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A defective signal peptide in the maize high-lysine mutant floury 2

(α-zein/endosperm/prolamin/storage protein)

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ABSTRACT **The maize floury 2 (*fl2*) mutation enhances the lysine content of the grain, but the soft texture of the endosperm makes it unsuitable for commercial production. The mutant phenotype is linked with the appearance of a 24-kDa α-zein protein and increased synthesis of binding protein, both of which are associated with irregularly shaped protein bodies. We have cloned the gene encoding the 24-kDa protein and show that it is expressed as a 22-kDa α-zein with an uncleaved signal peptide. Comparison of the deduced N-terminal amino acid sequence of the 24-kDa α-zein protein with other α-zeins revealed an alanine to valine substitution at the C-terminal position of the signal peptide, a histidine insertion within the seventh α-helical repeat, and an alanine to threonine substitution with the same α-helical repeat of the protein. Structural defects associated with this α-zein explain many of the phenotypic effects of the *fl2* mutation.**

Between 50% and 60% of the protein in maize (*Zea mays* L.) kernels consists of a mixture of prolamin storage proteins known as zeins. These proteins are rich in proline and glutamine but lack lysine, making the seed nutritionally inferior for monogastric animals. The lysine deficiency of maize spurred extensive efforts to identify mutants with higher levels of this essential amino acid. Thirty years ago the opaque 2 (*o2*) and floury 2 (*fl2*) mutants were shown to have elevated levels of lysine in the endosperm protein (1, 2). These observations generated a great deal of optimism regarding the potential development of high-lysine corn, but the soft, starchy endosperm of these mutants causes the kernels to be susceptible to mechanical damage and creates a higher susceptibility to insect and fungal damage. Consequently, neither mutant gained wide commercial application.

Despite their phenotypic similarities, distinctive biochemical differences distinguish *o2* and *fl2* mutants. The *o2* mutation is recessive, while the *fl2* mutation is semidominant, with the severity of the phenotype correlated to the dosage of the mutant allele. Both mutants exhibit reduced zein accumulation in the endosperm, but the *o2* mutation specifically affects the 22-kDa α-zeins (3), while the *fl2* mutation equally affects synthesis of all classes of zeins (4, 5). Protein bodies in *o2* and *fl2* endosperm are smaller than normal, but *fl2*-encoded protein bodies are asymmetrical and misshapen compared to the spherical protein bodies of normal and *o2* endosperm (6). The zein protein profile and the morphology of the protein bodies in *o2fl2* double mutants more closely resemble those found in *o2* alone, indicating that *o2* is epistatic to *fl2* (7, 8). The *O2* gene has been isolated and shown to encode a leucine zipper-type transcription factor that controls expression of the 22-kDa family of α-zein genes (9), but the basis of the *fl2* defect is unknown.

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The *fl2* mutation is associated with enhanced levels of binding protein (BiP), the 75-kDa endoplasmic reticulum (ER) molecular chaperone, that becomes localized near the periphery of ER-derived protein bodies (10–13). Only one *fl2* allele has been identified (14), and this, taken together with its semidominant pattern of inheritance, suggests that it is a unique gain-of-function mutation. The *fl2* mutation maps to the short arm of chromosome 4, within a cluster of α-zein genes.

A unique feature of *fl2* endosperm is the appearance of an unusual 24-kDa polypeptide in the zein fraction (5, 15). Recently, we demonstrated that this 24-kDa protein is the product of an α-zein gene tightly linked to the *fl2* locus. We also identified a restriction fragment length polymorphism (RFLP) associated with the *fl2* locus that cross-hybridizes to a 22-kDa α-zein cDNA (8). Here we report the cloning of that RFLP and show that it includes the coding sequence for an α-zein precursor that is not processed in *fl2* maize endosperm.¶

MATERIALS AND METHODS

Plant Materials and Chemicals. Maize (*Z. mays* L.) kernels of the normal inbred line W64A+, the mutant inbred line W64A*fl2*, and crosses between the two were obtained from plants grown in the field at the University of Arizona or North Carolina State University. Leaf material used for extraction of DNA was obtained from plants grown in a growth chamber.

Restriction endonucleases were purchased from Bethesda Research Laboratories, Life Technologies (Gaithersburg, MD), digoxigenin 11-UTP was from Boehringer Mannheim, deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate was from DuPont/NEN, bisacrylamide was from Bio-Rad, acrylamide was from Amresco (Solon, OH), and nylon membranes were from Micron Separations (Westboro, MA). All other chemicals were reagent grade and were purchased from Sigma or Bethesda Research Laboratories unless otherwise indicated.

Construction of a Maize Genomic Library and Analysis of the Cloned DNA. Genomic DNA was extracted from lyophilized leaf tissue according to an established protocol (16). A 10-μg aliquot of genomic DNA from W64A*fl2* was digested with 50 units of *Sst* I restriction enzyme for 4 h at 37°C and electrophoresed on a 0.7% agarose gel at 15 V/cm for 16 h. After staining the gel with ethidium bromide to visualize the DNA, a slice of the gel containing fragments in the range 7.5–8.0 kb was removed and the DNA was purified with a GeneClean kit (Bio 101). A maize genomic library was constructed with the size-fractionated DNA inserted into the λ ZAP II vector according to the manufacturer's instructions

Abbreviations: BiP, binding protein; ER, endoplasmic reticulum; RER, rough ER; RFLP, restriction fragment length polymorphism.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L34340).

(Stratagene). The resulting library of $\approx 2 \times 10^5$ plaque-forming units was screened with a 22-kDa α-zein cDNA probe and a single clone was isolated.

For Southern blot analysis, each lane was loaded with 10 μg of genomic DNA that was digested with 50 units of *Sst* I for 4 h at 37°C. For reconstruction of single copy sequence, 30 pg of the cloned DNA was added to the genomic DNA prior to digestion. The gels were transferred to a nylon membrane and the blots were hybridized to the specified probes. The probes were prepared by labeling with digoxigenin 11-UTP (17) and bands were detected by a chemiluminescent assay (18). DNA sequence was obtained with a Sequenase version 2.0 sequencing kit (United States Biochemical). Comparison of α-zein sequences was performed with the GCG software package (Genetics Computer Group, Madison, WI).

Zein Protein Purification and N-Terminal Sequencing. Protein bodies were isolated from endosperms collected 20 days after pollination (DAP) (19) and solubilized in a buffer consisting of 9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 1.6% Bio-Lyte 6/8 ampholyte (Bio-Rad), and 0.4% Bio-Lyte 3/10 ampholyte. Samples containing 50 μg of total protein were analyzed by two-dimensional isoelectric focusing SDS/polyacrylamide gel electrophoresis according to an established protocol (20), except that they were loaded at the acidic end of the tube gels in order to maintain the solubility of the zeins. For protein purification, zeins were prepared from 20 DAP kernels for reversed-phase chromatography as described (21), except that 2-mercaptoethanol was omitted from the extraction buffer. Reversed-phase chromatography was performed as described (22) on an RP-300 column (Sci-con, Atlanta), with a 45–65% acetonitrile column gradient, and peaks containing 24-kDa α-zeins from W64A*fl2* were separated by two-dimensional electrophoresis as described above. The gel was stained with Pro Green (Integrated Separation Systems, Natick, MA) and the 24-kDa α-zein was eluted from gel slices for N-terminal sequencing using a Hewlett-Packard G1005S protein sequencing system (23).

RESULTS

The 22-kDa α-zein gene in the inbred line W64A*fl2* is distinguished as a 7.7-kb DNA fragment after restriction enzyme digestion with *Sst* I. This fragment is marked with an arrow in the *fl2* lane of Fig. 1. Size-fractionated *fl2* genomic DNA between 7.5 and 8.0 kb was inserted into the λ ZAP II vector and a single clone, designated pCC515, was obtained by screening the resulting library with a 22-kDa α-zein cDNA probe. To confirm that the proper fragment had been cloned, a quantity of pCC515 DNA equivalent to a single copy gene was added to genomic DNA from the normal inbred and the mixture was subjected to Southern blot analysis (Fig. 1, lane 515). The banding pattern obtained from the DNA mixture was indistinguishable from that of W64A*fl2*, except for a 7-kb band not present in the mutant genome (compare lanes *fl2* and 515).

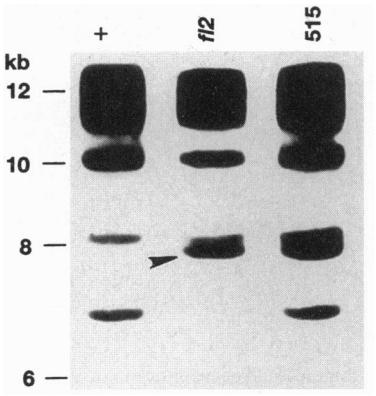

FIG. 1. Southern blot of genomic DNA from the normal inbred W64A (lane +), the mutant inbred W64A*fl2* (lane *fl2*), and W64A with pCC515 plasmid DNA equivalent to a single copy gene (lane 515). DNA in each lane was digested with *Sst* I and bands were detected after hybridization to a 22-kDa α-zein cDNA probe. Arrowhead in lane *fl2* identifies a 7.7-kb RFLP linked to the *fl2* locus. Size of markers is given in kb on the left.

Linkage of pCC515 to the *fl2* locus was assessed by probing *Sst* I-digested genomic DNA from plants grown from normal F_2 seed with a 700-bp *Pst* I/*Eco*RI fragment of pCC515. Reduced homology between the probe and the 8.0-kb band that forms a doublet with the 7.7-kb band on Southern blots allowed us to distinguish the two. None of 79 normal plants analyzed had the 7.7-kb fragment, suggesting tight linkage between the *fl2* locus and the cloned gene of pCC515 (Fig. 2; data not shown).

The nucleotide sequence was obtained for a 1.6-kb *Eco*RI fragment of pCC515 that cross-hybridizes to the 22-kDa α-zein cDNA probe (data not shown). As shown in Fig. 3, the gene contained in this DNA encodes an α-zein of the 22-kDa subfamily. The deduced amino acid sequence gives a polypeptide of 262 amino acids, including a 21-amino acid signal peptide. Comparison of the amino acid sequence of this protein with those of other 22-kDa α-zeins revealed three potentially significant alterations: an alanine to valine substitution at the C-terminal (−1) residue of the signal peptide; an insertion of a histidine following the seventh residue of the seventh α-helical repeat of the protein; and an alanine to threonine substitution within the same α-helical repeat (Fig. 4). The 5′-TCCACGTAGA-3′ motif, common to genes regulated by the *O2* transcriptional activator, is located 297 bp upstream of the translation start site (data not shown) (26).

To determine the relationship between this gene and the 24-kDa polypeptide in *fl2* endosperm, we purified the protein by two-dimensional isoelectric focusing SDS/polyacrylamide gel electrophoresis. Protein bodies obtained from developing endosperm of W64A and W64A*fl2* were extracted and the zeins were separated by two-dimensional gel electrophoresis (Fig. 5). Comparison of the pattern of proteins from the normal and mutant inbreds identified one 22-kDa α-zein polypeptide specific to normal endosperm and two 24-kDa polypeptides unique to *fl2*. The 24-kDa polypeptide indicated with an open arrow in Fig. 5*B* was isolated and the N-terminal sequence was determined. The sequence of the first 45 amino acids of this protein is identical to the deduced N terminus of pCC515. Interestingly, the signal peptide that targets the protein to the lumen of the rough ER (RER) is retained, indicating an absence of processing and providing an explanation for the shift in molecular mass of the molecule.

DISCUSSION

The alanine to valine substitution at the −1 position of the signal peptide provides a plausible explanation for its retention

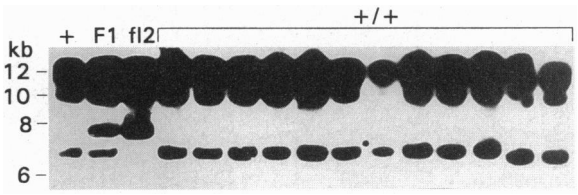

FIG. 2. Assessment of linkage of the insert in pCC515 to the *fl2* locus by Southern blot analysis. Blot contains *Sst* I-digested genomic DNA from W64A (lane +), W64A*fl2* (lane fl2), the F_1 progeny of these two (lane F1), and normal progeny from a segregating F_2 population (lanes +/+). Bands were detected by hybridization to a 0.7-kb *Pst* I/*Eco*RI fragment of pCC515. The larger band of the doublet shown in Fig. 1 is not present on this blot because of a lack of homology with the shorter probe. Size of markers is given in kb on the left.

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-21  M  A  T  K  I  L  A  L  L  A  L  L  A  L  L  V  S  A  T  N
     ATG GCT ACC AAG ATA TTA GCC CTC CTT GCG CTT CTT GCC CTT TTA GTG AGC GCA ACA AAT
 -1  V  F  I  I  P  Q  C  S  L  A  P  S  A  I  I  P  Q  F  L  P
     GTG TTC ATT ATT CCA CAG TGC TCA CTT GCT CCT AGT GCC ATT ATT CCA CAG TTC CTC CCA
 20  P  V  T  S  M  G  F  E  H  P  A  V  Q  A  Y  R  L  Q  L  V
     CCA GTT ACT TCA ATG GGC TTC GAA CAT CCA GCC GTG CAA GCC TAT AGG CTA CAA CTA GTG
 40  L  A  A  S  A  L  Q  Q  P  I  A  Q  L  Q  Q  Q  S  L  A  H
     CTT GCG GCG AGC GCC TTA CAA CAA CCA ATT GCC CAA TTG CAA CAA CAA TCC TTG GCA CAT
 60  L  T  L  Q  T  I  A  T  Q  Q  Q  Q  H  F  L  P  S  L  S  H
     CTA ACC CTA CAA ACC ATC GCA ACG CAA CAA CAA CAA CAT TTT CTG CCA TCA CTG AGC CAC
 80  L  A  V  V  N  P  V  A  Y  L  Q  Q  Q  L  L  A  S  N  P  L
     CTA GCA GTG GTG AAC CCT GTC GCC TAC TTG CAA CAG CAG CTG CTT GCA TCC AAC CCA CTT
100  A  L  A  N  V  A  T  Y  Q  Q  Q  Q  Q  L  Q  Q  F  M  P  A
     GCT CTG GCG AAC GTA GCT ACA TAC CAG CAA CAA CAA CAG CTG CAA CAG TTT ATG CCA GCG
120  L  S  Q  L  A  M  V  N  P  A  V  Y  L  Q  L  L  S  S  P
     CTC AGT CAA CTA GCC ATG GTG AAC CCT GCC GTC TAC CTA CAA CTG CTT TCA TCT AGC CCG
140  L  A  V  G  N  A  P  T  Y  L  Q  Q  Q  L  L  Q  Q  I  V  P
     CTC GCT GTG GGC AAT GCA CCT ACG TAC CTA CAA CAA CAG TTG CTG CAA CAG ATT GTA CCA
160  A  L  T  H  Q  L  A  M  A  N  P  A  T  Y  L  Q  Q  L  L  P
     GCT CTA ACT CAT CAG CTA GCT ATG GCA AAC CCT GCT ACC TAC TTA CAA CAG TTG CTT CCA
180  F  N  Q  L  A  V  S  N  S  A  A  Y  L  Q  Q  R  Q  Q  L  L
     TTC AAC CAA TTG GCT GTG TCG AAC TCT GCT GCG TAC CTA CAA CAG CGA CAA CAA TTA CTT
200  N  P  L  A  V  A  N  P  L  V  A  T  F  L  Q  Q  Q  Q  L  L
     AAT CCA TTG GCA GTG GCT AAC CCA TTG GTC GCT ACC TTC CTG CAG CAG CAA CAA TTG CTG
220  P  Y  N  Q  F  S  L  M  N  P  A  L  Q  Q  P  I  V  G  G  A
     CCA TAC AAC CAG TTC TCT TTG ATG AAC CCT GCC TTG CAG CAA CCC ATC GTT GGA GGT GCC
240  I  F  *
     ATC TTT TAG
```

FIG. 3. Nucleotide sequence of the coding region of the α-zein gene from pCC515 and its deduced amino acid sequence. Numbers on the left correspond to position of the first amino acid of each line beginning with −21 to reflect the signal peptide, so that the −1 position is occupied by the C-terminal residue of the signal peptide and the +1 position is occupied by the first amino acid of the predicted mature polypeptide.

on the mutant α-zein protein and would explain many of the phenotypic effects of the *fl2* mutation. According to von Heijne's "−3−1 rule" for signal peptides, the −1 position is critical for recognition by signal peptidase and is generally occupied by an uncharged amino acid with a small side chain (27). Of 161 eukaryotic signal peptides surveyed, none had valine at the −1 position, while nearly half those surveyed had alanine in that position (28).

Previous studies showed that a normal zein profile is produced when poly(A)$^+$ RNA from wild-type endosperm was incubated in the presence of microsomes from *fl2* endosperm (29). These results demonstrate that the ER in *fl2* endosperm is competent for processing zein polypeptides and are consistent with the defect arising from an unusual zein gene transcript in *fl2* endosperm. Furthermore, preliminary results show that when an alanine codon is substituted for the valine codon of the mutant α-zein gene by site-directed mutagenesis, the *in vitro* translated protein product is processed correctly in the presence of maize microsomes (data not shown).

Mutant proteins that retain their signal peptide have been described in humans (30, 31) and yeast (32); these proteins remain attached to the luminal surface of the ER membrane and fail to proceed through the secretory pathway. It has been found that a zein signal peptide will anchor alcohol dehydrogenase (ADH1) to the ER membrane when it is not cleaved from the chimeric protein (33). The 24-kDa α-zein described here represents an observable phenotype associated with a defective signal peptide in plants.

We propose that the 24-kDa α-zein in *fl2* endosperm remains anchored to the RER membrane and thereby disrupts the normal biogenesis of protein bodies. In normal protein body development, zein proteins are retained within the ER where they coalesce into spherical bodies in which α-zeins are localized to the interior of a shell of cross-linked β- and γ-zeins (34). Attachment of an α-zein to the RER membrane would inhibit its movement into the interior of the protein body. Interaction of the RER-attached α-zein with the shell of β-

```
A   -21                                                          -1
pCC515 M A T K I L A L L A L L A L L V S A T N V
pZ22.1 . . . . . . . . . . . . . . . . . . . . A
pZ22.3 . . . . . . S . . . . . . F A . . . . . A
ZSF4C1 . . . . . . S . . . . . V F A . . . . . A
ZSF4C2 . . . . . . . . . . . . . F . . . . . A

B   156                                                          174
pCC515 Q I V P A L T H Q L A M A N P A T Y L
pZ22.1 . . . . . . . . . . . . V . . . . A . .
pZ22.3 . . . . . . . . . . . . V . . . V A . .
ZSF4C1 . . . . . . . . . . . . V V . . . A . .
ZSF4C2 . N L . . . . . . . . . . . . . . A . .
```

FIG. 4. Amino acid sequence alignment of the signal peptide (*A*) and the seventh α-helical repeat from α-zein proteins encoded by pCC515 and four additional maize 22-kDa α-zein clones (*B*): pZ22.1 and pZ22.3 (24) and ZSF4C1 and ZSF4C2 (25). Valine residue at the −1 position, histidine at position 163, and threonine at position 172 of the protein encoded by pCC515 are in boldface to highlight these changes with respect to the other four polypeptides. Number above the first amino acid indicates its position within the protein as shown in Fig. 3.

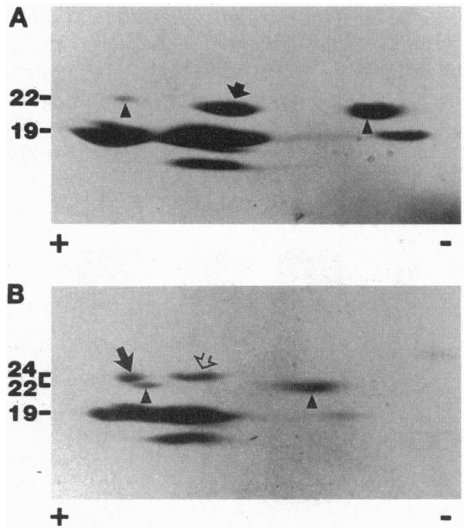

FIG. 5. Two-dimensional gel separation of α-zeins from the normal inbred W64A (*A*) and the mutant W64A*fl2* (*B*). Numbers on the left are apparent molecular masses in kDa based on known values for α-zeins. The anode is to the left and the cathode is to the right. In *A*, large arrow marks a 22-kDa α-zein that is absent in *B*. Two arrows in *B* mark ≈24-kDa α-zein proteins unique to *fl2* endosperm; protein marked with the open arrow was purified and the sequence of the first 45 amino acids was determined. Arrowheads identify polypeptides that are common to both genotypes.

and γ-zeins may disrupt the spatial organization of proteins in developing protein bodies by forming multiple foci for α-zein aggregation near the surface of the ER membrane.

Retention of the signal peptide could explain the overexpression of BiP in *fl2*, since this RER-resident chaperone is known to bind nonnative polypeptide chains (35, 36) and has been implicated in the normal translocation of proteins into the ER (37, 38). Induction of BiP in *fl2* endosperm may also reflect the insertion or substitution of amino acid residues in the seventh α-helical repeat of the protein. The 22-kDa α-zeins consist of 10 repeated peptide segments that are believed to form α-helical structures important to the packaging of the protein into protein bodies through intra- and intermolecular interactions (39–41). Changes in amino acid sequence within one of these repeats could alter the secondary structure of the protein and/or its interactions with other zeins. Induction of BiP has been observed in response to changes in protein structure or oligomerization of subunits in other systems (35).

The structural defect in the 24-kDa α-zein is the result of a specific point mutation and is consistent with the fact that only one *fl2* allele has been identified. This explains the difficulty in generating new *fl2* alleles by mutagenesis, especially transposon tagging. A defect in a structural protein as we describe here is also consistent with the observed pattern of semidominant inheritance. Furthermore, epistasis of *o2* to *fl2* can be explained by the presence of the *O2* binding site in the promoter region of the 24-kDa α-zein gene. In the absence of a functional *O2* transcriptional activator, the 24-kDa α-zein gene would not be transcribed, and thus the phenotypic effects of the mutation would not be observed. These data in combination with the genetic linkage data shown here provide compelling evidence that the 24-kDa α-zein gene is the causative agent of the *fl2* phenotype.

A defect in zein processing and folding could profoundly affect the secretory pathway, which would in turn lead to a generalized reduction in storage protein synthesis. Nevertheless, in the case of neither *fl2* nor *o2* is it obvious why these genetic defects lead to an enhanced synthesis of lysine-containing proteins. In both *o2* and *fl2* kernels, decreased zein synthesis and changes in protein body morphology are accompanied by increased abundance of several cytoplasmic proteins. The physiological function of these proteins is unknown, but several are lysine-rich (42). With the identification of a molecular defect associated with the *fl2* phenotype, we are better able to study the biochemical and physiological interactions that are responsible for the pleiotropic effects of the mutation.

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