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Expression of a mutant α-zein creates the *floury2* phenotype in transgenic maize

(endosperm/prolamin)

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ABSTRACT **The maize *floury2* mutation results in the formation of a soft, starchy endosperm with a reduced amount of prolamin (zein) proteins and twice the lysine content of the wild type. The mutation is semidominant and is associated with small, irregularly shaped protein bodies, elevated levels of a 70-kDa chaperone in the endoplasmic reticulum, and a novel 24-kDa polypeptide in the zein fraction. The 24-kDa polypeptide is a precursor of a 22-kDa α-zein protein that is not properly processed. The defect is due to an alanine-to-valine substitution at the C-terminal position of the signal peptide, which causes the protein to be anchored to the endoplasmic reticulum. We postulated that the phenotype associated with the *floury2* mutation is caused by the accumulation of the 24-kDa α-zein protein. To test this hypothesis, we created transgenic maize plants that produce the mutant protein. We found that endosperm in seeds of these plants manifests the *floury2* phenotype, thereby confirming that the mutant α-zein is the molecular basis of this mutation.**

Zeins are prolamin storage proteins that accumulate in the endosperm of maize (*Zea mays* L.) seeds. They are composed of four different types of polypeptides, classified as α-, β-, γ-, and δ-zeins (1). Accretions of zein proteins form spherical protein bodies within the lumen of the endoplasmic reticulum (ER), and there is a distinct spatial arrangement of these proteins within a protein body: β- and γ-zeins are located on the periphery, whereas α- and δ-zeins are found in the interior (2, 3). Collectively, the zein proteins are rich in glutamine and proline, but they lack lysine and tryptophan. Because zeins constitute such a large proportion of the total seed protein (60–70%), the amino acid composition of these proteins causes the grain to be of inferior nutritional quality for monogastric animals.

Efforts to improve the protein quality of maize seed have focused on mutants in which zein synthesis is reduced and the lysine content is increased. The first "high-lysine" mutants to be identified were opaque2 (*o2*) and floury2 (*fl2*) (4, 5). Unfortunately, the favorable nutritional quality of these mutants is offset by the inferior physical properties of their soft, starchy endosperms. It appears that the starchy endosperm of the *o2* and *fl2* mutants is caused by changes in the nature of their protein bodies. The *o2* mutation affects a transcriptional activator of a subset of α-zein genes, leading to a reduction in α-zein protein synthesis and the formation of protein bodies that are significantly smaller than normal (6–9). The *fl2* mutation, which is semidominant, causes a decrease in synthesis of all classes of zeins, and the resultant protein bodies are not only smaller than normal, but they are also asymmetrical and misshapen (10, 11). Another feature of *fl2* endosperm is the overexpression of the ER-resident binding protein (BiP), which becomes deposited at the periphery of the mutant protein bodies (12–15).

We have postulated that the phenotype associated with *fl2* is caused by the accumulation of a novel 24-kDa α-zein protein (16, 17). This hypothesis is partially based on tight linkage between the gene encoding the 24-kDa protein and the *fl2* locus, but it is also consistent with the abnormal structure of the protein. The mutant protein is 2 kDa larger than expected, because of a defect in its processing following targeting to the ER lumen. An alanine-to-valine substitution at the C-terminal position of the signal peptide prevents its removal, thereby anchoring the protein to the lumenal face of the ER membrane (18). To investigate whether this protein is responsible for the mutant phenotype, we transformed normal maize plants with the gene encoding the mutant 24-kDa α-zein protein. We show here that seeds of these plants manifest the key phenotypic characteristics associated with the *fl2* mutation.

MATERIALS AND METHODS

Transformation of Maize Plants. Maize embryos were cotransformed with the 24-kDa α-zein gene in plasmid pCC515 (17) and the bacterial bialaphos (*BAR*) gene (19) as a selectable marker. The 24-kDa α-zein gene in pCC515 is flanked by 3.0 kb of 5′ and 3.7 kb of 3′ noncoding sequences. The selectable marker gene plasmid is of the form *ubi*::*ubiintron*::*BAR*::*pinII*, where *ubi* is a maize ubiquitin promoter and first intron and *pinII* is the potato protease inhibitor II 3′ noncoding sequence. Plasmid DNAs were delivered by microprojectile bombardment to embryogenically responsive, immature embryos from the maize variety High Type II (20). Embryos were recovered from herbicide-resistant calli and grown to maturity. Transgenic plants were outcrossed as the female parent to an inbred line, and the progeny were scored for the floury kernel phenotype.

PCR Amplification of the 24-kDa α-Zein Gene. Genomic DNA was extracted from seedlings germinated from normal and floury kernels (21). The DNA was amplified by PCR using primers corresponding to the signal peptide coding sequences of the 24-kDa α-zein gene (5′-GCCCTTTTAGTGAGCG-CAACAAATGTG-3′) and coding sequences for the seventh α-helical repeat of the protein (5′-GCAGGGTTTGCCAT-AGCTAGCTGATG-3′). Products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed with a Polaroid DS-34 camera.

Protein Extraction from Maize Flour and Immunoblotting. A portion of each endosperm was cut from the seed prior to

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Abbreviations: BiP, binding protein; ER, endoplasmic reticulum.

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germination and converted into a fine flour using a ball mill. Proteins were extracted from the meal and separated according to their solubility in 70% alcohol (22). Alcohol-soluble (zein) proteins were separated by SDS/PAGE, blotted onto nitrocellulose, and immunoreacted with rabbit anti-α-zein polyclonal antibody (23). Alcohol-insoluble proteins were separated by SDS/PAGE, blotted, and immunoreacted with a rabbit anti-BIP polyclonal antibody (12). Goat anti-rabbit alkaline phosphatase conjugate was used for indirect detection of α-zein and BiP on the immunoblots (24).

Fixation and Embedding of Endosperms and Electron Microscopy. Seeds were harvested 18 days after pollination from a self-fertilized maize plant that was hemizygous for the 24-kDa α-zein transgene. Endosperms were fixed, embedded, sectioned, and viewed with a transmission electron microscope as described elsewhere (11).

RESULTS

Detection of the Transgene in Transformed Maize Seedlings. Transgenic maize plants were generated by a biolistic method using microprojectiles coated with plasmid pCC515 (17), which contains the 24-kDa α-zein gene within a genomic DNA fragment, and a plasmid containing the *BAR* gene (20), which confers resistance to the herbicide Basta. Plants from 25 herbicide-resistant events that were recovered from the transformation were crossed as females to an untransformed inbred line. F_1 progeny from 17 of these crosses segregated approximately 1 to 1 for floury-appearing kernels, consistent with the presence of a single site of transgene integration. To determine whether this phenotype was associated with the insertion of the 24-kDa α-zein gene, we used PCR primers for the coding region of the 24-kDa α-zein gene to amplify genomic DNA from F_1 seedlings. A 560-bp fragment was produced from DNA of the floury seedlings (Fig. 1*A*, lanes 4, 6, and 8), but not the wild-type seedlings (Fig. 1*A*, lanes 3, 5, and 7). A fragment of similar size was amplified from W64A*fl2* DNA (Fig. 1*A*, lane 9), but not from DNA of untransformed seedlings (Fig. 1*A*, lanes 1 and 2).

Immunodetection of α-Zein Proteins from Seeds of Transgenic Plants. An immunoblot of α-zein proteins from mature seeds was prepared to determine whether insertion of the 24-kDa α-zein gene resulted in synthesis of this protein (Fig. 1*B*). The blot shows that the appearance of a 24-kDa protein band, indicated by arrowheads in lanes 4, 6, and 8 of Fig. 1*B*, was always associated with the floury phenotype and insertion of the transgene. The 24-kDa protein band in these samples is similar to a band of identical molecular mass that was found in W64A*fl2* zein (indicated by an arrowhead in lane 9 of Fig. 1*B*). The 24-kDa α-zein was not present in samples from normal progeny or seeds of untransformed plants (Fig. 1*B*, lanes 1, 2, 3, 5, and 7). Three bands with molecular masses greater than 24 kDa were detected in all of the samples. In W64A*fl2,* these polypeptides are glycosylated forms of 19-kDa α-zein proteins that are products of a gene(s) closely linked to the *fl2* locus (J. W. Gillikin and R. S. Boston, personal communication). Apparently, the same glycoproteins are present in the maize line used for the transformation, although they had no effect on the kernel phenotype.

Analysis of BiP Expression in Seeds of Transgenic Plants. One phenotypic characteristic associated with the *fl2* mutation is overexpression of the 70-kDa ER-resident chaperone, BiP. Using anti-BiP antibody, an immunoblot of the alcohol-insoluble proteins showed that the amount of BiP in mature

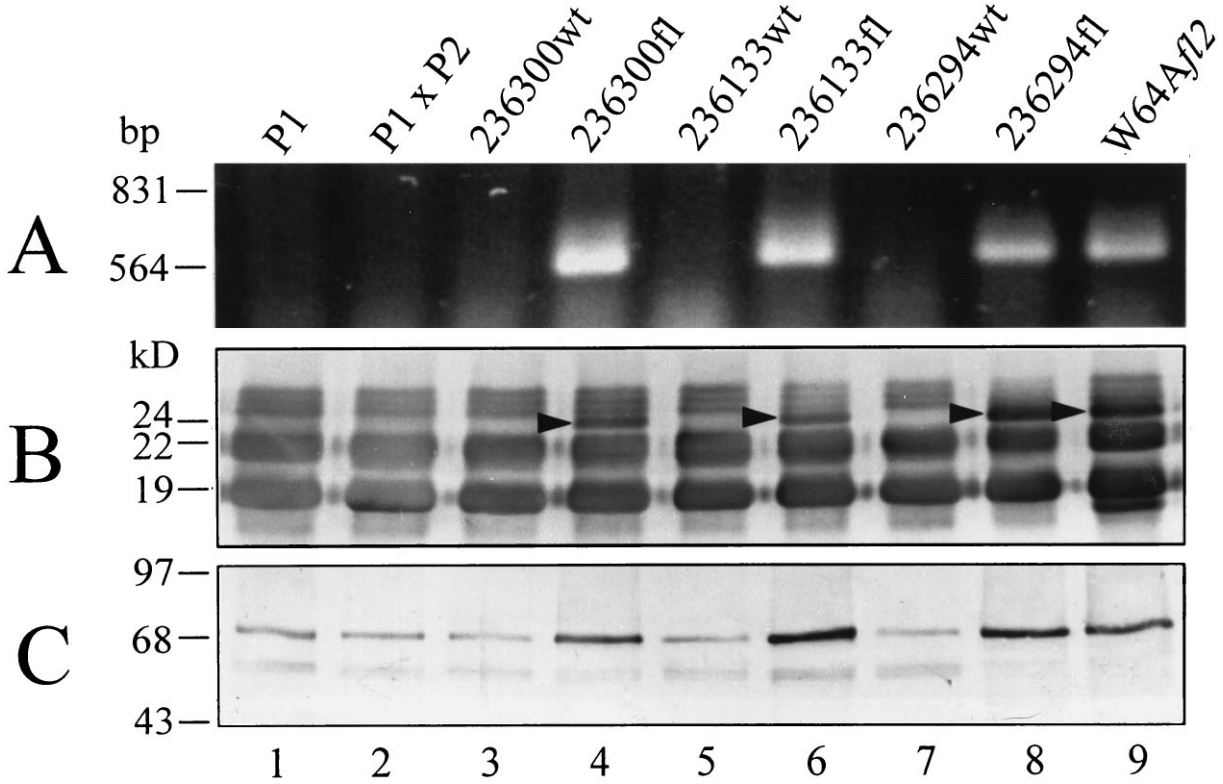

FIG. 1. Transformation of the 24-kDa α-zein gene into transgenic maize plants leads to synthesis of the encoded protein and overexpression of BiP. Analysis of 3 of 17 independent transgenic lines is shown. One normal and one floury kernel from transgenic lines 236300 (lanes 3 and 4), 236133 (lanes 5 and 6), and 236294 (lanes 7 and 8) were analyzed and compared with kernels from the untransformed (lane 1, P1), the untransformed parent outcrossed to a normal inbred (lane 2, P1 × P2), and W64A*fl2* (lane 9) plants. (*A*) Using PCR primers specific to the 24-kDa α-zein gene, a 560-bp DNA fragment was amplified from genomic DNA of seedlings germinated from floury kernels, but not wild-type kernels. (*B*) An immunoblot of α-zeins shows a 24-kDa protein band (indicated by arrowheads) in samples from floury kernels, but not wild-type kernels. (*C*) An immunoblot of the 70-kDa maize homolog of BiP.

transgenic kernels was comparable to that of W64A*fl2* (Fig. 1*C*, lanes 4, 6, 8, and 9). Normal quantities of BiP were detected in wild-type siblings and untransformed kernels (Fig. 1*C*, lanes 1, 2, 3, 5 and 7).

Protein Body Structure in the Endosperms of Seeds of Transgenic Plants. Another phenotypic characteristic associated with the *fl2* mutation is the formation of misshapen protein bodies. Protein bodies in the developing endosperm of seeds not expressing the 24-kDa α-zein gene are circular in cross-section and are relatively discreet, similar to the wild type (Fig. 2*A*; ref. 2), whereas those from seeds expressing the gene have a convoluted shape and aggregate into large masses of protein, similar to W64A*fl2* (Fig. 2*B*; ref. 11). Analysis at higher magnification revealed that protein bodies in the developing endosperm of seeds not expressing the 24-kDa α-zein gene contain darkly staining zein proteins primarily on the periphery of the protein bodies, as is seen in wild type (Fig. 2*C*, arrowheads; ref. 2). In contrast, those from endosperms expressing the gene have many regions of darkly staining zein in their interior (Fig. 2*D*, arrowheads; ref. 11), indicating an abnormal organization of α-, β-, and γ-zeins within the protein body.

DISCUSSION

Previous studies provided strong genetic evidence implicating a mutant 24-kDa α-zein protein as the cause of the phenotype

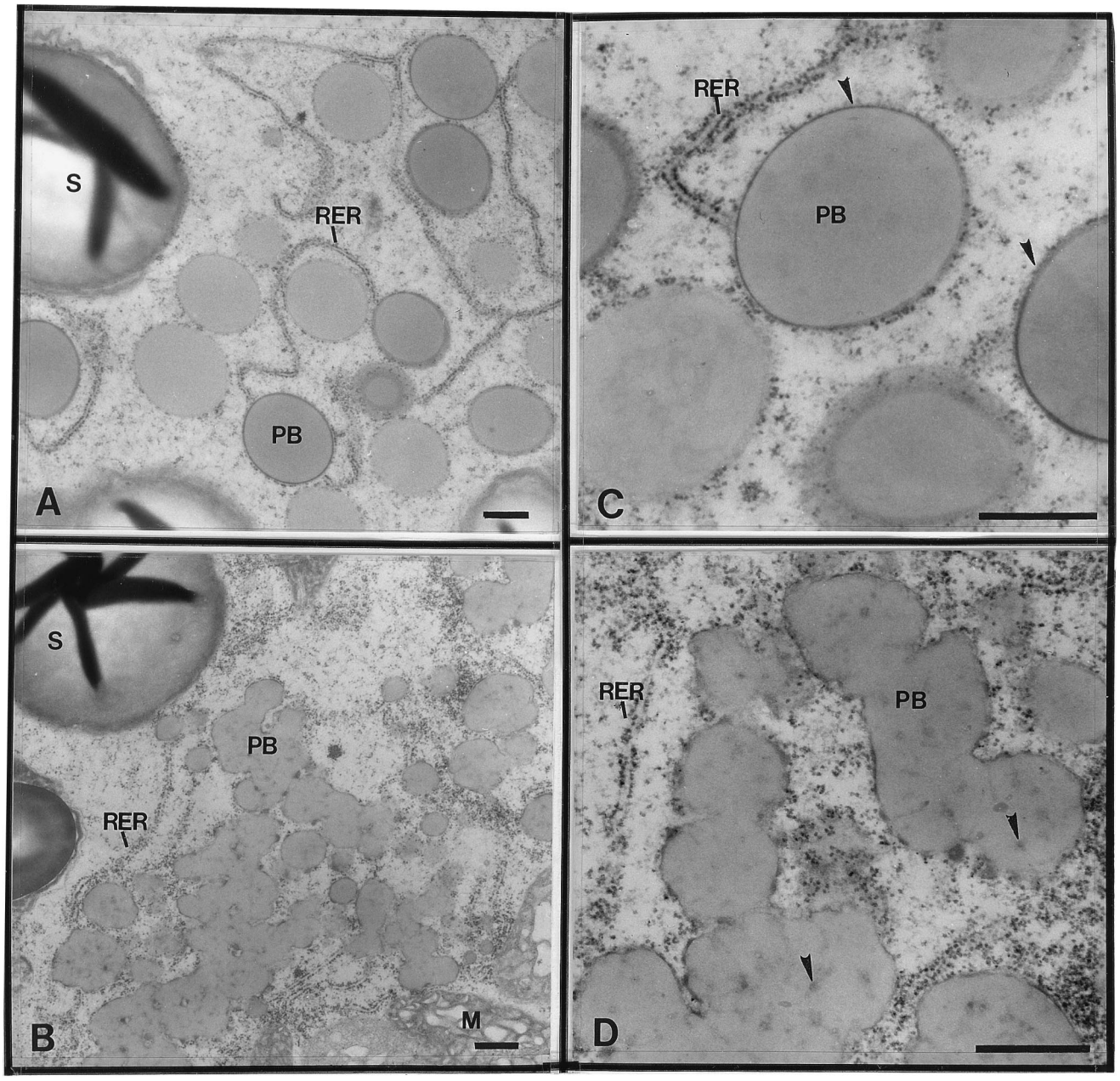

FIG. 2. Comparison of protein bodies from wild-type and transgenic maize endosperm. Protein bodies in the endosperm of wild-type seeds are round (*A* and *C*) compared with the misshapen protein bodies in the endosperm of seeds expressing the 24-kDa α-zein gene (*B* and *D*). Comparison of these protein bodies at lower magnification revealed that they appear as discreet spheres (circles in cross-section) in cells not expressing the 24-kDa α-zein gene (*A*), whereas they have a convoluted shape and are aggregated in cells expressing this gene (*B*). Comparison of these protein bodies at higher magnification highlights their differences in the internal structure. In cells not expressing the gene, the darkly staining zein proteins are found primarily on the periphery of the protein bodies (*C*, arrowheads), whereas in cells expressing the gene, the protein bodies have many locules of darkly staining zein in their interior (*D*, arrowheads). The irregularities seen in *B* and *D* are similar to those found in protein bodies of W64A*fl2*. PB, protein body; RER, rough endoplasmic reticulum; S, starch grain. Bars = 0.5 μm.

associated with the maize *fl2* mutation (16, 17). The experiments described here show that expression of the mutant gene in transgenic maize plants leads to the accumulation of the 24-kDa α-zein protein, which is correlated to the overexpression of BiP and the appearance of malformed protein bodies. Because these characteristics of the seed from the transgenic plants resemble the phenotype of the *fl2* mutant, our observation conclusively demonstrates that *fl2* is a structural mutation in a 22-kDa α-zein gene.

The mechanism whereby the 24-kDa α-zein protein disrupts the normal development of the protein body, leading to an altered texture of the mature endosperm, is not known. One possible explanation is that the mutant hydrophobic α-zein protein is anchored to the ER membrane and remains on the outer surface of the protein body, thereby disrupting protein interactions that are important to the maintenance of the spherical shape. Normally, α-zein proteins become sequestered in the interior of the protein body (2). This hypothesis is supported by evidence showing that the 24-kDa α-zein protein is associated with the membrane fraction following translation and translocation in the presence of microsomes (18).

Our results provide evidence of the utility of maize transformation for the analysis of genetic mechanisms. The amount of the 24-kDa α-zein protein detected in the transgenic floury kernels and the W64A*fl2* kernel was similar, suggesting that the expression of the transgene may be comparable to that of the native gene. This result is encouraging, because it demonstrates that transgene expression in tissues of transformed maize plants can be adequately controlled by native promoters. Furthermore, the 3 kb of 5′ and 3.7 kb of 3′ noncoding sequence included in the α-zein genomic clone must be sufficient for directing appropriate temporal and spatial expression in the endosperm; however, it is not known whether the gene is transcribed in other tissues of the transgenic plants.

Traditionally, techniques for stable transfer of DNA to monocotyledonous cereals have lagged behind similar work with dicotyledonous plants (25). Transformation of dicot species usually involves gene delivery through infection with *Agrobacterium tumefaciens*, but monocots are not readily amenable to infection by this bacterium. Although Ishida *et al.* (26) reported stable maize transformants following infection by *A. tumefaciens*, most successful transformations of maize plants have used microprojectile bombardment to deliver DNA to embryogenically responsive cells from immature embryos. Since the first report of the generation of stable transgenic maize plants using the biolistic method (27), this technique has been used to introduce a number of agronomically important traits into corn, such as insect resistance (20, 28), viral resistance (29), and fructan production (30). These advances, along with our report, attest to a promising future for the use of transgenic technologies in the genetic study and agronomic improvement of maize.

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