shift. Out of twelve inbreds and varieties seven had one or both mutated delta zeins, providing a perfect correlation between genotype and protein presence and absence (Table 1). A total of three different mutant alleles have been detected within twelve lines, indicating that the combination of a limited number of haplotypes provide a range of methionine levels in the maize seed.

Allelic variations of a regulatory factor for both delta zein genes

Although the genotypes of the two delta zein genes correlate well with their phenotypes in inbreds, there appear to be differences in the level of expression, indicating allelic variation of their regulatory loci. If this is the case, then hybrid crosses between inbreds exhibiting quantitative differences should provide answers to the nature of trans-regulation. For instance, expression of *Dzs10-W64A* is lower than that of *Dzs10-BSSS53* (Fig. 1a, d). Therefore, reciprocal crosses were performed between W64A and BSSS53 (Chaudhuri and Messing 1994). Because these genes are expressed in triploid endosperm, maternal alleles would provide two copies and paternal ones one copy. Given that expression is lower with *Dzs10-W64A*, one would then expect 10-kDa protein level to be lower when W64A is the maternal parent, but higher when it is the paternal parent. Indeed, 10-kDa delta zein accumulation exhibited a dosage-dependence in reciprocal crosses of BSSS53 and W64A (Chaudhuri and Messing 1994). Because both inbreds have *dzs18* frame shift mutant alleles, dosage dependence of the *dzs18* allele cannot be tested in protein gels. However, when their transcript levels were compared in reciprocal crosses, they also exhibited the same dosage-dependent pattern as the *dzs10* gene (Fig. 4). The simplest explanation would be that both genes are regulated by the same *trans*-acting factor DZR1 that was previously characterized, but that W64A and BSSS53 possess different *dzr1* alleles. The different DZR1 products confer different stability to the d*zs10* and *dzs18* mRNAs in a semi-dominant fashion. A more complex explanation would involve a paralogous gene copy of *dzr1*.

An inbred that has normal levels of both delta zeins is B73. To determine whether *dzr1* alleles would differ between B73 and BSSS53, reciprocal crosses were performed. No apparent differences in expression levels of mRNA and protein were observed. Although in hybrids the dosage of a normal *dzs18* gene differs in reciprocal hybrids due to the frame shift in *dzs18-BSSS53*, no significant

Table 1 *dzs18* and *dzs10* null alleles

	BSSS53	B73	B37	Mo17	W64A	W22	p1-ww-1112	A69Y	ILLIZE	A188	SD-Purple	A654
dzs10	+	+	+	+	+	+	−	+	+	+	−	−
dzs18	−	+	+	+	−	−	+	+	−	+	−	−

Out of ten inbred lines and two genetic varieties (p1-ww-1112, SD-Purple), seven are mutated in one or both delta zein genes. While mutations in *dzs18* are caused by a "TTAT" insertion or "C" deletion, *dzs10* is mutated by an insertion of the transposable element *Misfit*. Both delta zein genes are mutated in SD-Purple and A654. + intact gene; − mutated gene

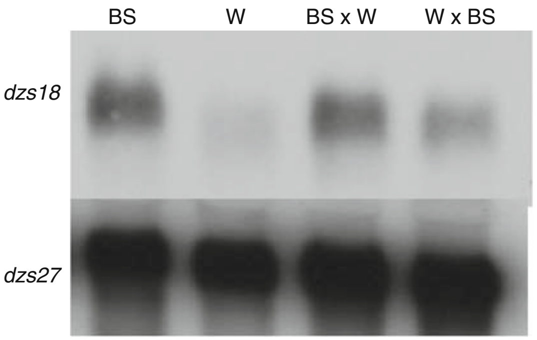

Fig. 4 Differential expression of *dzs18* in hybrids of BSSS53 and W64A: *dzs18* transcript levels are shown in the *upper panel*; *dzs27* expression, which was used as a control is shown in the *lower panel*. Specific probes are marked in the composite Northern blot. *BS* BSSS53, *W* W64A

difference occurs in the accumulation of DZS18-B73. Therefore, in contrast to W64A and BSSS53 the *dzr1* allele of B73 provided saturated levels of mRNA stability (Fig. 5a, b).

Uniparental inheritance of negative dominance of the *Dzr1-Mo17* allele

Although Mo17 had intact delta zein genes like B73, gene expression levels were reduced for both genes even further than *dzs10* in W64A. Therefore, one would expect that the *dzr1* allele of Mo17 would differ from W64A, B73, and BSSS53. Indeed, reciprocal crosses of BSSS53 and Mo17 exhibit a parent-of-origin rather than a dosage effect (Fig. 6a), as previously shown (Chaudhuri and Messing 1994). Because BSSS53 does not produce any level of 18-kDa protein, it was interesting to see that the same interactions of *dzr1* alleles between BSSS53 and Mo17 observed for the expression of *dzs10* from both parents applied also to the uniparental contribution of the *dzs18* gene of Mo17. When Mo17 is the male parent, expression of the *dzs18* is higher than in the inbred Mo17 itself, indicating a trans-regulation by the *Dzr1-BSSS53* allele and a muted *Dzr1-Mo17* allele, which does not occur when Mo17 is the female parent. Again, the simplest explanation is that both delta zein genes are regulated by the same regulatory locus, but that different alleles of this locus confer different modes of regulation on both genes simultaneously. Alternatively, an allele of a paralogous gene copy of *dzr1* exists that is not imprinted but sensitive to dosage of the uneven contribution of parental genomes to the endosperm. On the other hand, expression patterns of *dzs18* are also consistent with the hypothesis that *Dzr1-Mo17* is negative dominant when transmitted through the female parent. It also would suggest that *Dzr1-Mo17* is imprinted and female gametogenesis can activate *Dzr1-Mo17*.

To confirm that the trans-regulatory effect is not an effect of the *dzs10* allele over the *dzs18* allele, we took advantage of the double mutant A654, where neither 10- nor 18-kDa protein accumulates. Indeed, reciprocal crosses of A654 and Mo17 exhibit the same parent-of-origin effect on the expression of both delta zein genes as reciprocal crosses between BSSS53 and Mo17 (Fig. 6b, c). Therefore, these results are consistent with a trans-regulatory effect on both delta zein genes of Mo17 by *Dzr1-A654* and the parent-of-origin effect of *Dzr1-Mo17* on *Dzr1-A654*.

Based on expression patterns, one expects that the *dzr1* allele of B73 would be different to BSSS53 and W64A because of the absence of a dosage effect in hybrid crosses. Still in reciprocal crosses of B73 and Mo17, we can observe again a parent-of-origin effect on gene expression on both delta zein genes at the mRNA and protein level (Fig. 6d, e). Therefore, the *Dzr1-Mo17* allele exercises the same parent-of-origin effect on *Dzr1-B73* as it does on *Dzr1-BSSS53* and *Dzr1-A654*. Furthermore, *Dzr1-Mo17* is still dominant over *Dzr1-B73*. Another storage protein gene, the 27-kDa gamma zein gene is constantly expressed in both inbreds and crosses, indicating that *dzr1* does not regulate the *dzs27* gene and is possibly specific for a subset of prolamin genes.

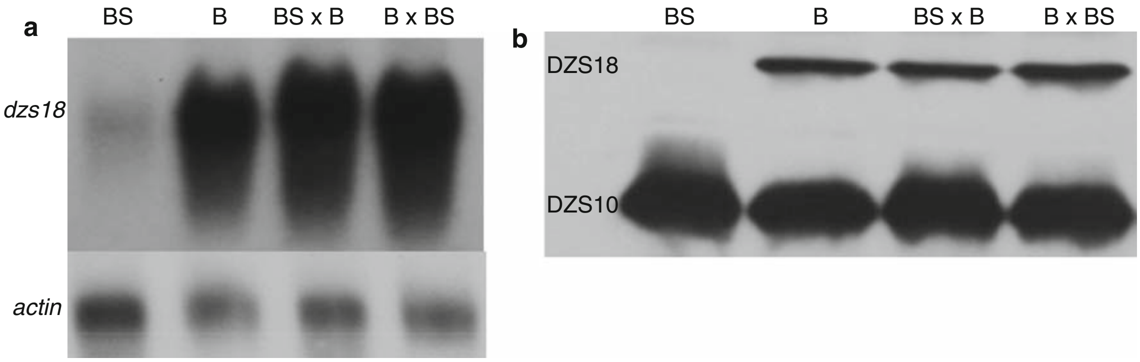

Fig. 5 Expression of delta zeins in hybrids of B73 and BSSS53: **a** and **b** High accumulations of *dzs18* transcript (**a**) and protein (**b**) were achieved by introduction of *Dzs18-B73* regardless of its parent of origin. *BS* BSSS53, *B* B73

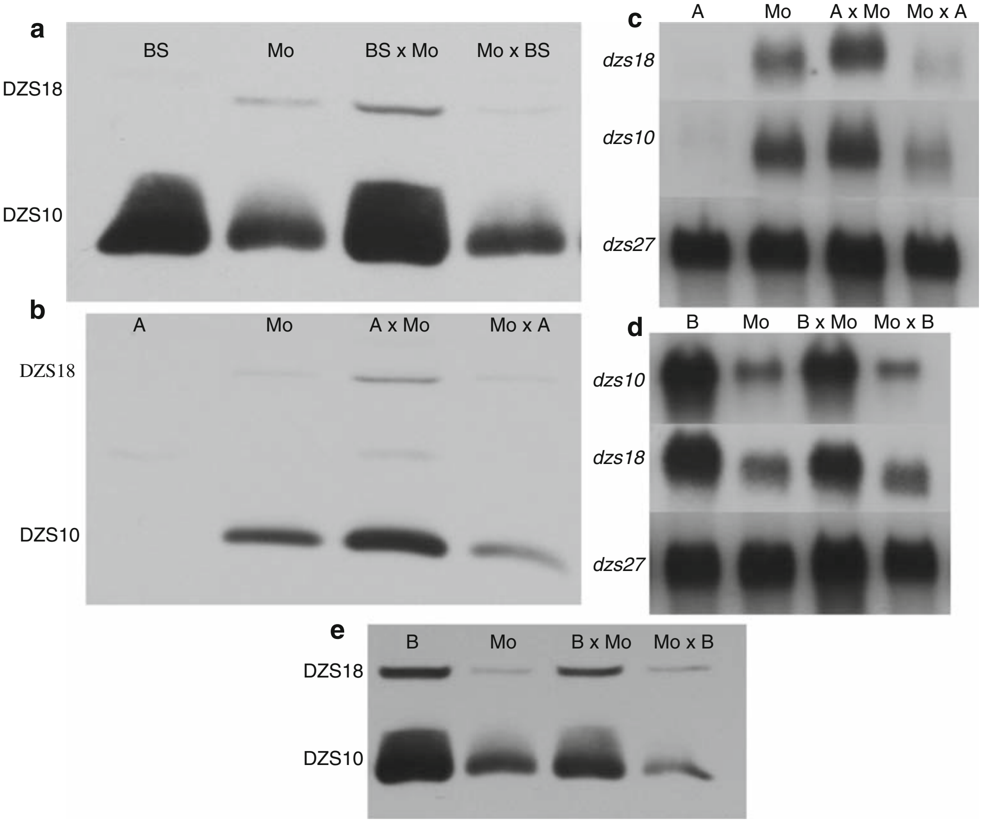

Fig. 6 Examination of the *Dzr1-Mo17* allele in hybrid crosses: **a** Western blot analysis of 10- and 18-kDa zeins in BSSS53 and Mo17 and their reciprocal crosses. **b** Western blot analysis of 10- and 18-kDa zeins in A654 and Mo17 and their reciprocal crosses. **c** Northern blot analysis of 10- and 18-kDa zeins in A654 and Mo17 and their reciprocal crosses; the same materials were used in **b**. *dzs27* was used as a control. Specific probes are also marked in this composite Northern blot; **d** Northern blot analysis of 10- and 18-kDa zeins in Mo17 and B73 and their hybrids; **e** Western blot analysis of 10- and 18-kDa zeins in B73 and Mo17 and their reciprocal crosses, the same materials were used in **d**. *BS* BSSS53, *Mo* Mo17, *A* A654, *B* B73

Loss of negative dominance in hybrid crosses with B73

If derepression of negative dominance were only activated during female gametogenesis, one would expect that such a change of state would not transmit to the next generation because all genetic changes destined to the endosperm are terminally differentiated. Indeed, when B73-Mo17 hybrids were used to pollinate B73, expression of both *dzs10* and *dzs18* is high and, when B73-Mo17 hybrids were used to pollinate Mo17, expression is low. However, when either of the reciprocal hybrids was pollinated with either Mo17 or B73, one would have expected that only half of the hybrids would provide negative dominance. That is not the case (Table 2; Fig. 7a). Therefore, it appears that *Dzr1-B73* and *Dzr1-BSSS53* alleles have different properties. When a hybrid of BSSS53 × Mo17 was pollinated with BSSS53, negative dominance was activated and segregated as expected, permitting the mapping of *dzr1* to chromosome 4S (Chaudhuri and Messing 1995). If *Dzr1-Mo17* is an allele that provides negative dominant regulation of both delta zein genes and it is imprinted, then it appears that, when it encounters *Dzr1-B73* in a single generation, the *Dzr1-Mo17* allele no longer can be activated. To monitor whether sequences tightly linked to *dzr1* in B73 and Mo17 properly segregate, progeny were genotyped accordingly. Indeed, linked markers of *Dzr1-B73* and *Dzr1-Mo17* segregated properly (Fig. 7b). This type of allelic interaction is reminiscent of paramutation, where *Dzr1-B73* would be the paramutagenic allele, *Dzr1-Mo17* the paramutable allele, and *Dzr1-BSSS53* the neutral allele.

Table 2 DZS10 and DZS18 expression in *Dzr1-Mo17* plants

	B73	Mo17	B73 × Mo17	Mo17 × B73
B73 × Mo17	H	H	H	Not done
Mo17 × B73	H	H	Not done	H
B73	H	H	H	H
Mo17	L	L	L	L

Phenotypes for all crosses are tabulated. Top row indicates male parent; left column indicates female parent. For each group, 20 single kernels were analyzed. *H* high expressions of DZS10 and DZS18; *L* low expressions of DZS10 and DZS18

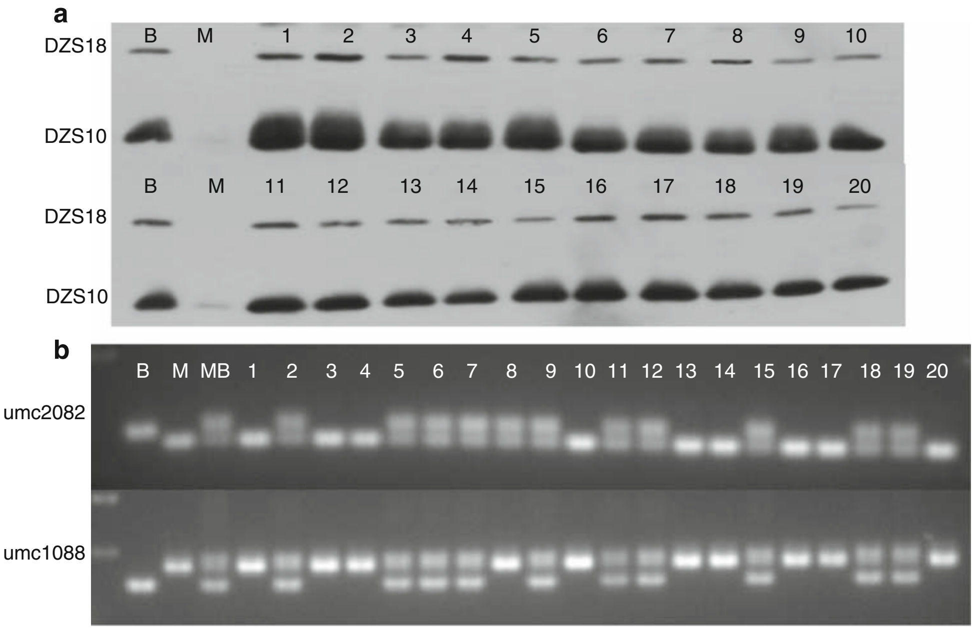

Fig. 7 Phenotype and genotype analyses of *Dzr1-Mo17* alleles in a backcross population: twenty individual kernels of a backcross between (Mo17 × B73) and Mo17 were analyzed for phenotype and genotype segregation. For each kernel, endosperm and embryo were dissected for protein extraction and genomic DNA extraction, respectively. **a** Western blot analysis was performed for both 10- and 18-kDa zeins; **b** Two SSR markers linked to *dzr1* on chromosome 4S were used to monitor the segregation of the *Dzr1-Mo17* and *Dzr1-B73* alleles. As shown in **b**, *Dzr1-Mo17* and *Dzr1-B73* were equally transmitted to the next generation. *B* B73 inbred line, *M* Mo17 inbred line, *MB* Mo17 × B73; 1–20, individual kernels of (Mo17 × B73) × Mo17

Discussion

Methionine levels in maize seeds range from a level of deficiency to a level that can completely replace synthetic methionine in animal feed with natural methionine (Messing and Fisher 1991). The accumulations of the two methionine-rich delta zeins vary in different inbred lines, explaining in part the differential methionine levels in maize seeds. It was demonstrated that BSSS53 with the highest methionine levels among tested inbred lines also expresses the highest amount of DZS10 (Phillips and McClure 1985). Over-expression of *dzs10* driven by the 27-kDa gamma zein promoter indeed increased the total methionine level in the kernel (Lai and Messing 2002). Here, we can show that the varied expressions of delta zeins are due to a combination of various alleles of two genes that represent the delta zein gene subfamily and different alleles of a post-transcriptional regulator. Employing null alleles of either delta zein gene in reciprocal crosses, alleles of the regulator seem to have the same specificity in trans for either delta zein gene. Interestingly, the negative dominance of the *Dzr1-Mo17* allele provides the same effect on the *dzs18* as on the *dzs10* gene. Moreover, the effect on both genes requires activation of the allele through parent-of-origin transmission.

Our findings of a common regulation of the *dzs10* and *dzs18* mRNA accumulation is not unexpected because these genes were duplicated by a whole genome duplication event compared to the single copy in the sorghum genome (Xu and Messing 2008). Still, 18-kDa protein and mRNA levels are significantly lower than those of the 10-kDa delta zein product. Indeed the UTRs of both genes are sufficiently diverged that one can envision differential mRNA accumulation through differential RNA protein interactions (Supplementary Fig. 2). Another explanation could be the lack of the prolamin box in *dzs18* compared to *dzs10* promoter region (Xu and Messing manuscript in preparation). In that case differential enhancement of transcription could be responsible for the lower 18-kDa mRNA accumulation. Because the 18-kDa delta zein has a higher nutritional value than the 10-kDa delta zein, it would be not unreasonable to swap the regulatory regions of *dzs10* with *dzs18* to achieve higher levels of expression of the 18-kDa delta zein in transgenic seeds.

Differentiation that occurred after allotetraploidization of duplicated genes is not unusual as illustrated by the *r1* and *b1* genes, which encode transcription factors with the same specificity but in different tissues at different times of development (Chandler et al. 1989; Coe 1959; Ludwig et al. 1989; McClintock and Hill 1931). Here, differentiation mainly is affected by null mutations and because the two genes are expressed to different degrees, the range of expression is more dramatic than for other prolamin genes. Indeed, we do not know of any natural null mutations for the other prolamin subfamilies. It could well be that basic levels of methionine, required for proper germination of the seed (Gallardo et al. 2002), are provided by the beta zein gene, which has a more moderate level of methionine codons (Pedersen et al. 1986). Moreover, the beta zein and other prolamins seem to play an essential role in the compartmentalization of the prolamins. The 27- and 16-kDa gamma zein genes, also the result of the whole genome duplication event, are thought to prime protein body formation and are both transcribed earlier than alpha and delta zein genes (Lending and Larkins 1989), though their accumulations are not at the same level (Woo et al. 2001). The synchronized expression regulated by various factors seems to be crucial for spatial organization of the components of protein bodies. Disturbance of the proper spatial arrangement of the zeins could result in irregular shape of the protein body, exemplified by the *floury-2* mutant (Coleman et al. 1997, 1995; Gillikin et al. 1997). Furthermore, it has been shown in heterologous transgenic plants that expression of either alpha or delta zein is stabilized and enhanced when co-expressed with gamma and beta zein, respectively (Bagga et al. 1997; Coleman et al. 1996).

Identification of null mutants in only one subfamily of prolamin genes also indicates that probably the others provide a selective advantage for kernel development. Indeed, mutants that affect the accumulation of alpha zeins usually have an opaque phenotype. These kernels are also softer and more susceptible to disease and less suitable for storage. Furthermore, even if one gene copy is knocked out there are other gene copies that can take over. Therefore, one wonders if the delta zein genes are dispensable and DZR1 is a specific regulator of them, why would a regulator of these genes acquire properties of an imprinted gene? Imprinting in mammals has been linked to differential weight of parental genes in the provision of nutrients to offspring (Moore and Haig 1991). Although we cannot exclude such a role in plants (Feil and Berger 2007), we also can find imprinted genes that have non-imprinted alleles and therefore the mechanism of imprinting also affects genes not essential for development of offsprings like *dzr1* and *r1*. On the other hand, a series of mutant genes has been isolated that are critical for seed development and exhibit a parent-of-origin phenotype (Chaudhury et al. 2001; Huh et al. 2007). Such mutants typically segregate 1:1, when selfed, rather than 3:1 as usually recessive mutations would do (Messing and Grossniklaus 1999). Interestingly, the *r1* allele that is imprinted correlates with a methylation pattern that suggests demethylation during female gametogenesis in the central cell before fertilization (Alleman and Doctor 2000). Indeed, such a mechanism has been demonstrated by the isolation of the *DEMETER* gene in Arabidopsis (Gehring et al. 2006). It is therefore conceivable that an allele of a gene that is epigenetically silenced can respond to a *DEMETER*-like gene although the interaction is not critical for seed development.

There is potentially another interesting parallel feature of epigenetic regulation between the *r1* and the *dzr1* locus, paramutation (Brink 1959). Paramutation is defined as an epigenetic change that occurs when two alleles of a gene interact in a single generation in such a way that one allele can impose its expression properties on the other one in a heritable way. Therefore, contrary to normal Mendelian segregation of two alleles, the offspring all have the same expression properties as the paramutagenic allele. The paramutable allele can acquire thereby also the property of paramutagenicity and paramutate the paramutable allele in a single generation as well. This test is also referred to as secondary paramutation. Recent investigations have shown that DNA methylation is also involved in paramutation as in genomic imprinting but involve different alleles of the *r1* locus (Walker and Panavas 2001). Therefore, imprinted alleles of the *r1* locus are not necessarily paramutagenic (Kermicle 1970). In case of *dzr1*, there are striking differences compared to the properties of *r1* alleles, although common properties could still emerge upon further investigations. The *Dzr1-Mo17* deviates from other *dzr1* alleles in two properties; one is its negative dominance, the other its epigenetic regulation. The first one could be explained if DZR1 heterodimerizes, the second is if the gene is silenced. The reversal of gene silencing through the female gametophyte would be consistent with genomic imprinting at the *r1* locus. However, when *Dzr1-Mo17* is exposed to *Dzr1-B73*, the active allele is not silenced, but activation of the silenced allele during female gametogenesis no longer occurs. Moreover, this maintenance of silencing occurs after fertilization and is transmissible. Therefore, it would be quite different to the mechanism that provides the parent-of-origin effect, which operates only in the central cell. To better understand the non-Mendelian inheritance of the *Dzr1-Mo17* allele, it would then be useful to isolate alleles that are also neutral to epigenetic changes, possibly *Dzr1-BSSS53* or *Dzr1-A654* and also investigate secondary paramutation. In summary, the characterization of naturally occurring *cis*- and *trans*-acting alleles affecting methionine storage in maize provide an invaluable system, with which to study various mechanisms of gene expression from post-transcriptional regulation to epigenetic factors.

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