Role of Zein Proteins in Structure and Assembly of Protein Bodies and Endosperm Texture

PROGRESS REPORT and APPENDIX 1-preliminary data

Summary Report

Although funding for this project was initiated less than two years ago, we have made significant progress with our research objectives. We have cloned the gene responsible for the *fl2* mutation. In *fl2*, the mutant phenotype appears to result from a defective signal peptide in an alpha-zein protein. As a consequence, the signal peptide remains attached when the protein accumulates in the protein body. A mutation like *fl2* could explain other semidominant and dominant opaque mutants on the basis of abnormal zein polypeptides. A manuscript describing the research that led to the cloning of *fl2* is in press, and a second manuscript on the characterization of this gene has been prepared for publication (Appendix 2).

We found that increased amounts of the 27-kD gamma-zein protein enlarge the proportion of vitreous endosperm and increases the hardness of *o2* mutants (Geetha *et al.*, 1991). This protein also enhances these properties in wild type seeds (Moro *et al.*, [Appendix 2]). The mechanism by which the gamma-zein protein brings about these changes is unclear, and is under investigation. We have found and characterized several mutants that reduce gamma-zein synthesis. The mutations do not significantly affect synthesis of any other type of zein protein. They appear to create an opaque phenotype by reducing the number rather than the size of protein bodies. Interestingly, the mutant seeds fail to germinate. A manuscript describing one of these mutants, *o15*, has been prepared for publication (Appendix 2).

We have created a number of transgenic tobacco plants that can produce alpha-, beta-, gamma(27-kD)-, or delta-zeins, as well as combinations of these proteins. Analysis of seeds from these plants and crosses of these plants has shown that tobacco endosperm can serve as a heterologous system to study zein interactions. We have obtained evidence that interactions between alpha- and gamma-zein proteins are required for stable accumulation of alpha-zeins in the endosperm. These and other preliminary results are illustrated in Appendix 1.

Identification and Cloning of the fl2 Gene

The *fl2* gene was one of the first mutations found to affect zein synthesis and cause a high lysine phenotype (Nelson *et al.*, 1965). The soft, starchy endosperm of this mutant is associated with a reduction in zein mRNA and protein synthesis, and consequently *fl2* has long been considered to correspond to a regulatory gene, like *o2* (Motto *et al.*, 1989; Schmidt, 1993). However, several features distinguish *fl2* from *o2*. In *fl2* mutants there is a reduction in synthesis of all four zein types that is dosage dependent, as well as a general reduction in the mRNAs encoding these proteins (Lopes *et al.*, 1994). Protein bodies in *fl2* are small, like *o2*, but they grow irregularly and the organization of alpha-, beta- and gamma-zeins is asymmetric compared to the wild type (Lending and Larkins, 1992). Furthermore, the ER as well as the protein bodies contain abnormally high concentrations of BiP (Zhang and Boston, 1992). It was known for many years that *fl2* endosperm contains a novel zein polypeptide that migrates on SDS-PAGE slightly slower than the 22-kD alpha-zeins (Jones, 1976), but the significance of this protein was unclear. Both the gene encoding this protein and *fl2* map to chromosome 4S in a cluster of 22-kD alpha-zein genes (Soave *et al.*, 1981). It was also found that *o2* is epistatic to *fl2* (DiFonzo *et al.*, 1990).

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Based on the above observations, we hypothesized that *fl2* corresponds to an abnormal 22-kD alpha-zein protein. We investigated this using a bulk segregant analysis (Michelmore *et al.*, 1991) of F2 progeny created with a cross of W64A by W64A/*fl2*. We were able to identify a novel 7.7 kb DNA fragment in the *fl2* progeny using a 22-kD alpha-zein gene probe with an *Sst* I digestion. Subsequent Southern analysis of DNA from 79 normal plants of the F2 population did not detect this genomic fragment, whereas it was always found in the *fl2* plants.

To characterize the gene contained in the 7.7 kb *Sst*I fragment, a genomic clone was isolated, and a 1.6 kb *Eco* R1 fragment subcloned and sequenced (Coleman *et al.*, [Appendix 2]). The gene was found to encode a 22-kD alpha-zein protein of 262 amino acids, including a 21 amino acid signal peptide. The deduced protein does not differ in size from the consensus for 22-kD alpha-zeins, but there are two significant amino acid changes. One is the terminal residue of the signal peptide, where a single nucleotide substitution changes an alanine to a valine. The other is an insertion of a histidine residue at position 184, which is in the middle of the seventh alpha-helical repeat.

Shortly after this gene was isolated, we shared the sequence of the protein with Dr. Becky Boston, whose lab has been studying the role of chaperone proteins in zein synthesis and deposition. Concurrent with our work, they had been purifying the novel zein protein in *fl2* by 2-D IEF-PAGE. A few weeks later they discovered that our gene matched the first 45 amino acids of the novel *fl2* protein. Surprisingly, the mature protein contained an intact signal peptide. Thus, it appears that the point mutation altering the cleavage site for signal peptidase plays an important role in the *fl2* defect. At the very least, this accounts for the 2-kD larger size of the polypeptide. It is not difficult to imagine how retention of the signal peptide would cause deformity of the protein bodies, and why it would increase production of the 70-kD chaperone and promote its association with the protein body. Signal peptides have been shown to traverse the ER membrane, with the NH₂-terminal end on the cytoplasmic face and the COOH-end in the lumen of the membrane (Muller, 1992). Failure to cleave the signal peptide has been found to cause proteins to stick to the ER membrane. In the case of *fl2*, we expect the mutant alpha-zein would remain near the surface of the protein body, which would affect the spatial distribution of beta- and gamma-zeins. Interestingly, immunogold labeling of BiP in *fl2* endosperm shows most of the gold particles just beneath the ER surface. (Zang and Boston, 1992). It is possible that the insertion of the histidine residue in the seventh repeated peptide could also be partially responsible for the phenotype, since it changes the orientation of the alpha-helix. Considering that only one *fl2* allele has been found (Emerson, *et al.*, 1935), it is clear that it is a unique gain of function mutation. Experiments are now in progress to verify that this gene causes the *fl2* phenotype, and determine if one or both of these amino acid changes are responsible.

Role of gamma-zein in development of vitreous endosperm

A 2- to 3-fold increase in gamma-zein in *o2* mutants can convert the soft, starchy endosperm to a vitreous phenotype (Wallace *et al.*, 1990a). It is therefore possible that an increased concentration of this protein in normal genotypes could enhance their proportion of vitreous endosperm. To evaluate this, we created a series of modified normal inbreds (Moro *et al.*, [Appendix 2]). These materials were examined for protein content (zeins and non-zeins), density, hardness, and amino acid composition. Some of these lines had gamma-zein contents similar to the modified *o2* parent, while maintaining high concentrations of alpha-zeins. The phenotype of the modified normal seeds was more vitreous than the parental wild type, but the higher gamma-zein content did not increase hardness beyond a threshold level. Nevertheless, all the inbreds exhibited a very hard texture. We interpreted these results to indicate that gamma-zein plays a role in the

development of vitreous endosperm, but other protein components are also involved.

To further investigate the role of gamma-zein in the formation of vitreous endosperm, we began a search for mutations that affect its synthesis, as well as for mutations affecting synthesis of the other cysteine-rich protein, beta-zein. Mutations affecting beta- and gamma-zein synthesis have not previously been described. Because these proteins are encoded by essentially single copy genes, it seemed possible that mutants might exist. We predicted they would have an opaque phenotype, because of the role the proteins play in initiating protein body formation, and based on the fact that high levels of gamma-zein can convert *o2* mutants to a vitreous phenotype (Wallace *et al.*, 1990a). To initiate our search, we analyzed 30 EMS-induced opaque mutants isolated by Dr. Gerry Neuffer at the University of Missouri, as well as 30 *Mutator* (*Mu*)-induced opaque mutants obtained by Dr. Jeff Bennetzen (Purdue University) from Dr. Don Robertson, at Iowa State University. Subsequently, we screened about 4000 *Mu* lines at Pioneer HiBred for opaque mutants, and just recently we have screened an additional 14,000 *Mu* lines created by Dr. Steve Briggs at Pioneer HiBred. The latest screen identified more than 200 opaque mutants, which are currently being analyzed.

Among the 30 EMS-induced opaque mutants identified by Dr. Neuffer, we found three with significantly reduced levels of the 27-kD gamma-zein (Appendix 1, Fig. 1). MGN10 and MGN13 (*o15*) cause approximately a 90% and a 60% reduction, respectively, in the 27-kD gamma-zein. Synthesis of the 16-kD gamma-zein also appears to be reduced. MGN25 causes about a 45% reduction in the 27-kD gamma-zein, but the seed is also missing the embryo. Consequently, we don't know if the reduction in gamma-zein in this mutant is a pleiotropic effect, or if there are two mutations. We have now grown MGN25 for three generations, and in the last planting we obtained opaque seeds with embryos. So, MGN25 may yet prove useful to our studies.

We have begun to characterize the MGN10 and MGN13 (*o15*) mutants biochemically and genetically. The mutations have been crossed into more vigorous lines in order to develop inbreds and to test for allelism. Both mutants fail to germinate, even though the seeds are relatively robust. (Opagues typically are somewhat smaller and less dense than wild-type seeds.) It is somewhat difficult to rationalize the lethality of the phenotype, since the seeds contain significant amounts of storage protein. It could be that gamma-zein provides critical amino acids (sulfur) during the early stages of germination. This protein is rapidly hydrolyzed following imbibition (de Barros and Larkins, 1990), so its deficiency could affect embryo vigor. However, there could be more cryptic effects of the mutation. Because protein differences in mutant seeds can be recognized on segregating ears by 20 days after pollination, we have been able to characterize some of the mutant's biochemical features.

Based on ELISA measurement of zeins in developing and mature endosperm, we estimate there is between 60% and 70% reduction in gamma-zein synthesis in the *o15* mutant compared to the wild-type (Bostwick *et al.*, [Appendix 2]). However, the total amount of alpha-, beta- and delta-zeins is relatively unaffected. We can first detect differences in endosperms at mid-development, so it seems that the reduction in gamma-zein protein synthesis becomes more pronounced as the seed matures. The reduction in this protein leads to a smaller number of protein bodies in the subaleurone cell layers, or so it appears with light microscopy. When normal and mutant endosperm are examined by electron microscopy, immunogold labeling of thin sections with alpha- and gamma-zein antibodies revealed no unusual distribution of zeins in the protein bodies. Furthermore, the protein bodies in normal and mutant endosperm are nearly the same size. Since *o15* endosperm contains at least 30% to 40% the level of wild type gamma-zein, these results are perhaps not surprising. If gamma-zein plays a role in initiating protein body formation,

as well as the retention/organization of alpha-zeins, a reduction in gamma-zein of this magnitude might not have a dramatic effect on the size and structure of protein bodies, although it might affect their number. Since the reduction in gamma-zein does not become significant until mid- to late development (after 18 DAP), the smaller number of protein bodies would be most noticeable in the subaleurone layers. Perhaps it is the smaller number of protein bodies that causes the opaque phenotype.

The impact of the *o15* mutation on protein synthesis is reflected at the mRNA level. RNA blots probed with clones of the 22-kD and 19-kD alpha-zeins, and the 14-kD beta-zein show little difference between the normal and mutant genotypes; however, the 27-kD gamma-zein RNA is reduced by nearly 70%. In many maize genotypes there are two genes (A and B) that encode the 27-kD gamma-zein protein. These genes produce identical polypeptides, but there are differences in their mRNA levels. With the exception of W22 (Das and Messing, 1987), the ratio of A:B transcripts in most inbreds is approximately 2:1 (Or *et al.*, 1993). This is also what we found in the wild type (*O15*) background, but in the *o15* mutant the ratio is reduced to almost 1:1.

We have recently characterized a complex set of genes called "*o2* modifiers" (Or *et al.*, 1993). These genes act through a posttranscriptional mechanism and increase the level of gamma-zein mRNA by 2- to 3-fold. Interestingly, they alter the ratio of A to B mRNAs, increasing it from 2:1 to nearly 4:1. We have mapped one of the *o2* modifier genes near the telomere of chromosome 7L, and this is where *o15* maps. Based on the phenotype of *o15* and its effect on gamma-zein mRNA accumulation, it is plausible that it corresponds to a mutation of an *o2* modifier gene.

We have not yet examined the ultrastructure of the MGN10 mutant, nor characterized its effect on gamma-zein gene expression. Our initial plantings of this mutant did not flower synchronously with the *o15* mutant, so we have been unable to test for allelism. Since MGN10 reduces gamma-zein synthesis by nearly 90%, it may have a more profound effect on protein body formation and number. We plan to more fully characterize this mutant with materials grown this season.

Characterization of Other Opaque/Floury Mutants

During the past two years, we have begun to introduce the previously identified opaque and floury mutations into a single inbred line in order to compare their phenotypic effects in a uniform background. We are within one or two generations of having most of these mutants in W64A, an inbred for which we have cloned many, if not most, of the zein genes (Marks and Larkins, 1982; Shen *et al.*, 1994). Preliminary studies of several of the semidominant and dominant mutations suggest they contain altered alpha-zein polypeptides. SDS-PAGE analysis of *f13*, *h1* (soft starch), *Mc* and *DeB*30* shows novel zein polypeptides in these mutants (Appendix 1, Fig. 2). The *f13* mutation differs from *f12* in that an opaque phenotype is only easily distinguished when there are two or three doses of the mutant allele. *DeB*30* is dominant, while the *h1* mutation is recessive. We are currently developing F2 populations of these mutants to determine if unique RFLPs can be identified that segregate with the mutation. If these relationships hold true, we may be able to clone the mutant genes much the same way *f12* was isolated.

Most of the original opaque and floury mutants were identified as spontaneous mutations, or were found following treatment of pollen with chemical mutagens. In recent years large populations of maize mutants have been created with transposons, such as *Mu* (Bennetzen *et al.*, 1993). We are making an extensive analysis of *Mu*-induced mutants, since this element appears to effectively move about the genome and because such mutants offer the possibility of being able to identify an RFLP associated with the disrupted gene.

This is especially important with mutants for which there is no obvious change in protein phenotype. This broad survey also increases the possibility of finding previously unidentified opaque mutants. From an initial set of 50 *Mu*-induced opaque mutants we obtained from Pioneer HiBred, we identified four that have a strong opaque phenotype, but no noticeable change in zein composition. We are currently growing segregating populations of two of these mutants, PMT10 and PMT11 (Appendix 1, Fig. 2), and they will be analyzed with *Mu* probes later in the summer to determine if an element is linked with the mutation.

Expression of Zein Genes in Transgenic Plants

To study how zeins interact during protein body assembly, we made two sets of expression vectors that direct zein gene expression in transgenic tobacco and rice plants. These are based on rice glutelin promoters, which were shown to function in tobacco endosperm (Takaiwa *et al.*, 1987; Takaiwa *et al.*, 1991). The *gluB-1* and the *gluA-2* promoters we used contain sequences extending approximately 1300 bp 5' from the start of transcription. Gene constructs were made with the rice *gluB-1* promoter and a 22-kD alpha-zein gene and a 27-kD gamma-zein gene. The rice *gluA-2* promoter was fused to the 14-kD beta and the 10-kD delta-zein genes. Constructs with two different zein genes were made by combining the *gluB-1*/22-kD alpha-zein gene and the *gluA-2*/delta-zein gene, and the *gluB-1*/27-kD gamma-zein gene with the *gluA-2*/beta-zein gene. The gene constructs were transferred to a pBIN-19 derived vector, pBI101 (Bevan, 1984), and transformed into tobacco cells using the leaf disk procedure (Horsch *et al.*, 1985). We also sent these constructs to Dr. Nicki Ayres in Dr. Bill Park's lab at Texas A&M University for transformation into rice. Dr. Ayres was able to create transgenic rice plants that produce the 27-kD gamma-zein protein, but most of her other transformants were lost due to a fungal infection. Consequently, we do not yet have extensive data on the rice plants.

A number of transgenic tobacco plants were generated with each of the zein gene constructs, but based on PCR amplification only a small number had intact gene inserts: there are three alpha-zein (alpha-plants B-D), two beta-zein (beta-plants A-B), five gamma-zein (gamma-plants A-E), four delta-zein (delta-plants A-D), six beta-/gamma-zein (505 plants A-F), and two alpha-/delta-zein (501 plants A-B) transgenic plants. None of the alpha-zein plants contained detectable alpha-zein protein in mature seeds (Appendix 1, Fig. 3A); however, zein proteins were present in variable amounts in mature seeds of all the gamma- and delta-zein plants (Appendix 1, Fig. 3B and C). The two beta-zein plants did not appear to contain protein in the mature seed, but of the six beta-/gamma-zein plants tested, three accumulated both proteins (Appendix 1, Fig. 3D). The amount of beta-zein in these seeds was significantly lower than the gamma-zein, suggesting differences in activity of the two rice promoters or variation in protein stability. Assuming similarity in the avidity of the two proteins, which may not be the case, the level of expression of the *gluB-1* promoter is approximately 25-times higher than the *gluA-2* promoter. Regardless of differences in the activity of the rice promoters, this does not explain the marked difference in accumulation of alpha- and gamma-zein proteins, since both hybrid gene constructs have the same promoter.

To examine the basis for the difference in accumulation of the alpha- and gamma-zein proteins in tobacco endosperm, developing seeds were harvested and analyzed for RNA and protein content. The results shown in Appendix 1, Fig. 4A indicate that the *gluB-1* promoter directs expression of both alpha- and gamma-zein genes. RNA transcripts for each gene appeared approximately seven days after pollination (DAP). With the alpha-zein gene, the RNA concentration peaked around 9 DAP and declined until 17 DAP; by 25 DAP it was not detected. With the gamma-zein construct, the RNA concentration peaked between 9 and 11 DAP, but it persisted throughout seed development. Immunoblotting

showed that both proteins were present in developing seeds (Appendix 1, Fig. 4B). However, there was clearly more gamma- than alpha-zein protein, since about 20-times more extract was necessary to detect the alpha-zein. The variation in RNA and protein levels for the alpha- and gamma-zeins could reflect structural differences between the mRNAs. Nevertheless, these results show that while the alpha-zein protein can be synthesized, it is not accumulated in mature seeds.

In order to determine whether interactions between zein proteins affect their accumulation in tobacco endosperm, we developed T-1 progeny by selfing T-0 plants and making reciprocal crosses between the alpha (B) and gamma (A) plants. We also made crosses between the gamma (A) and delta (A) plants and between the beta-/gamma (F) and alpha (B) plants. Proteins recovered from mature seeds of these crosses are shown in Appendix 1, Fig. 5. The effect of producing alpha-zein in the presence of gamma-zein is striking. Reciprocal crosses between the alpha- and gamma-zein plants accumulated alpha-zein protein in mature seed in amounts proportional to the number of alpha-zein genes contributed by the parent (Appendix 1, Fig. 5A). Thus, there is a dramatic stabilizing effect on the alpha-zein protein when gamma-zein is present. The gamma-zein did not appear to cause a similar enhancement in delta-zein accumulation (Appendix 1, Fig. 5B). The cross of the beta-/gamma- zein plant with the alpha-zein plant gave results similar to those with gamma-zein alone (Appendix 1, Fig. 5C). However, our data are insufficient to conclude that there is no effect of beta-zein on alpha-zein accumulation. We need to examine the cross of an alpha-zein and a beta-zein plant, but this must await the creation of a new set of transgenic beta-zein plants

Our experiments to date suggest that interactions between zein proteins are important for their accumulation and, presumably, assembly into protein bodies. Furthermore, it appears that tobacco endosperm will provide a suitable system with which to examine the nature of these interactions. However, additional analyses of seeds from transgenic plants are necessary before conclusions are warranted.

PUBLICATIONS ATTRIBUTABLE TO DOE FUNDING

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