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Improving Nutritional Quality of Maize Proteins by Expressing Sense and Antisense Zein Genes

SHIHSHIEH HUANG,*,† WHITNEY R. ADAMS,† QING ZHOU,†
KATHLEEN P. MALLOY,† DALE A. VOYLES,† JAN ANTHONY,†,‡
ALAN L. KRIZ,§ AND MICHAEL H. LUETHY†

Mystic Research, Monsanto Company, 62 Maritime Drive, Mystic, Connecticut 06355, and
Renessen LLC, 3000 Lakeside Drive, Bannockburn, Illinois 60015

The predominant proteins in maize grain are a family of alcohol-soluble prolamin storage proteins called zeins. They account for >50% of total seed proteins but are deficient in several essential amino acids. As a result, the corn grain is considered to be nutritionally poor for monogastric animals with respect to key essential amino acids, most notably lysine, tryptophan, and methionine. Thus, corn mutants with reduced levels of zeins, such as *opaque-2* (*o2*), have been demonstrated to possess grain with improved nutritional quality characteristics. The *o2* mutant has a superior amino acid composition and has been used through conventional breeding to develop Quality Protein Maize (QPM) for human and animal consumption in developing countries. With the understanding of molecular genetics of zeins and progress in biotechnology, an alternative approach to zein reduction is explored here. Through the targeted reduction of the 19-kDa α-zeins, increased levels of lysine, tryptophan, and methionine have been engineered in grain of transgenic hybrids. Currently, the agronomic properties and nutritional values of these transgenic lines are being evaluated.

KEYWORDS: Zein reduction; high lysine; high tryptophan; quality trait; antisense; maize; nutritional quality

INTRODUCTION

Maize originated in the New World and, through the transcontinental navigation of the 16th and 17th centuries, was rapidly adopted in Africa, Europe, and Asia. With the introduction of high-yielding hybrids in the 1950s, maize has become one of the world's largest crops and is now a major vehicle for delivering nutrients to humans and livestock. However, like most cereal proteins, maize proteins are nutritionally poor because of their reduced content of essential amino acids. They contain <3% of lysine, well below the Food and Agriculture Organization's (FAO) recommended concentration of 5.5% for human nutrition (*1*). In addition to lysine, tryptophan, threonine, and methionine are also limiting amino acids in maize protein (*2*, *3*).

Maize seed proteins are classified as albumins (3%), globulins (3%), prolamins (60%), and glutelins (34%) (*4*). With the exception of prolamins, all fractions are relatively balanced in amino acids. Maize prolamins, also called zeins, can be subdivided into distinct categories, α-, β-, γ-, and δ-zeins, based on their solubility and related protein structure as deduced from nucleotide sequences (*5*, *6*). Zeins are rich in glutamine, proline, alanine, and leucine and are almost completely devoid of lysine and tryptophan (*7*). As a result of their abundance, the contribution of lysine and tryptophan from other endosperm protein fractions is diluted. It is not surprising that attempts to isolate mutants with altered endosperm nutritional quality have led to the identification of mutations which affect zein levels.

The best-characterized zein mutants are *opaque-2* (*o2*) and *floury-2* (*fl2*) (*8*, *9*). These mutants contain elevated levels of lysine and tryptophan due to a decrease in zein synthesis and an increase in accumulation of non-zein proteins (*7*). Initially, the potential use of *o2* and *fl2* mutants to improve nutritional quality generated considerable excitement. Unfortunately, the soft chalky endosperm, characteristic of these mutants, led to increased mechanical damage, greater susceptibility to diseases, and lower yields, which made them unsuitable for broad-acre commercial production. Despite the development of genetic modifiers to restore the endosperm texture of *o2* to a normal hard and vitreous phenotype, the resulting Quality Protein Maize (QPM) hybrids have yet to gain broad popularity in developed countries. This may due to the relatively larger number of modifier genes required to achieve a hard endosperm phenotype and concomitant difficulty in integrating the modifiers into commercial breeding programs.

Analysis of these mutant lines has, however, yielded important information regarding the molecular genetics of zeins. The

* Corresponding author [telephone (860) 572-5201; fax (860) 572-5240; e-mail shihshieh.huang@na1.monsanto.com].
† Mystic Research, Monsanto Co.
‡ Present address: 92 Bayfield Drive, Wakefield, RI 02879.
§ Renessen LLC.

α-zeins, which account for ~70% of the total zein fraction, are thought to be the products of a complex multigene family and yet appear to be represented by only a few abundant transcripts (*10*). In this study, we demonstrate how the expression of antisense/sense 19-kDa α-zein nucleotide sequences is able to significantly reduce this class of α-zeins, which results in a change in amino acid composition of transgenic kernels.

MATERIALS AND METHODS

Vector Construction and Plant Transformation. DNA fragments including the coding and the 5′ and 3′ noncoding regions of an α-zein gene (*Z4*, GenBank accession no. V01472) were isolated by PCR based on the sequence published by Hu et al. (*11*). These fragments were inserted, in either sense or antisense orientation, into an expression cassette containing the γ-zeinA promoter (corresponding to the 19−1118 region of a GenBank sequence, accession no. S78780) and the 3′UTR of the nopaline synthase gene. Microprojectile bombardment mediated transformation and regeneration procedures described by Gordon-Kamm et al. (*12*) were followed.

Plant DNA and RNA Isolation and Analysis. Genomic DNA was isolated from leaves of corn plants using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Genomic DNA (20 μg) was digested with restriction enzymes, separated on a 0.8% (w/v) agarose gel, and transferred to positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN). Total RNA was isolated from developing kernels (20 days after pollination) using TRIzol reagent (Life Technologies, Gaithersburg, MD), and 10 μg was electrophoresed on a 1.2% (w/v) agarose/formaldehyde gel and transferred to the same type of membrane described in the DNA gel-blot. Prehybridization, hybridization, washing, and detection of the membranes were conducted using a non-radioactive fluorescein-based system (Amersham Pharmacia, Piscataway, NJ) following the manufacturer's protocols. PCR-labeled DNA fragments corresponding to the sequences of the γ-zein promoter and the *Z4* coding region were used as the probes for DNA and RNA gel-blot, respectively.

α-Zein Extraction and Sodium Dodecyl Sulfate— Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis. α-Zeins were selectively extracted according to a modified procedure described by Lund et al. (*13*). Single kernels were ground to a fine powder, the oils were extracted with 5 mL of hexane, and the powder was dried overnight. Twenty-five milligrams of the dry powder from each kernel was then weighed into a 96-well plate with 750 μL volume wells (Whatman Polyfiltronics, Clifton, NJ). The powder was extracted with 0.3 mL of deionized water containing 1 mM phenylmethanesulfonyl fluoride (PMSF) with vigorous agitation at 4 °C for 30 min. The homogenate was centrifuged for 5 min at 600*g*. Four further extractions were carried out on the resulting pellet: one extraction with 0.2 mL of 0.5 M NaCl and 1 mM PMSF, two extractions with 0.2 mL of deionized water and 1 mM PMSF, and the last extraction with 0.3 mL of 70% ethanol at 50 °C for 2 h. The last solution was spun as before, and the ethanolic extracts were stored at −20 °C. The bicinchoninic acid (BCA) method (*14*) was used to eliminate interfering substances during the determination of protein concentration. SDS-PAGE was carried out as described by Viotti et al. (*15*). Three micrograms of protein was loaded onto Bio-Rad (Hercules, CA) Criterion 18% polyacrylamide gels with 26 wells. The gels were stained with Coomassie R-250 (Bio-Rad). In the extraction of total zeins of hybrid kernels, PQ015/PQ015, PQ071/PQ071, 90DJD28/PQ015, and 90DJD28/91INH2, 80 mg of kernel powder was extracted with 4 mL of 60% acetonitrile, 10 mM dithiothreitol (DTT), and 25 mM ammonium hydroxide for 1 h at 60 °C with shaking every 10 min. The homogenate was centrifuged for 5 min at 600*g*. Twenty-five microliters of the resulting supernatant was evaporated at 78 °C, and then 50 μL of 1× SDS-PAGE sample buffer (0.042 M Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.008% bromophenol blue, and 1.7% v/v 2-mercaptoethanol) was added and heated to 95 °C for 5 min. Ten microliters of the sample, which was an equivalent to 100 μg of initial kernel powder, was loaded per well. This procedure allows all zein fractions to be present in the protein extracts. Unless indicated otherwise, all chemicals were purchased from Sigma (St. Louis, MO).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Analysis. To isolate all classes of zeins, the following zein extraction procedure was used. Twenty-five milligrams of powder from individual kernels was placed in the wells of a 96-well microplate with 2 mL volume wells (Whatman Polyfiltronics). The kernel powder was extracted twice with 250 μL of hexane to remove lipids and allowed to dry overnight. Samples were extracted with 1.2 mL of 60% acetonitrile containing 25 mM ammonium hydroxide and 10 mM DTT for 1 h in a 60 °C water bath. The samples were sealed and shaken every 10 min during the 1 h extraction period. The microplates were then centrifuged for 5 min at 600*g*. Five microliters of each sample was then mixed with 45 μL of matrix with an Applied Biosystems (Framingham, MA) SymBiot I robot, and 0.6 μL of the sample/matrix mixtures was deposited on a 384-position MALDI target plate (Applied Biosystems). The matrix mixture consisted of 5.2 mg/mL of 2-(4′-hydroxyazobenzene)benzoic acid (HABA) (Pierce, Rockford, IL) in 70% acetonitrile with 0.3% trifluoroacetic acid. In some cases, carbonic anhydrase (Sigma) was included in the matrix mixture (1 μM) as an internal standard to normalize the spectra. MALDI-TOF MS analyses were performed using an Applied Biosystems Voyager-DE PRO Biospectrometry Workstation with a 337 nm nitrogen laser. The mass spectrometer was operated in the linear mode with a delayed extraction of 425 ns and 25 kV accelerating voltage, with an ion flight path of 1.3 m. Spectra were averaged over 260 laser shots using 20 laser shots at 13 positions on each sample spot. The laser intensity was set at 200 units above the threshold laser intensity. Identification of the zein classes in the spectrum was accomplished by matching their molecular mass to deduced molecular weights derived from published sequences (*10*) and confirmed by using an iodoacetamide derivatization procedure. This procedure produces predictable mass increases through derivatization of cysteine residues that allowed the determination and confirmation by the number of cysteines corresponding to each zein. A detailed description of the development of analyzing zeins by MALDI-TOF MS will be published elsewhere (see accompanying paper elsewhere in this issue).

Biochemical and Amino Acid Composition Analyses. Two transgenic lines, PQ015 and PQ071, that demonstrated the 19-kDa α-zein reduction phenotype were backcrossed for two generations into Elite inbreds, 90DJD28 (*16*) and 91INH2 (*17*) (Dekalb Genetics, Dekalb, IL), and were used as the female and male lines, respectively, to produce hybrids. These F1 hybrids including two transgenic homozygotes (PQ015/PQ015 and PQ071/PQ071), a transgenic hemizygote (90DJD28/PQ015), and a wild-type control (90DJD28/91INH2) were planted in randomly mixed blocks at Monsanto's experimental station (Monmouth, IL) in the summer of 2001. Plants were hand-pollinated. MALDI-TOF MS analysis was performed on individual kernels from each of 20 ears selected from each line to confirm the 19-kDa α-zein reduction phenotype prior to biochemical and amino acid composition analyses. The proximate contents of bulked kernels from each ear were determined by near-infrared transmission (NIT) analysis described by Dyer and Feng (*18*) using an Infratec 1221 grain analyzer (Foss, Eden Prairie, MN). Samples of ground kernels were sent to the Experiment Station Chemical Laboratories at University of Missouri−Columbia for total amino acid composition analysis following Association of Official Analytical Chemists (AOAC) official method 982.30 E(a,b,c), Chapter 45.3.05.2000. The total protein content described in **Table 3** was determined by an FP-528 protein/nitrogen determinator (LECO, St. Joseph, MI) following American Oil Chemical Society (AOCS) official method Ba 4f-00. Systat version 10 (SPSS Inc., Chicago, Il) was used to determine the statistical significance of the differences observed in biochemical and amino acid compositions.

RESULTS

Vector Construction and Selection of Zein Reduction Transgenic Lines. DNA fragments isolated by PCR, including the coding and the 5′ and 3′ noncoding regions of a 19-kDa α-zein gene, *Z4* (*11*), were inserted into an expression cassette in either sense or antisense orientation. The γ-zein promoter was used to drive a high level of mRNA production, and the

Table 1. Genetic Characterization of Selected Transgenic Lines Displaying the 19-kDa α-Zein Reduction Phenotype[a]

line	harbored construct[b]	copy no. of transgene[c]	transgene segregation (+/–)[d]
7407	530 (1346–381)	ND[e]	28/26
8400	530 (1346–381)	3	22/31
9311	531 (381–1346)	7	24/27
9514	531 (381–1346)	>9	25/27
PQ015	763 (965–868)	3	ND
PQ071	763 (965–868)	6	ND
10616	879 (521–131)	5	27/24
10707	879 (521–131)	4	21/27
11354	878 (131–521)	4	19/25
11367	878 (131–521)	3	17/25
11440	877 (437–131)	>7	23/28

[a] F1 plants of 11 independent transgenic lines harboring 6 different constructs were analyzed by DNA gel-blot and PCR for the transgene segregation analysis. [b] The numbers specified in parentheses are the regions used in these sense/antisense constructs corresponding to the maize zein sequence, *Z4*, deposited in GenBank (accession no. V01472). The coding sequence starts at 459 and ends at 1262. [c] Because both endogenous genes and transgenes contained the γ-zein promoter sequence, when it was used as a probe, the copy number of the transgene was determined by number of bands that appeared on the DNA gel-blot excluding two bands that were common to all transgenic as well as wild-type plants. In transgenic lines 9514 and 11440, ">" indicates stronger intensity was observed in some of the bands and suggests that the actual copy number of the transgene may exceed the number of bands. The DNA gel-blot analysis for line 7407 is not available. [d] "+" and "–" donate the number of F1 plants that were tested positive and negative for the transgene. [e] ND, not determined.

3′UTR of the *A. tumefaciens* nopaline synthase gene was added to provide polyadenylation sequences. These expression cassettes were introduced into immature maize embryos by microprojectile bombardment (*12*). Regenerated R0 transgenic plants were pollinated with wild-type pollen to produce F1 seeds. These F1 kernels were screened for the opaque phenotype and analyzed by SDS-PAGE gels to evaluate the zein profiles. An opaque phenotype was observed to varying degrees in some transgenic kernels. The wild-type kernels analyzed by SDS-PAGE exhibited identical protein banding patterns, whereas a reduction in the band representing the 19-kDa α-zeins was observed among protein extracts from kernels of some of the transgenic lines (data not shown). When expression cassettes containing the coding regions of *Z4* were used to generate >100 independent R0 lines, ~30% exhibited the zein reduction phenotype in F1 seeds. However, the zein reduction phenotype was not detected among 50 transgenic lines containing only the 5′ or 3′ noncoding regions of *Z4*, with the exception of a single line, designated 11440 (**Table 1**).

Table 1 summarizes the transgenic lines with the zein reduction phenotype that were selected for further analyses. These lines were transformed with seven different constructs targeting various regions of *Z4*. DNA gel-blot analysis was performed to confirm the presence of the transgene in these lines. This analysis revealed complex integration patterns of the transgene in all of the lines tested. PCR analysis of segregating progeny indicates that the segregation ratios of transgenic versus wild-type segregants were nearly 1:1, suggesting that all of the transgenic copies were likely to be linked in each of these lines.

Reduction of 19-kDa α-Zein Expression in Kernels of Transgenic Plants. Experiments were conducted to examine levels of 19-kDa α-zein mRNA and protein. Transgenic and wild-type F1 plants identified by PCR and DNA gel-blot analysis were crossed with wild-type plants. Developing kernels were collected at 20 days after pollination from these F1 plants for RNA gel-blot analysis. Of all the lines tested, a reduced

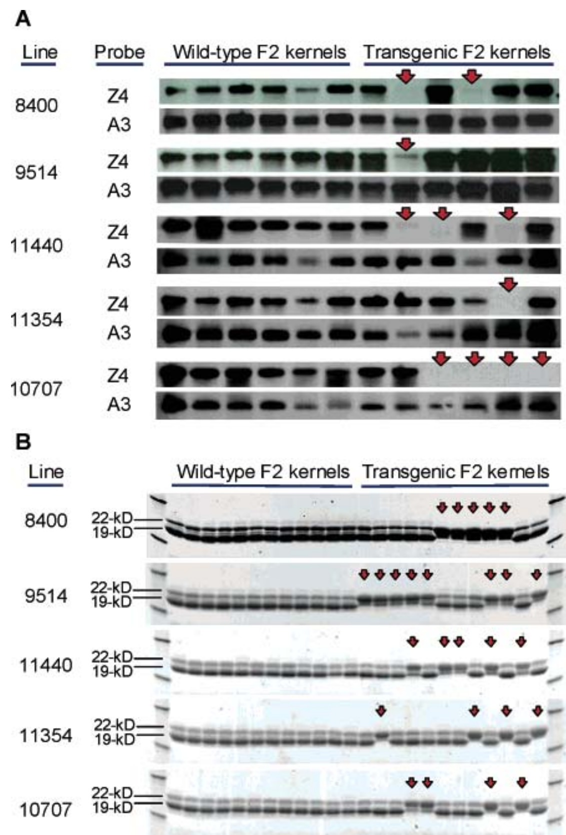

Figure 1. Reduction of the 19-kDa α-zein expression in transgenic kernels. (**A**) Approximately 10 μg of total RNA was loaded on each lane. The RNA gel-blot was first hybridized with a 19-kDa α-zein (*Z4*) probe. After removal of the zein probe, a probe encoding an RNA-binding protein, A3 (*29*), was applied. The arrows indicate the observed relatively reduced levels of 19-kDa α-zein transcripts in some of the kernels collected from the F1 transgenic plants. (**B**) Approximately 3 μg of α-zein protein extract was loaded on each lane. Kernels collected from wild-type F1 plants show a typical dominant 19-kDa α-zein band and a lesser 22-kDa α-zein band found in the wild type. In transgenic F1 plants, some kernels exhibit a reduction in 19-kDa α-zein and an increase in 22-kDa α-zein as indicated by the arrows. Two lines of molecular weight markers containing 20- and 30-kDa standard proteins flank each gel.

level of the 19-kDa α-zein mRNA was observed in some kernels segregated from transgenic F1 plants (**Figure 1A**). In contrast, the 19-kDa α-zein mRNA was relatively unchanged among individual kernels from the wild-type F1 plants (**Figure 1A**). In mature kernels, α-zein extracts analyzed typically contained a predominant 19-kDa α-zein band and a lesser abundant 22-kDa α-zein band on SDS-PAGE gels. This profile was observed with α-zein extracts from all kernels harvested from wild-type segregants (**Figure 1B**). Analysis of kernels derived from transgenic plants revealed segregation for a decreased 19-kDa α-zein band and an apparent increase in 22-kDa α-zeins (**Figure 1B**). These results suggest that the 19-kDa α-zein reduction phenotype in the transgenic plants is heritable and caused by the reduction of 19-kDa α-zein mRNA. The apparent increase in 22-kDa α-zeins in transgenic kernels on the SDS-PAGE gels was probably magnified because equal quantities of protein were loaded in each lane.

Analyses of the Zein Accumulation Pattern in F2 Seeds by MALDI-TOF MS. MALDI-TOF MS can separate proteins according to their true molecular weights and avoid the problems of anomalous migration and solubility associated with SDS-

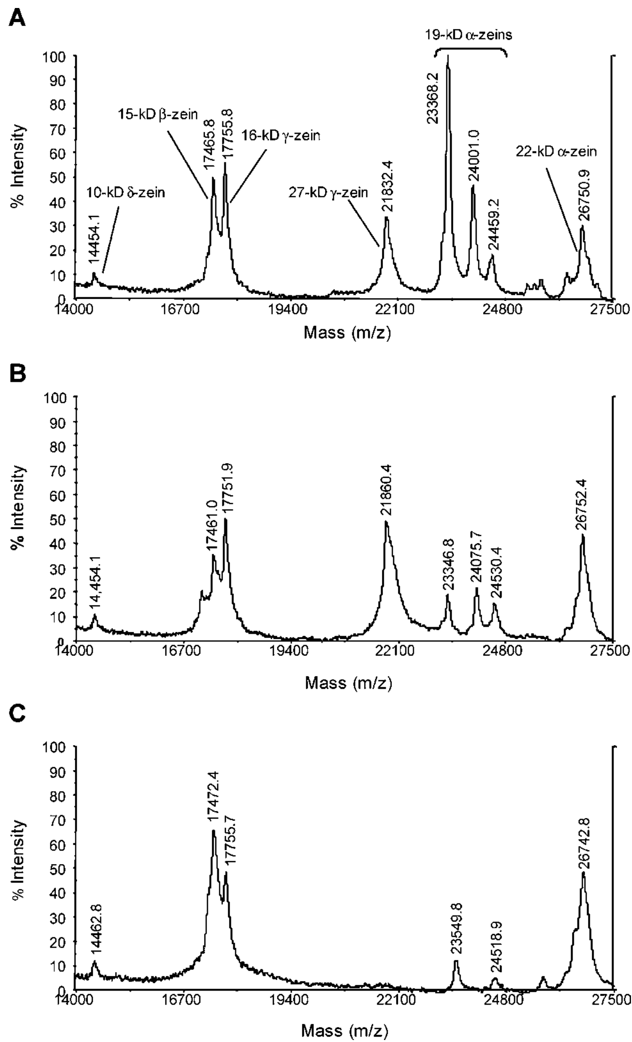

Figure 2. MALDI-TOF MS analysis of transgenic and wild-type kernels: (**A**) MALDI-TOF MS spectrum from a wild-type kernel shows the typical accumulation pattern for the major classes of zeins; (**B**) transgenic kernels display a specific reduction in 19-kDa α-zeins; (**C**) in most transgenic lines, 27-kDa γ-zein is also reduced.

PAGE (*19*). We used MALDI-TOF MS to semiquantitatively distinguish distinct zein expression patterns that are difficult to resolve by traditional SDS-PAGE methods in a high-throughput manner. Although variations can be observed in different genetic backgrounds, a typical spectrum from a single wild-type kernel is shown in **Figure 2A**. The major group of peaks, at 23,368, 24,001, and 24,459 Da, corresponds to the 19-kDa α-zeins, and peaks at 14,454, 17,466, 17,756, 21,832, and 26,751 Da are identified as the 10-kDa δ-zein, 15-kDa β-zein, 16-kDa γ-zein, 27-kDa γ-zein, and 22-kDa α-zein, respectively. All kernels produced by F1 wild-type segregants exhibited the typical zein accumulation pattern.

MALDI-TOF MS analysis of transgenic kernels revealed a specific reduction in the 19-kDa α-zein peaks, which was usually accompanied by an apparent increase in the 22-kDa α-zein peak (**Figure 2B**). This type of pattern appeared in ∼50% of the kernels examined and concurred with the results obtained from SDS-PAGE analysis. Reduction in the 27-kDa γ-zein peak was also observed in most lines (**Figure 2C**).

Composition of Grain Produced by Transgenic and Wild-Type Hybrids. To assess the effect of the 19-kDa α-zein reduction phenotype on grain composition, two transgenic lines, PQ015 and PQ071, were advanced into Elite inbred backgrounds, 90DJD28 (*16*) and 91INH2 (*17*). After two generations of backcross, they were self-pollinated to obtain lines that are homozygous for the transgenes. These transgenic homozygous inbreds were then used to produce hybrids. The F1 hybrids produced included two transgenic homozygotes (PQ015/PQ015 and PQ071/PQ071), a transgenic hemizygote (90DJD28/PQ015), and a wild-type control (90DJD28/91INH2). The F1 hybrids were self-pollinated to produce F2 ears. Twenty well-filled ears harvested from each hybrid were randomly selected for compositional analysis. Reduction of 19-kDa α-zein was confirmed by SDS-PAGE and MALDI-TOF MS. As expected, the reduction of 19-kDa α-zein was observed in all of the kernels produced by the two transgenic homozygous hybrids (PQ015/PQ015 and PQ071/PQ071) and segregated in kernels derived from the transgenic hemizygous hybrids (90DJD28/PQ015) (**Figures 3** and **4**). This demonstrates that the phenotype caused by the antisense *Z4* transgene can be stably introduced into hybrids and exhibited in F2 hybrid grain. The oil, protein, and starch contents along with the moisture and density of bulked seeds from individual ears were estimated by near-infrared transmission (NIT) analysis. No statistically significant difference was observed in the amount of total grain protein between the different hybrids (**Table 2**). The data would support the hypothesis that an increase in other seed proteins compensates for the reduction in 19-kDa α-zeins in the transgenic hybrids. Analysis of the relative amino acid content in kernels indicates noticeable differences among the four hybrids (**Table 3**). In general, transgenic kernels had increases in methionine, lysine, tryptophan, and aspartic acid, which are deficient in the 19-kDa α-zeins, and decreases in proline and leucine, which are abundant in the 19-kDa α-zeins. The homozygous transgenic hybrid (PQ015/PQ015) was found to have larger percentage modifications in amino acid composition than the hemizygous transgenic hybrid (90DJD28/PQ015). This was consistent with the fact that only 75% of the kernels produced by the hemizygous 90DJD28/PQ015 hybrid were transgenic due to the segregation of the transgene.

DISCUSSION

Unlike situations in animals where gene knockout by homologous recombination is easily achievable, gene silencing mediated by sense or antisense is one of the most useful tools in studying gene function in plants. Discovered more than a decade ago, it was immediately utilized to create one of the first genetically engineered products in agriculture (*20*). The recent improvement of using dsRNA not only makes gene silencing more effective but also consolidates the RNAi silencing studies from animal systems and will potentially expand its application to medicine (*21*, *22*). In this study, stable transgenic hybrids were demonstrated by using such technology to modify the protein composition of maize grain, resulting in an improvement in the nutritional quality of maize proteins.

One of the difficulties of studying zeins by transgenic approaches is the molecular and biochemical complexity of zein genes and gene products. A recent analysis of zein sequences present in developing endosperm cDNA libraries has provided additional insight into the abundance and organization of the zein gene expression (*10*). By using zein MALDI-TOF MS, we have examined the accumulation pattern of most major classes of zeins at the protein level in transgenic plants. The expression of sense/antisense *Z4* transcript (az19B2, GenBank accession no. AF371270), which normally represents only 0.1% of expressed sequence tags (ESTs) in the developing endosperm

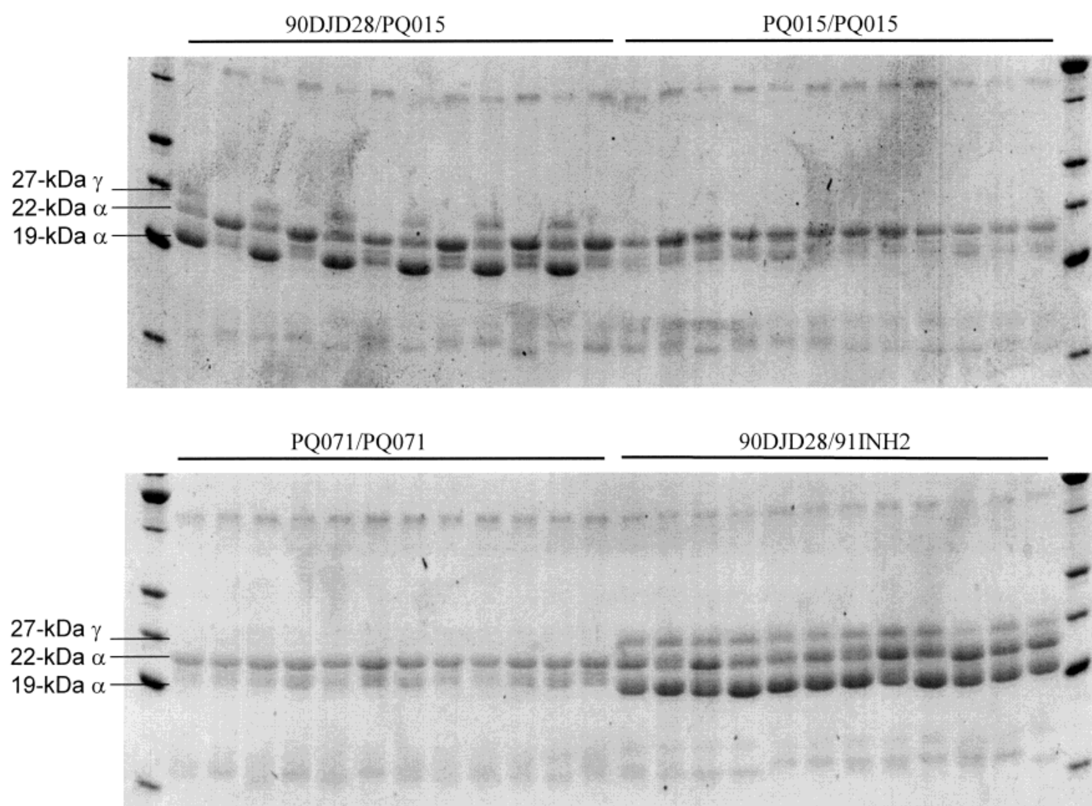

Figure 3. SDS-PAGE analysis of selfed hybrid kernels. Zein extracts of an equivalent of 100 μg of kernel powder were loaded per well. The reduction of 19-kDa α-zein and 27-kDa γ-zein is observed in all of the kernels produced by two transgenic homozygotes (PQ015/PQ015 and PQ071/PQ071) and segregated among the transgenic hemizygous kernels (90DJD28/PQ015), but not in the wild-type (90DJD28/91INH2) kernels. The protein molecular weight standards on each side of the gels are 10, 15, 20, 25, 30, 40, and 50 kDa from the bottom up.

Table 2. Biochemical Compositions (Percent ± Standard Deviation)[a] of Bulked Kernels Produced by Transgenic and Wild-Type Hybrids

	PQ015/ PQ015[b]	PQ071/ PQ071[b]	90DJD28/ PQ015[b]	90DJD28/ 91INH2[b]
oil[c]	5.2 ± 0.4	4.5 ± 0.3	4.7 ± 0.3	4.4 ± 0.2
protein	10.0 ± 1.1	11.0 ± 1.0	11.3 ± 0.9	10.6 ± 1.4
starch	68.4 ± 1.1	69.2 ± 0.7	68.7 ± 1.1	69.4 ± 1.1
moisture	10.4 ± 0.8	10.3 ± 0.6	10.9 ± 1.0	9.9 ± 0.5

[a] Data are means ± standard deviation ($n = 20$). [b] Two independent transgenic lines, PQ015 and PQ071, were backcrossed two generations into inbreds, 90DJD28 and 91INH2, which were used as the female and male lines, respectively. Bulked kernels harvested from two homozygous hybrids (PQ015/PQ015 and PQ071/PQ071), a hemizygous hybrid (90DJD28/PQ015), and a wild-type control (90DJD28/PQ015) were used for NIT analysis to determine their compositions. [c] The oil component is the only component that shows significant statistical difference among four hybrids ($p < 0.001$).

cDNA libraries, is able to greatly reduce the expression of the entire 19-kDa α-zein class, which accounts for >20% of ESTs. This is most likely due to the sequence similarities within the same class of zeins. In contrast, the inability to create the phenotype through the use of only the 5′ or 3′ noncoding sequences may be the result of insufficient shared sequence similarity in these regions. In addition to the reduction of 19-kDa α-zeins, in the majority of the transgenic lines, the phylogenetically distanced 27-kDa γ-zein is unexpectedly reduced, whereas the much more closely related 22-kDa α-zein remains unaffected or even appears to be increased in most instances. One potential explanation is that the expression of untranslated sequence derived from the γ-zein promoter, rather than the antisense/sense sequences of *Z4*, in the transgenic expression cassettes induces gene silencing of the 27-kDa γ-zein gene.

Table 3. Amino Acid Composition Analysis (Percent ± Standard Deviation)[a] of Bulked Kernels Produced by Transgenic and Wild-Type Hybrids

amino acid	PQ015/ PQ015	PQ071/ PQ071	90DJD28/ PQ015	90DJD28/ 91INH2
Asp[c]	8.10 ± 0.34	7.70 ± 0.33	7.02 ± 0.58	6.23 ± 0.30
Thr	3.94 ± 0.14	3.84 ± 0.12	3.56 ± 0.25	3.42 ± 0.18
Ser	4.78 ± 0.25	4.79 ± 0.14	4.44 ± 0.26	4.41 ± 0.24
Glu	19.90 ± 0.99	21.55 ± 0.89	19.90 ± 1.42	18.95 ± 1.14
Pro	7.13 ± 0.38	7.74 ± 0.44	7.51 ± 0.51	8.06 ± 0.50
Gly	4.83 ± 0.29	4.45 ± 0.25	4.03 ± 0.32	3.78 ± 0.25
Ala	7.62 ± 0.35	7.86 ± 0.27	7.33 ± 0.55	7.24 ± 0.35
Cys	2.48 ± 0.18	2.54 ± 0.39	2.31 ± 0.18	2.44 ± 0.13
Val	5.52 ± 0.22	5.50 ± 0.20	5.01 ± 0.40	4.49 ± 0.31
Met	3.43 ± 0.18	3.24 ± 0.27	2.82 ± 0.25	2.38 ± 0.20
Ile	3.57 ± 0.18	3.75 ± 0.17	3.45 ± 0.30	3.18 ± 0.25
Leu	11.04 ± 0.72	12.09 ± 0.68	11.72 ± 1.02	12.00 ± 0.86
Tyr	3.03 ± 0.12	3.06 ± 0.12	2.98 ± 0.19	2.77 ± 0.19
Phe	4.73 ± 0.14	4.85 ± 0.19	4.70 ± 0.37	4.71 ± 0.27
His	2.95 ± 0.08	3.02 ± 0.13	2.76 ± 0.20	2.76 ± 0.19
Lys	4.23 ± 0.29	3.87 ± 0.23	3.42 ± 0.30	2.94 ± 0.22
Arg	5.91 ± 0.27	5.69 ± 0.27	5.22 ± 0.41	4.70 ± 0.27
Trp	1.03 ± 0.10	0.97 ± 0.08	0.88 ± 0.08	0.79 ± 0.07
total protein measured[b] (g)	8.62 ± 1.24	9.74 ± 1.00	10.07 ± 1.07	9.27 ± 1.36

[a] Data are means of percentage of total protein measured ± standard deviation ($n = 20$). [b] Average grams per 100 g of dry sample ± standard deviation ($n = 20$) by nitrogen analyzer. [c] Amino acids in bold show significant statistical difference between transgenic and wild-type hybrids ($p < 0.001$).

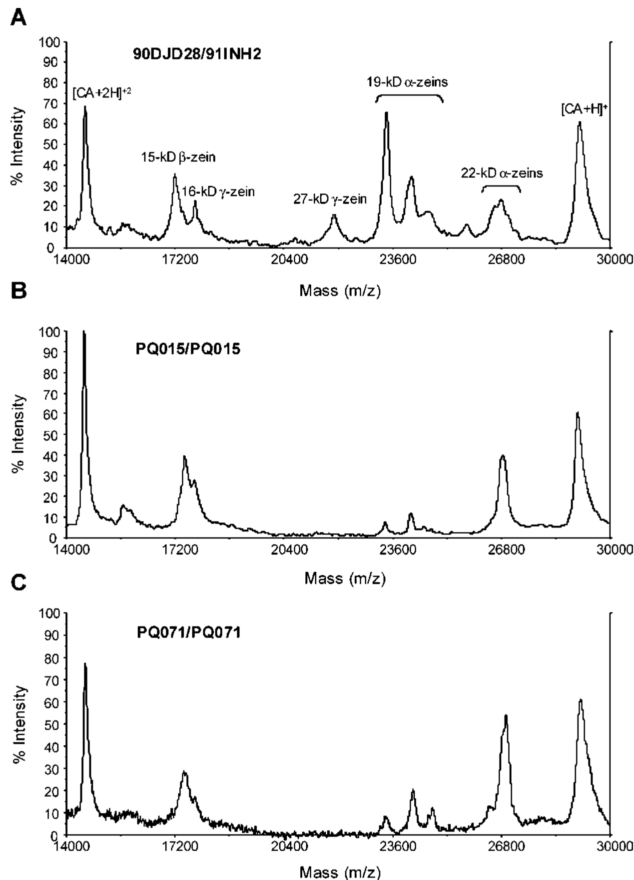

Figure 4. MADLI-TOF MS analysis of selfed hybrid kernels. The zein profiles of PQ015/PQ015 and PQ071/PQ071 displaying the reduction of 19-kDa α-zein and 27-kDa γ-zein phenotype are consistent with the SDS-PAGE results. These spectra were normalized by a standard protein peak, $[CA + H]^+$ (carbonic anhydrase), near *m/z* 29000.

The development of QPM varieties has established the possibility of modifying the soft, starchy phenotype of *o2* while maintaining its high levels of lysine and tryptophan. Earlier observations suggest that the accumulation of γ-zein is involved in the restoration of normal vitreous kernels in QPM (*23*). In our studies, we find that the opaque phenotype is not associated with all of the transgenic lines exhibiting reduced α-zein accumulation, nor can we correlate the level of γ-zein with the absence of an opaque phenotype. Many of the transgenic lines characterized display the reduction in both 27-kDa γ-zein and 19-kDa α-zeins; however, these same kernels sometimes display a vitreous phenotype. Because many of the transgenic lines were generated in various genetic backgrounds, we are now assessing whether these genetic backgrounds are influencing the occurrence of the opaque phenotype. The two transgenic lines that were advanced into the hybrid background exhibited the opaque endosperm phenotype (**Figure 5**). Additional measurements are required to determine the hardness of these opaque kernels as well as any other undesirable characteristics that may be associated with such a phenotype.

The changes in amino acid composition in transgenic zein reduction kernels are consistent with a reduction in 19-kDa α-zeins. In **Table 3**, lysine, methionine, aspartic acid, and tryptophan demonstrated the most consistent increases. These four amino acids are the least represented within the coding sequence of 19-kDa α-zeins (0, 0.5, 0.5, and 0%, respectively) (*24*). In contrast, proline and leucine, which correspond to 10.0 and 19.5%, respectively, of the amino acids comprising the 19-kDa α-zeins, have the largest percentage decrease in transgenic zein reduction kernels. It has been proposed that the elevated lysine and trytophan contents observed in the *o2* mutant grain are caused by significant increases in other lysine- or trytophan-containing proteins, such as elongation factor-1α (*25*) or globulin1 (*26*). In contrast, a recent study of protein fractions derived from *o2* endosperm suggests that the composition changes are largely attributable to the reduction in zein accumulation as well as an increase in free lysine (*27*). Although the mechanism behind the transgenic reduction of the 19-kDa α-zeins resulting in amino acid composition changes in these transgenic hybrids is still unclear, the lysine and trytophan contents of PQ015/PQ015 (4.23 and 1.03% in grain protein, respectively, **Table 3**) are comparable to those observed in QPM (*28*).

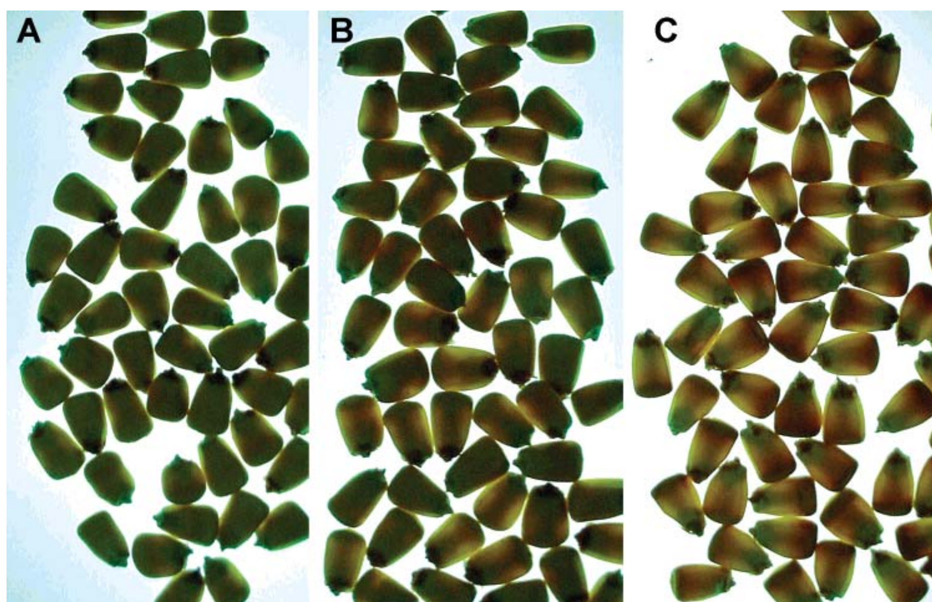

Figure 5. Opaque endosperm phenotype observed in transgenic hybrids. The transgenic kernels produced by (**A**) PQ015/PQ015 and (**B**) PQ071/PQ071 show various degrees of opaque phenotype, whereas the kernels of wild-type control (**C**) 90DJD28/91INH2 are vitreous.

We have demonstrated the feasibility of enhancing the nutritional quality of maize proteins through a genetic engineering approach designed to reduce the accumulation of α-zeins. It enables the specific reduction of zeins directly in the near Elite genetic background, which capitalizes on the relative simplicity of breeding with transgene when compared to the traditional breeding efforts required to develop the QPM varieties. In addition, the development of MALDI-TOF MS for zein separation facilitates the phenotypic analysis of transgenic plants. For example, we have been able to precisely identify which zeins are impacted by the transgenic zein reduction approach as well as which zein species could also be targeted for reduction. Finally, our studies suggest that transgenic plants with a specific deficiency in each class of zeins could be generated to examine protein function and provide new insights into the biochemistry and molecular biology of maize endosperm.

ABBREVIATIONS USED

QPM, Quality Protein Maize; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; MADLI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NIT, near-infrared transmission; EST, expressed sequence tag.

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