A New Opaque Variant of Maize by a Single Dominant RNA-Interference-Inducing Transgene

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ABSTRACT

In maize, α-zeins, the main protein components of seed stores, are major determinants of nutritional imbalance when maize is used as the sole food source. Mutations like *opaque-2* (*o2*) are used in breeding varieties with improved nutritional quality. However, *o2* works in a recessive fashion by affecting the expression of a subset of 22-kD α-zeins, as well as additional endosperm gene functions. Thus, we sought a dominant mutation that could suppress the storage protein genes without interrupting O2 synthesis. We found that maize transformed with RNA interference (RNAi) constructs derived from a 22-kD zein gene could produce a dominant opaque phenotype. This phenotype segregates in a normal Mendelian fashion and eliminates 22-kD zeins without affecting the accumulation of other zein proteins. A system for regulated transgene expression generating antisense RNA also reduced the expression of 22-kD zein genes, but failed to give an opaque phenotype. Therefore, it appears that small interfering RNAs not only may play an important regulatory role during plant development, but also are effective genetic tools for dissecting the function of gene families. Since the dominant phenotype is also correlated with increased lysine content, the new mutant illustrates an approach for creating more nutritious crop plants.

THE nutritional value of cereal grain protein is a critical constraint in its use for animal and human consumption. Protein deposits in the specialized tissue of cereal endosperm are mostly deficient in essential amino acids, particularly lysine, tryptophan, and methionine. Because protein supplementation to correct such deficiencies is costly and wasteful of energy in animal nutrition, it is not feasible in vast areas of developing countries that rely on cereals as a sole food resource. Therefore, the improvement of cereal storage proteins for increased amounts of protein-bound essential amino acids has been recognized as a primary objective in breeding.

In maize, several approaches have been undertaken to correct this problem (Mazur *et al.* 1999). A major breakthrough has been the isolation of the *opaque-2* mutant (*o2*), with superior nutritional properties, albeit undesirable agronomic traits (Mertz *et al.* 1964). More recently, the generation of quality-protein maize incorporated the enriched *o2* protein quality into breeding materials devoid of the endosperm soft texture and pest susceptibility that characterize *o2* (Cordova 2000). The molecular identification of the underlying defect in *o2* variants provided the first example of a transcriptional activator gene that directly controls the transcription of maize storage protein or zein genes (Schmidt *et al.* 1987; Lohmer *et al.* 1991). It is now well established that O2 plays an important role in multiple endosperm-specific metabolic pathways (Damerval and Le Guilloux 1998; Hunter *et al.* 2002).

In maize, zeins, the core of protein endosperm reserves, have been the subject of intense studies due to their abundance, complexity, and impact in the overall nutritional value of maize seed. They have been classified into four subfamilies of α-, β-, γ-, and δ-zeins on the basis of their primary structure and differential solubility (Ueda and Messing 1993). Zeins are encoded by single- or low-copy gene loci, with the exception of α-zeins. The large α-zein component, accounting for >70% of all zein protein, is composed of multiple active genes clustered in several chromosomal locations. Genomics approaches are best suited to identify and characterize the entire α-zein gene complement (Song *et al.* 2001, 2002; Woo *et al.* 2001; Hunter *et al.* 2002; Song and Messing 2002), which has been subdivided into 19- and 22-kD zein subfamilies. From a nutritional point of view, the exceedingly large proportion of codons for hydrophobic amino acids in α-zeins is mostly responsible for the imbalance of maize protein reserves. Therefore, the reduction in α-zein protein accumulation with biased amino acid content could provide a correction to this imbalance. The *o2* mutation differentially inhibits the transcription of the 22-kD α-zein component, which is thus greatly reduced in the endosperm (Kodrzycki *et al.* 1989). Therefore, *o2* mutants could be used to achieve such regulation. Unfortunately, several disadvantages have become apparent with the characterization of *o2*. First, O2 regulates genes other than 22-kD

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zeins. As mentioned above, the alteration of one or several of these transcripts generates deleterious phenotypes in the plant and grain product. Second, it has been reported that several 22-kD zeins are independent of *o2* (Ciceri *et al.* 2000; Song *et al.* 2001), preventing an efficient repression of all 22-kD zein genes. This prompted the question of whether this effect can be reproduced independently of the *o2* mutation.

Antisense and RNA interference (RNAi) technologies provide the means to target individual plant components for depletion (Waterhouse *et al.* 1998; Chuang and Meyerowitz 2000; Schweizer *et al.* 2000). However, in many cases reduction of gene expression is not complete and it is uncertain whether such an approach would be sufficient to achieve a visible phenotype. Since this has not been tested yet in maize seeds, we adopted a system for gene expression restricted to hybrid transgenic plants, first established in Arabidopsis (Moore *et al.* 1998). This system is composed of the introgression of sequences encoding a modified prokaryotic transcriptional activator from a first group of transgenic plants into a second set with a background of a reporter gene of interest downstream of a plant promoter restricted to a TATA box and modified *cis*-acting prokaryotic elements, which are recognized and bound by the heterologous transcriptional activator with high affinity. Only the cross between both kinds of plants activates the expression of the reporter transgene (Figure 1A). We report here the successful evaluation of these approaches by obtaining a dominant opaque phenotype through RNAi but not antisense constructs, the stable and heritable expression of recombinant sequences in maize that produce a specific reduction in 22-kD α-zein proteins. Moreover, the amino acid composition in transgenic opaque seeds had higher levels of lysine and reduced levels of leucine, alanine, and glutamine, confirming the shift in amino acid balance.

MATERIALS AND METHODS

Plant material: High type II callus maize (*Zea mays*) seed lines A and B were kindly provided by Kan Wang, Plant Transformation Facility, Iowa State University. W64A and W64A*o2* lines were from our own collection. All plants were grown under greenhouse conditions.

Transformation constructs: For the transactivation system, constructs were derived from those previously generated for *Arabidopsis thaliana* (Figure 1A; Moore *et al.* 1998). For the reporter lines of the 22-kD α-zein, the entire *azs22.8* coding region (Song *et al.* 2001) was purified as an *Eco*RI-*Nde*I fragment, filled in with Klenow enzyme, and cloned in the antisense orientation into the *Sma*I polylinker site of pOp (Moore *et al.* 1998), downstream of duplicated modified operator sequences of the *lac* operon followed by a minimal (−50) 35S cauliflower mosaic virus (CaMV) promoter and upstream of a 35S CaMV terminator. The entire insert was purified, subcloned into the *Sma*I site of pBluescript SKII, and reexcised with *Hin*dIII for ligation in the *Hin*dIII site of pUbi-bar with the transcription units in opposite orientation (Figure 1B, construct A3). The entire insert of pKI-His, including the 35S CaMV promoter, a coding region composed of the engineered version of a translational fusion between the *Escherichia coli lacI* and the *Saccharomyces cerevisiae Gal4* genes and the 35S CaMV terminator (Moore *et al.* 1998), was excised by *Pst*I digestion, end filled, and ligated to pUbi-bar (Christensen and Quail 1996), a maize expression cassette with the *Bar* gene as a selectable marker, driven by the maize ubiquitin1 promoter and first intron and 3′ end regulatory sequences of the nopaline synthase gene (Figure1C, construct KI).

Two constructs were designed for RNAi experiments. An inverted repeat (IR; Figure 3A) construct was generated following the guidelines of Tavernarakis *et al.* (2000). A 483-bp *Xba*I-*Nco*I including 5′ untranslated region (UTR) sequences and the amino-terminal half of *azs22;8* α-zein cDNA was ligated to *Xba*I-digested, dephosphatased pJM2170 (Lai and Messing 2002) between 1.1 kb of the 27-kD zein promoter sequence and the 35S CaMV terminator, so that the coding sequences established an inverted repeat without intervening sequences. *E. coli* Sure strain (Stratagene, La Jolla, CA) was transformed with this construct to reduce the frequency of rearrangements, and supercoiled plasmid DNA was purified and directly used for cotransformation experiments together with pUbi-bar. To increase the stability and ease of the detection of the transgene and to increase the efficiency of silencing (Levin *et al.* 2000), an additional construct with the green fluorescent protein (GFP) gene-coding region as intervening sequence was made (Figure 4A, LIN). A 313-bp fragment of the amino-terminal half of the α-zein cDNA was amplified by using primers R22-1 (5′-GGCGAGCGTCTACAACAACC) and R22-2 (5′-CACAACGAGAGGGCTAGATGAAAG) and directly cloned into pGEM-T Easy (Promega, Madison, WI). The insert was excised with *Sac*II and *Spe*I and subcloned into the corresponding sites of a pBluescript SKII-derived plasmid carrying the GFP-coding region (CLONTECH, Palo Alto, CA), cloned between the *Bam*HI and *Eco*RI sites. The same α-zein PCR fragment was excised with *Not*I and cloned into the *Not*I site immediately after the GFP stop codon and upstream of the terminator, recombinant colonies with the right orientation were selected, and the plasmid DNA was digested with *Sac*II to isolate the entire insert. This was gel purified, filled in with T4 DNA polymerase, and ligated to phosphorylated *Xba*I linkers (no. 1081, New England Biolabs, Beverly, MA). The excess linkers were eliminated by agarose gel electrophoresis followed by spin-columm agarose removal. The purified insert was digested with *Xba*I and then ligated to an *Xba*I-linearized, dephosphatased, pBluescript SKII-based vector between a 1.3-kb promoter fragment of the *zp22/6* zein gene (Song *et al.* 2001) and the 35S CaMV terminator. This promoter sequence has been determined to strongly activate the expression of transgenic reporter genes in maize endosperm (G. Segal and J. Messing, unpublished results). This construct was used as supercoiled DNA in cotransformation, together with pUbi-bar.

Maize transformation: Transgenic plants were generated by particle bombardment of highly embryogenic type II callus according to Frame *et al.* (2000). Immature 1.0- to 2.0-mm embryos were aseptically dissected, incubated in callus-inducing medium at 25° in the dark, and transferred every week. Once a friable, highly proliferating type II callus had developed, it was arranged in the center of osmotic-medium plates and shot with DNA-coated gold particles (Frame *et al.* 2000). We used single plasmids containing both the selection elements and the gene of interest (transactivator system) or cotransformation (RNAi experiments). After 2–4 months of bialaphos selection, rapidly growing callus lines were screened by the chlorophenol red assay (Kramer *et al.* 1993) and plants were regenerated. Transgenesis was confirmed by Southern analysis. T_0 plants were either selfed or outcrossed to untransformed B × A hybrids.

Molecular procedures: Southern analysis was performed as previously described (Das *et al.* 1990). As a preliminary characterization, all putative transgenic plant DNA was hybridized to a 444-bp PCR probe of the *Bar* gene-coding region. To further analyze the KI transactivation plants, a 1.6-kb *Xba*I fragment spanning almost the entire transactivator open reading frame (ORF) was used. LIN line DNA was analyzed by labeling a 763-bp *Xba*I sequence of the entire pGFP (CLONTECH) insert. Total zeins were extracted from mature kernels with 70% ethanol and 2% β-mercaptoethanol and fractionated by SDS-PAGE (Das *et al.* 1990). PCR reactions were carried out in 50 μl and 0.5 mm of each of the following primers: for KI, KI-1 (5′-CAGGGCCAGGCGGTGAAG) and KI-2 (5′-TTT TTGGGTTTGGTGGGGTATCT); for bar, Bar-for (5′-GACAT GCCGGCGGTCTGCACCATCG) and Bar-rev (5′-GCCAGAA ACCCACGTCATGCCAG); for GFP, GFP-1 (5′-CGTCCAGGA GCGCACCATCTTCTT) and GFP-2 (5′-ATCCGCGTTCTCGT TGGGGTCTTT); and for α-zein, R22-1 and R22-2 as described above. PCR conditions were 94° for 3 min, followed by 30 cycles of 30 sec at 94°, 30 sec at 57°, and 1 min at 72°, with a final extension for 10 min at 72°. A total of 10% DMSO was added in the PCR reactions to amplify Bar.

Amino acid composition analysis: Protein-bound amino acid seed composition was determined in samples submitted to the New Jersey Feed Laboratory, Trenton, New Jersey.

RESULTS

A transactivation system reduces 22-kD zein accumulation in hybrid transgenic plants: An outline of the components for a maize transgenic system regulated by transactivation is presented in Figure 1A. Two kinds of transgenic plants were required, one expressing a transcriptional activator that recognizes a unique synthetic promoter and the other with a chimeric gene consisting of the synthetic promoter controlling the gene of interest. When the transgenic lines are crossed, the synthetic promoter is recognized and the target transgene is transcribed. For the purpose of 22-kD antisense inhibition, we generated two sets of transgenic maize plants. One, termed A3, provided the target moiety of the system. Under an enhancerless 35S CaMV promoter, consisting mainly of the TATA box with two altered *lac* gene operator sequences for increased affinity, the entire *azs8* 22-kD zein cDNA (Song *et al.* 2001) in the antisense orientation was cloned (Figure 1B). The other set of lines involved the stable integration of sequences encoding a modified transcriptional factor (Moore *et al.* 1998) that is under the control of the constitutive 35S CaMV promoter and that can specifically bind to the altered *lac* operator sequences (Figure 1C, KI). However, since the *lac* operator is absent in the maize genome, expression of the transcriptional activator cannot result in transcription of any endogenous gene. This transgenic line should be useful for any future transactivation experiments in maize, which would require only the transformation of one line with a chimeric gene containing the synthetic promoter and a gene of interest. We did not expect to detect any antisense gene activity in these separated sets of transgenic plants. On the other hand, in a cross between both classes of plants, the transcriptional activator derived from KI should recognize and bind the upstream *cis*-acting elements from A3 and activate the transcription of the antisense α-zein, which would affect the accumulation of 22-kD protein in the hybrid transgenic endosperm. Several lines were established for each kind of construct, as shown by Southern blot analysis (Figure 2, A and B), with copy number ranging from 1 to >10. For transactivation experiments, 21 hybrid transgenic lines combining diverse transactivator and α-zein antisense events were screened by SDS-PAGE. Most seeds analyzed uncovered little or no reduction in the 22-kD zein level (data not shown). Line KI-GR-11 (designated by an asterisk in Figure 2B), which appears to have one entire copy of the transgene, crossed with A3-B, the line containing the antisense gene, revealed a significant reduction and was further analyzed. Individual T_1 seeds, originated from the cross of two selfed T_0 plants of these lines, were analyzed side by side from the parental lines by PCR and SDS-PAGE (Figure 2C). It is apparent that visible reductions in the 22-kD zein have occurred only in the seed resulting from the combination of KI with A3 in the same plant, while no change can be detected in the parental lines. No additional phenotype was detected in this progeny, which germinated and developed normally in every other respect. Inhibition of the 22-kD zein was not enough in this case to detect an opaque phenotype. These results show that, while the transactivation system works well in maize, the substantial residual level of 22-kD zein protein in hybrid transgenic plants proves that antisense constructs were unable to suppress 22-kD zein synthesis sufficiently to induce an opaque phenotype.

A simple inverted repeat RNAi construct triggers complete disappearance of the 22-kD zein: Since antisense RNA expression did not achieve the levels of 22-kD zein reduction equivalent to the knockout of O2, an alternative approach was undertaken. More recently, it has been shown that RNAi technology is an additional penetrant and an efficient way of depleting individual mRNAs in eukaryotes (Tavernarakis *et al.* 2000; Matzke *et al.* 2001). It is believed that in contrast to antisense RNA, RNAi triggers the post-transcriptional gene-silencing pathway, which seems to be conserved in plants. Therefore, we made a simple construct (see materials and methods), composed of an inverted repeat of a 22-kD zein cDNA fragment flanked by the endosperm-specific 27-kD γ-zein promoter and the 35S CaMV polyadenylation signal sequences (Figure 3A). Because of the concern that an RNAi construct with inverted repeats would be difficult to clone into a large plasmid, the silencing plasmid was used in cotransformation experiments with a second plasmid for expression of a maize selectable marker. The well-established pUbi-bar maize cassette of the 5′ maize ubiquitin gene regulatory sequences and the NOS 3′ UTR driving the expression of the phosphinothricin acetyltransferase (*Bar*) gene, for selection of

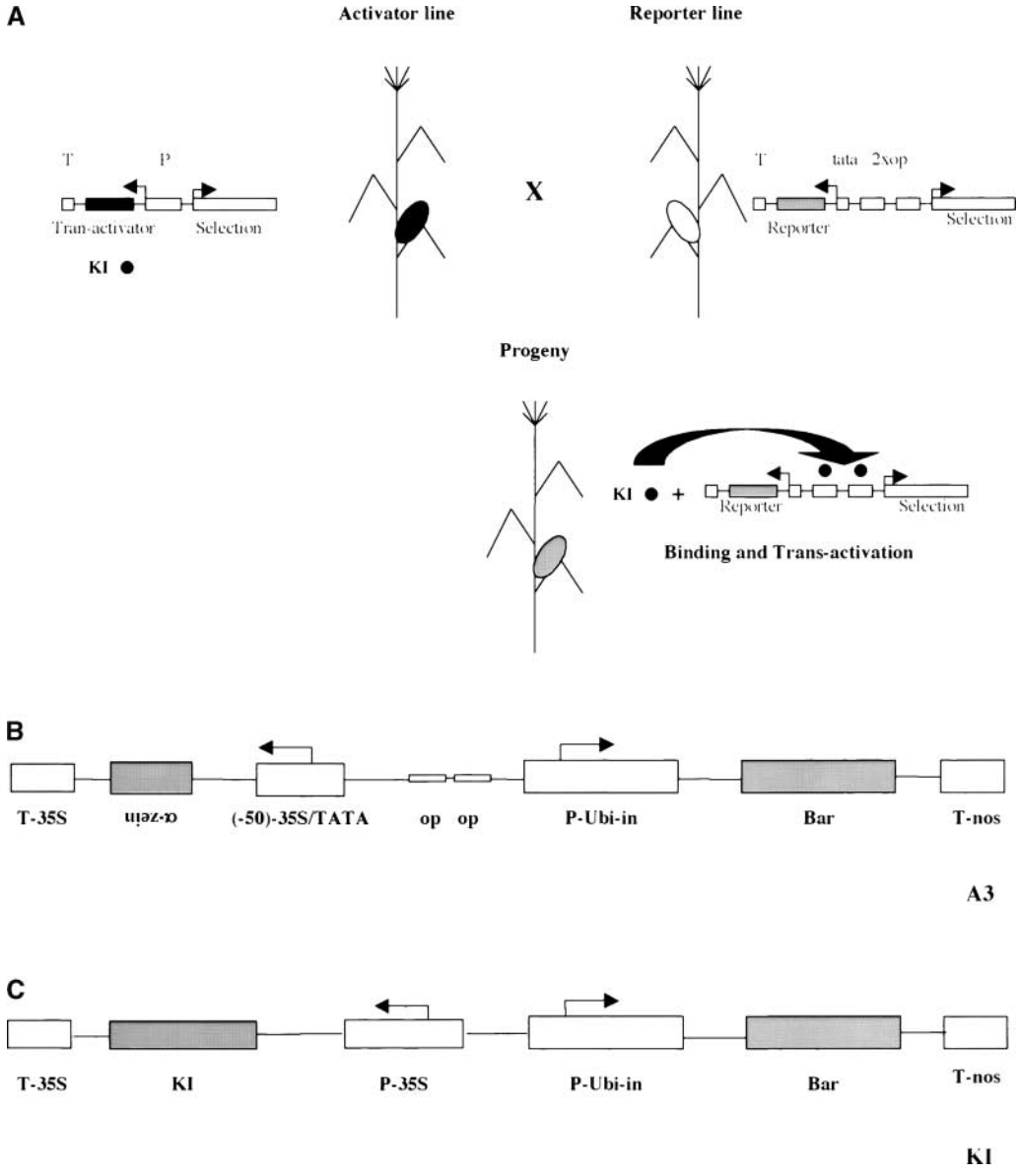

FIGURE 1.—Maize transactivation system. (A) Schematic representation of the transactivation system. Two sets of maize lines were produced: an activator line for constitutive expression of a chimeric transcriptional activator (KI), designed for high-affinity recognition and stimulation of a synthetic promoter (TATA box and 2x op, duplicated modified *lacI* operator binding sites; T, polyadenylation signal sequence), and a target line with no detectable transgene expression at first, since it is under the control of the synthetic promoter not recognized by any maize transcriptional activator (MOORE *et al.* 1998). In addition, both kinds of sequences include the elements for stable expression in maize. In the progeny of a cross between these lines where the active and passive sequences coexist, transactivation will take place with accumulation of the transcriptional activator and induction of the target gene. Regulation of the latter will depend on the characteristics of the promoter (P) used in the activator line. (B) Transactivation system antisense construct (A3). op, modified operator sequences of the *E. coli lac* operon (MOORE *et al.* 1998); (−50)-35S/TATA, (−50) promoter and TATA box of 35S CaMV; α-zein, 5′ UTR and coding region of the maize *azs22.8* α-zein cDNA, inserted in the antisense orientation in the *Sma*I site of the pOP polylinker; T-35S, 3′ UTR of 35S CaMV. The right half of the scheme shows the pUbi-bar plasmid (CHRISTENSEN and QUAIL 1996) used in all the experiments either as part of an entire construct (transactivation system) or in cotransformation experiments (RNAi experiments). P-Ubi-in, promoter and first intron of the maize ubiquitin gene; Bar, coding region of the phosphinothricin acetyltransferase gene; T-nos, 3′ UTR of the nopaline synthase gene. (C) Construct for constitutive expression of the hybrid modified *E. coli*/*S. cerevisiae* KI-His (MOORE *et al.* 1998). P-35S, promoter of 35S CaMV; KI, coding region of pKI-His; T-35S, 3′ UTR of 35S CaMV. ORFs are shaded or solid boxes; regulatory sequences are represented by open boxes. Arrows indicate the direction of transcription.

transgenic callus with the herbicide bialaphos (CHRISTENSEN and QUAIL 1996), was mixed with the RNAi-inducing plasmid for gold particle coating and bombardment of the embryogenic callus. Southern blot analysis of the regenerated plants with either the bar gene or the 35S CaMV 3′ UTR as a probe (Figure 3, B and C) revealed that a majority of the transgenic plants obtained had insertions of both the selectable and the cotransformed, nonselectable plasmids in 1–10 copies. Total zein extracts from T_0 mature seed products of self-fertilization or outcrosses to untransformed parental lines were analyzed by SDS-PAGE (Figure 3D). We could identify lines (IR-C and IR-D) with almost complete suppression of the 22-kD zein without compensatory changes in the other zein components. Differential levels of the larger γ-zein are due to segregation of the B and A lines that were used for transformation. The reduction of the 22-kD zein in these transgenic plants is much stronger than in those with antisense constructs and is at least as pronounced as the one observed with mutant *o2* seeds (Figure 3E). Residual protein detected at the position of the depleted 22-kD zeins is probably

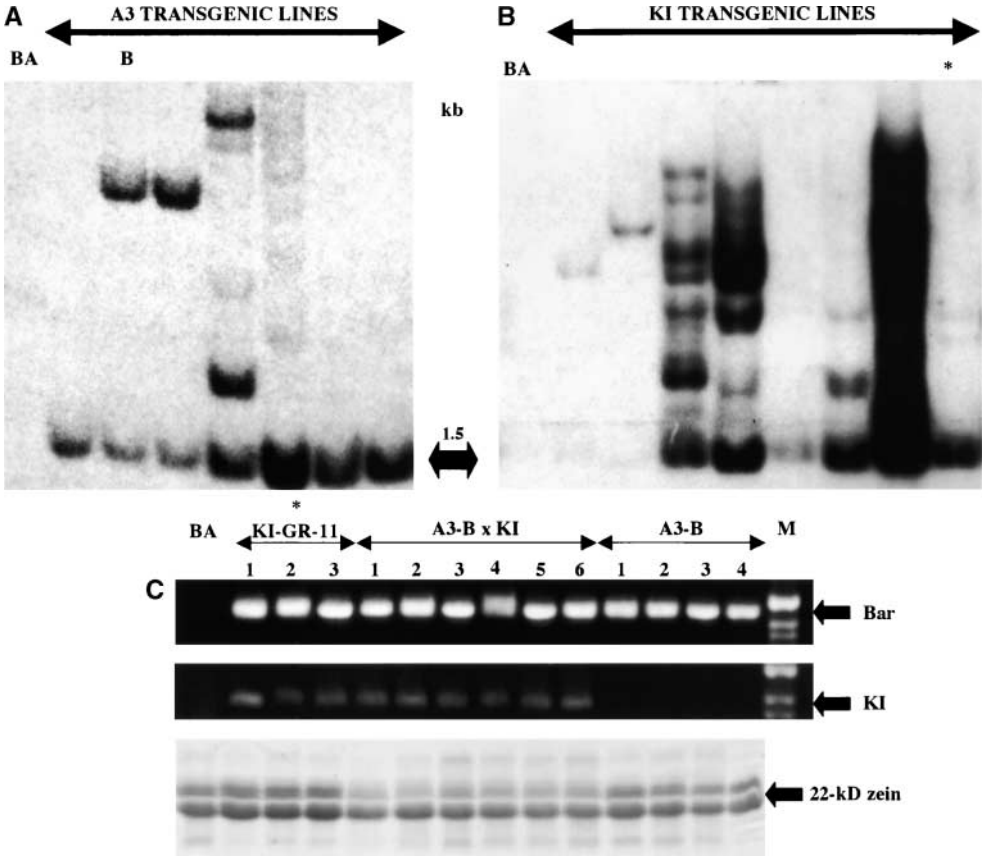

FIGURE 2.—Transactivation system in maize. (A) Southern blot analysis of A3 lines. Leaf DNA from putative transgenic plants was digested with *Eco*RI, blotted onto a nylon membrane, and hybridized with a 444-bp PCR fragment of the *Bar* gene. A 1.5-kb fragment is recognized by this probe if the plant is transgenic. Selected A3-B line is designed with B at the top. (B) Southern blot analysis of KI lines leaf DNA digested with *Eco*RI and hybridized with the *Bar* gene probe. A 1.5-kb fragment is recognized by this probe if the plant is transgenic. KI-GR-11 line characterized in C is identified by an asterisk at the top. Similar results were obtained with a 1.6-kb *Xba*I fragment of the coding region of KI-His (data not shown). (C) PCR and SDS-PAGE protein analysis of seed derived from lines KI-GR-11, expressing KI-His and A3-B, a target line with an antisense 22-kD zein sequence, and progeny from their cross. DNA and total zeins were extracted from individual seeds. PCR was conducted with specific primers for Bar (top) and KI (middle), and proteins were fractionated and stained. M, 1-kb ladder DNA molecular weight marker (Invitrogen, Carlsbad, CA). The migration of the 22-kD zein is indicated by an arrow. BA, untransformed parental line.

a divergent 19-kD α-zein, such as *z448F14* (SONG and MESSING 2002), that comigrates with the targeted proteins. From a total of 13 lines examined, 5 were found to segregate seeds with normal and reduced 22-kD zein phenotypes. We observed that in the case of progeny derived from selfed plants of the transgenic line IR-D, the affected seeds appeared at the ratio of ~3:1 relative to normal seeds, the expected frequency for a single dominant trait (data not shown). We next examined whether the transgene is stably transmitted. Seeds from T_0 IR-D plants were planted and the respective plants were either selfed or outcrossed. DNA and zein analysis from the resulting T_1 seeds (Figure 3E) provided evidence for 22-kD zein depletion in transgenic seeds from all these plants, indicating that transgene suppression is stable for at least two generations. More importantly, the quantitative changes in relative zein abundance were concomitant with the production of an opaque phenotype in the transgenic seed (Figure 3F). The appearance of a characteristic chalky texture across the entire endosperm cosegregated with protein alteration. Therefore, RNAi appears to be a useful genetic tool for studying the regulation of gene families in maize.

RNAi produced by 22-kD zein repeats separated by a linker sequence: Since RNAi appeared to simultaneously and specifically silence all members of a multigene family, two additional parameters of this approach were investigated. RNAi can be induced by either a direct inverted repeat or inverted repeats separated by a neutral sequence. In fact, repeats separated by either a silent coding region or an intron have consistently yielded deeper and more stable gene silencing (CHUANG and MEYEROWITZ 2000; WESLEY *et al.* 2001). If this is the case, then one might not need a strong promoter. Therefore, a different endosperm-specific promoter from the *zp22/6* zein gene (SONG *et al.* 2001) was selected for the synthesis of the RNAi transcripts. A 1.3-kb sequence comprising this promoter was fused to a RNAi sequence of inverted 22-kD coding sequences interrupted by the entire GFP-coding region as shown in Figure 4A. This RNAi construct was introduced into maize by the cotransformation method with a pUbi-bar plasmid as a selectable marker as described above. Southern analysis of DNA extracted from transgenic plant leaves (Figure 4B) corroborated the efficacy of cotransformation, since most of the plants selected presented one to multiple copies of the cotransformed, nonselectable transgene. There was no evidence of GFP expression in transgenic tissues, which is readily detectable when this reporter gene is placed under the control of the *zp22/6* promoter (G. SEGAL and

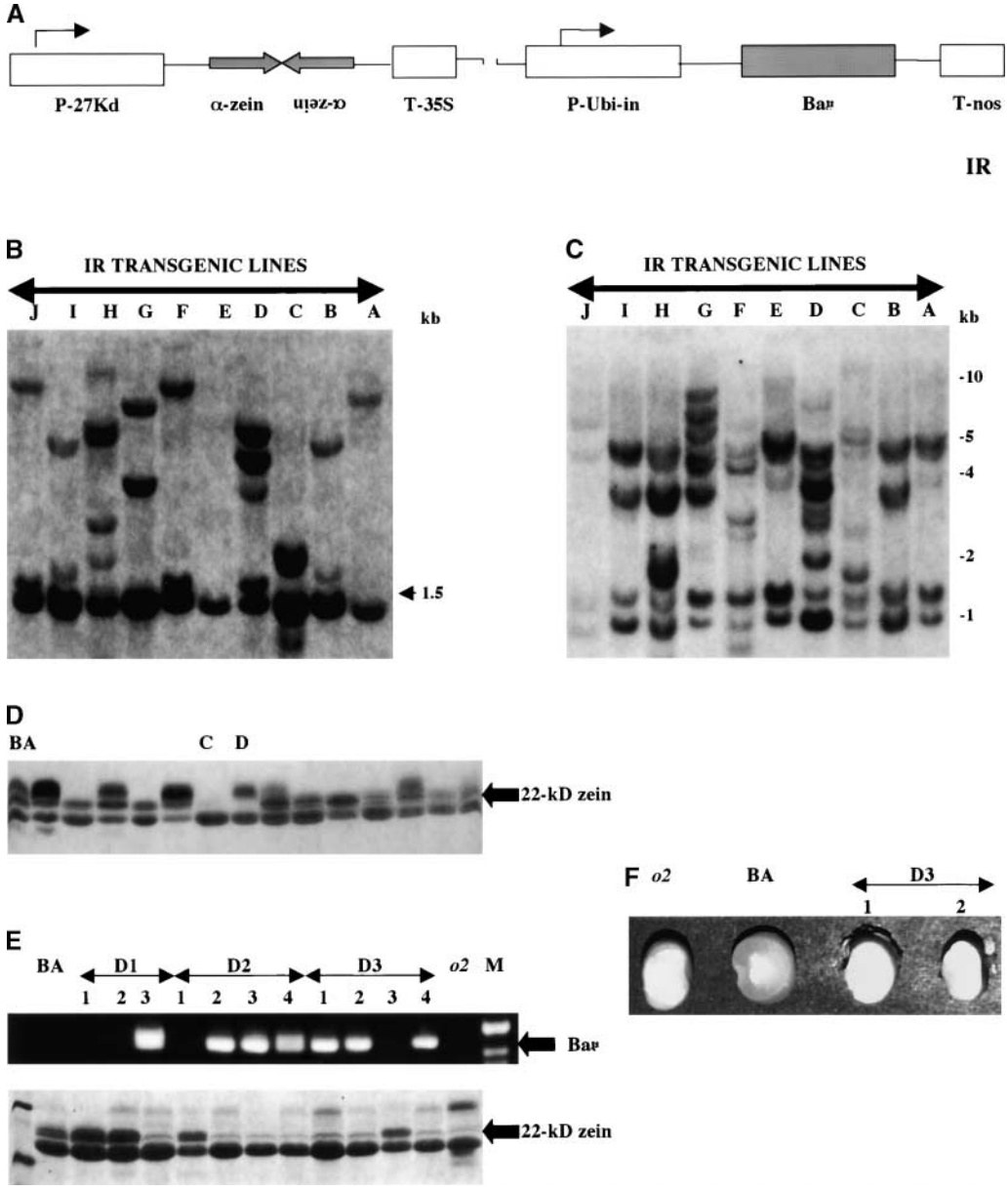

FIGURE 3.—Analysis of transgenic plants derived from a simple IR construct. (A) Construct with simple IRs. P-27kD, promoter of the maize 27-kD γ-zein gene (LAI and MESSING 2002). α-zein, *Xba*I-*Nco*I fragment of the 5′ UTR and coding region of the maize 22-kD α-zein cDNA, ligated to an *Xba*I site 3′ of P-27kD. Maize selection cassette is as in Figure 2. (B) Southern blot hybridization of DNA extracted from putative transgenic plants with the Bar probe. (C) The same blot as in A was stripped of signal and hybridized with a 203-bp *Xba*I-*Eco*RI probe of the 3′ UTR of the 35S CaMV gene. This probe is unique to the nonselectable plasmid of this experiment, indicating cotransformation. (D) SDS-PAGE analysis of total zeins extracted from T_0 seeds of putative transgenics. BA, untransformed parental line. The migration of the 22-kD zein is indicated by an arrow. Two seeds from lines IR-C and IR-D with suppressed 22-kD zeins are identified. (E) PCR and SDS-PAGE analysis of DNA and total zeins extracted from T_1 seeds derived from line IR-D in C. Three IR-D plants were either selfed or outcrossed to BA, and individual seeds from these progeny were analyzed. PCR was performed with primers specific to the Bar transgene. BA, untransformed parental line. *o2*, W64A*o2* seed. M, 1-kb ladder DNA molecular weight marker (Invitrogen). The migration of the 22-kD zein is indicated by an arrow. (F) Photograph of progeny seeds from IR-D, plant D3 in E, showing opaque phenotype. *o2*, W64A*o2*. BA, untransformed parental seed.

J. MESSING, unpublished results). The presence of GFP sequences in transgenic seed was established by PCR. SDS-PAGE of zeins extracted from mature seeds (Figure 4C) revealed an almost complete absence of 22-kD zeins in seeds from some lines, segregating with seeds with a normal phenotype in an apparent 3:1 ratio when originated from selfed plants, without appreciable change in the remaining zein components. The zein depletion pattern observed was indistinguishable from that of the IR lines with simple inverted repeats. At least three lines out of eight examined presented evidence of transgene-induced zein silencing. Again, the protein changes coincided with the appearance of an opaque endosperm (Figure 4D), segregating within families between normal and 22-kD zein-depleted seeds. These results show that gene silencing does not require high levels of RNAi transcripts and can be achieved with inverted repeats separated by other sequences, which are much easier to construct in *E. coli*. Again, the opaque phenotype can be achieved by a single, dominant Mendelian trait.

Amino acid composition of transgenic seeds: One of the hallmarks of the opaque seed phenotype is an increase in the amino acid lysine. Therefore, transgenic seeds with an opaque phenotype were chosen for amino acid analysis. T_0 seeds from two transgenic events, IR-D and LIN-G, exemplifying the two types of RNAi construct with strong opaque phenotype, were selected and subjected to a preliminary amino acid composition anal-

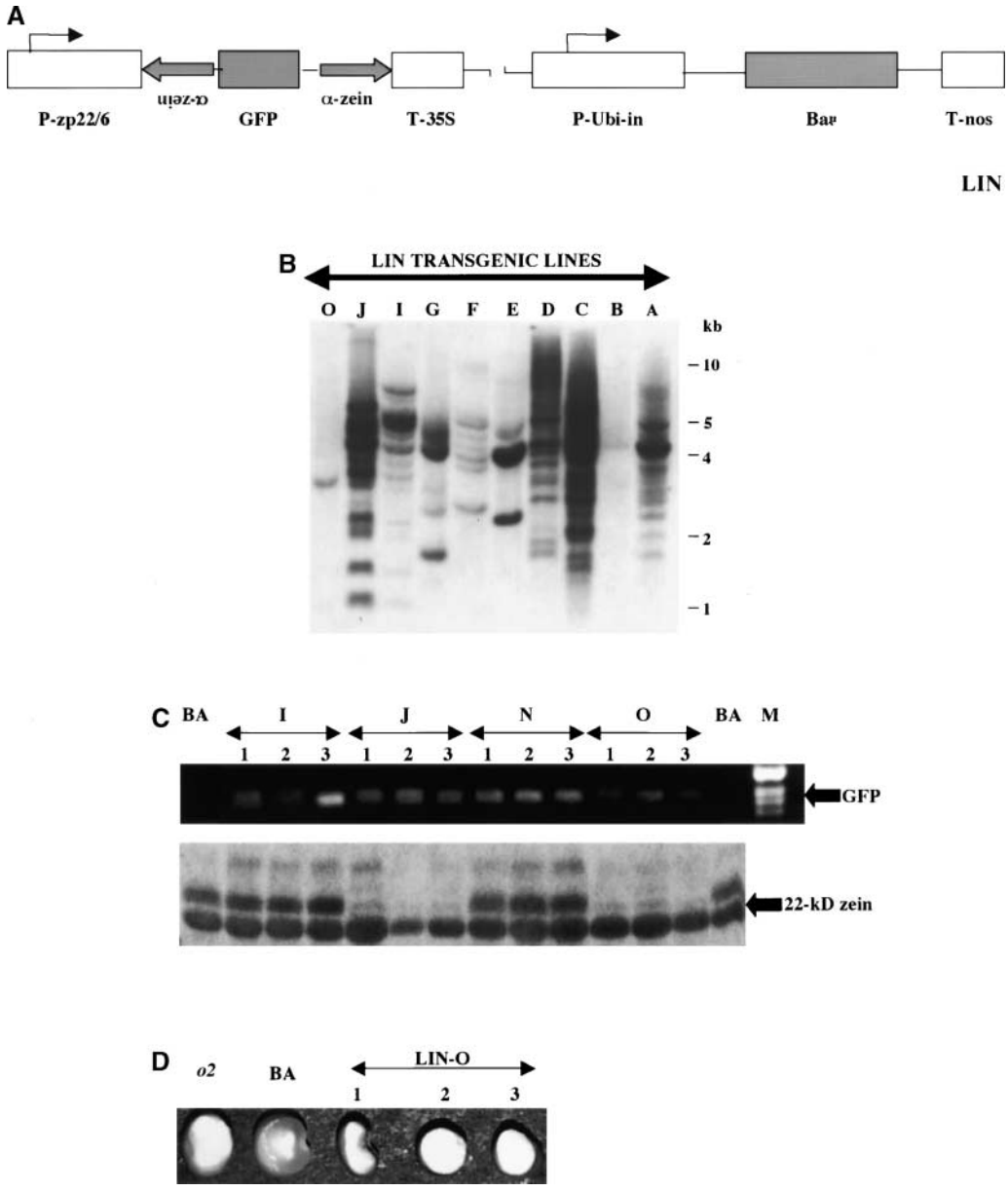

FIGURE 4.—Analysis of transgenic plants derived from a construct with inverted repeats separated by a linker (LIN). (A) RNAi construct with linker (LIN). P-zp22/6, 1.3-kb promoter fragment of the maize *zp22/6* gene (SONG *et al.* 2001; G. SEGAL and J. MESSING, unpublished results). α-zein, PCR fragment of the coding region (see MATERIALS AND METHODS), cloned in opposing fashion flanking the nonactive pGFP-coding region (CLONTECH). (B) Southern blot hybridization of DNA extracted from putative LIN transgenics, digested with *Bam*HI, and hybridized with a 763-bp *Xba*I fragment spanning the entire GFP coding region. Since the construct has a unique *Bam*HI site, this probe uncovers a different restriction fragment length polymorphism pattern for independent transgenic events. Migration of a MW marker (1-kb DNA ladder, Invitrogen) is indicated in kilobases on the right. (C) PCR and SDS-PAGE analysis of DNA and total zeins extracted from T_0 transgenic seed. PCR was performed with primers specific to the nonactive GFP sequence in the transgene. BA, untransformed parental line. M, 1-kb ladder DNA molecular weight marker (Invitrogen). The migration of the 22-kD zein is indicated by an arrow. (D) Photograph of seeds from line LIN-O in C showing an opaque phenotype. *o2*, W64Ao2. BA, untransformed parental line.

ysis. It is well established that seed chemical composition is very variable and dependent on the genetic background. Since transformation has been carried out with hybrids of A188 and B73, future analysis will depend on backcrossing the transgene with the RNAi construct to different genetic backgrounds. Still, a preliminary amino acid analysis provides the first indication of the total amino acid changes due to the reduction of the 22-kD zeins. To illustrate the impact of the two types of RNAi constructs, the deviation of the concentration of each amino acid relative to the untransformed line was determined. Prominent deviations are summarized in Table 1. We could detect specific changes characteristic of α-zein-deficient phenotypes. Indeed, the concentration of lysine was markedly higher in both transgenic lines. In general, the increase in basic and acidic amino acids and in glycine, and the relative reduction in glutamine, leucine, alanine, and serine, are expected if 22-kD zeins are reduced. However, some amino acid changes varied greatly between the two transgenic events (*i.e.*, methionine). Changes could have been compounded by the reduction of 22-kD zein proteins and the association of the transgenic event to two different segregating genetic backgrounds and/or specific regulatory mechanisms interacting with them (*e.g.*, CHIBA *et al.* 1999). Taken together, our data provide evidence that a new

TABLE 1

Amino acid composition analysis of selected opaque seeds

Amino acid	BA hybrid[a]: % sample (SD)	% variation from BA (SD)	
		IR-D line[b]	LIN-G line[b]
Met	0.31 (0.010)	−6.46 (0.010)	−25.81 (0.040)
Lys	0.27 (0.005)	+18.5 (0.025)	+11.11 (0.015)
Leu	1.92 (0.005)	−10.42 (0.100)	−16.14 (0.160)
Thr	0.43 (0.005)	+2.32 (0.005)	+2.32 (0.005)
Val	0.50 (0.010)	−2.00 (0.005)	−14.00 (0.035)
His	0.28 (0.000)	0.00 (0.000)	+3.58 (0.005)
Arg	0.46 (0.010)	+6.52 (0.025)	+13.04 (0.030)
Gly	0.38 (0.010)	+10.53 (0.010)	+10.53 (0.020)
Asx	0.90 (0.001)	+24.4 (0.110)	+13.34 (0.060)
Ser	0.69 (0.001)	−7.25 (0.025)	−4.35 (0.015)
Glx	3.17 (0.035)	−9.78 (0.160)	−8.52 (0.140)
Ala	1.13 (0.005)	−8.85 (0.050)	−9.73 (0.060)
Tyr	0.35 (0.00)	+2.86 (0.005)	+11.43 (0.020)
Total	13.52 (1.390)		

Only selected amino acids are shown.

[a] Untransformed parental hybrid.

[b] T_0 seeds from pooled individuals of selected transgenic lines.

dominant opaque phenotype has been created in maize that, unlike the recessive mutation that prevents the transcriptional activation of a subclass of α-zein storage protein promoters, uses a post-transcriptional gene-silencing approach.

DISCUSSION

Opaque-2 maize has attracted considerable attention due to its outstanding nutritional quality and the characteristic soft texture it confers to the endosperm. Once the molecular identity and the role of O2 as a transcriptional activator in 22-kD zein transcription were documented (Schmidt *et al.* 1992; Ueda *et al.* 1992), it was postulated that the alteration in the relative proportion of zeins was the cause of seed opacity. The changes in protein body structure detected in *o2* relative to normal maize and the effect of *o2* modifier genes, which restore the vitreous endosperm (Geetha *et al.* 1991), are in agreement with this interpretation. On the other hand, O2 is responsible for pleiotropic effects in a variety of endosperm metabolic pathways (Lohmer *et al.* 1991; Habben *et al.* 1995; Maddaloni *et al.* 1996; Kemper *et al.* 1999; Hunter *et al.* 2002). Whether one or more of these alterations indirectly cause this phenotype cannot be resolved from mutations affecting the expression of the *O2* locus. To separate effects on the transcription of storage protein genes from other genes by O2, a dominant mutation that would result in the selective repression of storage protein synthesis in the presence of normal O2 expression was needed.

We first used the expression of antisense sequences of 22-kD zein genes to block the expression of these storage proteins. Since we were concerned that constitutive expression of 22-kD antisense constructs might affect the regeneration and/or fertility of the resulting transgenic plants, we applied to maize for the first time a transgene expression concept that depends on crossing two different maize lines. Such a concept has previously been tested in Arabidopsis (Moore *et al.* 1998). Two transgenic components are stably integrated in the plant genome, one encoding a potent transcriptional activator, KI-His, and the other, pOp, with a passive reporter or gene of interest, under the control of an enhancerless promoter and with duplicated modified *cis*-acting operator sequences from the *E. coli lac* operon. Either after double transformation or as a result of the cross between both kinds of plants, the transcription factor binds the operator sequences with high affinity and activates transcription of the target gene. Thus, the process of transformation and generation of transgenic seeds becomes independent of the nature of the gene introduced. We demonstrated the feasibility of this approach by generating sets of maize transgenics, one ubiquitously expressing KI-His and the other with an antisense version of a 22-kD zein cDNA downstream of the operator with an appropriate distance to a TATA box sequence. Indeed, we could show that the 22-kD protein accumulation is reduced (Figure 2C). Moreover, this change is restricted to this zein component and the progeny of the seeds produced from the cross between the two kinds of plants in the system, whereas the parental transgenic lines remained unaltered compared to the untransformed parental line. The nature of these changes, although limited since no other phenotypic alteration is apparent, make us believe that this system will be a useful complement to other inducible expression systems in maize (Moore *et al.* 1998; Ouwerkerk *et al.* 2001; Shimizu-Sato *et al.* 2002). Furthermore, it would be possible to generate a variety of transactivator lines, driven by well-characterized promoters, for functional characterization of genes throughout development, as in the study of defective kernel mutant transgenes. Likewise, an allelic series of a target gene could be produced by crossing multiple target gene events with a particular transactivator line. Thus, variable levels of expression could be correlated with phenotypic variation. Since the elements included in the construction of pKI are generally recognized in transgenic monocots, it is likely that this system could be directly introduced in other cereals as well, together with suitable reporter lines.

The efficacy of antisense RNA for gene suppression was already known to be variable (Waterhouse *et al.* 1998; Levin *et al.* 2000). In our study, it does not constitute a genetic tool since transgenic seeds do not show a visible phenotype. In contrast, expression of DNA sequences, which generate RNA hairpin structures and double-stranded RNA, proved to be a consistent method to considerably reduce a gene product. Analysis of trans-

TABLE 2

Nucleotide sequence homology of α-zein genes

Gene	% identity to *azs22.8*[c]	Length of uninterrupted identity to *azs22.8* (bp)
Azs22.4[a]	94.1	88
Azs22.8[a]	100	801
Azs22.10[a]	94.6	67
Azs22.14[a]	93.6	66
Azs22.16 (*Fl2*)[a]	89.9	44
Zp22/6[a]	94.0	104
Zp22/D87[a]	95.7	109
Z1A subfamily[b]	42.1–49.0	
Z1B subfamily[b]	32.3–44.5	<15
Z1C subfamily[b]	38.0–47.6	

[a] Expressed 22-kD zein genes from inbred line BSSS53 (Song *et al.* 2001).

[b] 19-kD zein gene subfamilies from inbred line B73 (Song and Messing 2002).

[c] 22-kD zein cDNA used for transgenic constructs.

genic seeds revealed a higher frequency of events with strong target inhibition in the RNAi constructs compared to the regulated antisense experiment. A minimal requirement of a 26-nucleotide pairing for homology-based depletion (Parrish *et al.* 2000) makes it possible to control entire multigene families sharing at least short regions of almost identical sequence. With 90–100% of nucleotide sequence identity over the entire open reading frame (Table 2), 22-kD zein genes are *a priori* suitable targets for these manipulations. RNAi was very effective not only in reducing 22-kD zein expression, but also in giving rise to an opaque phenotype of transgenic seeds. Moreover, the opaque phenotype was correlated with the segregation of a single dominant Mendelian factor and the reduction of zein synthesis. So far, opaque phenotypes have been either recessive or semidominant. While *o2* requires a homozygous state of the allele, recreation of the *floury-2* phenotype by expression of a defective α-zein protein illustrates the potential of a unique transgenic change promoting deep endosperm modification in a semidominant fashion (Coleman *et al.* 1997). Here, we present the first maize dominant opaque phenotype generated by transgenic means. Together with *Mc* and *DeB30*, they are the only dominant opaque variants described thus far. The difference between 22-kD zein protein levels in mature seed of antisense and RNAi constructs is quite striking and at least partially based on the effectiveness of double-stranded RNA structures *in cis vs. in trans* to trigger post-transcriptional gene silencing (reviewed by Bosher and Labouesse 2000; Hsieh and Fire 2000; Hutvágner and Zamore 2002), which appears to be well conserved in evolution (reviewed by Matzke *et al.* 2001). Furthermore, we recently could show that plants produce specific double-stranded RNAs as well as a *Dicer* homolog as part of their normal repertoire of factors controlling gene expression (Park *et al.* 2002). These small transcripts are also referred to as microRNAs (miRNAs). The preservation of the degradation machinery for double-stranded RNA gives rise to the possibility that aspects of normal endosperm development depend on regulatory small interfering RNAs (Hamilton *et al.* 2002; Llave *et al.* 2002; Zamore 2002).

In contrast to recessive *o2* variants, our transgenic constructs triggered the disappearance of the entire 22-kD α-zein subfamily. In the maize inbred BSSS53, this subfamily consists of 22 + 1 clustered genes, 7 of them appearing to be active (Song *et al.* 2001), with >90% nucleotide sequence identity among their translated regions (Table 2). This level of homology makes possible the specific abrogation of the targeted proteins as if they were the product of one gene. Their closest relatives, the 19-kD zeins, with <50% identity (Table 2) and short regions of homology do not favor the accumulation of heterologous double-stranded RNA and are thus unaltered. Moreover, some of the 22-kD zeins are still synthesized in homozygous BSSS53(*o2*), indicating the ineffectiveness of *o2* variants in achieving a complete repression of all 22-kD zein genes (Song *et al.* 2001). Therefore, the simulation of miRNAs promises to be a particularly useful genetic tool in cases where redundancy of regulatory factors prevents typical genetic strategies, such as in many polyploid crop plants that differ from a typical diploid plant such as Arabidopsis.

The specificity of this approach is further illustrated by the synthesis and accumulation of protein from the other zein subfamilies. Surprisingly, there have been fewer compensatory effects from the lack of 22-kD zein proteins on the other storage proteins. Such changes were detected in maize opaque mutants (Hunter *et al.* 2002) as well as in rice seeds engineered for low glutelin accumulation (Maruta *et al.* 2001) and deemed important due to the strong interactions between zein proteins of different subfamilies during protein body formation (Bagga *et al.* 1997; Kim *et al.* 2002).

Frequently, the introduction of an opaque mutation improves dramatically the nutritional value of maize seed protein, but ultimately deleterious effects hinder either the agronomical or the industrial quality of the final product. Another appealing possibility for enhancing the nutritional value of cereals is to quantitatively modify individual seed components by genetic engineering (recently reviewed by Galili and Höfgen 2002). This includes the modification or replacement of metabolic enzymes to change their activity or regulatory properties (Karchi *et al.* 1994; Shaver *et al.* 1996; Chiba *et al.* 1999; Zhu *et al.* 2001) or the direct alteration of the amount or composition of structural proteins (Blechl and Anderson 1996; Maruta *et al.* 2001; Lai and Messing 2002). Potentially, other proteins, which appear correlated with enhanced amino acid seed composition, could become attractive targets for molecular intervention (Habben *et al.* 1995). Here, we show that it

is possible to produce intended changes in the chemical composition of the maize endosperm by stable disruption of one component. The amino acid changes in the lines with most intense silencing of the 22-kD zein (Table 1), although modest, are directly related to the relative abundance of the protein in untransformed maize and to Mendelian seed segregation of the transgenes analyzed. To our knowledge, this is one of the first reports of stable alteration of a cereal seed component by RNAi. In addition, we were successful in eliminating the abundant gene product derived from a large multigene family with at least two distinct chromosomal locations. It is unlikely that such a level of modification could be achieved in the present paradigm by regulation at the transcriptional level. In conclusion, considering the degree of gene silencing achieved, it would be advantageous to combine the transactivator hybrid system with constructs designed to generate transcript depletion by RNAi for functional dissection of seed metabolic pathways and biotechnological applications in maize.

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