Cytoskeletal Proteins Are Coordinately Increased in Maize Genotypes with High Levels of eEF1A[1][w]

Jose A. Lopez-Valenzuela, Bryan C. Gibbon, David R. Holding, and Brian A. Larkins*

Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721

The *opaque2* (*o2*) mutation increases the Lys content of maize (*Zea mays*) endosperm by reducing the synthesis of zein storage proteins and increasing the accumulation of other types of cellular proteins. Elongation factor 1A (eEF1A) is one of these proteins, and its concentration is highly correlated with the amount of other Lys-containing proteins in the endosperm. We investigated the basis for this relationship by comparing patterns of protein accumulation and gene expression between a high (Oh51A*o2*) and a low (Oh545*o2*) eEF1A inbred, as well as between high and low eEF1A recombinant inbred lines obtained from their cross. The content of α-zein and several cytoskeletal proteins was measured in high and low eEF1A inbred lines, and the levels of these proteins were found to correlate with that of eEF1A. To extend this analysis, we used an endosperm expressed sequence tag microarray to examine steady-state levels of RNA transcripts in developing endosperm of these genotypes. We identified about 120 genes coordinately regulated in association with eEF1A content. These genes encode proteins involved in several biological structures and processes, including the actin cytoskeleton, the endoplasmic reticulum, and the protein synthesis apparatus. Thus, higher levels of eEF1A in *o2* mutants may be related to a more extensive cytoskeletal network surrounding the rough endoplasmic reticulum and increased synthesis of cytoskeleton-associated proteins, all of which contribute significantly to the Lys content of the endosperm.

The discovery that the *opaque2* (*o2*) mutation almost doubles the Lys content of maize (*Zea mays*) endosperm (Mertz et al., 1964) led to extensive efforts to develop genotypes with better nutritional quality for human and animal diets. Unfortunately, the inferior agronomic traits associated with this mutation, including reduced yield and protein content, soft kernels, and pathogen susceptibility, made it difficult to develop *o2* in breeding programs (Glover and Mertz, 1987). To overcome these problems, maize breeders at International Center for Development of Maize and Wheat, Mexico (Villegas et al., 1992) and the University of Natal, South Africa (Gevers and Lake, 1992) developed modified *o2* mutants called Quality Protein Maize (QPM). These genotypes combine the improved nutritional quality trait of *o2* with those responsible for vitreous endosperm and normal yield. However, additional improvement of protein quality is required for Quality Protein Maize to meet the minimal Lys content (5 mg/100 mg of protein) recommended for human nutrition (Young et al., 1998).

Several studies have documented a broad range of variability in Lys content among normal, *o2*, and modified *o2* maize genotypes (Moro et al., 1996; Zarkadas et al., 2000), showing that the effect of the mutation is highly dependent on the genetic background. These studies suggested the Lys content of the kernel can be improved by appropriate selection. However, identification of more nutritional maize genotypes has been limited by knowledge of the genetic and biochemical basis for Lys accumulation in the endosperm. *O2* encodes a basic domain/Leu zipper transcription factor that regulates the expression of a number of genes, including the highly abundant α-zeins (Kodrzycki et al., 1989; Schmidt et al., 1990). Defects in O2 lead to a significant reduction in α-zein synthesis and a pleiotropic increase in the synthesis of several Lys-rich, nonzein proteins (Damerval and Devienne, 1993; Habben et al., 1993). Both of these effects contribute to the increased Lys content of the endosperm (Moro et al., 1996), but the molecular mechanisms responsible for the larger amount of Lys-rich proteins are unknown.

Habben et al. (1993) investigated the nature of the Lys-containing proteins that are increased in *o2* and found that elongation factor 1A (eEF1A), a protein synthesis factor, is highly increased compared to wild type. Analysis of a large number of normal and *o2* inbreds with extensive variability in Lys and eEF1A content showed this protein is highly correlated (r = 0.9) with the protein-bound Lys of the endosperm (Habben et al., 1995; Moro et al., 1996). However, the biological basis of this relationship is unclear. eEF1A contains 11% Lys, but this protein only accounts for 2.3% of the Lys in W64A*o2* endosperm (Sun et al., 1997). This suggests a stoichiometric relationship exists between eEF1A and the other major proteins contributing to the Lys content of the grain.

One possible explanation for the relationship between eEF1A and other Lys-rich proteins in maize

endosperm is that eEF1A is a component of the cytoskeleton (Durso and Cyr, 1994) that can bind both actin filaments (Yang et al., 1990) and microtubules (Kuriyama et al., 1990; Ohta et al., 1990). Biochemical characterization of eEF1A from maize endosperm revealed this protein binds F-actin (Sun et al., 1997; Lopez-Valenzuela et al., 2003) and immunolocalization showed eEF1A is associated with an F-actin network that surrounds the rough endoplasmic reticulum (RER) at sites where protein bodies are forming (Clore et al., 1996). Wang et al. (2001) found a quantitative trait locus linked with eEF1A content that mapped with a cluster of 22-kD α-zein genes. Based on this association, they proposed a positive correlation between eEF1A content and the surface area of protein bodies, hence the network of cytoskeletal proteins surrounding the RER in the endosperm. Thus, the level of eEF1A may reflect the amount of proteins that are components of the cytoskeleton in maize endosperm.

This study was initiated to identify proteins that vary in abundance in parallel with eEF1A. The inbred lines Oh51A$o2$ (high eEF1A) and Oh545$o2$ (low eEF1A), as well as high and low eEF1A recombinant inbred lines (RILs) derived from their cross, were used for ELISA measurement of endosperm proteins and for mRNA transcript profiling with cDNA microarrays. The results of this study revealed a number of genes that are coordinately regulated with eEF1A content, several of which encode proteins associated with the actin cytoskeleton and the endoplasmic reticulum (ER), as well as components of the protein synthesis machinery.

RESULTS

Development of Recombinant Inbred Lines with High and Low eEF1A Content

To monitor patterns of gene expression and protein synthesis in high and low eEF1A genotypes, we developed a set of 75 RILs by single seed descent from a cross between Oh51A$o2$, a high eEF1A inbred, and Oh545o2, a low eEF1A inbred. After six generations of self-pollination, each of the inbred lines appeared to be homogeneous based on phenotypic uniformity in the field. The level of eEF1A in these lines at the F_6 generation was measured by ELISA and found to exist in a 2-fold concentration range, similar to that observed in the F_3 (Wang et al., 2001). The variation in eEF1A content of these genetically fixed and related lines allowed us to study gene expression patterns and their relationship to endosperm Lys content in $o2$ mutants.

The parental inbreds, Oh545$o2$ and Oh51A$o2$, and a subset of 10 RILs representing the range of eEF1A variation were first compared for total zein and nonzein protein content in mature endosperm. Protein extracts from equal amounts of endosperm flour of each genotype were partitioned into zein and nonzein fractions (Wallace et al., 1990). Figure 1A illustrates a Coomassie Blue-stained gel showing the nature and

relative abundance of various zein proteins in the parental inbreds and the RILs. The accumulation of subfamilies of zein proteins is quite different between Oh545$o2$ and Oh51A$o2$; the amount of 19- and 22-kD α-zeins is much lower in Oh545$o2$ than Oh51A$o2$ (Fig. 1A), and this appears to account for most of the 50% decrease in total zein content in this inbred compared to Oh51A$o2$ (Fig. 1B). The amount of 50-kD γ-zein is less in Oh545$o2$ than Oh51A$o2$, while the amount of 27-kD γ-zein appears to be slightly greater in this inbred compared to Oh51A$o2$. The relative abundance of 16-kD γ-zein could not be estimated, because this protein migrated too close to the 19-kD α-zeins in the gel. The abundance of 15-kD β-zein was too low to be compared among these inbreds. The 10-kD δ-zein appears to be less abundant in Oh545$o2$ than Oh51A$o2$.

A diverse pattern of zein accumulation was observed in the RILs developed from the Oh51A$o2$ by Oh545$o2$ cross (Fig. 1A), but some of these lines closely resembled the parental inbreds. For example, the zein profiles of RILs 4-10, 31-5, and 39-1 are very similar to that of Oh545$o2$ (Fig. 1A), and the amount of total zein in these lines is also similar to Oh545$o2$ (Fig. 1B). On the other hand, the zein profiles of RILs 34-3 and 37-3 are qualitatively similar to that of Oh51A$o2$ (Fig. 1A), but the amount of total zein in these lines is 20% to 25% less than in Oh51A$o2$ (Fig. 1B). This is mainly because of a reduction in α-zeins, although there appeared to be a slight increase in the synthesis of the 27-kD γ-zein (Fig. 1A).

We measured the nonzein protein content in endosperms of these inbreds using the Bradford assay, and the level of eEF1A by ELISA (Fig. 2). There is about 2-fold more nonzein protein in Oh51A$o2$ (5.7 mg/100 mg of flour) than Oh545$o2$ (2.8 mg/100 mg of flour; Fig. 2A). The nonzein content of the RILs ranged from about 2.5 mg/100 mg of flour in 31-5 to about 4.5 mg/100 mg of flour in 34-3 (Fig. 2A). The pattern of eEF1A accumulation in the parental inbreds and the RILs (Fig. 2B) was similar to that observed for nonzein content (Fig. 2A). The difference in eEF1A content between Oh545$o2$ and Oh51A$o2$ is about 2-fold, and the range of eEF1A contents in the RILs was also about 2-fold. As with nonzein content, the lines with the lowest and highest eEF1A content were RILs 31-5 and 34-3, respectively (Fig. 2B). It is worth noting that these two lines also have zein profiles similar to those of the corresponding low and high eEF1A parents (Fig. 1A).

To test the hypothesis that proteins associated with the cytoskeleton were present in higher concentrations in genotypes with increased eEF1A content (Wang et al., 2001), the levels of eEF1A, actin, profilin, and α-zein were measured in mature endosperms of Oh545$o2$, Oh51A$o2$, and 10 of their RILs by ELISA (Fig. 3). This analysis showed that the relative contents of actin and profilin closely paralleled those of eEF1A. Actin and profilin proteins were about 1.8-fold higher in Oh51A$o2$ than Oh545$o2$, and the differences in their protein levels in RIL 34-3 compared to RIL 31-5 were about 1.9- and 1.7-fold greater, respectively. The
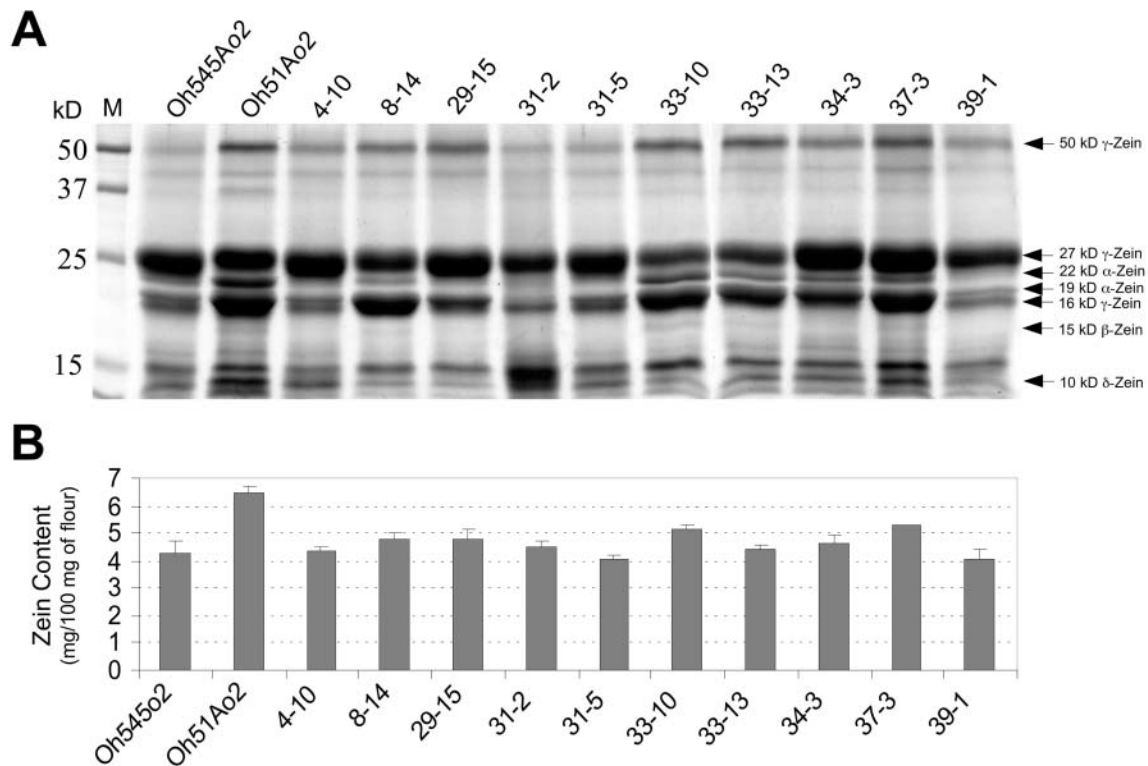
Figure 1. SDS-PAGE and quantification of zeins in mature endosperm of Oh545o2, Oh51Ao2, and 10 RILs with high and low eEF1A contents. A, Total zein extracts were prepared from 750 μg of endosperm flour of the indicated genotypes, separated by 12.5% (w/v) SDS-PAGE, and stained with Coomassie Brilliant Blue R-250. Prestained molecular mass standards are shown on the left. B, Total zein content was estimated using BCA reagent and BSA as standard. The values are the average of two independent extractions in which three measurements were taken.

relative amounts of eEF1A shown in Figure 3 correspond to those in Figure 2, but they are included here for reference. The levels of α-tubulin could not be determined in mature endosperms, perhaps because of protein instability. The relative content of α-zein was determined using antibodies that do not distinguish between the 19- and 22-kD subclasses. There was about four times more α-zein in Oh51Ao2 than Oh545o2 and about two times more α-zein in RIL 34-3 than RIL 31-5 (Fig. 3). Except for RIL 31-2, which had only about 50% of the α-zein content of Oh545o2, the levels of α-zein proteins correlated with those of eEF1A. In contrast, the levels of 27-kD γ-zein did not correlate with the levels of eEF1A (data not shown).

Microarray Analysis

To investigate the pattern of gene expression more broadly in high and low eEF1A genotypes, selected inbreds were analyzed using an endosperm cDNA microarray from the Maize Gene Discovery Project (Fernandes et al., 2002). Poly(A)$^+$ RNA was extracted from 18 d-after-pollination (DAP) endosperms of the high eEF1A inbred lines, Oh51Ao2 and RIL 34-3, and the low eEF1A inbreds, Oh545o2 and RIL 31-5. This stage of development (18-DAP) was chosen because most endosperm cells are differentiated and have high

levels of gene expression, as reflected by large amounts of starch and storage protein synthesis (Demason, 1997). For each genotype, two RNA samples were prepared from two different ears to account for biological variability. Following poly(A)$^+$ isolation, cDNA was synthesized and labeled with Cy3- and Cy5-dUTP. For comparison of each high and low eEF1A inbred, we used two RNA samples with reciprocal labeling, for a total of four slides that resulted in eight replicates because the array was duplicated on each slide. DNA microarray hybridization data were normalized as described in "Materials and Methods" and the fluorescence intensity ratio of the two probes was determined. The expression ratio from the eight replicates was used for statistical analysis. All the replicate slides were analyzed in parallel, and only the probes with an adjusted probability value $q < 0.05$ were considered significant.

We initially identified 540 probes that showed statistically significant differences in gene expression levels for both the parental inbreds and the RILs (see supplemental data, available at www.plantphysiol.org), of which about 343 corresponded to zein sequences, mainly 19-kD α-zeins. The 605 microarray is not normalized, and consequently these probes are highly redundant. A significant amount of redundancy was also found for a number of other genes.
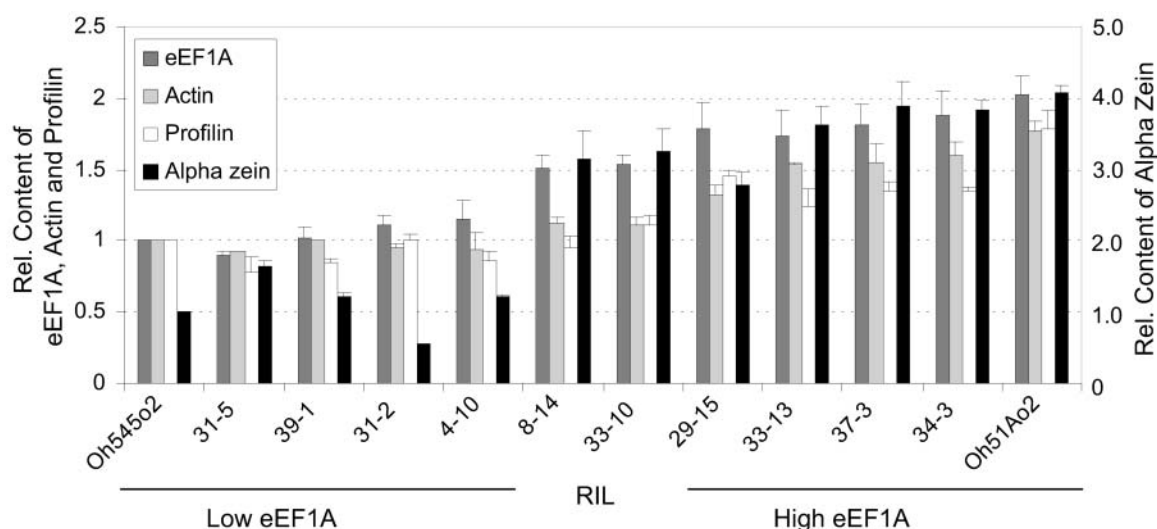
A

B
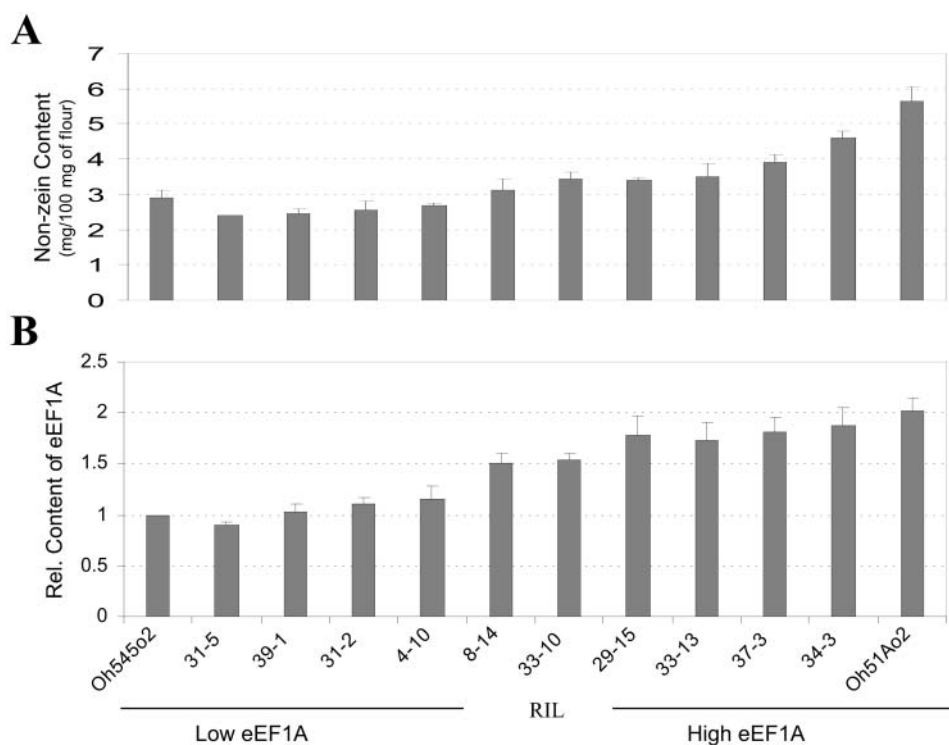
Figure 2. Nonzein quantification and ELISA measurement of eEF1A content in mature endosperm of Oh51Ao2, Oh545o2, and 10 RILs with high and low eEF1A content (see Fig. 1). Protein extracts were prepared from endosperm flour as described in "Materials and Methods." A, Nonzein content was estimated using Bradford reagent and BSA as standard. The order of samples is the same as that identified in B. B, ELISA measurement of eEF1A was conducted as described in "Materials and Methods," and the results were normalized to the values of the Oh545o2 inbred. In both A and B, the results are the average of two independent extractions in which three measurements were taken.

This array contains about 2,800 tentative unique genes among 5,534 expressed sequence tags (ESTs; Fernandes et al., 2002). However, the probe redundancy allowed us to accurately document some measurements, because in many cases different ESTs of the same gene showed similar changes in expression levels. In some cases, multiple ESTs corresponding to the same gene showed slightly different expression ratios, which may have resulted from differences in hybridization efficiency due to variable target/probe length and overall base-pair composition (Xu et al., 2001). After correction for redundancy, about 120 genes encoding proteins of known function were found to be differentially expressed, the majority of which were up-regulated in high eEF1A genotypes.

Table I shows a summary of the genes that changed expression in parallel with eEF1A content. The functional classification of these genes was based on their

Figure 3. ELISA measurement of eEF1A, actin, profilin, and α-zein content in mature endosperm of Oh51Ao2, Oh545o2, and 10 RILs with high and low eEF1A content. Protein extracts were prepared from endosperm flour. ELISA measurements of the indicated proteins were conducted as described in "Materials and Methods," and the results were normalized to the values of the Oh545o2 inbred. The results are the average of two independent experiments in which three measurements were taken.

Table I. *Summary of microarray results*

EST ID	Function	Oh51Ao2/Oh545o2 Fold Change	34-3/31-5 Fold Change	Top BLAST Hit BLASTN	Top BLAST Hit BLASTX
		Amino Acid Metabolism			
AI673901	Acetolactate synthase (EC 4.1.3.18) precursor	6.444	2.203	gi|21213093	gi|22139
AI664892	Putative ketol-acid reductoisomerase	5.618	1.831	gi|21206785	gi|34911874
AI676992	Putative lipoamide dehydrogenase	3.935	2.071	gi|21207606	gi|34894958
AI665281	Putative sarcosine oxidase	2.361	1.736	gi|21209418	gi|34894598
AI664857	Homo-Cys S-methyltransferase-1[e]	2.169	2.098	gi|10732784	gi|10732785
AI668313	Putative acetolactate synthase	2.131	1.718	gi|21208121	gi|30685071
		Carbohydrate Metabolism			
AI833375	Putative Suc cleavage protein	4.856	1.792	gi|21216346	gi|7488353
AI668434	Pyruvate dehydrogenase E1 α-subunit	3.227	1.846	gi|21209231	gi|3851005
AI677414	Putative phosphofructokinase α-subunit	3.210	1.776	gi|21209419	gi|29647436
AI746218	Pyruvate, orthophosphate dikinase	2.959	1.698	gi|21206738	gi|168586
AI677069	Suc synthase (Sus1)	1.751	1.630	gi|514945	gi|514946
		Cell Wall Synthesis/Turnover			
AI833405	Putative Hyp-rich glycoprotein	6.237	2.068	gi|21207317	gi|34394453
AI677486	Putative 2-dehydro-3-deoxyphosphooctonate aldolase	3.640	1.867	gi|21217159	gi|29367487
AI712112	Putative α-L-arabinofuranosidase/β-D-xylosidase	3.062	1.803	gi|21216968	gi|38344900
		Cytoskeleton			
AI740034	Putative actin	5.248	2.134	gi|21206665	gi|24496452
AI740030	Putative actin	4.535	1.787	gi|21210184	gi|28301928
AI677353	Actin-1 (*MAc1*)	0.307	0.243	gi|21207800	gi|113220
		Molecular Chaperonin			
AI668124	Putative 82-kD heat shock protein	5.310	1.714	gi|21206616	gi|7546186
AI833418	Putative heat shock hsp20 protein	4.404	1.601	AZM3_4963[a]	gi|15221027
AI833616	Protein disulfide isomerase	3.996	1.826	gi|21211194	gi|625148
AI745903	Putative cyclophilin ROC7	3.568	1.657	gi|21206974	gi|168461
AI665058	Putative calmodulin-binding heat shock protein	2.894	1.592	gi|21211155	gi|7489106
AI820388	DnaJ-related protein ZMDJ1	2.187	1.621	NH[b]	gi|2984709
		Membrane Transport/Function			
AI665354	Voltage-dependent anion channel protein 1a	5.115	2.257	gi|21207948	gi|5929928
AI670271	Putative translocase inner membrane-like protein	4.681	2.138	gi|21211219	gi|34394571
AI665496	Adenine nucleotide translocator	3.874	1.876	gi|22161	gi|22162
AI665590	Putative ethylene-responsive small GTP-binding protein	3.467	1.888	gi|21213302	gi|33146687
AI668193	H+-pyrophosphatase	3.294	1.760	gi|1049254	gi|1049255
AI668232	Putative nodulin N21 family protein	2.929	1.766	AZM3_44524	gi|15238103
AI714547	Putative YGL010w-like protein	2.906	1.822	gi|21211893	gi|2982301
AI820289	Putative acyl carrier protein	2.884	1.718	gi|21211812	gi|453189
AI668423	Putative lipid transfer protein	2.876	1.623	AZM3_33030	gi|21553541
AI820380	Toc34-2	2.346	1.797	gi|7259223	gi|7259224
AI712220	Putative phosphate/phosphoenolpyruvate translocator	2.233	1.694	gi|21213398	gi|27476065
AI668512	Small basic membrane integral protein ZmSIP2-1	2.114	1.684	gi|13447816	NH
AI676864	Putative amino acid transport protein	1.638	1.923	gi|21213436	gi|38345408
		Metabolism/Oxidoreductase			
AI665560	Putative cytochrome P450	6.384	1.772	gi|27464999	gi|17980361
AI712147	Met adenosyltransferase 1-like	4.426	2.381	gi|19172416	NH
AI668366	Putative succinyl-CoA ligase α-subunit	3.995	1.713	gi|21207397	gi|34393512
AI670285	DWARF3	2.866	1.840	gi|21209514	gi|987267
AI668359	Putative aluminum induced protein wali7	2.657	1.650	gi|21209487	gi|7489671

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

EST ID	Function	Oh51Ao2/Oh545o2 Fold Change	34-3/31-5 Fold Change	Top BLAST Hit BLASTN	BLASTX
AI759056	Putative nicotianamine aminotransferase A	2.370	1.967	gi|21207437	gi|6498122
AI746000	Putative oxidase	2.321	1.701	TC198800[c]	gi|21105122
AI739897	Putative 5′-phosphoribosyl-5-aminoimidazole synthetase	2.221	1.774	gi|21214521	gi|34897250
AI670559	Ferredoxin III (Fd) isoprotein	0.407	2.058	gi|168472	gi|168473
AI677085	Ferredoxin III, chloroplast precursor	0.405	1.731	gi|21207226	gi|168473
	Lipid Metabolism				
AI795367	Putative acyl-CoA synthetase	3.978	2.198	AZM3_44857	gi|27754483
AI665554	Putative GDSL family lipase	3.244	1.810	AZM3_16045	gi|7486138
	Protein Turnover				
AI711859	Putative 26S proteasome triple-A ATPase subunit4	5.336	1.950	gi|21213166	gi|11094192
AI746055	Putative 26S proteasome non-ATPase chain S5a	3.875	1.945	gi|21206642	gi|11281550
AI714537	Putative Clp protease ATP-binding subunit	3.764	1.649	gi|21212366	gi|18423503
AI665136	Ubiquitin conjugating enzyme	2.789	1.603	gi|21215571	gi|2668744
AI739846	Putative Clp protease	2.699	1.822	gi|21211614	gi|1168978
AI657385	Putative proteasome subunit α-type 1	2.349	1.661	gi|21208015	gi|1709758
AI668422	Putative ubiquitin-specific protease	1.907	1.670	gi|21207637	gi|11993471
AI714629	Putative SGT1	1.648	1.748	gi|21207770	gi|2984709
	Seed Protein				
AI657407	19-kD α zein[e]	10.663	1.830	gi|16305120	gi|16305121
AI664915	15-kD β zein[e]	6.062	0.421	gi|16305114	gi|16305115
AI668413	22-kD α zein[e]	5.255	2.651	gi|22215	gi|22216
AI711821	α Globulin	2.189	1.628	gi|16305141	gi|16305142
AI673885	18-kD δ zein	1.995	0.282	gi|16305116	gi|16305117
AI746160	16-kD γ zein	1.909	1.817	gi|16305110	gi|168666
AI657420	10-kD δ zein[e]	0.213	0.179	gi|340933	gi|511870
	Signal Transduction				
AI737044	Putative polo-like kinase	5.574	1.748	gi|21214227	gi|11340595
AI674011	Putative phosphoprotein phosphatase (EC 3.1.3.16)	5.517	1.850	gi|14333242	gi|11358619
AI740053	Putative GAST1-like protein	4.876	2.250	gi|21439749	gi|21439750
AI664994	Putative Ca^{2+}-binding EF-hand protein	4.570	1.736	gi|21214443	gi|18409649
AI665571	Putative phytosulfokine peptide precursor (PSK1)	3.887	2.169	gi|23466412	gi|23466413
AI670625	Putative phytosulfokine peptide precursor	3.531	1.784	gi|21211096	gi|23466413
AI711744	Protein phosphatase 2C	2.870	1.815	gi|21206843	gi|20146110
AI711829	Protein phosphatase type-2C	2.740	1.892	NH	gi|12003990
AI673979	Putative cell elongation protein DIMINUTO	2.387	2.045	gi|21207143	gi|37534052
AI746070	Putative protein phosphatase	2.152	1.635	gi|21210550	gi|26006493
AI746164	Putative transducin/WD-40 repeat protein	1.980	1.856	gi|21213317	gi|18416416
AI711873	VPS29-like phosphoesterase-related protein	0.243	0.188	gi|21207398	gi|7487365
	Stress/Defense Response				
AI668414	Putative proteinase inhibitor	5.514	2.227	gi|21211819	gi|7451428
AI665244	Cytosolic ascorbate peroxidase	4.341	2.037	gi|600115	gi|600116
AI657458	β-glucosidase aggregating factor	1.882	2.029	gi|21209445	gi|9313027
	Transcription/RNA Processing				
AI820389	Putative nucleolar autoantigen	7.101	1.983	gi|21215410	gi|15232342
AI664855	Putative RNA binding protein Rp120	6.557	1.803	gi|21217062	gi|32492578
AI677310	Putative heterogeneous nuclear ribonucleoprotein A1	5.713	2.112	gi|21211013	gi|38175737
AI746091	Translation initiation factor 5A	4.882	1.934	gi|2668737	gi|2668738
AI795508	Putative ribonucleoprotein F	4.455	2.010	gi|21211198	gi|10177116
AI833840	ABA-inducible gene for Gly-rich protein	3.941	1.640	gi|22312	NH

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

EST ID	Function	Oh51Ao2/Oh545o2	34-3/31-5	Top BLAST Hit	
		Fold Change	Fold Change	BLASTN	BLASTX
AI833456	Putative ETTIN-like protein 2	3.921	2.092	gi|21209173	gi|19352035
AI673982	Putative DNA-binding protein	3.488	2.126	gi|21207037	gi|7447216
AI711809	CRP1	3.479	1.685	NH	gi|3289002
AI746090	Putative CAF protein	3.295	1.652	gi|21216651	gi|34902240
AI833470	Putative RNA polymerase II subunit Rpb10	3.190	1.909	gi|21207629	gi|30682258
AI665397	Gly-rich protein[e]	3.170	1.802	gi|22292	NH
AI746156	Histone H3	3.143	2.135	AZM3_80817	gi|122087
AI759090	Putative OsNAC6-like protein	3.110	1.734	gi|21213270	gi|25458553
AI795298	Putative cohesin-like protein	2.949	1.856	gi|21213668	gi|32309542
AI833442	Putative zinc finger transcription factor	2.828	1.708	gi|21211573	gi|34906436
AI712222	Putative snRNP associated protein	2.826	1.815	gi|21209956	gi|33146808
AI667754	Histone H3 gene (H3C2)	2.730	1.792	gi|168494	gi|168493
AI833929	Putative nucleolar protein	2.710	2.054	gi|21209364	gi|34899158
AI665498	DNA binding protein S1FA	2.704	1.844	gi|21208641	gi|1173349
AI746026	Fertilization-independent endosperm protein 1	2.648	1.949	gi|13444304	gi|28192549
AI670631	Putative RNA polymerase II fifth largest subunit	2.631	1.737	AZM3_23172	gi|15233533
AI833836	Putative nuclear RNA binding protein A	2.430	1.768	gi|21206684	gi|34910712
	Translation				
AI668514	Putative eukaryotic initiation factor 3B	11.434	2.024	gi|21209497	gi|37537002
AI820279	Putative 60S ribosomal protein L37a	6.959	2.034	gi|14332189	gi|34911166
AI676990	Putative 60S ribosomal protein L13a	6.482	2.217	gi|21213066	gi|21326116
AI711802	Putative ribosomal protein S29	4.636	2.135	gi|21213327	gi|34915198
AI665388	Putative translational elongation factor Tu	4.229	1.819	gi|21208587	gi|11181616
AI711673	Putative ribosomal protein L27	3.433	1.694	gi|21207613	gi|37536910
AI665325	Putative 40S ribosomal protein S2	3.332	1.729	gi|21207125	gi|30103021
AI759043	Mitochondrial 26S rRNA	3.264	1.601	gi|342661	NA[d]
AI833872	Putative ribosomal protein S15	2.912	1.720	gi|21212912	gi|34897190
AI820275	Putative ribosomal protein L18a	2.899	1.832	gi|21206699	gi|34909590
AI676941	Acidic ribosomal protein P0	2.837	1.928	gi|1550813	gi|1550814
AI668357	Putative elongation factor EF-2	2.788	2.053	gi|21216529	gi|38344860
AI746166	25S rRNA	2.472	1.785	gi|13397940	NA
AI745842	Putative fibrillarin 2	2.256	1.943	gi|21206882	gi|18416588
AI668498	Ribosomal protein L39	1.985	1.878	gi|1177368	gi|1177369
AI746161	Putative translation elongation factor eEF-1 β[e]	1.799	1.714	gi|14332585	gi|38175744
AI677351	40S ribosomal protein S6	0.309	0.248	gi|21207495	gi|1172818
AI711882	Putative ribosomal protein L18a	0.244	0.174	gi|21206699	gi|34909590

[a]Accession numbers beginning with AZM3 were obtained from the maize genomics consortium genome survey build 3.0.　[b]NH, No significant hits obtained.　[c]Accession numbers beginning with TC are obtained from The Institute for Genomic Research maize gene index version 10.0.　[d]NA, Not applicable.　[e]Expression ratios represent an average value for more than one spot.

identification by BLAST, which corresponded to the most similar sequences in GenBank. Proteins encoded by these genes include several structural and metabolic proteins, such as enzymes involved in amino acid, carbohydrate, and cell wall metabolism, as well as storage proteins, proteins involved in stress responses, and components of the actin cytoskeleton, signal transduction pathways and the transcription/translation machinery.

Among genes encoding seed storage proteins, 19- and 22-kD α-zeins showed the largest difference in expression between high and low eEF1A genotypes. The transcript levels of these genes were about 10- and 5-fold higher, respectively, in Oh51Ao2 than Oh545o2

(Table I). The 19- and 22-kD α-zeins are encoded by multigene families, and our results most likely are representative of the gene subfamilies, due to cross-hybridization among the members (Marks et al., 1985). The transcript level of the 10-kD δ-zein correlated inversely with that of eEF1A; the amount of 10-kD δ-zein mRNA was about five times lower in high versus low eEF1A genotypes.

The mRNA levels of genes encoding enzymes involved in the synthesis of branched amino acids, such as acetolactate synthase and ketol-acid reductoisomerase, were severalfold greater in high compared to low eEF1A genotypes. Similarly, several genes involved in carbohydrate metabolism were up-regulated in the

high eEF1A genotypes. Genes encoding some of the proteins in these two groups were reported to be direct or indirect targets of *O2* regulation (Damerval and Le Guilloux, 1998).

Two genes encoding actin were significantly up-regulated in high eEF1A genotypes (Table I). Conversely, one actin probe (*MAc1*) was down-regulated in high eEF1A genotypes. The nucleotide sequence identity between *MAc1* and the other actin sequences is about 60%, so it is unlikely their transcripts cross-hybridized. Other actin genes represented in the array that were not altered in expression included *Maz83* (GenBank accession AI677580), *Maz87* (AI665295), *Maz89* (AI745940), and *Maz95* (AI677101). The transcript for *Maz87* was significantly increased in the high eEF1A parental inbred but not in the RILs. There were no significant differences in the expression levels of *Maz83* in high and low eEF1A genotypes, while the hybridization signals of *Maz89* and *Maz95* were similar to background.

One group of genes shown in Table I with altered expression associated with eEF1A has been implicated in stress responses and includes ascorbate peroxidase, a proteinase inhibitor, a Gly-rich (cell wall) protein and molecular chaperones, such as cyclophilin, heat shock proteins and protein disulfide isomerase. Other genes that were up-regulated in high eEF1A genotypes included several sequences encoding phosphatases, kinases, ribosomal proteins, histones, and protein synthesis factors.

RNA-Blot Analysis

To validate and extend the results of the microarray hybridization, the transcript levels of 13 genes were investigated by RNA gel-blot analysis using total RNA prepared from the same samples used for the microarray experiments (Fig. 4). The intensities of the radioactive signals were normalized to the 27-kD γ-zein RNA, because its level was found to be similar among these genotypes at 18-DAP. For most of these genes, the expression level determined by RNA gel-blot analysis correlated with that detected with microarrays. According to microarray data, the expression ratios of eEF1A, granule-bound starch synthase (*Waxy1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPC2*) were between 1.5- and 1.9-fold greater in the high versus the low eEF1A parental genotypes, while these genes were not found to be significantly changed in the RIL genotypes. The reason that the expression differences in the RIL genotypes were not statistically significant was related to the fact that the data obtained from one slide (no. 76) had markedly higher variance than the others, which reduced the ability to detect significant changes between the RILs. The expression ratios between 1.4- and 1.7-fold greater, measured by RNA gel-blot analyses of these genes, were consistent with the values from the microarray analysis of the parental genotypes. The expression levels determined by microarray hybridization

for actin (*Maz87*) and α-tubulin were 1.8- and 3.2-fold greater, respectively, in high compared to low eEF1A parental inbreds; however, the differences in gene expression detected by gel-blot analysis were approximately 1.2-fold larger. In the case of ascorbate peroxidase, the expression ratio obtained by microarray hybridization with Oh51A*o2* versus Oh545*o2* was 4.3, while the expression ratio obtained by gel-blot analysis was 1.9. In spite of the discrepancy in the RNA expression ratios for ascorbate peroxidase, the pattern of expression in high and low eEF1A genotypes was consistent. Conversely, according to microarray data, the expression ratio of Bip in RIL 34-3 versus 31-5 was not statistically significant, while by gel-blot analysis the ratio was 25.3. It is difficult to explain the unexpectedly high level of Bip mRNA in RIL 34-3, but this result was confirmed in multiple RNA blots.
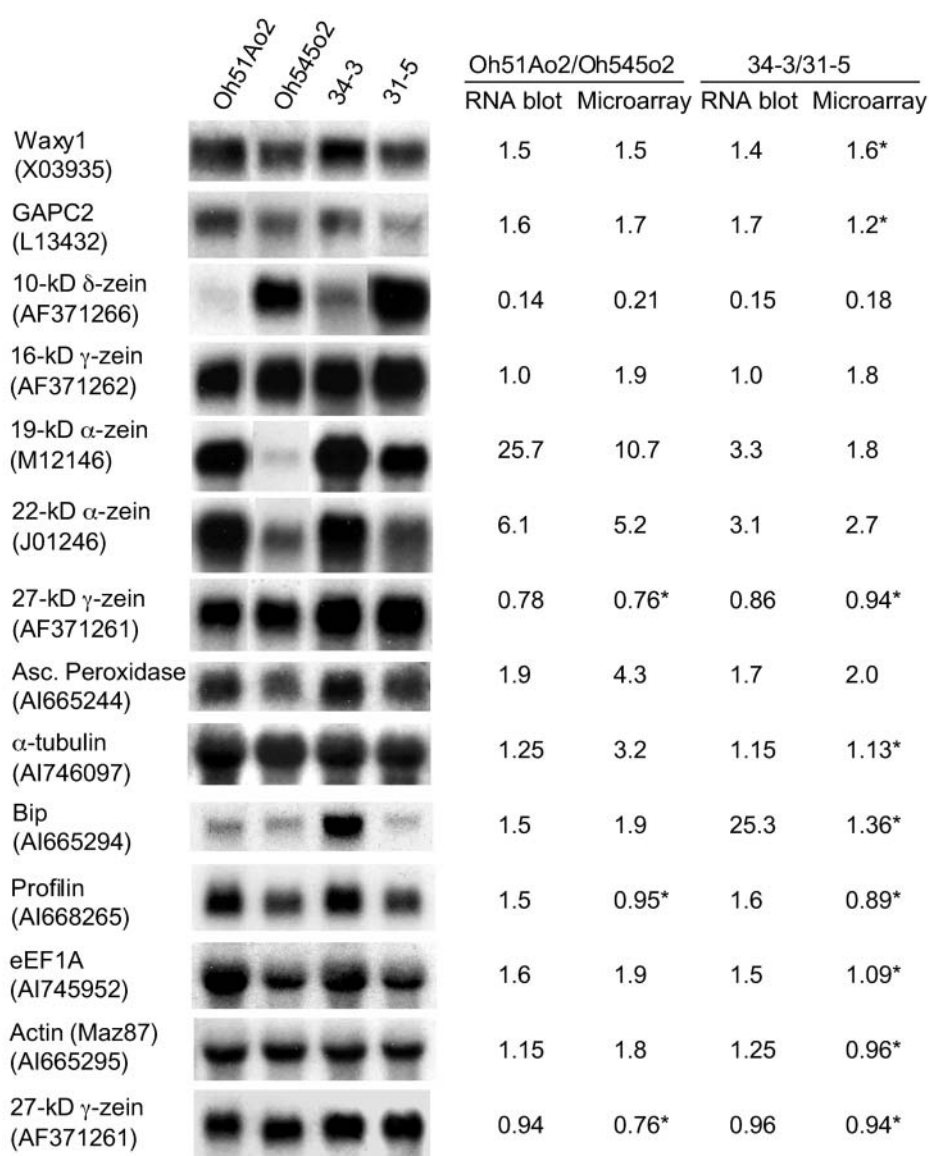
The expression of profilin was also determined by RNA gel-blot analysis, and it was found to be about 1.5-fold higher in Oh51A*o2* and RIL 34-3 compared to Oh545*o2* and RIL 31-5 (Fig. 4). Profilin transcript levels were not found to be significantly different between high and low eEF1A genotypes in the microarray experiments, because the signal was too close to background.

Patterns of Protein Synthesis in Developing Endosperm

To further assess the gene expression results obtained by RNA hybridization, we measured protein accumulation by ELISA in 18-DAP endosperms of the genotypes used for transcript profiling. In addition, we analyzed four RILs that differ in eEF1A content. Protein extracts from equal amounts of lyophilized endosperm of each genotype were partitioned into zein and nonzein fractions (Wallace et al., 1990) and analyzed using SDS-PAGE. Figure 5A illustrates a Coomassie Blue-stained gel showing the relative abundance of zein proteins in 18-DAP endosperm of the parental inbreds and six RILs. The level of 27-kD γ-zein appeared to be similar between Oh545*o2* and Oh51A*o2* and between RILs 31-5 and 34-3, although the amount of this protein was greater in the RILs. These observations were in agreement with the RNA levels obtained by microarray hybridization and RNA-blot analysis. The accumulation of 19- and 22-kD α-zein proteins was greater in Oh51A*o2* than Oh545*o2* (Fig. 5A) and correlated with the RNA hybridization results (Fig. 4). Based on Coomassie Blue staining, the differences in α-zein content in RILs 31-5 and 34-3 were not as obvious as for the parents, consistent with the smaller differences in RNA transcript levels measured for these lines compared to Oh545*o2* and Oh51A*o2* (Fig. 4). Except for RIL 33-13, the accumulation of zein proteins in RILs 39-1, 37-3, and 4-10 at 18-DAP appeared to be very similar. Figure 5B shows there were only small differences in total zein content at this stage of development for the six genotypes. The amount of total zein ranged from about 4.1 mg/100 mg

Figure 4. RNA gel-blot analysis for comparison with microarray hybridization data. Lanes contained 10 μg of total RNA extracted from 18-DAP endosperms of Oh51A*o2*, Oh545*o2*, and their RILs: 34-3 (high eEF1A) and 31-5 (low eEF1A). Blots were hybridized with ^{32}P-labeled probes corresponding to the genes indicated. GenBank accession numbers of EST-specific probes are indicated in parentheses. The values given are the ratio of signals between Oh51A*o2* and Oh545*o2*, as well as RILs 34-3 and 31-5, normalized relative to the 27-kD γ-zein RNA signal. For each gene, the expression ratio of high versus low eEF1A genotypes is compared to the ratio obtained by microarray hybridization for that accession (* expression ratio not significantly different according to Statistical Analysis for Microarray program).


	Oh51Ao2/Oh545o2		34-3/31-5	
	RNA blot	Microarray	RNA blot	Microarray
Waxy1 (X03935)	1.5	1.5	1.4	1.6*
GAPC2 (L13432)	1.6	1.7	1.7	1.2*
10-kD δ-zein (AF371266)	0.14	0.21	0.15	0.18
16-kD γ-zein (AF371262)	1.0	1.9	1.0	1.8
19-kD α-zein (M12146)	25.7	10.7	3.3	1.8
22-kD α-zein (J01246)	6.1	5.2	3.1	2.7
27-kD γ-zein (AF371261)	0.78	0.76*	0.86	0.94*
Asc. Peroxidase (AI665244)	1.9	4.3	1.7	2.0
α-tubulin (AI746097)	1.25	3.2	1.15	1.13*
Bip (AI665294)	1.5	1.9	25.3	1.36*
Profilin (AI668265)	1.5	0.95*	1.6	0.89*
eEF1A (AI745952)	1.6	1.9	1.5	1.09*
Actin (Maz87) (AI665295)	1.15	1.8	1.25	0.96*
27-kD γ-zein (AF371261)	0.94	0.76*	0.96	0.94*

of flour in RIL 33-13 to about 5.2 mg/100 mg of flour in Oh51A*o2*.

The nonzein content in 18-DAP endosperms of Oh545*o2*, Oh51A*o2*, and the six RILs was measured by Bradford assay and the relative levels of eEF1A, actin, profilin, and α-tubulin by ELISA. Figure 6A shows the nonzein content at this stage of development was similar in all genotypes and ranged from about 5 mg/100 mg of flour in RIL 31-5 to about 6 mg/100 mg of flour in Oh51A*o2*. Figure 6B shows the ELISA quantification of eEF1A, actin, profilin, and α-tubulin in these genotypes. The parental inbreds and the RILs were organized according to their relative eEF1A content. In general, the relative level of eEF1A, actin, and profilin in the RILs was lower than in the high and low eEF1A parents. There was about 1.3-fold more eEF1A in Oh51A*o2* than in Oh545*o2*. The difference in eEF1A content between the highest and lowest

RILs, 34-3 and 31-5, was also about 1.3-fold. The levels of actin, profilin, and α-tubulin protein paralleled those of eEF1A (Fig. 6B). The amount of these proteins in Oh51A*o2* compared to Oh545*o2* was about 1.2-, 1.3-, and 1.8-fold greater, respectively, and these ratios were significantly different (Student's t test, $P < 0.01$). Among the RILs, the greatest difference in the amount of these proteins was in RIL 34-3 (high eEF1A) and RIL 31-5 (low eEF1A), where the levels of actin, profilin, and α-tubulin were about 1.3-, 1.5-, and 1.4-fold higher in RIL 34-3 than in RIL 31-5, respectively, and were significantly different (Student's t test, $P < 0.01$).

DISCUSSION

Prior studies of the relationship between eEF1A and Lys content indicated that there might be an increase
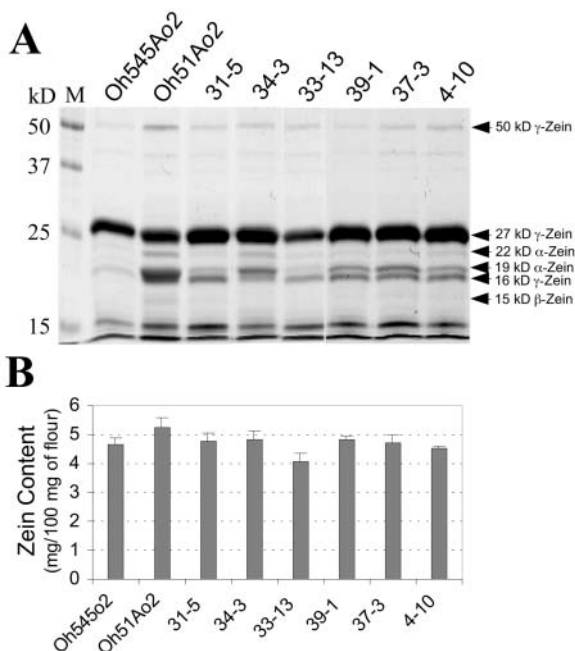
Figure 5. SDS-PAGE and quantification of total zein in developing endosperm of Oh545*o2*, Oh51A*o2*, and six RILs with high and low eEF1A content. A, For each lane, total zein extracts were prepared from 750 μg of lyophilized 18-DAP endosperms of the indicated genotypes, separated by 12.5% (w/v) SDS-PAGE, and stained with Coomassie Blue R. Prestained molecular mass standards are shown on the left. B, Total zein content was estimated using BCA reagent with BSA as standard. The results are the average of two independent extractions in which three measurements were taken.

in cytoskeleton and cytoskeleton-associated proteins in *o2* mutants. For this study, we selected the maize inbreds Oh51A*o2* and Oh545*o2*, because they have among the highest degree of variability reported for eEF1A content (Moro et al., 1996). We also analyzed RILs created from a cross between these inbreds, as they showed a range of variation for eEF1A content between the parental genotypes (approximately 2-fold). These differences allowed us to compare multiple, genetically fixed, and related inbred lines for patterns of protein accumulation and gene expression relative to eEF1A protein content. We measured the accumulation of selected proteins by ELISA in multiple high and low eEF1A RILs at 18-DAP and maturity, which confirmed that cytoskeletal proteins were increased in high eEF1A genotypes. Microarray analysis of these genotypes also showed changes in several cytoskeleton and cytoskeleton-associated proteins. Unfortunately, the cost of the microarray experiments prevented us from analyzing more genotypes than the parents and one high and one low eEF1A RIL.

Using an endosperm EST microarray, we were able to conduct a broad survey of genes that change in expression parallel with eEF1A in high and low eEF1A genotypes. Our goal was to identify genes with an expression level that was higher in Oh51A*o2* com-

pared to Oh545*o2*, and higher in RIL 34-3 compared to RIL 31-5. We tested the microarray results for several genes by RNA gel-blot analysis and generally found a good correspondence between the expression levels estimated by the two techniques. In a few cases, the differences in RNA levels detected by gel-blot analysis and microarray hybridization did not correspond. This may have been a consequence of inherent differences in the stringency of the two hybridization procedures or the specific alleles present, or absent, on the microarray. However, the general consistency between the results obtained by the RNA gel-blot analysis and microarray hybridization largely confirmed the utility of using the EST microarray to identify genes differentially expressed in high and low eEF1A genotypes.

The microarray hybridization identified about 120 genes coordinately increased with eEF1A (Table I). These genes encode proteins involved in a variety of cellular processes, and several of them have been previously reported to be differentially expressed at the transcript and protein level in wild type and *o2* mutant inbreds, although not necessarily in parallel with
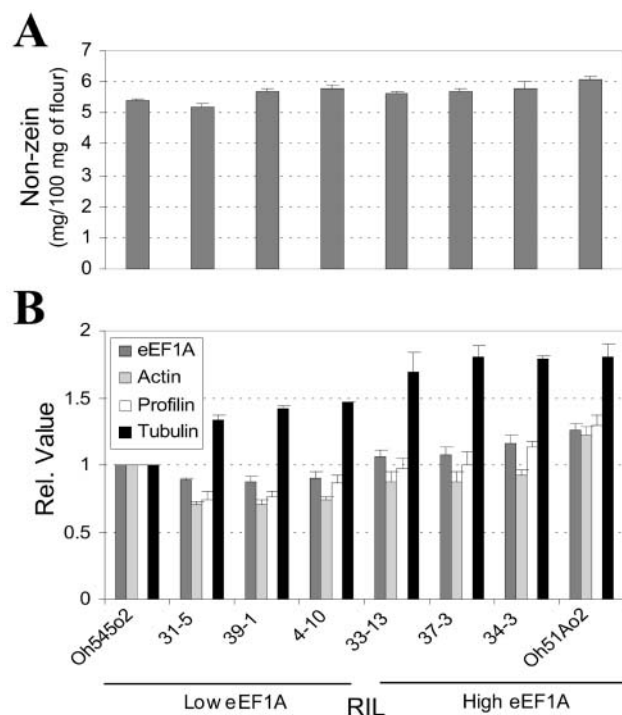
Figure 6. Nonzein quantification and ELISA measurement of eEF1A, actin, profilin, and α-tubulin content in developing endosperm of Oh51A*o2*, Oh545*o2*, and six RILs with high and low eEF1A content. Protein extracts were prepared from lyophilized 18-DAP endosperms. A, Nonzein content was estimated using the Bradford reagent with BSA as standard. B, ELISA measurements of the indicated proteins were conducted as described in "Materials and Methods," and the results were normalized to the values of the Oh545*o2* inbred. In both A and B, the results are the average of two independent experiments in which three measurements were taken.

eEF1A. For example, the levels of α-zeins, pyruvate orthophosphate dikinase, and acetolactate synthase were decreased in o2 mutants compared to wild type (Damerval and Le Guilloux, 1998; Hunter et al., 2002), while that of eEF1A was increased (Habben et al., 1993; Sun et al., 1997). For some enzymes involved in carbohydrate metabolism, such as glyceraldehyde-3-phosphate dehydrogenase and sorbitol dehydrogenase, earlier studies showed these proteins are increased in o2 mutants (Damerval and Le Guilloux, 1998).

One explanation for the relationship between eEF1A and endosperm Lys content is that eEF1A is part of an elaborate cytoskeletal network that surrounds the RER, particularly at sites where protein bodies are forming (Clore et al., 1996; Reuzeau et al., 1997). Other Lys-rich proteins that exist in association with this cytoskeletal network may be increased as the consequence of a greater protein body surface area (Sun et al., 1997). Oh51A*o2* has more α-zein and also appears to have a larger number of small protein bodies (larger RER surface area) than Oh545*o2* (Wang et al., 2001), which could result in a more extensive ER-associated cytoskeletal network. Consistent with this hypothesis, our microarray results indicated the expression of α-zeins and a number of genes encoding actin-associated and ER proteins are coordinately increased in high eEF1A genotypes (Table I). In addition, the levels of α-zeins and some cytoskeletal proteins corresponded well with those of eEF1A in the parental inbreds and some high and low eEF1A RILs (Figs. 3 and 6). This could explain a significant portion of the increased eEF1A content in Oh51A*o2* and its related high eEF1A RILs. Microscopy and biochemical studies will be required to examine the postulated ultrastructural differences in protein body size and number and ER development in RILs with differing levels of eEF1A.

The RNA expression data also showed that several genes encoding carbohydrate-metabolizing enzymes were up-regulated in high eEF1A genotypes. Interestingly, some of these enzymes, such as Suc synthase (Carneiro, 1998; Winter et al., 1998), glyceraldehyde-3-phosphate dehydrogenase, and Fru-1,6-bisphosphate aldolase were recently reported to be associated with the cytoskeleton (Azama et al., 2003). The Lys content of these proteins varies from about 5% to 8.5%, and their masses, together with those of structural cytoskeletal proteins (actin, tubulin, and eEF1A), were reported to contribute significantly (approximately 67%) to the total endosperm Lys content of W64A*o2* (Azama et al., 2003). Several endosperm proteins found to associate with actin in yeast two-hybrid interactions (Carneiro, 1998) included a Ser-Thr protein kinase, 60S acidic ribosomal protein, E2 ubiquitin-conjugating enzyme, and genes encoding these proteins were also up-regulated in high eEF1A genotypes (Table I).

In a previous study, we characterized isoforms of eEF1A from developing maize endosperm and found that they differ in their F-actin binding activities (Lopez-Valenzuela et al., 2003). The isoform that binds actin most efficiently was the predominant form accumulated in endosperm of the high eEF1A inbreds, Oh51*o2*, and RIL 34-3, which may reflect enhanced cytoskeleton formation and therefore increased synthesis of cytoskeleton-associated proteins in these genotypes. Consistent with this hypothesis, our results show the mRNA levels of several genes encoding ribosomal proteins and protein synthesis factors are significantly higher in Oh51A*o2* and RIL 34-3, compared to Oh545*o2* and RIL 31-5 (Table I). It is known that a significant proportion of polysomes in maize endosperm exist in a cytoskeleton-bound state and several plant translation factors have been shown to interact directly with cytoskeletal polymers (for review, see Browning, 1996; Hesketh and Pryme, 1991). Altogether, approximately 25% of the significantly changed genes shown in Table I have implied or proven associations with the cytoskeleton.

Proteins that directly regulate the dynamics of the actin (e.g. ARP2/3 complex, fimbrin, capping protein, or profilin) and microtubule cytoskeletal networks (e.g. katanin, p65, Mor1, or Tangled protein) are conspicuously absent from the microarray analysis. There are several factors that contributed to this. First, because the array was printed from randomly chosen cDNA clones from an endosperm library, many of these regulatory proteins were not represented. This is due to the great abundance of storage protein sequences, but it could also reflect that many of these proteins are not highly expressed in maize endosperm. Second, regulatory proteins that are present on the microarray, such as profilin, actin depolymerizing factor, katanin, and a putative centromeric microtubule binding protein, had signal intensities that were close to background levels; consequently, this made it difficult to discriminate significant differences in gene expression. Finally, some proteins, α- and β-tubulin for example, were significantly changed only in the parental inbred lines; it is unclear whether such proteins can be considered to contribute to the increased Lys content of high eEF1A lines, since they were not significantly different in the RILs.

Differences in transcript levels between genotypes are often consistent with variation in protein levels, although this need not be the case. Furthermore, differences in RNA levels in developing maize seeds need not necessarily be related to protein content in the mature seed. However, it is the latter that ultimately determines the Lys content of the grain. The microarray analysis was predicated on the hypothesis that by measuring differences in transcript levels at mid-development (18-DAP), we could identify proteins linked with eEF1A concentration in mature maize endosperm. To a large extent, this proved to be correct. At 18-DAP, the differences in eEF1A protein between Oh51A*o2* and Oh545*o2* and between RIL 34-3 and RIL 31-5 (approximately 1.3-fold; Fig. 6B) were similar to those measured at the RNA transcript level by microarrays and RNA gel-blot analysis

(1.6–1.9-fold; Table I; Fig. 4). According to microarray hybridization (Table I), two actin RNA transcripts were more than 2-fold increased in Oh51A*o2* and RIL 34-3 (high eEF1A) compared to Oh545*o2* and RIL 31-5 (low eEF1A), while only about 1.2-fold differences in actin transcript levels were measured by RNA-blot analysis for a third actin gene (Fig. 4). The protein measurements indicated there was about 20% to 30% more actin in Oh51A*o2* and RIL 34-3 (high eEF1A) compared to Oh545*o2* and RIL 31-5 (low eEF1A) at 18-DAP (Fig. 5B). The values obtained for α-tubulin protein content (1.8-fold greater in Oh51A*o2* than Oh545*o2* and 1.4-fold greater in RIL 34-3 than RIL 31-5) fell between the microarray results (approximately 3-fold; Table I) and the 1.2-fold difference obtained by RNA-blot analysis (Fig. 4). Differences in profilin RNA levels were not apparent by microarray hybridization, but differences in the amount of this protein in Oh51A*o2* versus Oh545*o2* and RIL 34-3 versus RIL 31-5, 1.3- and 1.5-fold, respectively, were in good agreement with the transcript levels determined by gel-blot analysis (approximately 1.5-fold; Fig. 3). In mature endosperm, the differences in eEF1A and actin protein content between Oh51A*o2* and Oh545*o2* and between RIL 34-3 and RIL 31-5 (approximately 2-fold; Fig. 6) were similar to those measured at the RNA transcript level by microarrays (Table I), while the differences in α-zein protein content (2- to 4-fold) were smaller than those measured at the transcript level in 18-DAP endosperm (Table I; Fig. 3). Thus, in general there was a good relationship between RNA transcript levels at 18-DAP and protein levels in developing and mature endosperm.

In conclusion, the results of this study support the hypothesis that eEF1A concentration in maize endosperm is related to the concentration of cytoskeleton-associated protein components, and this may contribute to the high correlation between eEF1A and the concentration of protein-bound Lys in endosperm (Habben et al., 1995). Cytoskeleton-associated proteins are not as abundant as zeins, but combined, their masses appear to make a substantial contribution to the Lys content of the grain (Azama et al., 2003). By selecting for maize genotypes, particularly Quality Protein Maize lines (Moro et al., 1996; Zarkadas et al., 2000) with high levels of eEF1A, it should be possible to create maize kernels that meet the Lys requirement for humans and other monogastric animals.

MATERIALS AND METHODS

Plant Materials

The maize (*Zea mays*) inbred lines Oh51A*o2* (high eEF1A content) and Oh545*o2* (low eEF1A content) were obtained from Crow's Hybrid Corn, Milford, IL. F$_1$ and F$_2$ progeny were created from a cross between these two inbreds (Wang et al., 2001), and the RILs were created by single seed descent from the F$_2$ progeny. Parental inbreds and RILs were grown at the University of Arizona West Agricultural Center in Tucson, AZ, and at the University of Florida, Gainesville, FL. Developing kernels were harvested at 18-DAP, frozen in liquid nitrogen, and stored at −80°C until use. Prior to RNA extraction, the

embryo and pericarp were removed by hand dissection and the endosperm placed in liquid nitrogen.

RNA Purification and Labeling

Total RNA was extracted from 18-DAP developing endosperms. Frozen endosperms were ground with a mortar and pestle, and the powder was homogenized in one volume of NTES buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% [w/v] SDS) and one volume of phenol/chloroform saturated with Tris pH 8.0. This extraction buffer allowed the precipitation and removal of starch, which is highly abundant in 18-DAP endosperm. Following centrifugation, nucleic acids were precipitated from the aqueous phase with three volumes of ethanol and resuspended in diethyl pyrocarbonate (DEPC) water. RNA was precipitated with three volumes of 4 M LiCl at −20°C for 4 h, resuspended in DEPC water, and purified using TRIzol (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was purified from total RNA using a Qiagen oligotex mRNA kit (Qiagen, Valencia, CA). RNA quantity was estimated by UV absorption at 260 nm, and its quality was assessed by visual inspection following agarose gel electrophoresis. For synthesis of fluorescently labeled cDNA targets, 2 μg of poly(A)$^+$ RNA was first hybridized to 1 μg oligo(dT) primer at 65°C for 10 min. The reaction was completed by addition of 1× reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$), 500 μM dNTPs (dATP, dCTP, dGTP), 100 μM dTTP, 50 μM Cy3- or Cy5-dUTP, 10 mM dithiothreitol (DTT), and 400 units Superscript II reverse transcriptase (Invitrogen), followed by incubation at 42°C for 90 min.

Microarray Hybridization and Analysis

Glass slides of the maize endosperm 605.03 microarray were obtained from the Maize Gene Discovery Project (Fernandes et al., 2002). A detailed description of the array design can be found at http://zmdb.iastate.edu/zmdb/microarray. Hybridization was conducted following the protocols described by Fernandes et al. (2002) and the details available at http://zmdb.iastate.edu/zmdb/microarray/protocols.html. The slides were analyzed with a ScanArray 3000 scanner (GSI Lumonics, Watertown, MA) for both channel 1 (Cy3) and 2 (Cy5) at 10-μm resolution. Photomultiplier settings were adjusted manually to reduce the percentage of spots on the array with saturated signal values and to obtain a Cy3:Cy5 signal ratio close to one for the control genes. For each high versus low eEF1A genotype comparison, we used two slides, one with direct and one with reverse labeling. Thus, for each probe a total of two RNA samples, two slides per sample and two arrays per slide, resulted in eight intensity values for analysis. The image files obtained were analyzed using Imagene software (Biodiscovery, Marina del Rey, CA). Quality control measurements produced by the Imagene software were used to ensure that only spots of good quality were used in the analysis. Further analysis and normalization of the data were performed using the BioConductor suite of microarray analysis programs (Dudoit et al., 2003). Net signal intensity was computed from the median signal minus the local background. The intensity data were normalized using the variance stabilized normalization function in BioConductor (Huber et al., 2002). The normalized data were exported to Microsoft Excel for statistical analysis of differential expression using the Statistical Analysis for Microarray Excel plug-in (Tusher et al., 2001) to minimize the false discovery rate due to multiple hypothesis testing. Prior to analysis of the data, the intensities for each subarray were placed in separate columns, resulting in a total of eight intensity values for each cDNA in the array. The cutoff value of the corrected probability (q) for significant differences was q < 0.05, which resulted in a median number of false positives less than 0.1% for each dataset.

The identity and function of each EST that was significantly changed was confirmed manually by BLAST analysis. BLASTN searches were performed against the GenBank plant nonredundant database release 138 with an expectation cutoff of 1×10^{-10}. BLASTX searches were performed against the nonredundant protein database with an expectation cutoff of 1×10^{-4}. In cases where the most significant nucleotide hit was to an uncharacterized maize sequence, unannotated mRNA sequences contributed by the maize mapping project for example, and the identity was greater than 95%, the complete sequence of the longer sequence in the database was used for a BLASTX search. Finally, any sequences that did not produce any significant hits were searched against the maize genomics consortium release 3.0 database of contigs from methyl-filtered and high-Cot sequences, as well as the Institute for Genomic Research maize gene index release 14. If the sequence identity of the hits from these databases were greater than 95%,

the contig sequence was used to search the nonredundant protein database as described above.

RNA-Blot Hybridization

RNA (10 μg of total RNA per sample) was separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (Lehrach et al., 1977) and blotted onto nylon membranes (MSI, Westboro, MA). DNA probes were obtained by labeling with α-^{32}P-dCTP using a random prime method, according to the manufacturer (Invitrogen). EST-specific cDNAs were obtained from the corresponding 605 EST clones by PCR amplification as described by Fernandes et al. (2002) and their identity confirmed by partial sequencing. Primer sequences used for amplification of the 605 EST clones were as follows: forward 5′-CTGCAGTAATACGACTCACTATAG-3′ and reverse 5′-CTATTC-GATGATGAAGATACC-3′. The cDNAs corresponding to zeins, glyceraldehyde 3-phosphate dehydrogenase (*GAPC2*), and granule-bound starch synthase (*Waxy1*) were obtained by PCR using gene-specific primers. Amplified DNA products were analyzed by agarose gel electrophoresis and purified using the GeneClean III kit from Amersham (Piscataway, NJ). The RNA gel blots were hybridized using the indicated ^{32}P-labeled probes. As a loading control, the blots were hybridized with a ^{32}P-labeled cDNA corresponding to the 27-kD γ-zein. Hybridizations were performed at 65°C in a solution containing 1.5× sodium chloride/sodium phosphate/EDTA, pH 7.7, 6% PEG 8000, 1% SDS, 1% nonfat dried milk, 0.1% (v/v) DEPC, and 30 μg mL^{-1} freshly denatured salmon sperm DNA (Sabelli and Shewry, 1993). After hybridization, the blots were washed once with 1× SSC and 0.1% SDS at room temperature, then twice with 1× SSC and 0.1% SDS for 15 min at 65°C, and then a final wash with 0.1× SSC and 0.1% SDS for 15 min at 65°C. Quantification of hybridization signals was obtained using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Blots were stripped between hybridizations with boiled 0.5% SDS, followed by a 20-min incubation at 65°C.

Zein and Nonzein Protein Extraction, Quantification, and SDS-PAGE Analysis

Protein fractions were obtained from mature endosperms or lyophilized, developing endosperms as described by Wallace et al. (1990). Fifty milligrams of flour was extracted overnight with 1 mL of borate buffer (12.5 mM Na$_2$B$_4$O$_7$, 1% [w/v] SDS, 2% 2-mercaptoethanol, pH 10) at 37°C, and the soluble proteins were partitioned into zeins (supernatant) and nonzeins (pellet) by the addition of absolute ethanol to a final concentration of 70% (v/v). Zein proteins (equivalent to an extract from 15 mg of flour) were lyophilized and resuspended in 100 μL of deionized water. Nonzein proteins obtained from the same amount of flour were resuspended in 100 μL of 8 M urea.

The concentration of zein proteins was determined by the bicinchoninic acid (BCA) method (Brown et al., 1989), using the BCA protein assay reagents from Pierce (Rockford, IL). The concentration of nonzein proteins was determined according to Bradford (1976), using the Bradford protein assay reagent from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin (BSA) was used as a standard for both zein and nonzein quantification. The results reported (mg of protein/100 mg of flour) correspond to the average of two independent experiments in which three measurements were taken. Five microliters of the original zein extract were used for analysis with 12.5% (w/v) SDS-PAGE, and the gel was stained with Coomassie Blue R.

ELISA Measurements

Endosperm protein extraction and zein/nonzein fractionation were performed as described by Wallace et al. (1990). ELISA of eEF1A was similar to that described by Habben et al. (1995), Moro et al. (1996), and Wang et al. (2001). Total endosperm protein extract was diluted 1,000-fold in carbonate coating buffer (CCB; 0.1 M Na$_2$CO$_3$/NaHCO$_3$, pH 9.6), and 150 μL of the sample was loaded in the well of an ELISA plate (Immulon2, Dynatech Laboratories, Chantilly, VA). A multichannel pipette was used to mix 50 μL of the sample with 100 μL of CCB to make four 3-fold dilutions into adjacent wells containing CCB. The protein was allowed to bind to the plate overnight at 4°C. Subsequently, the wells were washed twice using PBS plus Tween 20 (PBST, 0.05 M phosphate, 145 mM NaCl, 0.05% [v/v] Tween 20, pH 7.4) and 100 μL of rabbit eEF1A antiserum (Habben et al., 1995) diluted 1:1,000 in PBST was added and incubated for 3 h. After incubation, the primary antibody was removed, the wells were washed twice with PBST, and the secondary

antibody, goat anti-rabbit IgG alkaline conjugate (Sigma Chemical, St. Louis) diluted 1:1000 in PBST, was added and allowed to bind for 2 h. After removal of the secondary antibody, the wells were washed twice with PBST and 200 μL of the alkaline phosphatase substrate (Sigma) diluted in diethanolamine buffer (50 mM diethanolamine, pH 9.8) was added. The color reaction was allowed to develop for 30 to 45 min, and the absorbance was read at 420 nm with a micro plate reader (MRX, Dynatech).

An ELISA to measure actin, profilin, and α-tubulin was performed as for eEF1A, but with some modifications. Nonzein pellets from 15 mg of flour were resuspended in 100 μL of 8 M urea. The nonzein protein solution was diluted 2,500-, 4,000-, and 10-fold in CCB for actin, profilin, and α-tubulin, respectively. Two hundred microliters of the samples were loaded in the well of the ELISA plate, and 100 μL were mixed with 100 μL of CCB to make four 2-fold dilutions into adjacent wells. The rest of the procedure was similar to that described for eEF1A. The primary antibodies were from rabbit antiserum produced against actin and profilin from maize pollen (Gibbon et al., 1998; Gibbon et al., 1999) and recombinant α-tubulin of human origin (Santa Cruz Biotechnology, Santa Cruz, CA); they were diluted 1:500, 1:500, and 1:50, respectively, in PBST.

The ELISA for α-zein was similar to the procedure described by Moro et al. (1996), with some modifications. Following the initial extraction of endosperm flour, the protein extract was diluted 20,000-fold and used to make four 2-fold dilutions into adjacent wells of the ELISA plate. The dilution and antigen binding steps were done using a 40% (v/v) ethanol, 10% (v/v) acetic acid solution (Wallace et al., 1990), and the primary antibody (rabbit anti-α-zein) dilution was 1:2,000 in PBST. The range of protein concentration for all the ELISA assays was such that the relationship between absorbance and relative antigen concentration was linear, and a regression analysis was performed. The slope of the regression is proportional to the antigen concentration, and it was used to measure the relative protein content. The results were normalized to the values of the Oh545*o2* inbred and represent the average of two independent experiments in which three measurements were taken.

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