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THE DEVELOPMENT AND IMPORTANCE OF ZEIN PROTEIN BODIES IN MAIZE ENDOSPERM

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ABSTRACT - Research over the last fifty years has shown that the vitreousness of the maize kernel is influenced by the formation of zein protein bodies. Here we summarize what is known about the structure and importance of protein bodies, beginning with their first detailed characterization by Donald Duvick in the 1950s. We describe the proteins that compose zein protein bodies and explain how available data describing zein gene expression and zein protein interactions suggest a model for their initiation, expansion and structure. We also describe maize mutants with reduced kernel hardness and show that they can be explained as a result of a perturbation to zein protein structure and its effect on protein body formation. However, there are other soft kernel mutants that appear to be unrelated to zein proteins, indicating that much remains to be learned about the factors influencing the texture of mature maize endosperm.

KEY WORDS: Endosperm; Prolamin; Protein body; Storage protein; Zein.

INTRODUCTION

Maize is a crop is of immense importance that has many uses as food and livestock feed. These applications rely heavily on a high yield and optimal harvest and storage characteristics of the mature kernel. The development of a hard kernel texture is an essential aspect of these uses, and this property appears to be related to the formation of a layer of vitreous endosperm. Here we summarize what is known about the structure and importance of protein bodies, and of maize mutants with reduced kernel hardness and show that they can be explained as a result of a perturbation to zein protein structure and its effect on protein body formation.

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PROTEIN BODIES - THEIR DISCOVERY AND IMPORTANCE FOR KERNEL INTEGRITY

Granular protein inclusions in the maize aleurone and peripheral endosperm cells were observed as early as 1885 (HARZ, 1885). Later, Weatherwax also noted the appearance and growth of protein granules in the inner endosperm cells, though to a lesser extent than in the peripheral cells (WEATHERWAX, 1930). In his 1955 paper, Donald Duvick presented a detailed account of the appearance and growth of endosperm cytoplasmic inclusions. He described the initiation of starch grains from enlarged filament knobs, and at a later stage in the same region, the expansion of small granules into what he termed "protein granules" (DUVICK, 1955). These protein granules did not appear to increase in number in a given endosperm region, but rather became more readily visible during their expansion from pre-existing non-filamentous granules (DUVICK, 1961). Early indications were that starch grains were larger and more numerous in the central endosperm cells and protein granules were larger and more numerous in the peripheral layers (DUVICK, 1955). These factors were later proposed to be central to the formation of the "horny" or "vitreous" outer endosperm and the soft, floury central endosperm (DUVICK, 1961). Later observations of the outer starchy endosperm cell layers showed that there is actually an increase in the size of protein bodies from the first to the fifth subaleurone cell layer that reflects the developmental gradient of these cells (LENDING and LARKINS, 1989).

Duvick likened the microscopic appearance of the horny endosperm to a box of white marbles (starch grains) densely interspersed with buckshot (protein bodies) and all bound by a transparent glue (clear viscous cytoplasm) which forms a rigid conglomerate when dry (DUVICK, 1961). This rela-

tionship is apparent in a scanning electron micrograph showing developing maize endosperm cells (Fig. 1A). In theorizing what factors may be responsible for the abrupt partition between vitreous and floury endosperm, he suggested that there is a range of proportions of protein and starch granules and viscous cytoplasm which when desiccated will give rise to a rigid matrix. Moving towards the center of the kernel and out of this range, with progressively smaller protein granules and larger starch granules, the volume occupied by the cytoplasm is insufficient to prevent shattering of the matrix during kernel desiccation, giving rise to the floury appearance (DUVICK, 1961). Duvick also collated and presented evidence suggesting that protein granules are largely made up of zein proteins (DUVICK, 1961). He noted the fact that horny endosperm has more than double the percentage of zein as floury endosperm (HAMILTON *et al.*, 1951). Furthermore, increases in overall kernel protein that are observed when nitrogen fertilizer is applied are manifest as increased protein granule size, and the principle increase is in the zein protein fraction (HAMILTON *et al.*, 1951). These and other early observations of the structure and composition of the maize kernel laid the groundwork for subsequent studies that have characterized the nutritional and physical properties of maize seed. Protein granules or protein bodies (PBs), as they are now known, thus appear to be central to the generation of hard kernels that are resistant to insect and fungal attack during maturation and storage. However, the mechanism by which protein bodies form and the way in which they influence endosperm texture remain unknown despite many years of research.

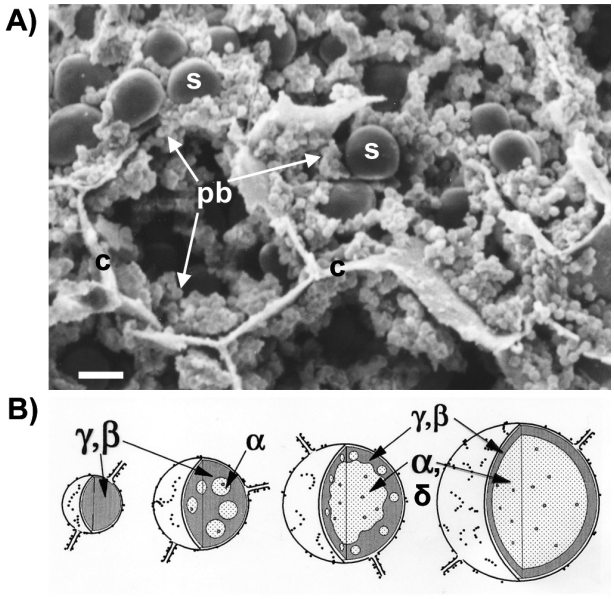

FIGURE 1

A. Scanning electron micrograph of endosperm cells in a developing maize kernel. The kernel was frozen and manually fractured prior to imaging and so some cellular contents may have been lost. Starch grains appear as grey spheres and adhering protein bodies are seen as smaller white spheres. Representative starch grains, protein bodies and cells walls are marked with **s**, **pb** and **c**, respectively. Scale bar in bottom left represents 10 μm.

B. Diagrammatic representation of the process of protein body development showing localization of α-, β-, γ- and d zein proteins. Adapted from LENDING and LARKINS (1989) with permission from the American Society of Plant Biologists.

ORIGIN OF PROLAMIN AND GLOBULIN PROTEIN BODIES

All storage proteins are initially synthesized on the surface of the rough endoplasmic reticulum (ER) and are imported into the lumen of the ER via an N-terminal signal peptide (VONHEIJNE, 1984). Storage proteins are then either directly assembled into PB accretions that remain within the ER, or they may be transported through the endomembrane system to protein storage vacuoles (PSVs) (VITALE and DENECKE, 1999). The prolamin storage proteins of maize and rice are retained in ER PBs (LARKINS and HURKMAN, 1978; LI *et al.*, 1993a,b). Globulins, the major class of storage proteins in dicotyledonous plants, as well as the prolamins of certain cereals such as wheat, are stored in PSVs. In wheat, prolamins in ER vesicles bud off into small cytoplasmic PBs that become sequestered into PSVs by autophagy (GALILI *et al.*, 1995). In recent years, a central focus of research has been on what governs the ultimate destination of ER-synthesized storage proteins, and considerable progress has been made in the study of rice (CROFTS *et al.*, 2004). Rice provides a good system for investigating protein targeting because unlike most other plants, it accumulates large amounts of both prolamins and a globulin homolog, glutelin, and so both types of protein storage are represented. It appears that targeting mRNAs to different regions of the ER plays a major role in governing the eventual location of storage proteins in rice (LI *et al.*, 1993a; CHOI *et al.*, 2000). It is probable that other plants employ similar mechanisms to target the synthesis of their storage proteins, although

rice could represent a particularly developed system given that two types of storage proteins (prolamin and glutelin) are produced in abundance and therefore the need to prevent inappropriate interactions may be greater. Other plants that predominantly synthesize a single type of storage protein may not require such specific targeting at the RNA level, but this remains to be demonstrated.

In general, the mRNA localization pathway involves the movement of ribonucleoprotein particles from the nucleus to the target ER (De Heredia and Jansen, 2004). These particles consist of targeted mRNAs containing cis-acting signals that bind specific trans-acting proteins involved in movement on the cytoskeleton (De Heredia and Jansen, 2004), target site anchoring and translation once the target site is reached (Wilhelm and Vale, 1993; Carson *et al.*, 1998). In rice endosperm and the majority of plant cells, the cortical ER is the predominant site of protein synthesis and consequently it is associated with abundant actin microfilaments (Muench *et al.*, 2000) and EF1α (Clore *et al.*, 1996), with these proteins presumably serving to anchor ribosomes and recruit aminoacyl tRNAs to the ribosomes. In rice, the cortical ER consists of the PB ER where prolamin mRNAs are targeted and translated and the proteins stored, and the cisternal ER, where glutelin mRNAs are targeted and translated (Crofts *et al.*, 2004). Studies that involve real time observations of movements of storage protein mRNAs fused to a green fluorescent protein (GFP) coding sequence have helped to identify the cis-elements that specify prolamin and glutelin RNA trafficking (Hamada *et al.*, 2003a). Initial findings indicated that although the prolamin signal-peptide sequence is not necessary, per se, for correct localization to the PB ER, it is necessary to initiate protein synthesis prior to mRNA anchoring (Hamada *et al.*, 2003b). By creating deletions of a prolamin cDNA fused to GFP, it was shown that cis-acting RNA localization-elements reside in the 5' coding sequence distal to the signal peptide and in the 3' untranslated sequence, both of which are essential for localization to the PB ER; deletion of these nucleotide sequences resulted in mistranslocation to the cis ER, thus implying that this is a default location. The glutelin 3' UTR appears to have at least one cis-element that is dominant over the prolamin cis-elements, since replacing the prolamin 3'UTR with that of glutelin also caused mistranslocation to the cis ER (Crofts *et al.*, 2004).

The high concentrations of specific types of storage proteins in defined ER regions resulting from these types of processes could be essential for the productive protein interactions that give rise to mature protein bodies. In maize, whilst it is likely that mRNA trafficking to the ER does occur, the distribution of zein mRNAs was found to be essentially random between the cis ER and the PB ER, indicating that there may be subtle differences between maize and rice endosperm in prolamin RNA trafficking (Kim *et al.*, 2002).

PROTEIN BODIES IN MAIZE

1. Storage proteins in maize

There are two predominant types of storage proteins in maize seeds: prolamins and globulins. The embryo contains a 7S globulin (Kriz, 1999), and the endosperm accumulates primarily zein prolamin proteins (Coleman and Larkins, 1999). The endosperm also accumulates an 18-kD α globulin and an 11S legumin-like protein in organelles that have a vacuole-like appearance (Woo *et al.*, 2001; Yamagata *et al.*, 2003).

The initial designating factor for classifying zein proteins was that, as with all types of prolamins, they are soluble in 70% ethanol. There are, however, differences in aqueous solubility and ability to form disulfide interactions, and the current nomenclature separates zeins into α-, β-, γ- and δ-types based on these properties (Esen, 1987; Coleman and Larkins, 1999). Protein body formation in maize is controlled at several levels, including the temporal and spatial regulation of zein gene expression, the level of transcription and interactions that occur between the different types of zein proteins (Woo *et al.*, 2001; Kim *et al.*, 2002).

Substantial differences exist in the copy number and relative expression level of the different zein genes and these properties are summarized in Table 1. The α-zein gene family is large and complex, and there has been considerable uncertainty as to the relative number of functional coding sequences and pseudogenes. However, it is now apparent that most of the approximately 30% of total endosperm transcripts that encode α-zeins come from a small number of genes (Song *et al.*, 2001; Song and Messing, 2002). Cluster analysis of ESTs from endosperm cDNA libraries indicated that there are nine distinct α-zein genes that fit into three distinct classes based on amino acid similarity: 19-kD "B" and "D" classes and the "Z" class of 22-kD α-zeins. In B73, most 19-kD α-zein transcripts are derived from only two

TABLE 1 - *Characteristics of zein genes and proteins.*

Adapted from Woo *et al.* (2001) with permission from the American Society of Plant Biologists.

Name (abbreviation)	GeneBank Acc#	Map loci (Pioneer composite map)	% EST in developing B73 endosperm cDNA libraries	Mature peptide (amino acid residues)	mw (calculated)	mw (SDS-PAGE)
27-kD gamma-zein (gz27)	AF371261	c7L, position 147, Bin 7.05 [1]	5.4	204	21,822	27 kD
16-kD gamma-zein (gz16)	AF371262	c2L, position 128.1, Bin 2.07 [2]	2.9	163	17,663	16 kD
50-kD gamma-zein (gz50)	AF371263	c7L, position 70.5, Bin 7.05 [2]	1.5	278	32,882	50 kD
15-kD beta-zein (bz15)	AF371264	c6S, position 28.9, Bin 6.01 [3]	4.7	160	17,458	15 kD
18-kD delta-zein (dz18)	AF371265	c6L, position 84, Bin 6.04 [4]	1.0	190	21,220	18 kD
10-kD delta-zein (dz10)	AF371266	c9L, position 67.6, Bin 9.03 [5]	0.5	129	14,431	10 kD
19-kD alpha-zein D1 (az19D1) [6]	AF371267	c1L, position 123.3, Bin 1.06 [2]	1.2	222	24,818	19 kD
19-kD alpha-zein D2 (az19D2)	AF371268	c1L, position 122.4, Bin 1.06 [2]	1.0	220	24,706	19 kD
19-kD alpha-zein B1 (az19B1)	AF371269	4L, 7S [7]	15.9	213	23,359	19 kD
19-kD alpha-zein B2 (az19B2)	AF371270	4L, 7S [7]	0.1	246	27,128	22 kD
19-kD alpha-zein B3 (az19B3)	AF371271	c4L, c7S, c10L [8]	5.7	219	24,087	19 kD
19-kD alpha-zein B5 (az19B5)	AF371272	4L, 7S [7]	0.05	truncated cDNA	na	na
19-kD alpha-zein B4 (az19B4)	AF371273	4L, 7S [7]	0.06	In-frame stop codon	na	na
22-kD alpha-zein Z1 (az22z1)	AF371274	c4S, position 27.3, Bin 4.02 [9]	4.9	242	26,359	22 kD
22-kD alpha-zein Z3 (az22z3)	AF371275	c4S, position 27.3, Bin 4.02 [9]	0.5	245	26,751	22 kD
22-kD alpha-zein Z4 (az22z4)	AF371276	c4S, position 27.3, Bin 4.02 [9]	0.5	246	26,923	22 kD
22-kD alpha-zein Z5 (az22z5)	AF371277	c4S, position 27.3, Bin 4.02 [9]	0.1	245	26,701	22 kD

[1] (Burr *et al.*, 1988; Lopes and Larkins, 1995); [2] (Woo *et al.*, 2001); [3] (Weerakoon *et al.*, 1993); [4] (Swarup *et al.*, 1995); [5] (Benner *et al.*, 1989); [6] zein subfamily z1D/ SF3 (Heidecker and Messing, 1986; Rubenstein and Geraghty, 1986); [7] zein subfamily z1B/ SF2 (Heidecker and Messing, 1986; Rubenstein and Geraghty, 1986); [8] zein subfamily z1A/ SF1 (Heidecker and Messing, 1986; Rubenstein and Geraghty, 1986); [9] zein subfamily z1C/ SF4 (Llaca and Messing, 1998; Rubenstein and Geraghty, 1986); na, not applicable.

genes of the B class and many of the 22-kD α-zein transcripts came from a single gene (Woo *et al.*, 2001). The β- (Pedersen *et al.*, 1980), γ- (Prat *et al.*, 1987), and δ- (Kirihara *et al.*, 1988; Chui and Falco, 1995) zein genes occur in only one or two copies per genome, but these genes are all expressed at high levels. The 27-kD γ-zein and the 15-kD β-zein account for about 5% each of total endosperm transcripts. The 15-kD β-zein is more correctly placed within the γ-zein family, based on an invariant stretch of 6 cysteine residues and other conserved polypeptide domains (Woo *et al.*, 2001). The 16- and 50-kD γ-zeins make up about 3% and 1.5% respectively of endosperm transcripts, and the sulfur-rich 10- and 18-kD δ-zeins, about 1% and 0.5%, respectively (Woo *et al.*, 2001). Because of their low abundance, the 50-kD γ-zein and the 18-kD δ-zein were difficult to identify at the protein level (Woo *et al.*, 2001).

2. Assembly of zein protein bodies

Prolamins in general contain 30-70% proline and glutamine, and are suggested to have evolved by amplification of small hydrophobic proline and glutamine-rich peptides (Herman and Larkins, 1999). Studies indicate that specific interactions between the different types of zeins influence the formation of discrete PB accretions (Coleman *et al.*, 1996; Bagga *et al.*, 1997). Although the exact nature of these protein interactions is unknown, PB structure has been well characterized (Fig. 1B) (Lending and Larkins, 1989) and progress has been made in identifying interactions between specific zein proteins (coleman *et al.*, 1996; Kim *et al.*, 2002) and gene expression patterns have been found to be consistent with such interactions (Woo *et al.*, 2001).

Immunolocalization with specific zein antibodies determined that young meristematic cells closest to the aleurone layer contain small PBs that are com-

posed entirely of β- and γ-zein (Fig. 1B) (LENDING and LARKINS, 1989; LOPES and LARKINS, 1991). More mature cells farther away from the aleurone layer contain larger PBs with central locules composed primarily of α-zein and small amounts of δ-zein (Fig. 1B). This suggests that these proteins penetrate the matrix of cysteine-rich and cross-linked β- and γ-zeins. In the largest PBs (1-2 μm), these locules fuse into a solid core. In these PBs, a thin layer of β- and γ-zeins completely surrounds the protein body, although these proteins can also be found internally as small central inclusions or stands (Fig. 1B).

Studies with transgenic plants demonstrated the importance of zein interactions in PB assembly. Genes encoding one or more types of zeins were expressed in tobacco plants so that interactions could be studied without the influence of endogenous zeins (COLEMAN *et al.*, 1996; BAGGA *et al.*, 1997). When synthesized individually, α- and δ-zeins formed small accretions similar to immature PBs, which were retained in the ER and were stable over prolonged periods. However, expression of α- or δ-zeins, either singly or together, did not result in protein accumulation; the proteins apparently entered the endomembrane system and were degraded. When α-zein was co-expressed with γ-zein (COLEMAN *et al.*, 1996) or β-zein (COLEMAN *et al.*, 2004) in transgenic tobacco cells, small ER-localized PB-like accretions accumulated. These results demonstrate that it is the γ-zein/β-zein proteins that nucleate and anchor the protein body in the ER. It has been suggested that the PPPVHL amino acid repeats in the N-terminal region of the 27-kD γ-zein could provide a means of attachment to the ER (GELI *et al.*, 1994) by forming an amphipathic helix that can interact with the ER surface (RABANAL *et al.*, 1993). Transgenic studies also suggest that the γ-type zeins are able to bind α-zein/δ-zein proteins.

Even though α-zein is by far the most abundant protein in the endosperm, its accumulation begins after the onset of γ-zein gene expression and protein accumulation (WOO *et al.*, 2001). It was found that in 10 days after pollination (DAP) kernels, all the γ-zein genes were expressed throughout the endosperm. Of this class, 27-kD γ-zein was the most highly expressed, 16-kD γ-zein was expressed at a lower level and 50-kD γ-zein and 15-kD β-zein were expressed at a still lower level. However, expression of the 19- and 22-kD α-zein genes at this same stage was not detectable, apart from a narrow stripe on the adgerminal (embryo proximal) face of the kernel. At later stages, expression of α-zeins was observed at high levels and in a more uniform pattern, although α-zein gene expression was not detected in the central cells of the starchy endosperm at any stage of development. This is consistent with Duvick's early observations of small, unexpanded PBs in the center of the endosperm.

A series of yeast two-hybrid experiments confirmed that there are specific and predictable interactions between different types of zeins, as well as weak or absence of measurable interactions between proteins that might be expected to associate (KIM *et al.*, 2002). Self-interaction of each type of zein protein was also measured. These interactions are summarized in Fig. 2. In general, strong interactions were found amongst the γ-zein family, including the 15-kD β-zein, and this is consistent with their co-localization at the periphery of the protein body. The interaction between the main constituents of the protein body core, the 19- and 22-α-zeins, was surprisingly weak, although both interacted strongly with the δ-zeins. These relative affinities may relate to protein abundance; a strong interaction between the α-zeins may be unnecessary if they are both strongly targeted to the PB core by their interactions with the γ-zein proteins and, in

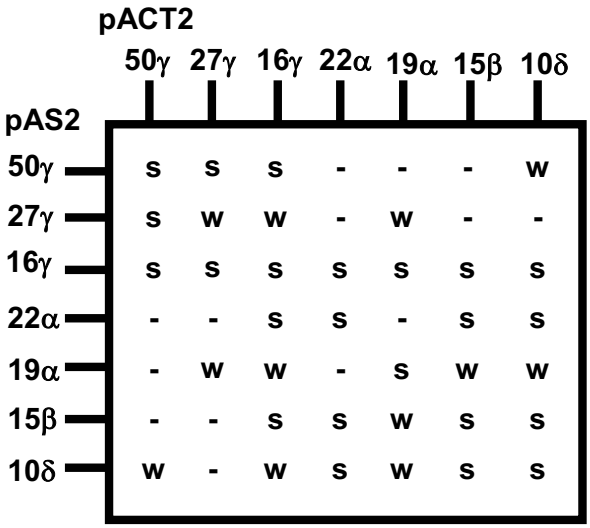

FIGURE 2 - Interactions between zein proteins. Modified from KIM *et al.* (2002) with permission from the American Society of Plant Biologists. The coding regions (without signal peptide sequences) were cloned into the pAS2 and pACT2 plasmids of the yeast two hybrid system and transformed reciprocally into yeast cells. For further experimental details, refer to KIM *et al.* (2002). **S**, **W** and **–** refer to strong, weak and immeasurable interactions.

light of their high abundance, their specific interaction could indeed disrupt assembly if it occurred prior to penetration into the PB core. The relatively low level of δ-zein accumulation could necessitate interaction with α-zeins, as well as with γ-zeins, in order to target it correctly. This low abundance and ability to interact with both 22-kD and 19-kD α-zeins may suggest that a primary function of the10-kD δ-zein is to mediate the interaction and accumulation of the α-zeins. δ-zeins are rich in methionine and vary in abundance, suggesting that they also function as a sulfur sink (SWARUP *et al.*, 1995).

The 16-kD γ- and the 15-kD β-zein both interact strongly with the α- and δ-zeins, but surprisingly, the 50-kD and 27-kD γ-zeins do not. Furthermore, although the 50-kD γ-zein interacts strongly with itself, as do the 16-kD γ- and 15-kD β-zeins, the 27-kD γ-zein does not. These results suggest an important role for 16-kD γ- and 15-kD β-zein in binding and assembly of α-zeins within the PB. They also make the properties of the 27-kD γ-zein somewhat difficult to reconcile with the observations that transgenically expressed 27-kD γ-zein promotes ER retention of transgenically expressed 22-kD α-zein. The early stages of PB assembly are suggested to be driven by interactions between unique N-terminal signal sequences in the 50-kD and 27-kD γ-zeins (GELI *et al.*, 1994; LEE, 1998). As the PB expands and α- and δ-zeins accumulate in the PB core, the 50-kD and 27-kD γ-zeins remain on the surface (LENDING and LARKINS, 1989; WOO *et al.*, 2001). Immunolocalization of the 16-kD γ- and 15-kD β-zeins reveals that they are not restricted to the surface but occur throughout the PB structure. Taken together, these observations suggest that the 50-kD and 27-kD γ-zeins allow the nucleation and early growth of PBs, and the 16-kD γ- and 15-kD β-zeins allow the retention of the α- and δ-zeins by physically linking them to the 50-kD and 27-kD γ-zeins. It is perhaps also surprising that the 27-kD γ-zein does not strongly interact with itself. Of the γ-zein class, the 27-kD γ-zein accumulates to the highest level, and it is predicted to nucleate protein body formation in the ER (COLEMAN *et al.*, 1996). Thus, it is possible that the lack of self interaction of 27-kD γ-zein may prevent its uncontrolled aggregation, and thus may be responsible for the even dispersal of PBs throughout endosperm cells that allows effective interactions with starch grains. Early PB expansion could be driven by the interaction of 50-kD and 27-kD γ-zeins as well as by the self-interaction of the 50-kD g-zein. Further expansion would then be driven by interactions between other γ-zein types and α-/δ-zeins. It is possible that when the 27-kD γ-and 22-kD α-zeins are co-expressed transgenic tobacco in the absence of other zeins (COLEMAN *et al.*, 1996), they are able to interact in a way that does not occur in developing maize endosperm. In maize endosperm, the sequestration 22-kD α-zein may be more efficiently performed by the 16-kD γ and 15-kD β-zeins, or by a complex consisting of all the γ-type zeins.

The 22-kD α-zein protein contains a distinct N-terminal leader of ~40 amino acids followed by a series of eight or nine 20 amino acid repeated peptides and a C-terminus of ~ 35 amino acids (ARGOS *et al.*, 1982). A series of deletion mutants of the 22-kD α-zein were constructed and used in yeast two-hybrid assays with the various other types of zeins with which the 22-kD α-zein had been shown to interact (KIM *et al.*, 2002). These experiments showed that the repeated peptides in the α-zein are responsible for binding to other zeins; specifically, one or two of the repeats nearer the C-terminus, as well as the N- and C-termini are necessary for the strongest interactions (KIM *et al.*, 2002).

PROTEIN BODIES AND ENDOSPERM TEXTURE

The development of a hard, vitreous endosperm is important for many aspects of the maturation, harvest, storage, handling and processing of the maize grain, and considerable evidence suggests that zein protein accumulation plays a central role in these processes. The vitreous portion of the wild-type maize kernel contains much more zein than the soft, starchy interior (DOMBRINK-KURTZMAN and BIETZ, 1993), which supports the hypothesis that zeins are involved in determining kernel hardness. Environmental conditions that cause reduced zein synthesis, such as nitrogen depletion, result in kernels that are soft and starchy throughout (TSAI *et al.*, 1978). Mutants that exhibit reduced kernel hardness offer the opportunity to dissect the various biochemical processes that govern the formation of a vitreous endosperm, and consequently their study is of significant agronomic importance.

There are a number of well characterized mutants that have soft, starchy kernels that do not transmit light and are thus termed opaque. Several of these mutants have been shown to alter some aspect of zein synthesis and protein body structure, thus providing further evidence for the role of zeins

in kernel hardness. However, some opaque mutants have apparently normal patterns of zein synthesis and PB structure and therefore suggest that additional factors are also important determinants of kernel texture.

1. Opaque mutations affecting zein synthesis and processing

The *opaque2* (*o2*) and *floury2* (*fl2*) mutants were originally reported in 1935 (EMERSON, 1935) and have been well characterized because it was discovered that they contain high levels of the essential amino acids lysine and tryptophan, both of which are limiting in wild-type maize (MERTZ *et al.*, 1964; NELSON *et al.*, 1965). The *OPAQUE2* gene encodes a transcriptional activator that positively regulates the expression of 22-kD α-zein genes as well as other genes (DAMERVAL and DEVIENNE, 1993). The abundance of other zein proteins, especially 19-kD α-zeins, is also reduced in *o2*, even though O2 does not appear to transcriptionally regulate those genes. The reduction of zeins in *o2* is generally accompanied by an increase in non-zein proteins, some of which are relatively rich in lysine and tryptophan. Among these proteins are cytoskeleton-associated carbohydrate metabolizing enzymes (AZAMA *et al.*, 2003) and eEF1A (LOPEZ-VALENZUELA *et al.*, 2004). At the molecular level, the main effect of the low level of α-zeins in *o2* is a reduction in PB size due to reduced protein synthesis.

Soon after the discovery of the improved nutritional quality of *o2*, it became apparent that this advantage was offset by undesirable kernel phenotypes such as impaired harvesting and handling characteristics, pathogen susceptibility and reduced yield. However, interest in *o2* was renewed when it was discovered that there are genetic modifiers that restore the vitreous kernel phenotype while maintaining the enhanced nutritional quality (PAEZ *et al.*, 1969; ORTEGA and BATES, 1983). Breeding programs in Mexico (VILLEGAS, 1992) and South Africa (GEEVERS, 1992) created a new type of *o2* mutant that was termed "Quality Protein Maize" (QPM). The molecular identities of the *o2* modifiers are mostly unknown, although there is ongoing work to map and characterize them. The *SUGARY 2* gene was shown to be a modifier of the *o2* phenotype (PAEZ, 1973), and it was found to encode starch synthase 2a (ZHANG *et al.*, 2004). Several studies have shown that 27-kD γ-zein is increased 2-3 fold in QPM (WALLACE *et al.*, 1990; GEETHA *et al.*, 1991; LOPES and LARKINS, 1991), and while it is not known if this is a primary modification or a secondary effect of another gene, the degree of endosperm modification is seen to closely correlate with the level of 27-kD γ-zein protein (LOPES and LARKINS, 1991). In light of the role of the 27-kD γ-zein described above, it is easy to imagine how up-regulation of this gene in QPM could increase kernel hardness. It could be the result of an increased number of PBs (LOPES and LARKINS, 1995; MORO *et al.*, 1995). In QPM, the PBs are small, due to the low level of α-zeins conferred by the *o2* mutation. It was suggested that compaction of these 27-kD γ-zein-rich PBs (DANNENHOFFER *et al.*, 1995), as well as their possible interaction with a network of amorphous non-crystaline amylopectin at the surface of starch grains (GIBBON *et al.*, 2003), result in a vitreous endosperm. Interestingly, another mutant, *opaque15* (*o15*), shows a 2-3 fold reduction in 27-kD γ-zein synthesis, resulting in a reduced number but not the size of PBs, which is consistent with the predicted role of the 27-kD γ-zein in the initiation of PB formation (DANNENHOFFER *et al.*, 1995). Although the *O15* gene has not been isolated, it maps to the same region on chromosome 7L as an o2 modifier gene (DANNENHOFFER *et al.*, 1995). Thus, these data suggest that *O15* is itself a modifier gene, or resides near and is possibly regulated by one.

The *fl2* mutant manifests a general reduction in zeins and an increase in non-zein proteins, similar to *o2*. PBs are small, as in *o2*, but they are also severely deformed (LENDING and LARKINS, 1992). *fl2* is a semi-dominant mutation that results from the expression of an abnormal 22-kD α-zein that interferes with protein body assembly. Since *FL2* is regulated by O2, *o2* is epistatic to *fl2* and the presence of the abnormal zein and abnormally shaped PBs depends on the presence of at least one functional *O2* gene (COLEMAN and LARKINS, 1999). The 22-kD α-zein is mutated to a ~24-kD form as a consequence of an alanine to valine substitution at the C-terminal cleavage site of the signal peptide. This results in the protein remaining attached to the ER membrane, which disrupts zein association in the PB, and leads to a lobed PB morphology (GILLIKIN *et al.*, 1997). Transgenic maize plants that expressed the mutant gene also produced opaque kernels and thus provided confirmation that the abnormal α-zein is the molecular basis of this mutant phenotype (COLEMAN *et al.*, 1997). Similar to *fl2*, another dominant opaque mutant, *Defective endosperm B30* (*DeB30*), also displays abnormally lobed PBs. This is the result of a serine to proline substitution at the

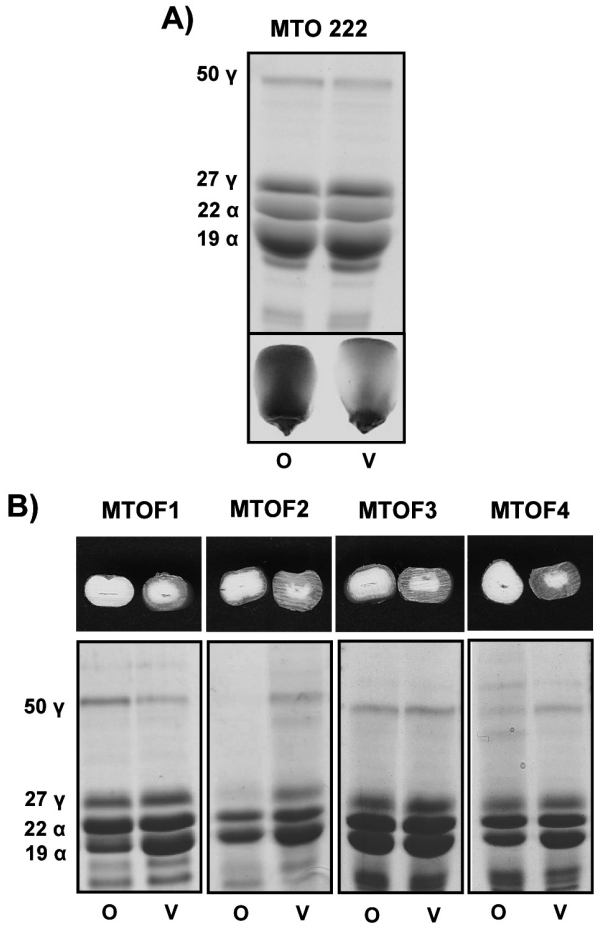

FIGURE 3

A. SDS-PAGE analysis of zein fractions extracted from equal amounts of flour from opaque and vitreous kernels taken from a segregating MTO222 ear. Zein extracts were prepared according to the method of WALLACE (1990). The kernel phenotype is shown using transmitted light.

B. SDS-PAGE analysis of zein proteins extracted from equal amounts of flour from opaque and vitreous kernels taken from segregating ears of MTOF-1, MTOF-2, MTOF-3 and MTOF-4. Thickness of vitreous endosperm was shown by grinding off the tops of kernels.

15th amino acid of the signal peptide of a 19-kD α-zein (KIM *et al.*, 2004). This results in a mutant protein that migrates between the 19- and 22-kD α-zeins during SDS-PAGE, but this protein is of low abundance and is detectable only by immunoblotting. This mutation was mapped to a cluster of 19-kD α-zeins (SOAVE and SALAMINI, 1984) and the defective gene sequence was recently identified by sequencing 19-kD α-zein cDNAs from an endosperm cDNA library (KIM *et al.*, 2004). The mutant gene was expressed in transgenic maize plants and opaque kernels were obtained, thus confirming that the abnormal 19-kDa α-zein is responsible for the mutant phenotype. Another dominant opaque mutant, *Mucronate* (*Mc*) (SOAVE and SALAMINI, 1984) results from a 38 base pair deletion that leads to a frame shift mutation in the 16-kD γ-zein (C.S. KIM *et al.*, unpublished data). As with *fl2* and *De-B30*, *Mc* has pleiotropic effects and results in a general reduction of all zeins and misshapen PBs.

It is unclear if the alterations in PB structure in *fl2*, *De-B30* and *Mc* disrupt vitreous endosperm formation directly, in ways similar to those described above for *o2*, or it occurs by some indirect effect or by a combination of both. The pleiotropic effects that reduce zein accumulation in *fl2*, *De-B30* and *Mc* are accompanied by a 5-10-fold increase in the level of a chaperone protein called BiP (BOSTON *et al.*, 1991). BiP is a member of a class of ER-localized chaperones that function to prevent protein folding intermediates from aggregating and also to stabilize energetically unfavorable intermediates during controlled folding, such that terminally incorrectly-folded proteins do not occur (KAUFMAN, 1999). When protein misfolding and aggregation occurs in the ER, there is a signal that activates the expression of numerous ER-localized proteins, and this has been termed the "unfolded protein response" (UPR) (KAUFMAN, 1999). The results of recent transcriptional profiling experiments indicate that a number of genes associated with the UPR are significantly upregulated in *fl2*, *De-B30* and *Mc* (HUNTER *et al.*, 2002). The significance of this is not known, but it may provide an explanation for the pleiotropic reductions in the synthesis of other zeins that are being targeted, folded and incorporated into PBs in the ER.

In light of the nutritional improvements that many opaque mutants manifest, another approach to improving protein quality is to alter zein synthesis and accumulation by transgenic means. RNA interference was used to reduce the expression of 22-kD (SEGAL *et al.*, 2003) and 19-kD α-zein genes (HUANG *et al.*, 2004) and this resulted in modest increases in lysine content, though nowhere near that observed in a typical *o2* mutant. Both RNAi lines manifest an opaque kernel phenotype. These studies illustrate the potential to alter nutritional value as well as to further investigate the role of zein proteins in kernel texture using transgenic maize.

2. Other opaque mutants

Whilst studies of *o2*, *fl2*, *De-B30*, and *Mc* make it clear that zeins are closely linked to PB structure and the formation of vitreous endosperm, some other opaque mutants suggest that there are additional factors influencing this phenotype. For example *opaque1* (*o1*) (NELSON *et al.*, 1965) has little effect on zein synthesis but its kernels have a soft, starchy endosperm. However, in light of the abnormal 19-kD γ-zein in De-B30 that is of low abundance, caution must be exercised before ruling out the direct involvement of a zein protein in the generation of this and other opaque mutant phenotypes.

MTO 222 is an opaque mutant for which the molecular identity has recently been determined (HOLDING and LARKINS, unpublished data). MTO 222 is a recessive mutant that has little or no observable effect on zein accumulation (Fig. 3A). The mutant gene is expressed at a low level in endosperm, and it encodes a plant-specific protein of unknown function. Experiments directed at the characterization of additional alleles of MTO 222 and their functional analyses are underway in our laboratory.

Recent screens of the UniformMu (www.uniformmu.org) population at the University of Florida identified four additional opaque mutants, MTOF-1, -2, -3 and -4 (HOLDING and LARKINS, unpublished data) (Fig. 3B). The UniformMu population was generated in a colored aleurone background making it impossible to identify mutants on the basis of their light transmission. Consequently, mutants were identified by grinding off the top third of the kernel to reveal the thickness of the vitreous endosperm. These mutants fall into two classes: fully opaque (MTOF-1 and MTOF-4), and partially opaque with a thin layer of vitreous endosperm (MTOF-2 and -3). The molecular identities of the genes responsible for MTOF-1-4 are currently unknown. One of the methods being employed to identify candidate genes in the UniformMu collection is the generation and high-throughput sequencing of libraries of Mu insertions for each mutant by Thermal Asymetric Interlaced (TAIL) PCR (LIU *et al.*, 1995). Theoretically, these libraries have approximately three-fold coverage of all Mu insertions, such that the causative insertion flanking sequence is likely to be represented in the library. In order to reduce the potential number of candidate Mu insertions, TAIL PCR sequences are put through a series of bioinformatics filters and clustering (SETTLES *et al.*, 2004). The MTOF 1-4 TAIL PCR libraries did not reveal Mu insertions in or near any zein genes or genes that are known to regulate zeins. However, prelimary analyses revealed significant effects on zein protein accumulation (Fig. 3B). MTOF-1 shows a generalized reduction in zeins, most prominently a reduction in the 19-kD α-zein. MTOF-4 has reduced levels of all zeins, especially 19-kD α-zeins, although not as pronounced as in MTOF-1. MTOF-2 also has reduced levels of all zeins, as well as a dramatic reduction in the 27-kD γ-zein. MTOF-3 appears to have little or no effect on zein accumulation. Whatever the identities of the mutant genes may be, results of these preliminary analyses support previous observations showing that the loss of vitreous endosperm can result from alterations in zein protein accumulation that may be secondary pleiotropic effects. Alternatively, the loss of vitreous endosperm can result from pathways unrelated to zein proteins. Analysis of PB structure as well as mapping of these mutant genes and determination of their molecular identities should provide insight into the nature of the relationship between zein synthesis and kernel texture.

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