Published November, 2011

NOTE

Maize Transgenes Containing Zein Promoters are Regulated by *opaque2*

Megan N. Schroder, Brice E. Floyd, and M. Paul Scott*

ABSTRACT

Understanding epistatic interactions between transgenes and native genes is critical for predicting the response of transgenes to different genetic backgrounds and environments. Our objective was to determine if predicted epistatic interactions occur between maize (*Zea mays* L.) transgenes carrying zein promoters and *opaque2*, a transcriptional regulator of zein genes. Expression of the transgenes was significantly decreased in kernels containing the *opaque2* mutation. Native zein proteins in these kernels were reduced as well. Thus, transgene expression mirrored expression of the native gene that contributed the promoter of the transgene. This work demonstrates that information about native gene expression may be useful for predicting transgene expression, reducing the risk of unintended consequences of transgene expression.

M.N. Schroder, unaffiliated, 130 S. Riverside Dr., Ames, IA 50010; B.E. Floyd, Interdepartmental Plant Biology Program, Agronomy Dep., Iowa State Univ., Ames, IA 50011; M.P. Scott, USDA-ARS, Corn Insect and Crop Genetics Research Unit, Ames, IA 50011. This work was carried out as part of the Iowa State University Interdepartmental Genetics Program degree requirements for MNS and is a joint contribution of the Iowa Agriculture and Home Economics and Experiment Station and the USDA-ARS. Received 25 Mar. 2011. *Corresponding author (paul.scott@ars.usda.gov).

Abbreviations: BT, *Bacillus thuringiensis*; GFP, green fluorescent protein; HPLC, high performance liquid chromatography.

Transgenes in agricultural crops have led to significant benefits in many countries. The use of transgenic cotton (*Gossypium hirsutum* spp.) in China, for instance, containing a Bt (*Bacillus thuringiensis* Berliner 1915) insecticidal toxin has increased yields while reducing exposure of workers and the environment to toxic pesticides (Pray et al., 2002). Economic advantages also have been observed. In the past 14 yr, nearly US$6.9 billion in benefits have been realized by both Bt and non-Bt maize (*Zea mays* L.) growers in the Midwest (Hutchison et al., 2010). The use of transgenes in crops has resulted in many gains and holds much promise for the future.

Understanding how expression of transgenes is regulated facilitates effective deployment of transgenes and reduces the risks of unpredicted and undesirable phenotypes. Interactions with native genes (i.e., epistasis) are a key component of transgene regulation; however, little information is available about these interactions. Such epistatic interactions could be responsible for the unexpected failure of transgenes and for variation in transgene expression in different genetic backgrounds. Genetic background

Published in Crop Sci. 51:2716–2720 (2011).
doi: 10.2135/cropsci2011.03.0168
Freely available online through the author-supported open-access option.
Published online 19 Aug. 2011.

affects the amount of toxin produced in a transgenic Bt cotton cultivar (Sachs et al., 1998), for example. Understanding how transgenes interact with native genes is an important step toward predicting how a transgene will perform in a given genetic background. In addition, it may be possible to capitalize on known epistatic interactions to regulate transgene expression in desired ways.

Reporter genes are used extensively in biological research to facilitate imaging (reviewed in Kang and Chung, 2008). In cases where transgenic reporter genes are used to study gene expression, the assumption is often made that a transgene will exhibit gene expression patterns similar to those of a native gene on which the transgene is based. This would require the epistatic interactions of the gene under investigation to be similar to those of the transgenic reporter gene. While a large body of evidence supports this assumption indirectly, direct tests of this assumption are rare.

Seed storage protein gene promoters have been used extensively to confer high-level seed tissue-specific expression to transgenes (Russell and Fromm, 1997; Wu et al., 1998). Furthermore, regulation of expression of seed storage protein genes is well understood due in part to the study of naturally occurring mutations that alter seed storage protein expression. For example, the *opaque2* mutation of maize is characterized by soft, floury endosperm that causes the kernel to appear opaque rather than translucent when placed on a light box. One effect of this mutation is to decrease the level of 19 and 22 kDa α zein seed storage proteins in the kernel (Kodrzycki et al., 1989; Langridge et al., 1982). The *opaque2* gene encodes a bZip transcription factor that binds to a 22 kDa α zein gene promoter, thereby having a direct impact on gene expression (Hartings et al., 1989; Schmidt et al., 1990). The bZip transcription factor has not been found to bind to 19 kDa α zein promoters (Schmidt et al., 1990) suggesting that the *opaque2* mutation has an indirect impact on this gene family. Surprisingly, it has been reported that the *opaque2* mutation does not impact the levels of the other zein proteins (Kodrzycki et al., 1989). Thus, the *opaque2* mutation exhibits epistatic interactions with some native zein genes and offers an opportunity to study the regulation of transgenes carrying zein promoters.

The objective of this project was to determine if the maize *opaque2* mutation exhibits epistatic interactions with two green fluorescent protein (GFP) transgenes containing different zein promoters. The transgenes used in this study express GFP using either a 27 kDa α or a 19 kDa α zein promoter (Shepherd et al., 2008). These promoters are of interest because the native zein genes from which they are derived are reported to be impacted differently by the *opaque2* mutation. The 19 and 22 kDa zeins have been reported to decrease while the 27 kDa zeins have been reported to be unaltered by the *opaque2* mutation (Kodrzycki et al., 1989; Hunter et al., 2002). We examined the effect of the *opaque2* mutation on expression of these two transgenes by comparing fluorescence in kernels with the wild-type and mutant alleles of *opaque2*.

MATERIALS AND METHODS

Maize plants were grown in the Iowa State University transgenic maize nursery on the Woodruff-Bennett farm near Ames, IA, under approved Animal and Plant Health Inspection Service (APHIS) notifications. Four ears segregating for both the *opaque2* mutation and either the 19zn-GFP transgene having a 19 kDa zein promoter (two ears) or the 27zn-GFP transgene having a 27 kDa zein promoter (two ears) were produced by self-pollination of plants that were heterozygous at the *opaque2* locus and hemizygous at the transgene locus. Plants that were heterozygous at the *opaque2* locus and hemizygous at the transgene locus were produced by crossing a plant produced from an opaque kernel (*opaque2/ opaque2*) by a plant produced from a GFP-expressing kernel (GFP/-) and selecting a GFP-expressing kernel for planting. The ears were shelled and kernels were separated based on the opaque or translucent phenotype using a light box. Individual kernels were not prescreened for fluorescence.

Fluorescence was measured using 48 translucent and 48 opaque individual kernels that were randomly selected from each of the four ears and individually ground using a tungsten-carbide ball bearing in a Talboys HT Homogenizer (Troemner, Thorofare, NJ). Twenty-five milligrams of flour from each kernel was extracted with 250 μL of 30 mmol Tris-HCl, 10 mmol ethylenediaminetetraacetic acid (EDTA), 10 mmol NaCl, and 5 mmol dithiothreitol for 1 hr. After centrifugation at 10,000 × *g* for 10 min, supernatants were randomized and placed into a black 96-well plate. Extracted GFP was quantified using a spectrofluorimeter (Tecan, Mannedorf, Switzerland) using an excitation wavelength of 485 nm and an emission wavelength of 535 nm with a gain of 70 using nine flashes per sample and an integration time of 40 μs. The mean of the 48 translucent kernels was compared to the mean of the 48 opaque kernels for each of the four ears using a Student's *t* test.

Zein composition of each ear was analyzed on two mass-based bulks of ground grain from 48 kernels with one bulk made from translucent kernels and the other made from opaque kernels. Zeins were extracted from 10 mg of each bulk using 400 μL of 70% ethanol containing 61 mmol NaOAc and 5% α-mercaptoethanol, incubated while shaking for 1 h, and centrifuged at 13,000 rpm for 10 min. Each sample was separated by high performance liquid chromatography (HPLC) using an injection volume of 25 μL, a flow rate of 1 mL min^{-1} on a Vydac C18 column (Grace Davison Discovery Sciences, Deerfield, IL) as described previously (Bietz, 1983). The eluent was monitored by HPLC ultraviolet absorbance at 200 nm and the areas under the peaks of the resulting chromatograms were determined. Three opaque and three translucent subsamples were analyzed from each ear, and the means of these three subsamples were compared using a Student's *t* test.

RESULTS AND DISCUSSION

To identify epistatic interactions between the *opaque2* mutation and two transgenes based on zein promoters, we crossed plants containing either the 19zn-GFP or the 27zn-GFP transgene to plants with the genotype *opaque2/opaque2*. The F_1 progeny from these crosses were translucent kernels that were

Table 1. Construct, transformation events, and genome composition for the nine ears analyzed.

Source†	Construct	Event	Genome composition (%)‡				
			B14A	B45	B73	B110	A188
9141-04	19zn-GFP§	29-1	50	–	43.75	–	6.25
9145-04	19zn-GFP	3-2	–	50	43.75	–	6.25
9143-03	27zn-GFP	71-1	–	50	21.87	25	3.13
9148-03	27zn-GFP	71-1	50	–	43.75	–	6.25

†Source is given as row-plant number. Plants in the same row are the same genotype. All plants are self pollinated.

‡Genome composition estimated from the pedigree of the source.

§GFP, green fluorescent protein.

Table 2. Comparison of mean fluorescence values between wild-type and *opaque2* kernels.

Construct	Ear	Phenotype	Mean fluorescence†
27zn-GFP§	9143-03	Translucent	1950
		Opaque	1076
	9148-03	Translucent	3849
		Opaque	1415
19zn-GFP	9145-04	Translucent	1599
		Opaque	269
	9141-04	Translucent	1752
		Opaque	564

†Mean fluorescence given in arbitrary fluorescence units. Translucent and opaque values are significantly different ($p \leq 0.01$) by Student's *t*-test in all four comparisons.

§GFP, green fluorescent protein.

fluorescent, verifying earlier observations that expression of neither transgene causes an opaque phenotype. Presumably, these plants were hemizygous at the transgene locus and heterozygous at the *opaque2* locus. These seeds were planted and the resulting plants were self-pollinated to produce F_2 ears. These ears segregated with phenotypic ratios that were not statistically different than three translucent kernels: one opaque kernel, as expected for the *opaque2* locus (data not shown). Visually, a range of fluorescence was observed on these ears. We selected one ear from each of two different plants for each transgene for further analysis. The two ears that contained transgene 19zn-GFP were from two different transformation events while the two ears that contained transgene 27zn-GFP were derived from the same transformation event (Table 1). Differences in the pedigrees of the ears used in the study also resulted in differences in the predicted genome compositions as described in Table 1. However, all of the lines in these pedigrees are derived from Iowa Stiff Stalk Synthetic except A188 and B45. Thus, one ear containing each can be considered to be in the Stiff Stalk heterotic group and one ear containing each construct is a hybrid between Stiff Stalk and the non-stiff stalk inbred line B45.

We measured fluorescence levels in extracts from 48 opaque kernels and 48 translucent kernels from each ear. The kernels were selected randomly from within each class (opaque or translucent). The mean fluorescence value of the opaque kernels from each ear was compared to the mean fluorescence values of the translucent kernels from the same ear, resulting in four comparisons. In all four comparisons, the mean fluorescence of the translucent kernels was significantly higher than the mean fluorescence of the opaque kernels (Table 2), suggesting an epistatic interaction occurs between both the 19 kDa zein-GFP and the 27 kDa zein-GFP transgenes and the *opaque2* gene. The ratio of fluorescence in translucent kernels to opaque kernels varied among the ears from 1.8 to 5.9. Most of this variation is due to the different effect of the *opaque2* mutation on the 19 kDa and the 27 kDa zein promoters. The 19 kDa promoter is more strongly downregulated in the mutant than the 27 kDa zein promoter.

If the transgenes exhibited normal Mendelian inheritance, about one fourth of the kernels in each phenotypic class (translucent or opaque) would not have inherited the transgene, one half would be hemizygous at the transgene locus, and one fourth would be expected to be homozygous at the transgene locus. Factors that alter the segregation ratios differentially between the phenotypic classes would interfere with our analysis by changing the ratio of transgenic to nontransgenic kernels in each phenotypic class. For example, genetic linkage of the *opaque2* and transgene loci would result in underrepresentation of the transgene in the opaque pool, with a concomitant overrepresentation of the transgene in the translucent pool because the transgene and the mutant *opaque2* allele would be linked in repulsion. This could explain the observed results. It is unlikely that all three transformation events examined resulted in linkage of the transgene to the *opaque2* locus. Thus, our data support the conclusion that there is epistasis between the transgene loci and the *opaque2* locus. Similarly, differential pollen transmission of the transgene between opaque and translucent kernels could result in a similar underrepresentation of the transgene in the opaque pool. While transgenes have been observed to exhibit abnormal pollen transmission (Scott et al., 2007), it is unlikely that the degree of pollen transmission would be altered by *opaque2*, a mutation that primarily effects seed phenotypes.

The observation that expression of the transgene construct based on the 27 kDa α zein promoter was reduced in opaque seeds was somewhat surprising because levels of the 27 kDa α zein have been reported to be unchanged in an *opaque2* mutant background (Kodrzycki et al., 1989; Hunter et al., 2002). The effect of *opaque2* on gene expression is complex and dependant on the genetic background (Jia et al., 2007; Paez et al., 1969; Gentinetta, 1975), so we examined the kernels to determine if levels of the native 27 kDa α zein gene were altered in the ears used in this study. Both the α and 27 kDa α zein proteins were reduced in the opaque kernels relative to the translucent kernels of all ears used in this study (Fig. 1; Table 3).

The two transgenes in this study exhibited expression patterns similar to the native genes on which the transgene promoters were based. In mature kernels, GFP was strongly expressed in endosperm tissue and was only weakly detectable

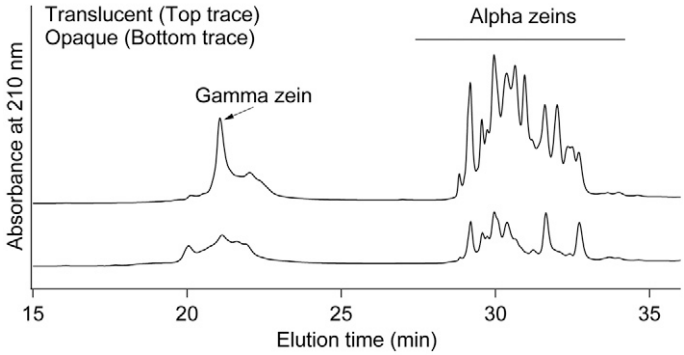

Figure 1. High performance liquid chromatography (HPLC) chromatogram showing a reduction in both α and γ zein levels in opaque maize kernels compared to translucent maize kernels. Each trace is the average of three HPLC runs using ear 9148-03, which showed the smallest reduction in zein levels compared to other ears.

(27 kDa promoter construct) or not detectable (19 kDaA promoter construct) in embryo (Shepherd et al., 2008). In addition to their well-characterized endosperm activity, zein promoters have recently been shown to have activity in aleurone cells (Reyes et al., 2011). Since we ground whole kernels, the vast majority of the observed fluorescence was from endosperm tissue, but a small amount of fluorescence may have been derived from aleurone or embryo tissue. If *opaque2* had different tissue specificity than the transgenes, this would complicate interpretation of these data. Examination of the tissue specificity of *opaque2* transcript accumulation (Sekhon et al., 2011) reveals a pattern of expression very similar to that of our transgenes, with high expression in endosperm, low expression in embryo, and expression in pericarp that may be attributable to aleurone expression. Since the tissue specificity of expression of both the transgene and *opaque2* are similar, the bias introduced by grinding whole kernels was small.

Many factors may influence the interaction of transgenes with native genes. We examined two transformation events from one construct and one event from the other, and this is not a sufficient number of events to rule out the possibility that interactions between transgenes and native genes vary by event. In fact, we observed variation in the degree of epistasis among the events studied. Similarly, such interactions may vary by genetic background (we tested two for each transgene) or the environment. It seems likely that the genomic context of a transgene would influence the regulation of its expression, but the scope of this study was not broad enough to estimate the frequency of unexpected expression patterns. The observation that at least some interactions between transgenes and native genes are predictable illustrates the potential for knowledge-based manipulation of biological traits using genetic engineering methods and provides an opportunity to reduce the risk of unintended consequences of transgene expression.

Table 3. Comparison of mean zein levels in four opaque and translucent kernels from four maize ears.

Ear	Phenotype	α-zein mean†	γ-zein mean
9143-03	Translucent	77.53	4.33
	Opaque	34.62	2.66
9148-03	Translucent	102.11	6.43
	Opaque	52.51	9.62
9145-04	Translucent	78.62	8.66
	Opaque	35.18	4.23
9141-04	Translucent	111.50	12.02
	Opaque	35.43	4.20

†Zein levels are expressed in arbitrary units reflecting the area under the peak in chromatograms similar to that shown in Fig. 1. All eight comparisons of Translucent vs. Opaque values are significantly different ($p < 0.01$) by Student's *t*-test except the α-zein values for 9148-03, which are significantly different at $p < 0.05$.

Acknowledgments

We gratefully acknowledge the skillful technical support of Dr. Adrienne Moran-Lauter, USDA-ARS. Names are necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

References

Bietz, J.A. 1983. Separation of cereal proteins by reversed-phase high-performance liquid chromatography. J. Chromatogr. A 255:219–238. doi:10.1016/S0021-9673(01)88286-8

Gentinetta, E. 1975. Protein studies in 46 opaque-2 strains with modified endosperm texture. Maydica 20:145–164.

Hartings, H., M. Maddaloni, N. Lazzaroni, N. Di Fonzo, M. Motto, F. Salamini, and R. Thompson. 1989. The O2 gene which regulates zein deposition in maize endosperm encodes a protein with structural homologies to transcriptional activators. EMBO J. 8:2795–2801.

Hunter, B.G., M.K. Beatty, G.W. Singletary, B.R. Hamaker, B.P. Dilkes, B.A. Larkins, and R. Jung. 2002. Maize opaque endosperm mutations create extensive changes in patterns of gene expression. Plant Cell 14:2591–2612. doi:10.1105/tpc.003905

Hutchison, W.D., E.C. Burkness, P.D. Mitchell, R.D. Moon, T.W. Leslie, S.J. Fleischer, M. Abrahamson, K.L. Hamilton, K.L. Steffey, M.E. Gray, R.L. Hellmich, L.V. Kaster, T.E. Hunt, R.J. Wright, K. Pecinovsky, T.L. Rabaey, B.R. Flood, and E.S. Raun. 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330:222–225. doi:10.1126/science.1190242

Jia, H., D. Nettleton, J.M. Peterson, G. Vazquez-Carrillo, J.-L. Jannink, and M.P. Scott. 2007. Comparison of transcript profiles in wild-type and o2 maize endosperm in different genetic backgrounds. Crop Sci. 47:S45–S59. doi:10.2135/cropsci2006-0002tpg

Kang, J.H., and J.-K. Chung. 2008. Molecular-genetic imaging based on reporter gene expression. J. Nucl. Med. 49:164S–179S. doi:10.2967/jnumed.107.045955

Kodrzycki, R., R.S. Boston, and B.A. Larkins. 1989. The *opaque-2* mutation of maize differentially reduces zein gene transcription. Plant Cell 1:105–114. doi:10.1105/tpc.1.1.105

Langridge, P., J.A. Pintor-Toro, and G. Feix. 1982. Direction of zein gene transcription in maize genomic clones. Biochem. Biophys. Res. Commun. 107:1236–1242. doi:10.1016/S0006-291X(82)80130-7

Paez, A.V., J.L. Helm, and M.S. Zuber. 1969. Lysine content of

opaque-2 maize kernels having different phenotypes. Crop Sci. 9:251–252. doi:10.2135/cropsci1969.0011183X000900020045x

Pray, C.E., J. Huang, R. Hu, and S. Rozelle. 2002. Five years of Bt cotton in China – The benefits continue. Plant J. 31:423–430. doi:10.1046/j.1365-313X.2002.01401.x

Reyes, F.C., T. Chung, D. Holding, R. Jung, R. Vierstra, and M.S. Otegui. 2011. Delivery of prolamins to the protein storage vacuole in maize aleurone cells. Plant Cell 23:769–784. doi:10.1105/tpc.110.082156

Russell, D.A., and M.E. Fromm. 1997. Tissue-specific expression in transgenic maize of four endosperm promoters from maize and rice. Transgenic Res. 6:157–168. doi:10.1023/A:1018429821858

Sachs, E.S., J.H. Benedict, D.M. Stelly, J.F. Taylor, D.W. Altman, S.A. Berberich, and S.K. Davis. 1998. Expression and segregation of genes encoding CryIA insecticidal proteins in cotton. Crop Sci. 38:1–11. doi:10.2135/cropsci1998.0011183X003800010001x

Schmidt, R., F. Burr, M. Aukerman, and B. Burr. 1990. Maize regulatory gene opaque-2 encodes a protein with a "leucine-zipper" motif that binds to zein DNA. Proc. Natl. Acad. Sci. USA 87:46–50. doi:10.1073/pnas.87.1.46

Scott, M.P., J.M. Peterson, D.L. Moran, V. Sangtong, and L. Smith. 2007. A wheat genomic DNA fragment reduces pollen transmission of maize transgenes by reducing pollen viability. Transgenic Res. 16:629–643. doi:10.1007/s11248-006-9055-x

Sekhon, R.S., H. Lin, K.L. Childs, C.N. Hansey, C.R. Buell, N. de Leon, and S.M. Kaeppler. 2011. Genome-wide atlas of transcription during maize development. Plant J. 66:553–563. doi:10.1111/j.1365-313X.2011.04527.x

Shepherd, C.T., N. Vignaux, J.M. Peterson, L.A. Johnson, and M.P. Scott. 2008. Green fluorescent protein as a tissue marker in transgenic maize seed. Cereal Chem. 85:188–195. doi:10.1094/CCHEM-85-2-0188

Wu, C.-Y., T. Adach, T. Hatano, H. Washida, A. Suzuki, and F. Takaiwa. 1998. Promoters of rice seed storage protein genes direct endosperm-specific gene expression in transgenic rice. Plant Cell Physiol. 39:885–889.